Neurotoxic morphological changes induced in the medial prefrontal cortex of rats behaviorally sensitized to methamphetamine*

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Summary. The present study examined whether the development in rats of behavioral sensitization to methamphetamine (MAP) is related to the development of neurotoxic morphological changes presumably induced in the medial prefrontal cortex (MFC). Male rats were intraperitoneally injected with MAP (5 mg/kg) once a day for 12 days (day 1–day 12), and then the drug was withdrawn for 7–42 days (WD7–WD42). The MAP- treatment caused hypersensitivity of a successive head-movement stereotypy, which reached a basic plateau level on day 4, and rose successively to a higher level by day 12. Morphological changes were histochemically and morphometrically examined in the MFC. In the strata covering layers II and III, the densities of tyrosine hydroxylase (TH)-immunoreactive axons decreased on a daily basis to 50% of the control on day 4 and then to 40% on days 6 and 12. The densities of dopamine- β -hydroxylase (DBH)-immunoreactive axons did not change during the injection period. A few TUNEL-positive cells were observed in a unit area (0.25 mm²) covering layers II–V on day 6 and they increased to 19 and 16 on day 12 and WD7, respectively. These observations demonstrate a role for the neurotoxic changes in the MFC in the processes of behavioral sensitization of a stereotypy to a low dose of MAP.

Introduction

Chronic intoxication to amphetamine (AMP) or methamphetamine (MAP) may induce schizophrenia-like symptoms in humans, such as paranoid states that relapse with single doses of the drug after abstinence periods (Utena, 1974; Machiyama, 1992; Sato, 1992; Angrist, 1994; Kratochvil *et al.*, 1996). Relapse-liability in AMP- or MAP-psychosis has been suggested to be mediated by plastic changes induced in brain tissues following intake of the drug (Ridley *et al.*, 1988). Acute toxic effects of AMP or MAP, usually resulting from overdose, bring about central stimulation and psychotic symptoms such as confusion, aggressiveness, delirium, paranoid hallucinations, and panic states (Angrist, 1994; Hoffman and Lefkowitz, 1996).

In rats or mice, the subchronic administration of lower doses (1–8 mg/kg) of AMP or MAP progressively enhances the potency of the drug to induce behaviors such as hyperactivity and stereotypy (Robinson and Becker, 1986). The developed hypersensitivity to AMP or MAP, called behavioral sensitization or reverse tolerance, persists for a longer duration, and relapses with a challenge with single lower doses of the drug after periods of withdrawal (Robinson and Becker, 1986; Robinson and Berridge, 1993; Segal and Kuczenski, 1994; Valadez and Schenk, 1994). Behavioral sensitization has been pharmacologically and biochemically investigated as a suitable model for relapse-liability of AMP- or MAP-psychosis. It has been shown to be associated with enduring changes in the dopaminergic signaling pathways in various brain regions, including the ventral tegmental area and nucleus accumbens (Kalivas and Stewart, 1991; Lillrank *et al.*, 1991; Paulson and Robinson, 1991; Steketee, 1994, 2003; Tolliver *et al.*, 1996; Karler *et al.*, 1997). Reduction in dopaminergic transmission in the medial prefrontal cortex (MFC) has been suggested to

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enhance glutamatergic output to the nucleus accumbens facilitating the development of behavioral sensitization (see Steketee, 2003). Gross morphological changes have not been reported in relapse-liability animals models (Robinson and Becker, 1986). However, a neurocytological study has implied correlations between behavioral sensitization and fine structural synaptic changes by demonstrating an increase in the densities of dendritic spines and in the numbers of branched spines in the MFC and nucleus accumbens in rats treated subchronically with lower doses of AMP (Robinson and Kolb, 1997).

Regimens to maintain high brain concentrations of AMP or MAP, either by continuous administration or frequent injections of higher doses of the drug (10–100 mg/kg) within one or a few days, exert toxic effects on the behavior and brain tissues of rats and mice (Robinson and Becker, 1986; Kita *et al.*, 2003). The neurotoxic regimen induces a long-lasting reduction in the levels of dopamine and tyrosine hydroxylase (TH) and also dopaminergic terminal degenerations in the striatum (Ricaurte *et al.*, 1980, 1982; Trulson *et al.*, 1987; Gibb *et al.*, 1994; Friedman *et al.*, 1998; Kita *et al.*, 2003). TUNEL-positive cells have been shown to be induced in the frontal cortex, striatum, nucleus accumbens, and other brain regions rich in dopaminergic innervations following the administration of large doses of MAP (Deng and Cadet, 2000; Asanuma *et al.*, 2002). The neurotoxic regimen induces intense, disorganized behaviors such as excessive grooming, self-injurious biting, or performance deficits in maze tests (Mueller *et al.*, 1982; Robinson and Becker, 1986; Friedman *et al.*, 1998; Kita *et al.*, 2000a). Behavioral sensitization of hyperactivity or stereotypy has been demonstrated in mice and rats a few days after neurotoxic treatment (Itzhak *et al.*, 1998; Wallace *et al.*, 2001).

The medial prefrontal cortex in rats functions as an integration center communicating with neocortical and subcortical areas and participates in the processing of working memory (Rosenkilde, 1983; Kolb, 1984, 1990; Takagishi and Chiba, 1991; Azuma and Chiba, 1996; Ishikawa and Nakamura, 2003). A lesion of the MFC, induced by ibotenate, was demonstrated to prevent the development of behavioral sensitization of hyperactivity to AMP, implicating a role for the MFC in the induction of persistent hypersensitivity to the drug (Wolf *et al.*, 1995). The MFC is densely innervated by dopaminergic fibers from the ventral tegmental area (VTA) (Björklund and Lindvall, 1984). A local injection of 6-hydroxydopamine (6-OHDA) to this brain region induces hypersensitivity to AMP in rats as well as performance deficits in delayed responses in maze tests (Bubser and Schmidt, 1990; Banks and Gratton, 1995). A neurotoxic oxidant, 6-OHDA, has been suggested to be generated from excess dopamine released by MAP or AMP

from dopamine nerve terminals and to induce various neurotoxic damages, including reduction in dopamine content and TH, nerve terminal degeneration, and apoptosis in brain tissues (Ricaurte *et al.*, 1980, 1982; Seiden and Vosmer, 1984; Trulson *et al.*, 1987; Karoum *et al.*, 1993; Gibb *et al.*, 1994; Deng *et al.*, 1999, 2001; Asanuma *et al.*, 2002; Gao *et al.*, 2003; Kita *et al.*, 2003). From the preceding findings, it is believed that the subchronic administration of lower doses of MAP may repeatedly produce small but significant amounts of oxidants in the MFC, which, in turn, exert neurotoxic effects on the dopaminergic axons and cells in the region, eventually participating in the gradual development of behavioral sensitization. Thus, the present study examined whether neurotoxic morphological changes were induced in the MFC of the rats that were treated daily with a relatively low dose of MAP (5 mg/kg) to develop behavioral sensitization to the drug. Dopaminergic impairments and apoptotic changes were monitored daily by TH and dopamine-beta-hydroxylase (DBH) immunohistochemistry and TUNEL histochemistry, and compared with the progressive enhancement of behavioral sensitivity to MAP.

Materials and Methods

Animal law

The present studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory animals, Chiba University, adopted by the Committee on Animal Research of Chiba University, which is accredited by the Ministry of Education, Science, Sports, and Culture of Japan.

Animals and administration of MAP

Male rats of the Wistar strain weighing 140–160 g, and aged 6 weeks at the starting time of the experiments were obtained from a local breeding house, SLC Co., Shizuoka. Three animals were housed per hanging metal cage at a room temperature of 21°C under a 12 h light: 12 h dark cycle. Solid rat chow and water were provided *ad libitum*. MAP hydrochloride was dissolved in physiological saline at a concentration of 5.0 mg/ml. The rats received a dose of 5.0 mg/kg of MAP intraperitoneally once a day for 12 days (injection period); 6 consecutive days (day 1–day 6), a 1-day interruption, and then a further 6 consecutive days (day 7–day 12). Animals injected with an equal volume of saline served as the control. Groups of rats were administered MAP for 12 days, and the drug was then withdrawn for 1–42 days (withdrawal period), WD1–WD42. The animals were challenged with a dose of 2 mg/kg of MAP for

assessing the retention of behavioral sensitization after periods of withdrawal.

Behavioral studies

Hyperactivity and successive head-movement stereotypy induced by MAP were rated according to a scale by a single observer for 2–3.5 h. The scale was as follows: 0, drowsy or sleepy; 1, slight increase in locomotor activity, fairly continual; 2, exploratory behaviors including frequent rearing and moderate increase in horizontal locomotion; 3, highly increased horizontal locomotion, frequent rearing, and continual sniffing-like behaviors; 4, stereotyped behaviors characterized by successive head-movements in which the animals remained at one location on the floor, and moved their heads back and forth or right and left, successively, and simultaneously, the animals moved their fore-legs, but not their rear-legs, from side to side; 5, an increase in the frequency of the successive head-movement stereotypy, standing still without the movements of all four legs; 6, excessive grooming, licking or biting—which was scarcely observed. Hyperactivity and stereotypy were defined as those behaviors that were rated with a score of 1–3 and 4–6 on the scale, respectively.

Tissue preparation

Animals were fixed on each experimental day within a few hours after completing the rating of MAP-induced behaviors. Some rats were fixed 24 h after the MAP administration on day 1, which aimed to observe early morphological changes prior to the injection on day 2. These rats were termed as the rats on “day 1.5.” All rats were deeply anesthetized with 6 ml/kg of 20% urethane, i.p., and transcardially fixed with 4% paraformaldehyde or Zamboni solution in a 0.1 M phosphate buffer at pH 7.4. After perfusion, the brains were dissected and postfixed with the same fixative for 16 h at 4°C. The samples were coronally cut into four blocks during fixation. Materials were dehydrated through an ascending series of ethanol concentrations and embedded in paraffin. Sections, 5 μ m in thickness, were cut on a microtome (Zeiss, Germany). Coronal sections were prepared from MFC; cingulate cortex, area 3 (Cg3), approximately 3.2 mm anterior to the bregma according to the stereotaxic atlas of Zilles (1985) (cf. Fig. 2 and 6).

Immunohistochemistry

Immunohistochemical staining was performed to examine the damage caused to the catecholaminergic axons. The staining was conducted sequentially. In brief, fixed brains were cut into 50 μ m thick sections on a microslice (Dosa-

ka EM, Japan), immersed in 0.3% Triton-X 100 in 0.05 M Tris buffered saline at pH 7.4 (TBS) for membrane permeabilization, and then rinsed with TBS. The materials were placed in TBS containing 0.3% Triton-X 100 (TBS-T) and 3% normal goat serum (DACO A/S, Denmark) for 30 min in order to block nonspecific binding at room temperature, washed with TBS-T, and then immersed in 0.3% hydrogen peroxide in methanol for 10 min to suppress endogenous peroxidase activity. Following the rinse with TBS-T, the sections were incubated in a rabbit anti-TH polyclonal antibody (1:500) (Chemicon International Inc., CA, USA) diluted with TBS-T containing 1% normal goat serum at 4°C for 48 h. After rinsing with TBS-T, the specimens were placed in a biotinylated secondary antibody (Vector, USA) at a dilution of 1:200 with TBS for 45 min at room temperature, and then in an ABC reagent (Vectastain Elite Kit, Vector) for 1 h at room temperature. Immunoreactivity was visualized using 0.05% diaminobenzidine containing 0.02% H₂O₂ in a 0.05 M Tris-HCl buffer, pH 7.6. Sections were mounted on Silane-coated glass slides, dehydrated in ethanol and xylene, and then coverslipped.

The staining with anti-DBH was essentially conducted in a manner similar to the one mentioned above. After the blocking procedures for nonspecific binding and endogenous peroxidase activity, the sections were incubated in a mouse anti-DBH monoclonal antibody (Chemicon International Inc.) (1:1000) for 48 h at 4°C, and then processed for visualization and mounting.

Morphometrical analyses

Changes in the densities of axons immunoreactive to TH or DBH were measured for assessing the degrees of damage to the dopaminergic or noradrenergic axons. Three sections were collected in each experiment, which was performed with three rats on the predetermined experimental day of MAP- treatment. One section was randomly selected from those prepared from each rat. Images of axons immunoreactive to TH or DBH were photographed with the aid of an Olympus BX51 microscope with a FUJI digital 3CCD camera system (type HC-2500) (FUJI FILM, Tokyo). The areas occupied by the immunoreactive axons were analyzed using the Win ROOF analysis system (Mitani Co., Fukui), and the density of the axons was expressed as a percentage relative to the total area within one definite division. In the present study, the density of TH- or DBH-immunoreactive axons was measured within a unit area, 200 μ m $\times$ 200 μ m, which covered layers II and III of the MFC. Three unit areas were randomly placed in the strata across these two layers. All results are expressed as means $\pm$ SEM. Data were analyzed by Student's *t* test. Differences were considered significant at $P < 0.005$.

Fig. 1. Development of behavioral sensitization to MAP. Rats received MAP (5 mg/kg) or saline (cont), intraperitoneally, once a day for 12 days. Behavioral sensitization develops gradually reaching a basic plateau level on day 4, successively reaches a slightly higher stage on day 6, and then progresses to a further advanced scale on day 12. Day 1, 4, 6, and 12: 1st, 4th, 6th, and 12th day during the period of daily injection of MAP. Each point represents mean $\pm$ SEM in 6 rats.

Assessment of apoptotic cells

Apoptotic changes were assessed using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method. In brief, the deparaffinized sections were treated with 20 μ g/ml proteinase K at 37°C for 20 min after rinsing with PBS. The TUNEL reaction mixture was prepared by mixing a TdT enzyme, buffer, and the substrate that contained biotinylated dUTP (Roche, Germany). Approximately 50 μ l of the mixed solution was added onto each section in a humidified chamber followed by incubation for 2 h at 37°C. After rinsing, the sections were incubated in an ABC reagent (Vector) for 30 min at room temperature, and then with DAB solution for 10 min. Stained sections were washed two times with PBS, dehydrated, and covered with mounting media (Mount-Quick, Daido Sangyo, Tokyo) and cover slips.

Morphometrical analysis of TUNEL-positive cells

The sampling of the sections for the morphometry was essentially carried out in a manner similar to that described in the morphometrical analysis to the axons immunoreactive to TH. The number of TUNEL-positive cells was counted within a unit area, 500 μ m $\times$ 500 μ m, which covered layers II–V of the MFC. Three unit areas were randomly selected in the strata across these layers. The populations of TUNEL-positive cells were expressed as mean $\pm$ SEM. Data were analyzed by Student's *t* test. Differences were considered significant at $P < 0.005$.

Results

Development of behavioral sensitization to MAP

The administration of MAP (5 mg/kg) first induced increases in locomotion and rearing, and, a short while later, sniffing-like behavior, or 1–3 on the behavioral scale. These initial hyperactivities were subsequently followed by a stereotyped behavior characterized by successive head and forelimb movement remaining in one position on the floor, which was 4 on the behavioral scale. The time of onset of the behaviors shortened day by day. Furthermore, the stereotyped behaviors gradually increased in intensity and duration. The head-movement stereotypy was increasingly enhanced as the daily MAP administration was repeated. The sensitization of the successive head-movement stereotypy reached a basic plateau level around day 4, showing slightly higher scores on day 6, and then further advanced to a score of 4–5 on the behavioral scale on day 12 (Fig. 1).

When a challenge dose of 2 mg/kg of MAP was administered to the rats after periods of drug withdrawal, they expressed sensitized hyperactivity and stereotypy comparable in quality and intensity to that attained on the last day of MAP injection (day 12). The challenge dose of MAP (2 mg/kg) did not induce the sensitized behavior in control animals treated with saline.

Immunohistochemistry

Immunohistochemical images to anti-TH or -DBH were examined in the MFC (Fig. 2). TH-immunoreactive axons

Fig. 2. Laminar distributions of TH- and DBH-immunoreactive axons in the MFC of rats. **A:** An atlas image of the MFC (medial prefrontal cortex); cingulate cortex, area 3 (Cg3), 3.2 mm anterior to the bregma (Zilles, 1985). Sample tissues for histochemical analyses were collected from this area. **B:** TH-immunoreactive axons. I–VI, 1st–6th layer of the MFC. Fibers are relatively low in density in layers II–IV, while high in layers V–VI. **C:** DBH-immunoreactive axons. Fibers extend parallel to the cortical surface in layer I. They are relatively low in density in layers II–IV. Bar = 100 μ m

ran rather parallel to the cortical surface in layer I while they were located vertically or reticularly in other layers. The densities of the axons were relatively low in the strata covering layers II and IV, while they were high in layers III, V, and VI (Fig. 2B). DBH-immunoreactive axons extended horizontally along the cortical surface in layer I, almost vertically in layers II–IV, and reticularly in layers V and VI (Fig. 2C).

The pattern of axon distribution obtained by subtracting the innervation profile of DBH-immunoreactive axons from that of TH-immunoreactive axons was consistent in distribution pattern and density with that of dopaminergic nerve fibers in the MFC displayed with fluorescence microscopy by Björklund and Lindvall (1984; Fig. 10A). Thus, it was suggested that TH immunohistochemistry was useful as a means to study changes in the dopaminergic axons induced by MAP in the MFC.

The densities of TH-immunoreactive axons clearly and gradually lessened in the strata covering layers II–IV as MAP was administered daily (Fig. 3, 4). The reduction in the density was rapid enough to be observed 24 h after the injection on day 1 (Fig. 4; d1.5). On the whole, the pattern of the density reduction was consistent in time course with that of the development of behavioral sensitization of the head-movement stereotypy (cf. Fig. 4 with Fig. 1). On the other hand, the densities of DBH-immunoreactive axons did not change throughout the entire period of MAP administration (Fig. 5). This observation was in contrast with the obvious and steady decreases in the densities of TH-immunoreactive axons shown in Figure 4. Thus, it was suggested that the daily density reduction of TH-immunoreactive axons reflects the density reduction of dopaminergic axons and terminals in layers II and III in the MFC.

Fig. 3. Density changes in TH-immunoreactive axons in MFC in MAP-treated rats. Layers II–IV. Animals were administered MAP (5 mg/kg) for 4 or 12 days, (B and C). A: saline treatments for 12 days. MAP induces a decrease in the axon densities in layers II–IV, which progresses with repeated injections of the drug. Bar = 100 μ m

Fig. 4. Reduction in the densities of TH-immunoreactive axons in the MFC in MAP-treated rats. Layers II and III. Rats were administered saline or MAP (5 mg/kg) once a day, and fixed by perfusion on predetermined days for histochemical examinations. Control, saline treatments for 12 days. d1.5, fixed 24 h after the MAP injection on day 1. d2, d4, d6, and d12, fixed within a few hours after the MAP injection on days 2, 4, 6, and 12. Densities of TH-immunoreactive axons (%), areas occupied by the axons within a unit area ($200 \times 200 \mu\text{m}^2$) covering layers II and III. Each bar is expressed as mean $\pm$ SEM. Data are analyzed by Student's *t* test. $P < 0.005$. Refer to "Material and Methods" for more details. Compare with Figures 2 and 3.

Fig. 5. No substantial changes in the densities of DBH-immunoreactive axons in MFC in MAP-treated rats. Layers II and III. Rats were treated with saline or MAP, as mentioned in Figure 4. Densities of DBH-immunoreactive axons (%), areas occupied by the axons within a unit area ($200 \times 200 \mu\text{m}^2$). The densities of the axons are maintained at an almost constant level resistant to the MAP treatments. Each bar is expressed as mean $\pm$ SEM. Data analyzed by Student's *t* test. No significant differences are detected. Compare with Figures 2–4.

Fig. 6. TUNEL-positive cells in MFC in MAP-treated rats.

A and C: Klüver-Barrera stain. **B and D:** TUNEL stain. Animals were injected daily with, intraperitoneally, MAP (5 mg/kg) for 12 days (**A, B**; d12) and the drug was then withdrawn for 7 days (**C, D**; WD7). MAP induces TUNEL-positive cells in layers II and III most frequently, and occasionally in layer V. **E:** An atlas image of MFC; cingulate cortex, area 3 (Cg3), 3.2 mm anterior to the bregma (Zilles, 1985). Bar = 100 μ m. Compare with Figure 2.

Induction of TUNEL-positive cells in MFC with MAP treatments

In the MFC, the induction and increase in the number of TUNEL-positive cells started a few days later than the beginning of the reduction in TH-immunoreactive axons. The cells were scarcely found on day 4, when TH-immunoreactive axons significantly decreased in density in association with a considerable development of behavioral sensitization (cf. Fig. 1, 3, 4). A few cells appeared on day

6. A significant number of the cells was observed on day 12 and WD7 (Fig. 6, 7). The TUNEL-positive cells were most frequently and almost selectively induced in layers II and III, in which the TH-immunoreactive axons were significantly reduced in density (cf. Fig. 6 with Fig. 3, 4). A few occasional TUNEL-positive cells were found in layer V on day 12 (Fig. 6). However, the cells were scarcely induced in layers I and VI. The TUNEL-positive cells were found to have disappeared on WD28, i.e., one month after the last injection of MAP. No TUNEL-positive cells were induced in control rats treated with saline.

Fig. 7. Increase in the number of TUNEL-positive cells in MFC in MAP-treated rats. Animals were treated daily with MAP (5 mg/kg) for 4, 6, or 12 days, and then the drug was withdrawn for 7 days; d4, d6, d12, and WD7. The number of cells is counted within a unit area, $500 \times 500 \mu\text{m}^2$, covering layers II–V. No TUNEL-positive cells are found 28 days after the last injection of MAP on day 12. Compare with Figure 6, and Figures 3–5. Data analyzed by Student's *t* test. $P < 0.005$

Discussion

The present study demonstrated the development of behavioral sensitization of the head-movement stereotypy in rats treated once a day with an intraperitoneal administration of MAP (5 mg/kg) for 12 days. This regimen for the behavioral sensitization simultaneously induced gradual histochemical changes in the MFC. The reduction in the densities of TH-immunoreactive axons started 24 h after the first MAP injection on day 1, and then progressed steadily as the drug was administered daily. In contrast, the density of DBH-immunoreactive axons did not change during the daily injection period. The induction of TUNEL-positive cells began on day 6 in the MFC, followed by a gradual increase up to day 12. They were present on WD7 and disappeared on WD28. The development of behavioral sensitization was parallel in time course to that of the morphological changes induced in the MFC.

The stereotypy, rather than the hyperactivity, was chosen as a behavior suited to the actual examination of the details of the daily changes in the behavioral sensitization using a behavioral scale. Stereotypic behaviors such as continuous sniffing and successive head-movements have been demonstrated as showing rapid and clear sensitization to the drug without neurotoxic damage following an administration of relatively low doses, 3–5 mg/kg, of AMP or MAP (Robinson and Becker, 1986; Robinson and Berridge, 1993). Our preliminary dose-response experiments demonstrated that a relatively low dose, 5 mg/kg of MAP, invariably induces a definite pattern of stereotypy in rats, easily distinguishable

in temporal pattern from others and characterized by repetitive head-movements while remaining at one position on the floor. Thus, the hypersensitivity of the successive head-movement stereotypy has been developed following the present sensitization procedures, which are compatible in drug dose (5 mg/kg) and the interval and route of injection with preceding behavioral sensitization regimens using repeated daily administrations of constant doses, 1–8 mg/kg of AMP or MAP (Robinson and Becker, 1986; Robinson and Berridge, 1993; Segal and Kuczenski, 1994). No self-injurious biting, characterized as a neurotoxic behavior, was induced under the present experimental conditions. Excessive grooming behaviors, classified as neurotoxic behaviors, were only rarely observed.

The present experiments have demonstrated that morphological deteriorations were brought about in the MFC of the rats subchronically treated with an ordinary sensitization regimen with a dose of 5 mg/kg of MAP. Furthermore, it is suggested that the daily reductions in the densities of TH-immunoreactive axons substantially reflect the decreases in the dopaminergic axons and terminals distributed in the MFC (cf. Fig. 2–5). Impairing effects of MAP on the dopaminergic fibers have been displayed in rats and mice treated with neurotoxic regimens, maintaining high brain concentrations of MAP to induce disorganized behaviors such as excessive grooming and self-injurious biting of the skin (Mueller *et al.*, 1982; Robinson and Becker, 1986; Gibb *et al.*, 1994; Friedman *et al.*, 1998; Kita *et al.*, 2000a). The administration of high doses of MAP in the range of 10–100 mg/kg with a single or frequent injections in rats or mice causes a reduction in dopamine contents and decreases in TH-immunoreactive axons in the striatum and other

brain regions under the dopaminergic innervation (Ricaurte *et al.*, 1980, 1982; Trulson *et al.*, 1987; Gibb *et al.*, 1994; Friedman *et al.*, 1998; Kita *et al.*, 2003). In addition, dopaminergic terminal degenerations and increases in TUNEL-positive cells have been demonstrated to be induced in the frontal cortex, nucleus accumbens, striatum, and other brain regions of rats that are rich in dopaminergic nerve fibers (Ricaurte *et al.*, 1982; Kita *et al.*, 2000a; Deng and Cadet, 2000; Asanuma *et al.*, 2002). These neurotoxic damages noted by preceding investigators are consistent in morphological characteristics with the histochemical changes revealed by the present study in the MFC of the rats subchronically treated with a relatively low dose (5 mg/kg) of MAP.

The present study demonstrated close relationships between the development of neurotoxic morphological changes induced in the MFC and that of behavioral sensitization to MAP. Oxidants such as 6-OHDA, reactive oxygen, and NO radicals are suggested to be generated from excess dopamine released by MAP or AMP from dopaminergic nerve terminals and to induce various neurotoxic damages including persistent reduction in dopamine content and TH, nerve terminal degeneration, and apoptosis in brain tissues (Ricaurte *et al.*, 1980, 1982; Seiden and Vosmer, 1984; Trulson *et al.*, 1987; Karoum *et al.*, 1993; Gibb *et al.*, 1994; Deng *et al.*, 1999, 2001; Kita *et al.*, 2000b, 2003; Asanuma *et al.*, 2002; Gao *et al.*, 2003). On the basis of this evidence, the present observations are compatible with the preceding findings in rats that suggest that the lesion of the MFC by a local injection of 6-OHDA, a neurotoxic agent, stimulates the development of behavioral sensitization to AMP (Banks and Gratton, 1995). In addition, other reports have demonstrated that neurotoxic regimens cause the development of behavioral sensitization of hyperactivity or stereotypy a few days after MAP treatment in rats (Itzhak *et al.*, 1998; Wallace *et al.*, 2001). In addition, the reduction in dopamine level in the MFC has been suggested to enhance the glutamatergic outputs to other nuclei leading to stimulation of the behavioral sensitization to AMP, MAP, or cocaine (Steketee, 2003). Based on all these studies, it can be stated that subchronic intraperitoneal administrations of lower doses of MAP may repeatedly generate reactive oxidants in rat brains, which eventually cause neurotoxic damage in the MFC to facilitate the gradual development of behavioral sensitization.

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