10. Robertson, J.D., Orrenius, S.: Molecular mechanisms of apoptosis induced by cytotoxic chemicals. Crit Rev Toxicol 2000, 30, 609–627.
11. Shih, J.C., Chen, K., Ridd, M.J.: Monoamine oxidase: From genes to behavior. Ann Rev Neurosci 1999, 22, 197–217.
12. Brunner, H.G., Nelen, M., Breakfield, X.O., Ropers, H.H., van Oost, B.A.: Abnormal behavior associated with a point mutation in the structure gene for monoamine oxidase A. Science 1993, 262, 578–590.
13. Riederer, P., Konrad, G., Habenstreit, G., Youdim, M.B.H.: Neurochemical perspectives to the function of monoamine oxidase. Acta Neurol Scand 1989, 126, 41–45.
14. Zeevalk, G.D., Bernard, L.P., Nicklas, W.J.: Role of oxidative stress and the glutathione system in loss of dopamine neurons due to impairment of energy metabolism. J Neurochem 1998, 70, 1421–1430.
15. Heikkila, R.E., Terleckkyj, I., Sieber, B.A.: Monoamine oxidase and the bioactivation of MPTP and related neurotoxins: relevance to DATATOP J Neural Transm 1990 (Suppl), 217–227.
16. Langston, J.W., Irwin, I., Langston, E.B., Forno, L.S.: Pargyline prevents 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonism in primates. Science 1984, 225, 1480–1482.
17. Birkmayer, W., Knoll, J., Riederer, P., Youdim, M.B.H.: (–)-Deprenyl leads to prolongation of L-DOPA efficiency in Parkinson's disease. Mol Probl Pharmacopsychiatr 1983, 19, 170–176.
18. Tetrud, J.W., Langston, J.W.: The effect of deprenyl (selegiline) on the natural history of Parkinson's disease. Science 1989, 245, 519–522.
19. De Zutter, G.S., Davis, R.J.: Pro-apoptotic gene expression mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Proc Natl Acad Sci USA 2001, 98, 6168–6173.
20. Naoi, M., Maruyama, W.: Neuroprotection by (–)-deprenyl and related compounds. Mech Age Devel 1999, 111, 189–200.
21. Maruyama, W., Akao, Y., Youdim, M.B.H., Naoi, M.: Neurotoxins induce apoptosis in dopamine neurons: protection by *N*-propargyl-1(*R*)- and -(*S*)-aminoindan, rasagiline and TV1022. J Neural Transm 2000, [Suppl] 60, 171–186.
22. Maruyama, W., Boulton, A.A., Davis, B.A., Dostert, P., Naoi, M.: Enantiospecific induction of apoptosis by an endogenous neurotoxin, *N*-methyl(*R*)-salsolinol, in dopaminergic SH-SY5Y cells: suppression of apoptosis by *N*-(2-heptyl)-*N*-methylpropargylamine. J Neural Transm 2001, 108, 11–24.
23. Maruyama, W., Akao, Y., Youdim, M.B.H., Davis, B.A., Naoi, M.: Transfection-enforced Bcl-2 overexpression and an anti-Parkinson drug, rasagiline, prevent nuclear accumulation of glyceraldehydes-3-phosphate dehydrogenase induced by an endogenous dopaminergic neurotoxin, *N*-methyl(*R*)salsolinol. J Neurochem 2001, 78, 727–735.
24. Akao, Y., Maruyama, W., Shimizu, S., Yi, H., Nakagawa, Y., Shamoto-Nagai, M., Youdim, M.B.H., Tsujimoto, Y., Naoi, M.: Mitochondrial permeability transition mediates apoptosis induced by *N*-methyl(*R*)salsolinol, an endo-

genous neurotoxin, and is inhibited by Bcl-2 and rasagiline, *N*-propargyl-1(*R*)-aminoindan. J Neurochem 2002, 82, 913–923.
25. Maruyama, W., Takahashi, T., Naoi, M.: (–)-Deprenyl protects human dopaminergic neuroblastoma SH-SY5Y cells from apoptosis induced by peroxynitrite and nitric oxide. J Neurochem 1998, 70, 2510–2515.
26. Sandler, M., Bonham Carter, S., Hunter, K.R., Stern, G.M.: Tetrahydroisoquinoline alkaloids: in vive metabolites of L-DOPA in man. Nature 1973, 241, 439–443.
27. Collins, M.A.: Neuroamine condensations in human subjects. Adv Exp Med Biol 1980, 126, 87–102.
28. Sjöquist, B., Borg, S., Kvande, H.: Salsolinol and methylated salsolinol in urine and cerebrospinal fluid from healthy volunteers. Subst Alcohol Actions Misuse 1981, 2, 73–77.
29. Dostert, P., Strolin Benedetti, M., Dordain, G., Vernay, D.: Enantiomeric composition of urinary salsolinol in Parkinsonian patients after Madopar. J Neural Transm [P-D Sect] 1989, 1, 269–278.
30. Strolin Benedetti, M., Bellotti, V., Pianezzola, E., Moro, E., Carminati, P., Dostert, P.: Ratio of the R and S enantiomers of salsolinol in food and human urine. J Neural Transm 1989, 77, 47–53.
31. Maruyama, W., Deng, Y., Dostert, P., Naoi, M.: Analysis of endogenous enantiomers of neurotoxins in clinical samples by a multiple-electrode detection system with a chiral column, In: Acworth, I.N., Naoi, M., Parvez, H., Parvez, S., editors. Coulometric electrode array detectors for HPLC, Progress in HPLC-HPCE Volume 6, Utrecht: VSP., 1997, 339–350.
32. Naoi, M., Maruyama, W., Dostert, P., Kohda, K., Kaiya, T.: A novel enzyme enantio-selectively synthesizes (*R*)salsolinol, a precursor of a dopamineergic neurotoxin, *N*-methyl(*R*)salsolinol. Neurosci Lett 1996, 212, 183–186.
33. Naoi, M., Maruyama, W., Matsubara, K., Hashizume, Y.: A neutral *N*-methyl-transferase activity in the striatum determines the level of an endogenous MPP^+-like neurotoxin, 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion, in the substantia nigra of human brains. Neurosci Lett 1997, 235, 81–84.
34. Naoi, M., Maruyama, W., Zhang, J.H., Takahashi, T., Deng, Y., Dostert, P.: Enzymatic oxidation of the dopaminergic neurotoxin, 1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, into 1,2-dimethyl-6,7-dihydroxyisoquinoli-nium ion. Life Sci 1995, 57, 1061–1066.
35. Maruyama, W., Sobue, G., Matsubara, K., Hashizume, Y., Dostert, P., Naoi, M.: A dopaminergic neurotoxin, 1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetra-hydroisoquinoline, *N*-methyl(*R*)salsolinol, and its oxidation product, 1,2(*N*)-dimethyl-6,7-dihydroxyisoquinolinium ion, accumulate in the nigrostriatal system of the human brain. Neurosci Lett 1997, 223, 61–64.
36. Yamada, Y.: Effects of salsolinol on rat brain and liver monoamine oxidase. Japan J Pharmacol 1971, 21, 833–835.
37. Collins, M.A., Cashaw, J.L., Davis, V.E.: Dopamine-derived tetrahydroisoquinoline alkaloids-Inhibitors of neuroamine metabolism. Biochem Pharmncol 1973, 22, 2337–2348.

38. Dostert, P., Strolin Benedetti, M., Dordain, G.: Dopamine-derived alkaloids in alcoholism and in Parkinson's and Huntchington's diseases. J Neural Transm 1988, 74, 61–74.
39. Bembenek, M., Abell, C.W., Chrisey, L.A., Rozwadowska, M.D., Gessner, W., Brossi, A.: Inhibition of monoamine oxidase A and B by simple isoquinoline alkaloids: Racemic and optically active 1,2,3,4-tetrahydro-, 3,4-dihydro-, and fully aromatic isoquinolines. J Med Chem. 1990, 33, 147–152.
40. Thull, U., Kneubühler, S., Gaillard, P., Carrupt, P.-A., Testa, B., Altomare, C., Carotti, A., Jenner, P., McNaught, K.: Inhibition of monoamine oxidase by isoquinoline derivatives. Qualitative and 3D-quantitative structure-activity relationship. Biochem Pharmacol 1995, 50, 869–877.
41. Minami, M., Maruyama, W., Dostert, P., Nagatsu, T., Naoi, M.: Inhibition of type A and B monoamine oxidase by 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinolines and their N-methylated derivatives. J Neural Transm [GenSect] 1993, 92, 125–135.
42. Naoi, M., Maruyama, W., Sasuga, S., Deng, Y., Dostert, P., Ohta, S., Takahashi, T.: Inhibition of type A monoamine oxidase by 2(*N*)-methyl-6,7-dihydroxyisoquinolinium ions. Neurochem Int 1994, 25, 475–482.
43. Langston, J.W., Ballard, P., Tetrud, J.W., Irvin, I.: Chronic parkinsonism in humans due to a product of meperidine-analog synthesis. Science 1983, 219, 979–980.
44. Naoi, M., Maruyama, W., Dostert, P., Hashizume, Y., Nakahara, D., Takahashi, T., Ota, M.: Dopamine-derived endogenous 1(*R*),2(*N*)-dimethyl-6,7-dihydroxy-1,2,3,4-tetra-hydroisoqunoline, *N*-methyl-(*R*)-salsolinol, induced parkinsonism in rat: biochemical, pathological and behavioral studies. Brain Res 1996, 709, 285–295.
45. Maruyama, W., Abe, T., Tohgi, H., Dostert, P., Naoi, M.: A dopaminergic neurotoxin, (*R*)-N-methylsalsolinol, increases in parkinsonian cerebrospinal fluid. Ann Neurol 1996, 40, 119–122.
46. Naoi, M., Maruyama, W., Nakao, N., Ibi, T., Sahashi, K., Strolin Benedetti, M.: (*R*)Salsolinol *N*-methyltransferase activity increases in parkinsonian lymphocytes. Ann Neurol 1998, 43, 212–216.
47. Akao, Y., Nakagawa, Y., Maruyama, W., Takahashi, T., Naoi, M.: Apoptosis induced by an endogenous neurotoxin, *N*-methyl(*R*)salsolinol, is mediated by activation of caspase 3. Neurosci Lett 1999, 267, 153–156.
48. Naoi, M., Maruyama, W., Akao, Y., Zhang, J., Parvez, H.: Apoptosis induced by an endogenous neurotoxin, N-methyl(R)salsolinol, in dopamine neurons. Toxicology 2000, 153, 123–141.
49. Naoi, M., Maruyama, W.: Future of neuroprotection in Parkinson's disease. Parkin Rel Dis 2001, 8, 139–145.
50. Olanow, C.W., Schapira, A.H., Agid, Y.: Neuroprotection for Parkinson's disease: prospects and promises. Ann Neurol 2003, 53 (Suppl 3), S1-S2.
51. Magyar K., Szende B., Lengyel J., Tarczali J., Szatmary I.: The neuroprotective and neuronal rescue effects of (–)-deprenyl. J Neural Transm 1998, [Suppl] 52, 109–123.

52. Tatton, W.G., Greenwood, C.E.: Rescue of dying neurons: a new action for deprenyl in MPTP parkinsonism. J Neurosci Res 1991, 30, 666–672.
53. Finberg, J.P.M., Lamensdorf, I., Commissiong, J.W., Youdim, M.B.H.: Pharmacology and neuroprotective properties of rasagiline. J Neural Transm 1996, [Suppl] 48, 95–101.
54. Maruyama, W., Weinstock, M., Youdim, M.B.H., Nagai, M., Naoi, M.: Anti-apoptotic action of anti-Alzheimer drug, TV3326 [(N-propargyl)-(3R)-aminoindan-5-yl]-ethyl methyl carbamate, a novel cholinesterase-monoamine oxidase inhibitor. Neuosci Lett 2003, 341, 233–236.
55. Kuwabara, I., Kuwabara, Y., Yang, R.-Y., Schuler, M., Gren, D.R., Zuraw, B.L., Hsu, D.K., Liu, F.-T.: Galectin-7 (PIG1) exhibits prop-apoptotic function through JNK activation and mitochondrial cytochrome c release. J Biol Chem 2002, 277, 3487–3497.
56. Kitazawa, M., Wagner, J.R., Kirby, M.L., Ananthram, V., Kanthasamy, A.G.: Oxidative stress and mitochondrial-mediated apoptosis in dopaminergic cells exposed to methylcyclopentadienyl manganese tricarbonyl. J Pharm Exp Therap 2002, 302, 26–35.
57. Kunduzova, O.R., Bianchi, P., Pizzinat, M., Escourrou, G., Seguelas, M.-H., Parini, A., Cambon, C.: Regulation of JNK/ERK activation, cell apoptosis, and tissue regeneration by monoamine oxidases after renal ischemia-reperfusion. FASEB J 2002, 16, 1129–1131.

Fos Expression Associated with the Discriminative Stimulus Effects of Methamphetamine in Rats

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ABSTRACT: **Methamphetamine, a psychostimulant drug, produces both acute psychomotor stimulation and long-lasting behavioral effects including addiction and psychosis. To identify anatomical substrates for the discriminative stimulus effects of methamphetamine in rats, we examined the drug discrimination-associated c-Fos expression in the brains of rats that were trained to discriminate methamphetamine from saline under a two-lever fixed ratio (FR-20) schedule of food reinforcement. c-Fos expression in the brains of rats trained to discriminate methamphetamine from saline was significantly increased in the nucleus accumbens (NAc) and the ventral tegmental area (VTA) as compared with the expression in the control rats that were maintained under the FR-20 schedule, but no alternation was observed in other areas including the cerebral cortex, caudate putamen, substantia nigra, hippocampus, amygdala, and habenulla. Methamphetamine treatment in the trained rats caused a significant increase in c-Fos expression in the VTA, and a decrease in the NAc core, as compared to saline treatment. However, c-Fos expression in the NAc and VTA of rats that received chronic intermittent methamphetamine administration without discrimination training, did not differ from the expression in saline-treatment animals. These results suggest that the VTA and the NAc play an important role in the discriminative stimulus effects of methamphetamine.**

KEYWORDS: **drug dependence; drug discrimination; c-Fos; methamphetamine; nucleus accumbens; ventral tegmental area**

INTRODUCTION

Methamphetamine (MAP), an addictive drug, produces various behavioral effects that are mainly mediated by the dopaminergic (DA) neuronal system.[1] It has been known that the discriminative stimulus effects of psychostimulants in experi-

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mental animals are related to their subjective effects in humans.[2] Therefore, the drug discrimination procedures have been proven to be a valuable means for elucidating the mechanism of action underlying the unique properties of addictive drugs.[1,3]

Quantification of the changes in the expression of the immediate early gene *c-fos* has been proven to be a very useful method by which the distribution of neurons that are activated by physiological or pharmacological stimuli may be mapped.[4] Several studies have shown that acute methamphetamine administration dose-dependently produces c-Fos-like immunoreactivity in wide areas of the brains including the nucleus accumbens and striatum,[5] and that chronic methamphetamine or amphetamine administration abolishes the inducibility of *c-fos* in the striatum.[6,7] In the present study, to identify potential anatomical substrates of the discriminative stimulus effects of methamphetamine in rats, we examined the c-Fos expression in the brain of rats trained to discriminate methamphetamine from saline.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (7 weeks old, Charles River Japan, Yokohama), weighing 230 ± 10 g at the beginning of experiment, were used in this study. They were under controlled laboratory conditions (12-h light/dark cycle with lights on at 9:00 h, 23 ± 0.5°C, 50 ± 0.5% humidity). Their body weights were gradually reduced to approximately 80% of the free-feeding weight by limiting daily access to food. Water was available *ad libitum*. All animal care and use were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Methamphetamine Discrimination Procedure

Methamphetamine discrimination procedures were conducted as described in detail by Mori *et al.*[3] Rats were injected 10 min before the session with either saline or methamphetamine (0.5 mg/kg, s.c.). Discrimination training sessions were conducted 5 days per week under a double-alternation schedule (i.e., MMSSMMSS, etc., where M is methamphetamine; S, saline).

c-Fos Immunohistochemistry

A total of 44 of rats were prepared. Four groups were prepared in order to investigate the neural circuitry underlying the discriminative stimulus effects of methamphetamine: (1) naive rats that were subjected to neither food restriction nor drug discrimination training, (2) control rats that were maintained on the FR-20 schedule of food reinforcement without drug discrimination training, and (3) saline- and (4) methamphetamine-injected trained rats that had met the criteria for learning methamphetamine discrimination. Control rats were subjected to the FR-20 schedule of food reinforcement, while saline- and methamphetamine-injected rats were subjected to the test session of methamphetamine discrimination. Accordingly, the three groups of animals except naive rats obtained the same number (20 pellets) of food reinforcement by almost equal numbers of lever pressing. The saline- and methamphetamine-injected rats had the same drug history during the drug discrim-

ination training sessions, but received different drug treatment (methamphetamine vs. saline) on the test day. Rats were killed 2 h after the drug discrimination test for c-Fos immunohistochemistry. An additional four groups of rats were prepared in order to examine the effects of acute and chronic intermittent methamphetamine treatment on c-Fos expression—two groups of rats received chronic intermittent methamphetamine treatment at a dose of 0.5 mg/kg under a double-alternation schedule (i.e., MMSSMMSS etc., where M is methamphetamine; S, saline) without discrimination training. On the final day, half of these rats were challenged by methamphetamine 0.5 mg/kg or saline, respectively, and killed 2 h after the challenge. Another two groups of rats were injected with saline daily to examine the acute effects of methamphetamine. On the final day, half of the rats were injected with methamphetamine 0.5 mg/kg, while the others were treated with saline and killed 2 h after the treatment. Three separate groups of rats were treated with single saline or methamphetamine (0.5 mg/kg or 2 mg/kg, s.c.) to examine the dose-dependent effects of acute methamphetamine treatment on c-Fos expression, and killed 2 h after the treatment.

The c-Fos immunohistochemical procedure was the same as previously described methods.[8] Quantitative analysis of c-Fos immunohistochemistry was conducted by a computer-assisted image analysis system (C. Imaging Systems; Compix Inc., Mars, PA) as described previously.[9]

RESULTS

c-Fos Expression Associated with the Discriminative Stimulus Effects of Methamphetamine

c-Fos expression were observed in all examined areas of the brain. Significant differences in c-Fos expression among the four groups of rats were observed in four brain areas including the cingulate cortex, the core and shell of NAc, and VTA (TABLE 1). There was a marked difference in c-Fos expression between control and trained groups in the core and shell of the NAc, and the VTA. Moreover, the number of c-Fos–positive cells was significantly smaller in the NAc core of methamphetamine-injected trained rats than in that of saline-injected trained rats, whereas it was increased in the VTA of methamphetamine-injected trained rats compared with saline-injected trained rats. No alteration in c-Fos expression was observed in other brain areas examined.

Effects of Acute and Chronic Intermittent Methamphetamine Treatment on c-Fos Expression

No significant alteration of c-Fos expression was observed in rats treated with chronic intermittent methamphetamine (0.5 mg/kg), whereas the number of c-Fos–positive cells in the NAc core of acute methamphetamine-treated rats was significantly higher than that of saline-treated rats.

Single administration with methamphetamine (0.5 and 2 mg/kg) produced a dose-dependent and significant increase in the number of c-Fos–positive cells in the NAc core, while methamphetamine 2 mg/kg, not 0.5 mg/kg, produced a significant increase in the NAc shell and the VTA.

TABLE 1. c-Fos expression in the brain subregions of rats trained for methamphetamine discrimination

Brain area	Naive ($n = 3$)	Control ($n = 4$)	Saline ($n = 4$)	Methamphetamine ($n = 4$)
Cerebral cortex				
Cingulate	72.3 ± 2.8*	102.4 ± 5.9	110.0 ± 3.4	115.7 ± 6.9
Motor	54.1 ± 3.2	66.7 ± 4.5	68.3 ± 3.4	68.0 ± 1.9
Somotosensory	38.8 ± 3.5	48.7 ± 1.8	51.4 ± 1.2	49.2 ± 2.7
Nucleus accumbens				
Core	36.8 ± 1.2	50.6 ± 4.3	21.9 ± 10.9***	90.3 ± 6.7*,#
Shell	30.6 ± 3.0	33.6 ± 5.8	86.0 ± 7.9***	71.2 ± 4.3**
Ventral tegmental area				
	16.7 ± 1.3	21.0 ± 4.7	57.4 ± 7.1**	90.4 ± 5.9***,##

Rats were trained to discriminate methamphetamine (0.5 mg/kg) from saline under the two-lever FR-20 schedule of food reinforcement. The trained rats were subjected to the drug discrimination test after either saline or methamphetamine (0.5 mg/kg) treatment. Control rats were maintained under the FR-20 schedule of food reinforcement without drug discrimination training. Naive rats were subjected to neither food restriction nor the methamphetamine discrimination training. c-Fos expression in each area is indicated as the number of c-Fos–positive cells per mm^2. Each value represents the mean ± SE. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ versus control. $^{\#}P < .05$, $^{\#\#}P < .01$ versus saline.

DISCUSSION

It is well known that DA plays a major role in the discriminative stimulus effects of methamphetamine.[1] In the discrimination test in rats, DA uptake inhibitors and D1 or D2 receptor agonists substituted for methamphetamine, whereas their antagonists completely blocked the discriminative stimulus effects.[1] In the present study, we demonstrated immunohistochemically that development of the ability to discriminate methamphetamine from saline in rats is associated with increases in c-Fos expression only in the VTA and NAc. This activation is unlikely due to lever-press behavior or food reinforcement because c-Fos expression in these brain areas did not increase in the control group maintained on the FR-20 schedule of food reinforcement (TABLE 1). Further, it is also unlikely that the changes are due to chronic intermittent methamphetamine treatment because such treatment without discrimination training did not increase c-Fos expression in the VTA and NAc.

Dopaminergic projections from the VTA to the NAc are involved in investigatory behavior evoked by novel stimuli and the reinforcement of adaptive investigatory approaches evoked by naturally occurring rewards and by addictive drugs.[10–12] It has also been suggested that the NAc core is more important than the NAc shell in response-reinforcement learning[13] and in behavioral response to motivationally significant stiumuli in general.[14,15] Accordingly, our findings suggest that the development of methamphetamine discrimination is associated with a selective activation of the VTA-NAc, probably the dopaminergic neuronal system. Compared to the shell region, c-Fos expression in the NAc core is more extensive, indicating that the

core region of the NAc is more important for the discriminative stimulus effects of methamphetamine.

Interestingly, both saline and methamphetamine treatment in rats that fulfilled the criteria of the discrimination led to an increase in c-Fos expression in the VTA and the core and shell of the NAc. Therefore, it is suggested that once the animals acquired the ability to discriminate methamphetamine from saline, these brain areas were selectively activated even after saline challenge. The mechanism underlying this activation is unclear, but it will provide an insight into the mechanisms for the discriminative stimulus effects of methamphetamine.

In conclusion, our findings suggest that the VTA and the NAc as possible neuronal substrates play a role in the discriminative stimulus effects of methamphetamine. To prove the hypothesis, further related experiments will be necessary.

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REFERENCES

1. MUNZAR, P. & S.R. GOLDBERG. 2000. Dopaminergic involvement in the discriminative-stimulus effects of methamphetamine in rats. Psychopharmacology **148:** 209–216.
2. KAMIEN, J.B. *et al.* 1993. Drug discrimination by humans compared to nonhumans: Current status and future directions. Psychopharmacology. **111:** 259–270.
3. MORI, A. *et al.* 2001. Phencyclidine-induced discriminative stimulus is mediated via phencyclidine binding sites on the N-methyl-D-aspartate receptor-ion channel complex, not by sigma1 receptors. Behav. Brain Res. **119:** 33–40.
4. MORGAN, J.I. & T. CURREN. 1991. Stimulus-transcription coupling in the nervous system: involvement of inducible proto-oncogenes fos and jun. Annu. Rev. Neurosci. **14:** 421–451.
5. UMINO, A. *et al.* 1995. Methamphetamine-induced nuclear c-Fos in rat brain regions. Neurochem. Int. **26:** 85-90.
6. NAMIMA, M. *et al.* 1998. Lithium inhibits the reverse tolerance and c-Fos expression induced by methamphetamine in mice. Brain Res. **782:** 83–90.
7. COLE, R.L. *et al.* 1995. Neuronal adaptation to amphetamine and dopamine molecular mechanisms of prodynorphin gene regulation in rat striatum. Neuron **14:** 813–823.
8. HE, J. *et al.* 2002. A role of Fos expression in the CA3 region of the hippocampus in spatial memory formation in rats. Neuropsychopharmacology **26:** 259–268.
9. YAMADA, K. *et al.* 1996. Reduction in the number of NADPH-diaphorase-positive cells in the cerebral cortex and striatum in aged rats. Neurosci. Res. **24:** 393–402.
10. MUNZAR, P. *et al.* 1999. Effects of dopamine and serotonin-releasing agents on methamphetamine discrimination and self-administration in rats. Psychopharmacology **141:** 287–296.
11. SCHULTZ, W. 1998. Predictive reward signal of dopamine neurons. J. Neurophysiol. **80:**1–27.
12. HOLLERMAN, J.R. & W. SCHULTZ. 1998. Dopamine neurons report an error in the temporal prediction of reward during learning. Nat. Neurosci. **1:** 304–309.

13. KELLY, A.E. *et al.* 1997. Response-reinforcement learning is dependent on N-methyl-D-aspartate receptor activation in the nucleus accumbens core. Proc. Natl. Acad. Sci. USA **94:** 12174–12179.
14. BASSAREO, V. & G. DI CHARA. 1999. Differential responsiveness of dopamine transmission to food-stimuli in nucleus accumbens shell/core compartments. Neuroscience **89:** 637–641.
15. PARKINSON, J.A. *et al.* 1999. Dissociation in effects of lesions of the nucleus accumbens core and shell in appetitive Pavlovian approach behavior and the potentiation of conditioned reinforcement and locomotor activity by D-amphetamine. J. Neurosci. **19:** 2401–2411.

Nefiracetam Attenuates Methamphetamine-Induced Discriminative Stimulus Effects in Rats

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ABSTRACT: **Nefiracetam has been reported to increase intracellular cyclic AMP levels and enhance calcium channel current. Since the cAMP cascade is involved in the development of drug dependence, we investigated whether nefiracetam attenuates the methamphetamine (MAP)-induced discriminative responses in rats. Nefiracetam (50 mg/kg) inhibited MAP-induced discriminative responses. Furthermore, rolipram, raclopride, and L-745870, all of which can enhance cAMP activity, disrupted MAP-paired lever press of rats. Nifedipine and neomycin, which are blockers of voltage-sensitive calcium channels (VSCCs), decreased MAP-induced discriminative responses. However, pretreatment of these VSCC blockers failed to affect the inhibitory effects of nefiracetam on MAP-induced discriminative responses. Our findings suggested that nefiracetam inhibits MAP-induced discriminative responses, which may be at least partly associated with the changes in intracellular cAMP levels.**

KEYWORDS: **drug discrimination; methamphetamine (MAP); nefiracetam; voltage-sensitive calcium channel blockers; cAMP pathway; rolipram; D_2-like receptor antagonists; rats**

INTRODUCTION

Previous studies have reported that nefiracetam ameliorates the impairment of learning and memory in animal models of aging, Alzheimer's disease, and head trauma.[1] It has been suggested that the pharmacological effects of nefiracetam are mediated by the elevation of intracellular cAMP levels and enhancement of the calcium ion channel current.[2,3] Nefiracetam also attenuates the development of morphine dependence and tolerance by increasing the cAMP content in the cerebral cortex of mouse brains.[4]

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Methamphetamine (MAP) induces an efflux of dopamine, which activates D_2-like receptors that are coupled with Gi/Go proteins. Gi/Go proteins trigger signal transduction pathways, such as the cAMP-PKA-CREB cascade, which are accompanied by neuronal adaptations and synaptic plasticity. It is suggested that the cAMP-PKA-CREB cascade is important in drug addiction.[5,6]

Nefiracetam, a compound originally developed as a cognitive enhancer, increases intracellular cAMP levels and enhances calcium channel current in brains. Thus, this compound may have some therapeutic effects against drug dependence. Previous reports have shown that nefiracetam and other compounds that modulate cAMP activity attenuate locomotor sensitization, withdrawal symptoms, conditioned place preference, and self-administration of drugs of abuse.[4,7,8] Discriminative stimulus effects of psychostimulants are most consistently associated with their subjective effects in the human. Drug discrimination procedures in rats have been used to clarify the mechanism underlying the unique properties of drugs of abuse. In the present study, we investigated the effects of nefiracetam on MAP-induced discriminative stimulus effects in rats.

MATERIALS AND METHODS

Subjects

Fourteen male Sprague-Dawley rats (Nihon SLC Co., Ltd., Shizuoka, Japan), initially 8 weeks old and weighing 260 ± 10 g, were used in this study. All animal care and use was in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Procedures for MAP-Induced Discrimination Training and Drug Testing

The procedures were previously described in detail.[6] Lever selection was examined after the administration of MAP at the various doses (0.1–0.5 mg/kg) or the coadministration of MAP (50 mg/kg) and the tested drugs. MAP was injected 10 min before the sessions. The rats were administered with nefiracetam, rolipram, and raclopride 30 min before MAP treatment, and with L-745870 and the VSCC blockers 40 min before MAP treatment.

Drugs

The following compounds were used in this study: nefiracetam (Daiichi Pharmaceutical, Tokyo, Japan); MAP hydrochloride (Dainippon Pharmaceutical, Japan); rolipram, L-745870 hydrochloride, and raclopride tartrate salt (Sigma Chemicals Co., St. Louis, MO); flunarizine 2HCl (RBI Research Biomedicals International, Natick, MA); and neomycin sulfate and nifedipine (ICN Biomedicals Co., Aurora, OH). MAP, nefiracetam, raclopride, L-745870, and neomycin were dissolved in saline immediately before use. Rolipram was first dissolved in dimethyl sulfoxide (DMSO) and diluted with saline immediately before use. Flunarizine and nifedipine were initially dissolved in polyethylene glycol and then diluted with saline immediately before use.

TABLE 1. Effects of voltage-sensitive calcium channel (VSCC) blockers, nefiracetam, and other cAMP-related compounds on MAP-induced discriminative responses in rats (n = 11–14)

Tested Drug	Type	Effect (P Value)	Dose
Nefiracetam	—	inhibition ($P < .01$)	50 mg/kg p.o.
Nifedipine	L-type VSCC blocker	inhibition ($P < .05$–$.001$)	0.25–1.0 mg/kg i.p.
Neomycin	VSCC blocker	inhibition ($P < .05$)	2.0–10.0 mg/kg i.p.
Flunarizine	nonselective VSCC blocker	tendency to inhibit	0.2–2.0 mg/kg i.p.
Any of above VSCC blockers + nefiracetam	—	no antagonistic or synergistic effect	
Rolipram	phosphodiesterase inhibitor	inhibition ($P < .05$–$.01$)	0.05–0.3 mg/kg i.p.
Raclopride	D_2 antagonist	inhibition (($P < .001$)	0.3–1.0 mg/kg i.p.
L-745870	D_4 antagonist	inhibition ($P < .001$)	0.025–0.5 mg/kg i.p.

RESULTS

Rats discriminated MAP from saline after 60 ± 10 training sessions. MAP induced drug-paired responses in a dose-dependent manner (0.1–0.5 mg/kg, s.c.), while the response rate was constant at all the doses examined. When 0.2 mg/kg of MAP was used, MAP-paired lever response was reached at 90%. To increase the sensitivity of MAP-induced discriminative response to the tested compounds, the MAP dose used for all the testing sessions was fixed to 0.2 mg/kg (s.c); whereas 0.5 mg/kg of MAP (s.c) was used for all the training sessions.

The results are summarized in TABLE 1. Nefiracetam and other cAMP-related compounds including rolipram, raclopride, and L-745870 inhibited MAP-induced discriminative stimulus effects in rats. Nifedipine and neomycin inhibited the discriminative stimulus effects of MAP, but flunarizine had no significant effects. The inhibitory effects of nefiracetam on MAP-induced responses were not affected by coadministration of VSCC antagonists.

DISCUSSION

Previous studies have demonstrated that nefiracetam has two pathways of downstream signal transduction, including cAMP/PKA and PKC, which are involved in the ameliorating effects of nefiracetam on dysfunction of learning and memory.[3] It has also been reported that rolipram, which inhibits phosphodiesterase IV and maintains a higher intracellular concentration of cAMP, attenuates the behavioral sensitization induced by MAP and the initiation of cocaine self-administration.[7,8] Our present findings demonstrate that nefiracetam is as effective as rolipram in inhibiting

discriminative stimulus effects induced by MAP in rats. Similarly, inhibition by D_2-like receptor antagonists on MAP-induced discrimination in our study further supported the hypothesis that cAMP activity was negatively modulated by long-term intermittent MAP injection in discrimination procedures.

On the other hand, it has been strongly proposed that VSCCs are implicated in the ameliorating effects of nefiracetam in the various amnesia animal models. Nefiracetam triggers influx of calcium ion and then enhances the transmitter release, the mechanism being attributable to the improvement of impaired learning and memory.[2,3] However, VSCC blockers showed the inhibitory effects on MAP-induced discriminative responses in our study. Furthermore, coadministration of nefiracetam with VSCC blockers did not show any antagonistic effect. These results suggest that activation of VSCCs induced by nefiracetam may have little or no role in MAP-induced discriminative stimulus effects. Consistently, several studies have demonstrated that VSCC blockers also modulate psychostimulant-induced self-administration, conditioned place preference, and locomotor activity in a similar manner.[9,10]

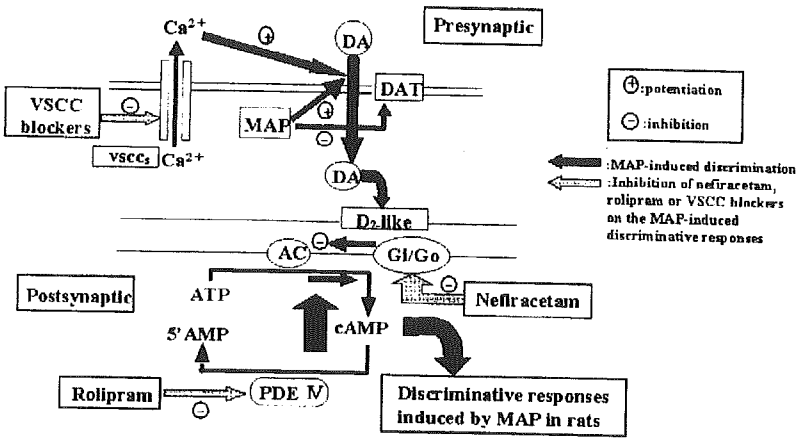

FIGURE 1. Hypothesis for the inhibitory effects of nefiracetam on MAP-induced discrimination in rats. VSCCs are involved in the dopamine release induced by long-term intermittent MAP injection. The VSCC blockers show the inhibitory effects via attenuating influx of calcium ions and then reduce intermittent MAP-induced dopamine release. Nefiracetam enhances intracellular cAMP activity via coupling to Gi/Go proteins and increasing adenylyl cyclase activity, by which nefiracetam shows the inhibitory effects on MAP-induced discriminative responses. Similarly, rolipram, D_2 and D_4 antagonists decrease the activity of cAMP, by which they exert inhibitory effects on discriminative stimuli in rats. ABBREVIATIONS: MAP, methamphetamine; VSCCs, voltage-sensitive calcium ion channels; DA, dopamine; DAT, dopamine transporter; D_2, dopamine receptor 2; AC, adenylyl cyclase; Gi, Gi protein; Go, Go protein; cAMP, adenosine 3′,5′-cyclic monophosphate; ATP, adenosine triphosphate; 5′AMP, 5′-adenosine monophosphate; PDEIV, phosphodiesterase IV.

In conclusion, the cAMP system may play an important role in MAP-induced discriminative stimulus effects. Nefiracetam may exhibit the inhibitory effects on MAP-induced discriminative stimulus effects by increasing intracellular cAMP levels. The suggested hypothesis is illustrated in FIGURE 1. Further experiments are under way.

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REFERENCES

1. NABESHIMA, T. 1994. Ameliorating effects of nefiracetam (DM-9384) on brain dysfunction. Drugs of Today **30:** 357–379.
2. WATABE, S. & M. YOSHII. 1993. Ion channels and cognitive enhancers. Jpn. J. Neuropsychopharmacol. **15:** 249–257.
3. NISHIZAKI, T., T. MATSUOKA, T. NOMURA, *et al.* 1998. Nefiracetam modulates acetylcholine receptor currents via two different signal transduction pathways. Mol. Pharmacol. **53:** 1–5.
4. ITOH, A., T. SHIOTANI, S. NAKAYAMA, *et al.* 2000. Attenuation of the development of morphine dependence/tolerance by nefiracetam: involvement of adenosine 3′:5′-cyclic monophosphate system. Behav. Brain Res. **115:** 65–74.
5. SELF, D.W., L.M. GENOVA, B.T. HOPE, *et al.* 1998. Involvement of cAMP-dependent protein kinase in the nucleus accombens in cocaine self-administration and relapse of cocaine-seeking behavior. J. Neurosci. **18:** 1848–1859.
6. MORI, A., Y. NODA, T. MAMIYA, *et al.* 2001. Phencyclindine-induced discriminative stimulus is mediated via phencyclidine binding sites on the *N*-methyl-D-aspartate receptor-ion channel complex, not by sigma 1 receptors. Behav. Brain Res. **119:** 33–40.
7. IYO, M., Y. BI, K. HASHIMOTO, *et al.* 1996. Prevention of methamphetamine-induced behavioral sensitization in rats by a cyclic AMP phophodiesterase inhibitor, rolipram. Eur. J. Pharmacol. **312:** 163–170.
8. KNAPP, C.M., M.M. FOYE, D.A. CIRAULO & C. KORNETSKY. 1998. The type IV phosphodiesterase inhibitors, Ro 20-1724 and rolipram, block the initiation of cocaine self-administration. Pharmacol. Biochem. Behav. **62:** 151–158.
9. ROSENWEIG-LIPSON, S. & J.E. BARRETT. 1995. Modification of the behavioral effects of (±)BAY 8644, cocaine and d-amphetamine by L-type calcium channel blockers in squirrel monkeys. J. Pharmacol. Exp. Ther. **274:** 842–851.
10. MARTELLOTTA, M.C., A. KUZMIN, P. MUGLIA, *et al.* 1994. Effects of the calcium antagonist isadipine on cocaine intravenous self-administration in rats. Psychopharmacology **113:** 378–380.

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Oxidative Stress in Mitochondria

Decision to Survival and Death of Neurons in Neurodegenerative Disorders

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Abstract

In mitochondria, oxidative phosphorylation and enzymatic oxidation of biogenic amines by monoamine oxidase produce reactive oxygen and nitrogen species, which are proposed to cause neuronal cell death in neurodegenerative disorders, including Parkinson's and Alzheimer's disease. In these disorders, mitochondrial dysfunction, increased oxidative stress, and accumulation of oxidation-modified proteins are involved in cell death in definite neurons. The interactions among these factors were studied by use of a peroxynitrite-generating agent, *N*-morpholino sydnonimine (SIN-1) and an inhibitor of complex I, rotenone, in human dopaminergic SH-SY5Y cells. In control cells, peroxynitrite nitrated proteins, especially the subunits of mitochondrial complex I, as 3-nitrotyrosine, suggesting that neurons are exposed to constant oxidative stress even under physiological conditions. SIN-1 and an inhibitor of proteasome, carbobenzoxy-*L*-isoleucyl-γ-*t*-butyl-L-alanyl-L-leucinal (PSI), increased markedly the levels of nitrated proteins with concomitant induction of apoptosis in the cells. Rotenone induced mitochondrial dysfunction and accumulation and aggregation of proteins modified with acrolein, an aldehyde product of lipid peroxidation in the cells. At the same time, the activity the of 20S β-subunit of proteasome was reduced significantly, which degrades oxidative-modified protein. The mechanism was proved to be the result of the modification of the 20S β-subunit with acrolein and to the binding of other acrolein-modified proteins to the 20S β-subunit.

Increased oxidative stress caused by SIN-1 treatment induced a decline in the mitochondrial membrane potential, $\Delta\Psi m$, and activated mitochondrial apoptotic signaling and induced cell death in SH-SY5Y cells. As another pathway, p38 mitogen-activated protein (MAP) kinase and exracellular signal-regulated kinase (ERK)-mediated apoptosis induced by SIN-1. On the other hand, a series of neuroprotective propargylamine derivatives, including rasagiline [*N*-propargyl-1(*R*)aminoindan] and (–)deprenyl, intervened in the activation of apoptotic cascade by reactive oxygen species–reactive nitrogen species in mitochondria through stabilization of the membrane

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potential, $\Delta\Psi m$. In addition, rasagiline induced antiapoptotic Bcl-2 and glial cell line-derived neurotrophic factor (GDNF) in SH-SY5Y cells, which was mediated by the ERK–nuclear factor (NF)-κB pathway. These results are discussed in relation to the interaction of oxidative stress and mitochondria in the regulation of neuronal death and survival in neurodegenerative diseases.

Index Entries: Oxidative stress; mitochondria complex I; proteasome; 3-nitrotyrosine; acrolein; Parkinson's disease; apoptosis; rasagiline; transcription factors.

Oxidative-Modified Protein as the Marker of Oxidative Stress

Oxidative stress has been proposed as one of the major causes inducing neuronal death in aging and age-associated disorders *(1)*, and mitochondria produce most of reactive oxygen and nitrogen species (ROS and RNS, respectively) in the cells. The superoxide anion radical is generated by oxidative phosphorylation in mitochondria and reacts with nitric oxide (NO) to produce peroxynitrite ($ONOO^-$), one of the most potent radicals. On the other hand, oxidation of biogenic amines by monoamine oxidase in the mitochondrial outer membrane generates hydrogen peroxide. Mitochondria are now considered to play a pivotal role in apoptosis *(2)*, which is the common death type of neurons in Parkinson's (PD) and Alzheimer's diseases (AD) *(3)*. The role of mitochondria in the process of apoptotic commitment is recognized, and impairment of energy charge and redox, permeability transition (PT), disruption of membrane potential ($\Delta\Psi m$), and release of cytochrome-*c* are observed prior to fragmentation of nuclear DNA, a hallmark of apoptotic morphological features.

Neurodegenerative disorders are characterized by a decline of specified neurons in selected brain regions, associated with protein deposits specific for each disease. In PD, dopamine neurons in the substantia nigra degenerate progressively with formation of Lewy bodies (LBs). The pathogenesis of PD remains as an enigma, but PD is considered a consequence of various genetic and environmental interactions. The vulnerability of the neurons might be the result of increased generation of ROS and RNS, reduced antioxidant capacity, high contents of iron and dopamine, and mitochondrial dysfunction in nigral dopamine neurons. ROS and RNS generated in mitochondria modify bioactive molecules, such as lipids, proteins, DNA, and carbohydrates, either directly or indirectly with peroxidation products of lipid or carbohydrates. 4-Hydroxynonenal (4-HNE) and acrolein, aldehyde products of lipid peroxidation, are cytotoxic, and aldehyde-modified proteins were increased in dopamine neurons of the nigro-striatum in PD *(4)*, neurofibrillary tangles in AD *(5)*, and the spinal cord in amyotrophic lateral sclerosis (ALS) *(6)*. Figure 1 shows the accumulation of 4-HNE-modified protein in the substantia nigra of parkinsonian brain. Dopamine neurons containing neuromelanin were selectively modified with 4-HNE more markedly in the PD brain than those in the control or in cells other than dopamine neurons. This suggests the essential role of dopamine in increased oxidative stress.

One of the most active RNS, $ONOO^-$, nitrates sulfhydryl and hydroxyl residues in cysteine, methionine, phenylalanine, and tyrosine, and the modification inactivates the membrane function and key enzymes *(7)*. 3-Nitrotyrosine (3-NT) is synthesized by nitration of tyrosine residues in protein and a marker for the oxidative stress induced by $ONOO^-$ in vivo *(8)*. An increase in 3-NT-containing protein (3-NT protein) was observed in atherosclerosis *(9)*, ALS *(6)*, AD *(10)*, and PD *(11)*. Another oxidation product of tyrosine is dityrosine, which is produced from free and protein-bound tyrosine in the presence of hydrogen peroxide and myeroperoxidase and was detected in atherosclerotic plaques and lipofuscin pigments in the aged human brain *(12)*.

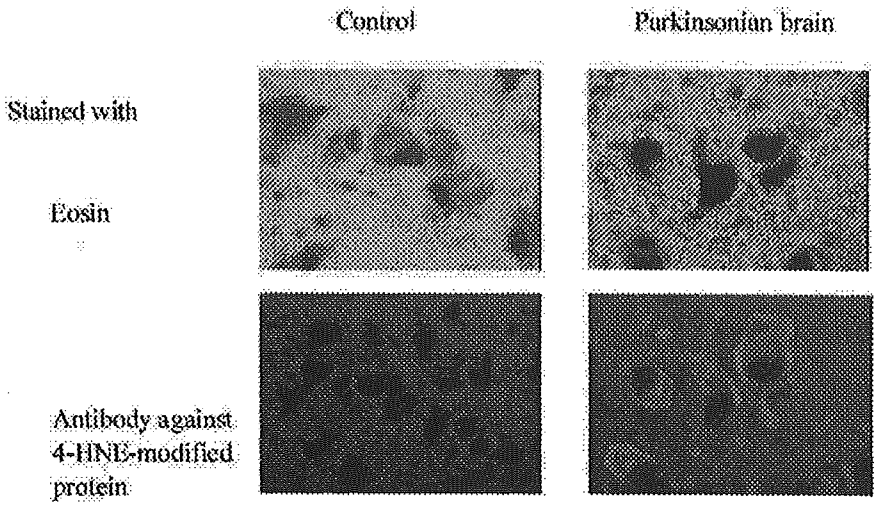

Fig. 1. 4-HNE-modified protein in the substantia nigra of the parkinsonian brain. In dopamine neurons containing neuromelanin, proteins stained with the anti-4-HNE antibody increased in the parkinsonian brain, but were not detected in the age-matched control.

Oxidative modification produces aggregated and crosslinked proteins, which are resistant to proteolytic degeneration and difficult to removed from cells. Accumulation of modified proteins might impact on a variety of cellular functions by changing the enzymatic, regulatory, and transporting potencies of specific proteins, in addition to occupying space in the limited cellular volume. The level of oxidized proteins might reflect the balance between the generation of ROS-RNS and degradation of modified protein, in which the ubiquitin–proteosome system plays a key role *(13)*, as discussed in the following sections.

Mitochondrial Complex I Subunits Are Major Targets of $ONOO^-$

In the brain, NO has been thought to be produced in microglia and astrocytes and transported to neurons, where it reacts with superoxide, yielding $ONOO^-$. However, NO is synthesized also *in situ* in the neurons and functions as a neuromodulator *(14)*. Using an antibody against 3-NT protein *(15)*, nitrated proteins were detected in control brains. In brains from AD and PD patients, the same proteins stained with anti-3NT antibody as in control increased markedly, indicating that $ONOO^-$ preferentially modified selective proteins in neuronal cells under physiological and pathological conditions. Figure 2 shows the presence of 3-NT protein in control SH-SY5Y cells almost at the same levels as in the cells treated with $ONOO^-$-generating *N*-morpholino sydnomine (SIN-1), indicating that the cells are exposed to constant oxidative stress. However, the control cells are intact in growth and proliferation, and under physiological conditions, an active mechanism functions to eliminate modified protein from the cells and to protect cellar function in neuronal cells.

As shown in Figure 3, the presence of nitrated protein in control cells was confirmed further by the Western blot analysis of the subcellular fractions of SH-SY5Y cells. In mitochondrial fraction, 3-NT protein was detected in the subunits of complex I stained with that against mitochondria complex I, II, III, and IV,

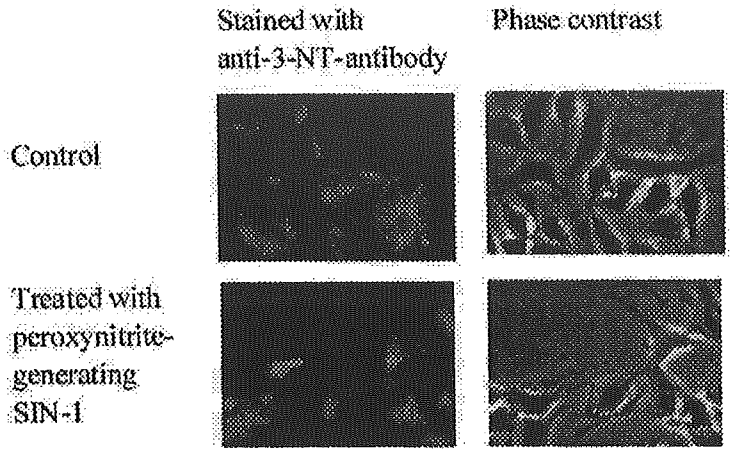

Fig. 2. 3-NT protein detected in control SH-SY5Y cells and cells treated with $ONOO^-$-generating SIN-1. In cytoplasm, 3-NT proteins were detected by staining with anti-3-NT antibody *(15)*. Note that the 3-NT protein was detected also in the control.

respectively *(16)*, (Fig. 3). These results suggest that complex I subunits are nitrated preferentially and the modification might contribute to mitochondrial dysfunction observed in the nigro-striatum of the parkinsonian brain *(17)*.

$ONOO^-$-generating SIN-1 induced apoptosis in SH-SY5Y cells *(14,18–20)* and inhibited ATP synthesis in mitochondria. The inhibition might be the result of the binding of NO and $ONOO^-$ to cytochrome oxidase or inactivation of complexes II and III and ATPase *(21)*. However, SIN-1 treatment did not increase 3-NT protein as markedly. These seemingly contradicting results indicate again that the levels of oxidative stress and oxidative-modified protein are regulated by the degradation rate rather than the production of ROS-RNS.

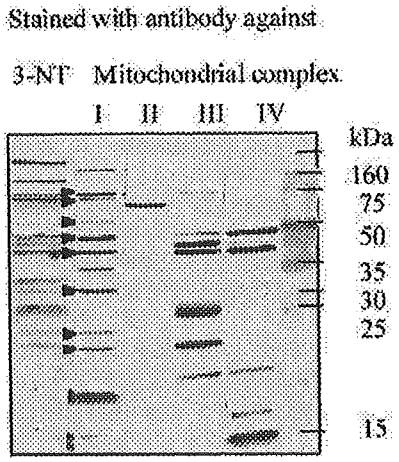

Fig. 3. 3-NT proteins in the subunits of mitochondrial complex I. Mitochondria were prepared from SH-SY5Y cells and subjected to Western blot analysis using antibody against 3-NT protein or mitochondrial complex I, II, III, and IV, respectively.

Proteasome Plays a Key Role in Accumulation of Oxidized Proteins

Insoluble protein aggregates, such as LBs in PD and senile plaques composed of β-amyloid in AD, are hallmarks of neurodegeneration. However, it remains to be clarified whether protein aggregates cause neuronal cell death directly or are the results of deteriorated cellular homeostasis in dying neurons. Protein aggregation is considered a manifestation of

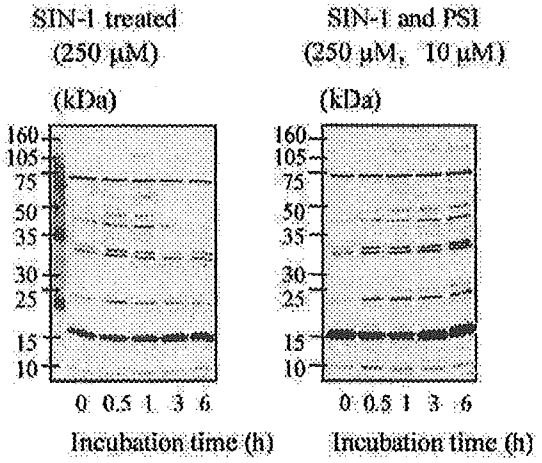

Fig. 4. Effects of $ONOO^-$-generating SIN-1 and a proteasome inhibitor, PSI, on 3-NT levels in SH-SY5Y cells. After incubated for 0.5, 1.3, and 6 h, the cells were subjected to the immunoblotting analysis with anti-3-NT antibody. PSI increased 3-NT in the same proteins as in control and SIN-1-alone-treated cells.

disturbed cellular protein-folding homeostasis maintained by the ubiquitin–proteasome system. Ubiquitinated proteins and proteasome subunits *(22,23)*, in addition to α-synuclein, Parkin and ubiquitin C-terminal hydrolase-L1 (UCH-1) *(24–27)*, are the components of LBs and are sometimes modified with ROS-RNS.

To clarify the interactions among oxidative stress, dysfunction of the proteasome system, and formation of the inclusion body, the effects of a proteasome inhibitor, carbobenzoxy-L-isoleucyl-γ-*t*-butyl-L-alanyl-L-leucinal (PSI) were examined on the aggregation of oxidative-modified proteins and the cell vulnerability *(20,28)*. PSI increased the amount of 3-NT proteins in SH-SY5Y cells, but the number of 3-NT proteins was almost the same as in the control (Fig. 4). At the same time, the number of apoptotic cells increased significantly by PSI, but that of necrotic cells was not (Fig. 5). These results indicate that inhibition of proteasome activity might play a key role in the accumulation of oxidative-modified protein and the induction of cell death.

Rotenone, a Complex I Inhibitor, Inactivates Proteasome by Oxidative Modification

The activity and protein of complex I of the mitochondrial electron transfer chains reduced in the nigro-striatum of patients with PD *(17)*. The systemic administration of rotenone, an inhibitor of complex I, induced parkinsonism in rodents, and fibrillar cytoplasmic inclusions containing ubiquitin and α-synuclein were detected in dopamine neurons *(29)*. The effects of mitochondrial dysfunction on the proteasome system were studied by use of rotenone *(28)*. Apoptosis was induced in the cells after 4–5 d treatment with rotenone. The oxidative modification of proteins was followed by the use of an antibody against acrolein-modified protein *(30)*. As shown in Fig. 6, the levels of acrolein-modified protein increased markedly by the rotenone treatment. In the lysate of rotenone-treated cells, aggregation of acrolein-modified protein with high molecular mass was also detected.

The ubiquitin–proteasome system is a major site for removal of damaged or modified proteins and also regulatory proteins controlling cell cycle and signal transduction. In the nigro-striatum of the parkinsonian brain, the decreased activity of proteasome was reported, suggesting its involvement in the pathological features *(31)*. Oxidized protein is preferentially degraded in vitro by 20S proteasome in an ATP-independent way. Binding of the regulatory subunit, 19S complex (ATPase, PA700), to both ends of the 20S cylinder produces 26S proteasome with higher catalytic activity than 20S proteasome. 26S proteasome degrades polyubiquitinated proteins and ornithine decarboxylase in an ATP-dependent process.

In SH-SY5Y cells, the rotenone treatment increased ROS-RNS levels detected with 2,7-dichlorofluorescence diacetate only transitionally and slightly, whereas the oxidized protein levels increased progressively, suggesting that protein might function as "the second scavenger" of ROS-RNS and that degradation of modified proteins might be impaired. The