

Delta Opioid Peptide [D-Ala²,D-leu⁵]Enkephalin Blocks the Long-term Loss of Dopamine Transporters Induced by Multiple Administrations of Methamphetamine: Involvement of Opioid Receptors and Reactive Oxygen Species

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ABSTRACT

Delta opioid peptide [D-Ala²,D-leu⁵]enkephalin (DADLE) can prolong organ preservation and increases myocardial tolerance to ischemia. Our study examined the protective property of DADLE against methamphetamine- (METH) induced dopaminergic terminal damage in the central nervous system. Because the neurotoxicity of METH involves reactive oxygen species, we also examined if DADLE might be an antioxidative agent *in vitro*. DADLE at 2 and 4 mg/kg (i.p.), given 30 min before each METH administration (5 or 10 mg/kg, i.p., four injections in a day at 2-hr intervals), dose-dependently blocked the METH-induced long-term dopamine transporter loss. The opioid antagonist naltrexone blocked this action of DADLE in both aspects of striata but tends not to affect the effects of DADLE in the nucleus accumbens. DADLE did not alter changes in body

temperature induced by METH. The reduction of striatal dopaminergic content and tyrosine hydroxylase activity caused by METH, however, were not blocked by DADLE. *In vitro*, DADLE was approximately equipotent to glutathione in inhibiting both superoxide anion formation induced by xanthine oxidase and hydroxyl radical formation evoked by ferrous/citrate complex. DADLE was only slightly less potent than glutathione in inhibiting the iron/ascorbate-induced brain lipid peroxidation. These results suggest that DADLE can protect the terminal membranes of dopaminergic neurons against METH-induced insult but not the loss of dopaminergic content and tyrosine hydroxylase activity and that this action of DADLE might involve opioid receptors as well as the sequestration of free radical.

Endogenous opioid peptides and their receptors are known to participate in many pharmacological and physiological functions. For example, DADLE, a metabolically stable analog of the endogenous *delta* opioid peptide enkephalin, can induce hibernation when injected into summer-active ground squirrels—in the months when they do not usually hibernate (Oeltgen *et al.*, 1988). Moreover, DADLE can dramatically extend the organ survival time in a multiorgan block preparation, including the heart, lung, liver, spleen and kidney, from an average of 14 to 46 hr—the longest in the history of organ preservation (Chien *et al.*, 1994). Lungs preserved in

such a fashion functioned normally when transplanted into the host animals 24 hr after the preservation (Oeltgen *et al.*, 1996). DADLE also enhanced the hypothermic preservation time of isolated rat lungs (Wu *et al.*, 1996). Further, using isolated rabbit hearts, a recent report demonstrated that DADLE can promote myocardial tolerance to ischemia in a fashion far superior to the standard cardioplegic procedure (Bolling *et al.*, 1997). The exact mechanism(s) underlying the protective property of DADLE in peripheral organs is(are) not completely understood at present. However, because the survival of organs depends largely on the oxidative state of the tissue, it is possible that the protective property of DADLE may involve, via as yet unknown mechanism, reactive oxygen species (ROS). As such, it is not unreasonable to speculate that DADLE might be a tissue protective agent

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ABBREVIATIONS: BPT, bathophenanthroline disulfonic acid; CNS, central nervous system; DA, dopamine or dopaminergic; DADLE, [D-Ala²,D-leu⁵]enkephalin; DAT, dopamine transporter(s); 2,3-DHBA and 2,5-DHBA, 2,3- and 2,5-dihydroxybenzoic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; DTT, dithiothreitol; GSH, glutathione; HPLC-ECD, high performance liquid chromatography utilizing electrochemical detection; HVA, homovanillic acid; METH, methamphetamine; 6-MPH₄, 6-methyl-5,6,7,8-tetrahydropterin; PTBN, α -phenyl-N-tert-butyl nitron; PD, Parkinson's disease; ROS, reactive oxygen species; RTI-121, 3 β -(4-[¹²⁵I]iodophenyl)tropane-2 β -carboxylic acid isopropyl ester; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TH, tyrosine hydroxylase; ANOVA, analyses of variance.

even in the central nervous system. This speculation is in alignment with the observation that endogenous opioid system is implicated in increasing the length of survival of mice which have been subjected to hypoxic preconditioning before prolonged hypoxic shock (Mayfield and D'Alecy, 1994). We decided therefore to extend this tissue protective property of DADLE from the peripheral organs to the CNS and to test if DADLE might provide protection against certain models of CNS damage.

METH ("ice," "crank," "speed") is an analog of the psychostimulant amphetamine and is a major drug of abuse in many parts of the world including the United States (Bai, 1997). METH causes terminal degeneration of monoaminergic nervous systems in rodents (Ricaurte *et al.*, 1982; O'Callaghan and Miller, 1994), nonhuman primates (Seiden *et al.*, 1975; Woolverton *et al.*, 1989) and humans (Wilson *et al.*, 1996). Although the mechanisms involved in METH-induced dopaminergic (DA) terminal loss remain to be completely clarified, they are known to involve ROS (Seiden and Vosmer, 1984; Cadet *et al.*, 1994; Albers and Sonsalla, 1995) and excitotoxic damage (Sonsalla *et al.*, 1991). As stated above, because DADLE prolongs organ survival and because the survival of organs *ex vivo* might depend on oxidative mechanisms, we decided to test if DADLE could affect METH-induced DA terminal damage in the nigrostriatal and mesolimbic DA neurons. The purpose of this study was therefore 2-fold: 1) to demonstrate that *delta* opioid peptide DADLE might provide protection against METH-induced long-term damage to the DA neuronal terminals as indicated by the level of the dopamine transporter (DAT); 2) to examine if the *delta* opioid peptide DADLE might be an antioxidative agent *in vitro*.

Methods

Drug Treatments and Autoradiographic Procedures

Male CD-1 mice, 8 wk old weighing about 30 g, were used. All animal procedures were carried out according to the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care and Use Committee. Mice received *i.p.* injections of 5 or 10 mg/kg of METH four times, given 2 hr apart. This procedure has been shown to effectively cause terminal damage and depletion of DA content in DA neurons in this strain of mice (Albers and Sonsalla, 1995; Ali *et al.*, 1994; Sonsalla *et al.*, 1991). Some animals received saline 30 min before each METH injection; although others received one of three different doses of DADLE (Multiple Peptide Systems, CA or Research Triangle Institute, NC, 1, 2, 4 mg/kg), *i.p.*, 30 min before each METH injection. Enkephalins and their analogs are known to cross the blood-brain barrier (Kastin *et al.*, 1976). For core temperature measurement, animals were gently pushed into an acrylic cage for 2 min before measurement. Rectal temperature was measured with a digital thermometer with the probe lubricated with mineral oil. Data from the temperature experiments were analyzed by a 2-way ANOVA followed by the Scheffé's test for the *post hoc* analysis. The significance level was set at $P < .05$. Whenever the opioid receptor antagonist naltrexone was used, it was either administered alone (0.1 or 1 mg/kg; *i.p.*) or coadministered with DADLE in the same solution. All drugs were dissolved in saline. Two weeks later, animals were killed by decapitation and their brains removed and dipped into 2-methylbutane on dry ice for 10 sec. The frozen brains were stored at -75°C before autoradiographic examination using a DAT marker [^{125}I]RTI-121 (*i.e.*, 3 β -(4-[^{125}I]iodophenyl)tropane-2 β -carboxylic acid isopropyl ester; Boja *et al.*, 1995; Hirata *et al.*, 1996). The brains were cut into 20- μm sections

and mounted on gelatine coated slides. After drying, the slides were incubated with 0.073 nM of [^{125}I]RTI-121 (2200 Ci/mmol; New England Nuclear, Boston, MA) at room temperature for 60 min to label the DAT. Nonspecific binding was determined in the presence of 10 μM GBR-12909. After incubation, unbound radioligand was removed by rinsing the slides in ice-cold buffer (137 mM NaCl, 2.7 mM KCl, 10.14 mM Na_2HPO_4 , 1.76 mM KH_2PO_4 , 10 mM NaI) twice for 20 min. The slides were then washed with distilled water and dried overnight. The slides were apposed to Hyperfilm βmax (Amersham Corp., Arlington Heights, IL) for 53 hr together with radioactive standards. The films were developed and the results were quantified by densitometry. The following three areas of the brain were examined in this study: medial striatum, lateral striatum and the nucleus accumbens. In each autoradiographic examination, brain slices (typically at least eight slices) chosen for each animal represented sections encompassing the rostral to caudal structure of each brain region under study. A Macintosh computer-based analysis system (Image, NIH) using standard curves generated from the [^{125}I] microscales (Amersham) was used for the quantification of [^{125}I]RTI-121 binding to the brain. Data were first analyzed by ANOVA. *Post hoc* analyses were performed using Scheffé's test (see "Results") for comparison of differences between individual groups. The criteria for significance were set at the 0.05 level.

Neurochemical Analysis

Two weeks posttreatment, mice were killed by cervical dislocation and their brains were rapidly removed and dissected on ice to obtain striatum for neurochemical analysis. Brain region samples were analyzed for DA, its metabolites, and a stable metabolite of 5-hydroxytryptamine [5-hydroxyindoleacetic acid (5-HIAA)] by HPLC-ECD at +0.8V with minor modifications (Andrews and Murphy, 1993). Briefly, individual samples were sonicated in 400 to 500 μl of 0.1 M perchloric acid and centrifuged at $7200 \times g$ (12,000 rpm) for 10 min. Fifty μl of each resulting supernatant were injected onto a 10 cm by 4.6 mm Advantage 3 μm ODS reversed-phase chromatography column (Thomson Instruments, Springfield, VA) in a mobile phase containing 0.1 M monochloroacetic acid, 8% acetonitrile, 0.55 g/liter octanesulfonic acid, 0.3% triethylamine and 10 μM EDTA at a flow rate of 0.5 ml/min. DA, its metabolites DOPAC and HVA and 5-HIAA, the stable metabolite of 5-hydroxytryptamine, were separated and quantitated as relative peak areas versus the internal standard, 5-hydroxy-N-methyltryptamine. Protein was determined by the method of Lowry *et al.* (Lowry, 1951). Concentrations are expressed as a percentage of the control group mean (ng/mg protein) \pm S.E.M. For each compound, a one-way ANOVA was used to detect overall statistically significant differences among groups. *Post hoc* analysis after the ANOVA was performed by using Scheffé's test comparing difference between individual groups. Level of significance was set at $P < .05$.

TH Activity Assay

The TH enzymatic activity assay was adapted from the procedure of Reinhard *et al.* (1986) and Vrana *et al.* (1992) measuring the stoichiometric release of [^3H]H $_2\text{O}$ from L-[3,5- ^3H]tyrosine. On the day of the experiment, the striatal tissues were homogenized with a Teflon/glass homogenizer in 15 volumes (w/v) of ice-cold 10 mM sodium phosphate, monobasic, containing 0.2% Triton X-100. The homogenate was then used directly without centrifugation for TH activity determination. The stock reaction mixture containing 100 μM tyrosine, 2 mM 6-MPH $_4$, 4 mM DTT and 5 $\mu\text{g}/\mu\text{l}$ catalase was prepared by adding these components to lyophilized L-[3,5- ^3H]tyrosine (1 $\mu\text{Ci}/\text{reaction}$). For each reaction, 25 μl of striatal homogenates were added to 25 μl of reaction mixture. After incubation at 37°C for 20 min, unreacted L-tyrosine and L-dihydrophenylalanine were adsorbed with 500 μl of 7.5% charcoal (Sigma Chemical Co., 100–400 mesh) in 1 M HCl. The mixture was vortexed thoroughly and centrifuged at $14,000 \times g$ for 5 min. Supernatant was removed

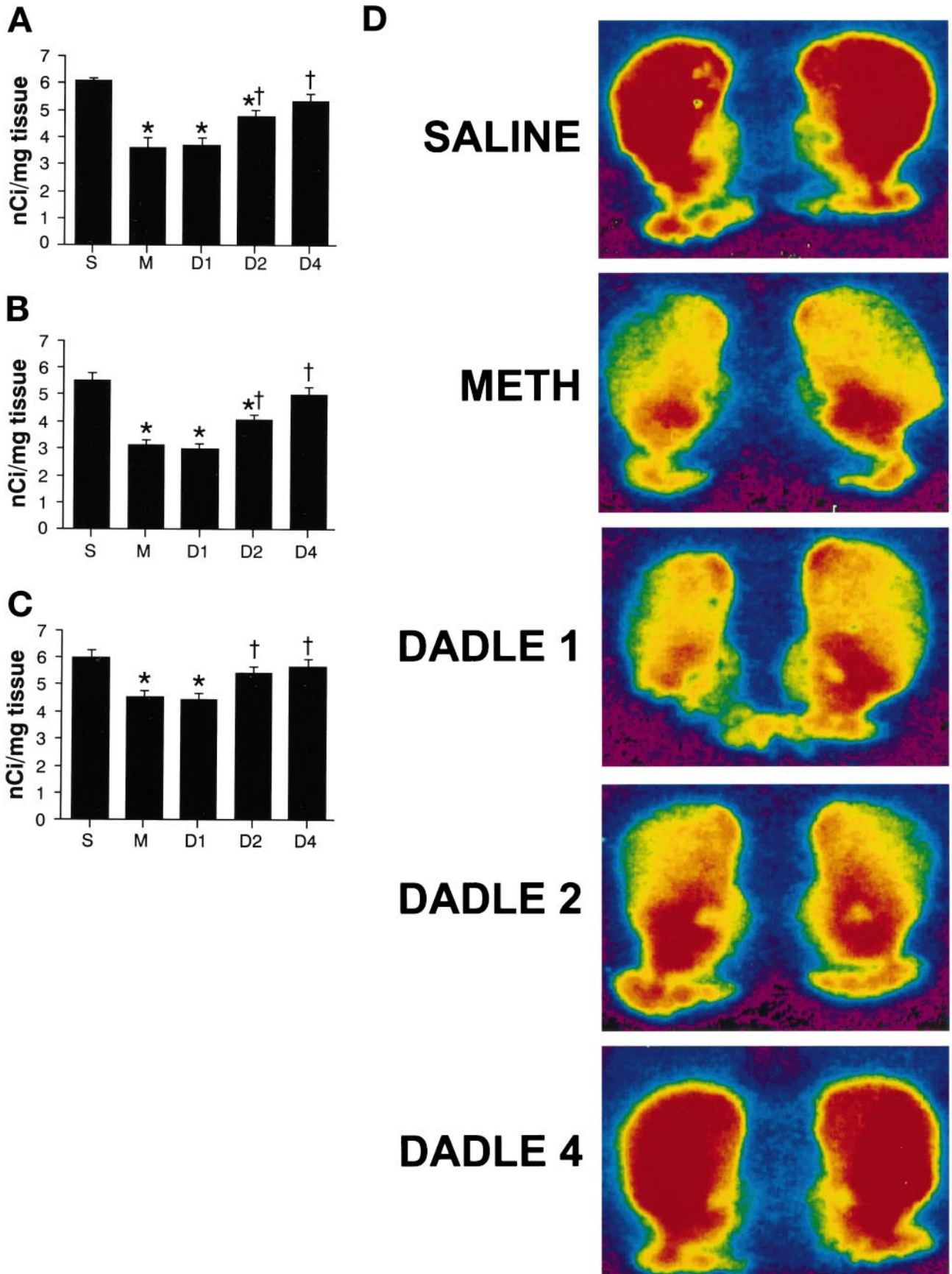


Fig. 1. Delta opioid peptide DADLE protects against long-term loss of DAT induced by multiple administrations of METH. METH (5 mg/kg, i.p.) was given every 2 hr for four times. DADLE was given in different doses (i.p.) 30 min before each METH injection. Data represent the densitometric quantification of the [¹²⁵I]RTI-121 labeled DAT in the medial (A), lateral (B) sides of the striata and the nucleus accumbens (C) of mice. A color-coded

into a fresh tube and recentrifuged at $14,000 \times g$ for 5 min. Of the resulting supernatant, 300 μ l were added to 3 ml of scintillation fluid (Poly-fluor; Packard Meriden, CT). Blank values were obtained by performing the reaction in the absence of 6-MPH₄, DTT and catalase. The fraction of tritiated water recovered (generally 90%) was determined and was used to calculate TH activity for each reaction. Protein content was measured by the Pierce Micro BCA method (Pierce Chemicals, Chicago, IL) using bovine serum albumin as the protein standard. The enzymatic activity is expressed as pmol of [³H]H₂O formed per mg protein per min. Data were analyzed by a 1-way ANOVA examining the overall significance. *Post hoc* analysis was done by using the Scheffé's test with the significance level set at $P < .05$.

Methods for Measuring Reactive Oxygen Species

Measurement of the generation of superoxide anions *in vitro*. Hypoxanthine and xanthine oxidase were used to generate superoxide anions (Roubaud *et al.*, 1997). The generation of superoxide anions was followed spectrophotometrically by measuring the reduction of ferric ions using BPT (Atlante *et al.*, 1997) as a chelator. A total of 150 μ l of 1.5 mM BPT, 400 μ l of various concentrations of DADLE or glutathione, 50 μ l of 1.4 mM ferric chloride and 50 μ l of 1.4 mM hypoxanthine in 1.12 mM potassium chloride were added together in a test tube. A total of 100 μ l of xanthine oxidase (0.677 unit/ml) was added to the tube to initiate the reaction. Control tubes contained no xanthine oxidase. The absorbance was measured 2 hr after the addition of xanthine oxidase. The data are reported as superoxide anion formation calculated on the basis of the stoichiometry of the reaction using a ferrous-BPT extinction coefficient of 19.4 $\text{mM}^{-1} \text{cm}^{-1}$ at 535 nm. The IC₅₀ value in each experiment was determined by the linear regression analysis of $[\log \text{control absorbance/experimental absorbance}] \times 100$ vs. log molar concentration of test compound according to a published report (Hirayama and Yida, 1997).

Measurement of the generation of hydroxyl radicals *in vitro*. A salicylate hydroxylation trapping method (Mohannakumar *et al.*, 1994) was used to monitor the generation of hydroxyl radical produced by iron complex. All reactions were performed in a total volume of 1000 μ l by mixing 100 μ l of 10 mM sodium salicylate in Ringer solution, 100 μ l of 250 μ M citric acid, 100 μ l of 250 μ M ferrous chloride, various concentrations of DADLE or glutathione, and water. After incubation at 37°C for 4 hr, the formation of hydroxyl adducts of salicylate such as 2,3-DHBA and 2,5-DHBA were assayed using an HPLC-EC procedure (Chiueh *et al.*, 1992). The method for calculating the IC₅₀ values were the same as that used for the superoxide anion experiments except that amounts of salicylate adducts were used instead of absorbance.

Measurement of brain lipid peroxidation *in vitro*. A previously described method was used to initiate the brain lipid peroxidation (Omodeo-Sale *et al.*, 1997). Lipid peroxidation was induced by incubating brain homogenates (2.5 mg brain tissue from CD-1 mice) with 80 μ M of FeSO₄ and 400 μ M of ascorbic acid in a 500- μ l sample solution that contained 1 mM Tris-HCl, PH 7.4, 154 μ M NaCl and 0.1 mM EDTA. The FeSO₄ and ascorbic acid solutions were prepared fresh immediately before use. The sample solution was incubated in a water bath at 37°C for 2 hr. After incubation, a 500 μ l of TBA in 50 mM NaOH and a 500 μ l of 2.8% trichloroacetic acid were added to the sample solution to propagate the formation of TBARS (Hess and Dix, 1992). The resultant solution was heated at 100°C for 10 min. After filtration using glass wools, the TBARS in the supernatant was quantified spectrophotometrically by measuring the absorbance at 532 nm. DADLE and GSH were added to the test tube just before the

addition of FeSO₄ and ascorbate. The concentration of TBARS was calculated as malondialdehyde equivalents by using an extinction coefficient of the 1,1,3,3-tetraethoxypropanethiobarbituric acid complex of 177,391 $\text{M}^{-1} \text{cm}^{-1}$ at 532 nm that was established in this laboratory and was found comparable to a published value of 156,000 $\text{M}^{-1} \text{cm}^{-1}$ (Nourooz-Zadeh *et al.*, 1994). The method for calculating the IC₅₀ values were the same as that used for the superoxide anion experiments.

Measurement of the reactivity of hydrogen peroxide *in vitro*. The method using a mixture of titanium (IV) and 4-(2-pyridylazo)resorcinol was used for spectrophotometric determination of hydrogen peroxide (Matsubara *et al.*, 1983; Haavik *et al.*, 1997). A 400- μ l sample containing 43.75 or 62.5 μ M hydrogen peroxide was first incubated with various concentrations of DADLE or catalase respectively at room temperature for 2 hr. Then, 200 μ l of 100 mM Tris-HCl (pH 8.0), 100 μ l of 1 mM of 4-(2-pyridylazo)resorcinol in 5 mM sodium chloride, and 100 μ l of 1 mM titanium chloride in 5 mM hydrochloric acid were added to each tube. The solution was heated at 37°C for 10 min. After cooling to room temperature, the concentration of hydrogen peroxide was estimated by measuring the absorbance at 508 nm.

Results

The first experiment examined the effects of different doses of DADLE in blocking the long-term DAT loss induced by 5 mg/kg of METH (four injections at 2-hr intervals). DADLE was given 30 min before each METH injection. Data were analyzed by a 1-way ANOVA with drugs and doses treated equally. *Post hoc* analysis was done using Scheffé's test after the one way ANOVA to compare the difference of effects caused by METH and various doses of DADLE. Criteria for significance were set at .05 level. There were significant main effects of drug on the levels of DAT in all three areas of brain examined ($F = 14.46$, $P < .001$ for medial striatum; $F = 12.79$, $P < .001$ for lateral striatum; $F = 8.777$, $P < .003$ for nucleus accumbens). Multiple administrations of METH (5 mg/kg, i.p.) caused a significant reduction about 40% ($P < .05$) of DAT levels in both aspects of striata when compared to saline controls, as visualized by [¹²⁵I]RTI-121 (fig. 1, A, B and D). The autoradiographic data indicated that the nucleus accumbens was less affected (to about 25% of controls; $P < .05$) by METH (fig. 1C). The lowest dose of DADLE tested (1 mg/kg) did not change the DAT level altered by 5 mg/kg of METH (fig. 1, A–D). However, both 2 and 4 mg/kg of DADLE, given before each administration of METH, significantly attenuated the DAT loss induced by METH in a dose-dependent fashion with the highest dose causing a complete protection ($P < .05$; fig. 1, A–D).

To test whether the protective effects of DADLE against METH-induced DAT loss occurred through opioid receptor mediated events, the universal opioid receptor antagonist naltrexone was used in an attempt to antagonize the actions of DADLE. In this experiment, 10 mg/kg instead of 5 mg/kg of METH was used to cause a higher degree of DAT loss to allow a more clear detection of naltrexone antagonism, if any. Toward that end, 4 mg/kg of DADLE (given 30 min before each METH injection) was used to protect against the dele-

transform of the autoradiograms depicting the densities of [¹²⁵I]RTI-121 in mouse brains from different treatment groups is also shown (D). In D, areas with highest densities of [¹²⁵I]RTI-121 are in red. Decreasing densities of the radioligands are indicated in yellow and green. The data represent means \pm S.E.M. of four (D4) or five (S, M, D1, D2) animals per group. See "Results" for details of treatments. S, Saline-saline; M, saline-METH; D1 or DADLE 1, 1 mg/kg DADLE-METH; D2 or DADLE 2, 2 mg/kg DADLE-METH; D4 or DADLE 4, 4 mg/kg DADLE-METH. * $P < .05$ compared to S. † $P < .05$ compared to M.

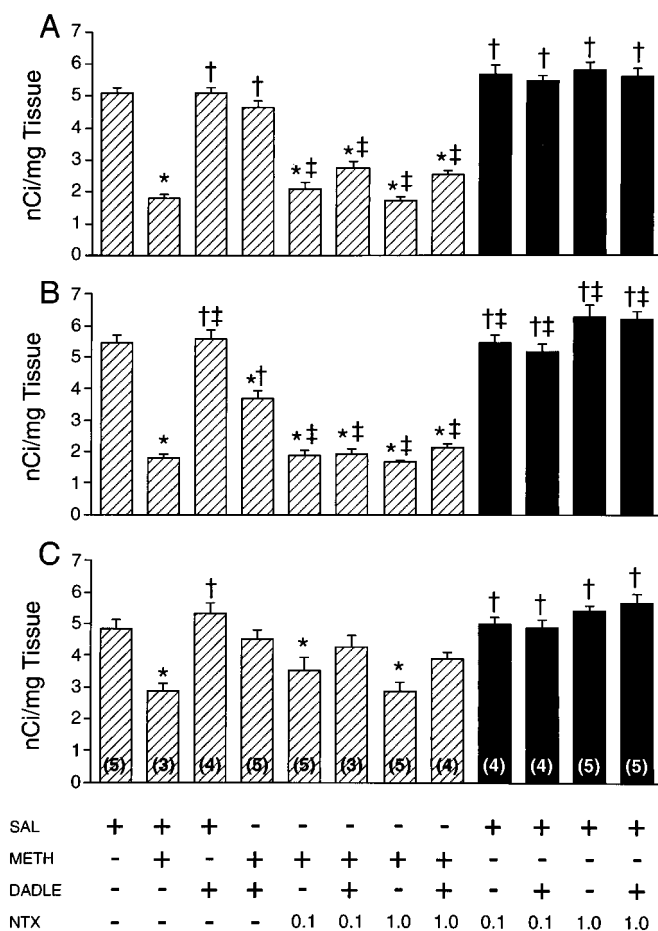


Fig. 2. Naltrexone blockade of the protective effects of DADLE against DAT loss in METH-treated mice. METH (10 mg/kg, i.p.) was given every 2 hr for four times. DADLE (4 mg/kg, i.p.) was given 30 min before each METH injection. Whenever naltrexone was used, it was coadministered with DADLE. See "Results" for details of treatments. Quantitative data are from the medial (A), lateral (B) sides of the striata and the nucleus accumbens (C). DAT were labeled with [¹²⁵I]RTI-121 as described (Hirata *et al.*, 1996). The data represent means \pm S.E.M. of animals in each group. Numbers of animals are indicated at the base of each bar in C. Darkened bars are controls containing naltrexone. SAL, Saline; NTX, naltrexone (numbers are mg/kg). * $P < .05$ compared to saline; † $P < .05$ compared to METH alone; ‡ $P < .05$ compared to the METH+DADLE group.

terious effects caused by 10 mg/kg of METH (four injections at 2-hr intervals); two doses of naltrexone (0.1 and 1 mg/kg) were used to antagonize the effect of DADLE. The data obtained were analyzed by a 1-way ANOVA with drugs and doses treated equally. Scheffé's test was used as a *post hoc* analysis to compare differences between individual groups. Criteria for significance was set at the .05 level. As can be

seen in figure 2, there were significant main effects of drugs in all three brain areas ($F = 52.347$, $P < .0001$ for medial striatum; $F = 60.935$; $P < .0001$ for lateral striatum; $F = 11.745$, $P < .0001$ for nucleus accumbens). When 10 mg/kg of METH was used, 4 mg/kg of DADLE completely and significantly attenuated the DAT loss in the medial striatum ($P < .05$; fig. 2A) but only partially attenuated the DAT loss in the lateral striatum ($P < .05$; fig. 2B). Naltrexone at both 0.1- and 1.0-mg/kg doses completely blocked the effects of DADLE in both aspects of striata ($P < .05$; fig. 2A and B). In the nucleus accumbens, the DAT level was reduced significantly (25%) by METH ($P < .05$) when compared to saline controls. DADLE blocked the DAT loss caused by METH in the nucleus accumbens because animals receiving "DADLE+METH" exhibited a DAT level not different from that seen in the saline controls ($P = .99$). However, the DAT level in the "DADLE+METH"-treated animals did not differ from that receiving METH alone ($P = .23$). Naltrexone at both 0.1 and 1.0 mg/kg did not appear to affect the effect of DADLE in the nucleus accumbens: animals receiving "naltrexone+DADLE+METH" showed a DAT level not different from those receiving "DADLE+METH" ($P = .99$).

The striatal contents of DA, DA metabolites (DOPAC and HVA), 5-HIAA and striatal TH enzymatic activity were measured. Data from the neurochemical analysis were analyzed by a 1-way ANOVA (General Linear Models Procedure) examining the overall significance of drug effect. *Post hoc* analysis was done by using the Scheffé's test with the significance level set at $P < .05$. For DA, DOPAC and HVA there were significant main effects of drug treatment (DA: $F = 25.25$, $P < .0001$; DOPAC: $F = 17.51$, $P < .0001$; HVA: $F = 8.88$, $P < .0001$). However, there was no significant drug effect on the level of HIAA ($F = 1.61$, $P = .1545$). METH (10 mg/kg, i.p., four injections at 2-hr intervals) depleted striatal DA by approximately 60% ($P < .05$) and its metabolites DOPAC and HVA by 25 to 40% ($P < .05$) (table 1) when compared to controls. The reduced levels of DA and its metabolites caused by METH were not affected by pretreatment with DADLE ($P = .3396$; table 1). The level of 5-HIAA was not affected by METH ($P = .2219$) or DADLE ($P = .3439$) treatment (table 1). The HIAA levels in the METH group was not significantly different from those in the DADLE+METH group ($P = .6781$; table 1). In the TH enzymatic assays, the TH activity was significantly altered by the drug treatment ($F = 32.24$, $P < .0001$). In the METH-treated animals, the TH activity was reduced to 45% of that of the controls ($P < .05$; table 2). DADLE pretreatment did not prevent the reduction of TH activity caused by METH ($P = .8149$; table 2). The DADLE treatment per se did not significantly alter the TH activity ($P = .1478$; table 2).

Because the pharmacological effects of METH have been

TABLE 1
Changes of DA, DOPAC, HVA, and HIAA levels in striata

Treatment	DA	DOPAC	HVA	HIAA
Saline + saline (7)	100.00 \pm 9.24	100 \pm 5.49	100 \pm 6.81	100 \pm 12.17
DADLE + saline (7)	99.43 \pm 5.12	123.03 \pm 6.13 ^a	116 \pm 8.01	89.79 \pm 6.50
Saline + METH (7)	35.53 \pm 3.98 ^a	58.33 \pm 4.85 ^a	74.51 \pm 7.80 ^a	86.78 \pm 4.37
DADLE + METH (8)	31.80 \pm 6.45 ^a	47.80 \pm 4.42 ^a	69.54 \pm 4.19 ^a	91.10 \pm 6.3

CD1 mice received 4 mg/kg of DADLE (i.p.) before each i.p. injection of 10 mg/kg of METH which was given four times in a day at 2-hr intervals. Animals were killed 2 wk later and striata removed for analysis of DA, DOPAC, HVA and HIAA by HPLC with electrochemical detection as described in "Materials and Methods." Results are mean \pm S.E.M. percentages of the following control group (i.e., saline + saline) values (in ng/mg protein): DA, 101.39 \pm 9.37; DOPAC, 36.96 \pm 2.02; HVA, 21.46 \pm 1.46; HIAA, 4.49 \pm 0.54. Number of animals per group is indicated in parentheses.

^a $P < .05$ compared to the saline + saline.

TABLE 2
Striatal tyrosine hydroxylase activity

Treatment	TH Activity (pmol/mg Protein/min)
Saline + saline (5)	17.44 ± 0.99
Saline + METH (5)	7.88 ± 0.65 ^a
DADLE + saline (5)	15.50 ± 1.08
DADLE + METH (5)	7.58 ± 0.80 ^a

CD1 mice received 4 mg/kg of DADLE (i.p.) before each i.p. injection of 10 mg/kg of METH which was given four times in a day at 2-hr intervals. Animals were killed 2 wk later and striata removed for analysis of TH activity after the previously described methods (Reinhard *et al.*, 1986; Vrana *et al.*, 1992). Numbers in parentheses indicate numbers of animals examined.

^a P < .05 compared to the saline + saline group.

associated with increased body temperature and some drugs that show protective effects against METH-induced toxicity have been shown to prevent METH-induced increase in temperature (Bowyer *et al.*, 1994), we evaluated whether DADLE could affect METH-induced changes in temperature. Data were analyzed by a 2-way ANOVA with time and drug treatment considered as factors. *Post hoc* analysis was done by Scheffé's test with the significance level set at P < .05. There were significant main effects of drugs (F = 9.499, P < .0001) and time (F = 50.358, P < .0001). METH (4 × 10 mg/kg) significantly caused an elevation in rectal temperature of approximately 1.5°C when compared to saline-saline controls (P < .006) (fig. 3). The effect of DADLE alone was not different from saline-saline controls (P = .9667). DADLE did not affect the temperature change caused by METH: No difference was found in body temperature between the METH-treated animals and those receiving a combined treatment of DADLE and METH (P = .9993) (fig. 3). Animals receiving DADLE-METH combination exhibited body temperatures significantly higher than those receiving DADLE alone (P < .002) (fig. 3).

DADLE was examined for a potential antioxidative property in this study because in the nucleus accumbens the effects of DADLE appeared to be not affected by naltrexone (see fig. 2C). These results suggested that DADLE might protect tissue against METH insult in the nucleus accumbens

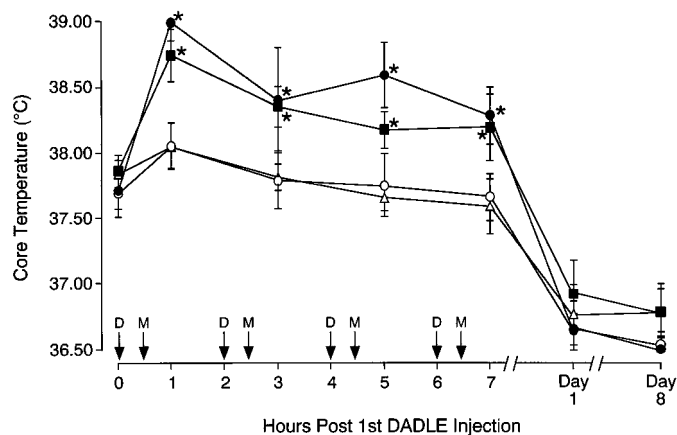


Fig. 3. Time-course curves comparing the effect of DADLE (4 mg/kg, i.p.) on METH- (10 mg/kg, i.p.) induced changes on rectal temperature. Arrows indicate time points when DADLE (D) and METH (M) were administered. Rectal temperature was measured 30 min after each METH injection as well as on days 1 and 8 post-DADLE treatment. The ambient temperature was 21°C. Each data point represents the mean ± S.E.M. of six animals. Open triangles, Saline+saline; open circles, DADLE+saline; closed circles, saline+METH; closed squares, DADLE+METH. * P < .05 compared to saline+saline group on the same time point of measurement.

via a nonopioid mechanism. Because the neurotoxicity of METH involves ROS, it was decided therefore to examine a possibility that DADLE might act as a free radical scavenger *in vitro*. In antioxidative experiments *in vitro*, DADLE inhibited the formation of superoxide anions produced by xanthine oxidase. Hirayama and Yida's method (1997) was used to estimate IC₅₀ values. The DADLE's inhibitory potency (IC₅₀ = 101.3 ± 4.9 μM; N = 9) for superoxide formation was not significantly different from that of GSH (IC₅₀ = 77.9 ± 4.7 μM; N = 3; P = .08, Student's *t* test) (fig. 4). DADLE dose-dependently inhibited the formation of hydroxyl radicals induced by ferrous citrate (25 μM) that were trapped by sodium salicylate (1 mM) and detected as 2,3- and 2,5-DHBA (fig. 5). The inhibitory potency of DADLE (IC₅₀ = 447.1 ± 42.7 μM; N = 5) for hydroxyl radical formation was not significantly different from that of GSH (IC₅₀ = 366.6 ± 15.9 μM; N = 3; P = .259, Student's *t* test) (fig. 5). DADLE (IC₅₀ = 1012 ± 64.9 μM; N = 3) inhibited FeSO₄/ascorbate-induced brain lipid peroxidation with a potency close to but significantly less than that of GSH (IC₅₀ = 755.7 ± 29.7 μM; N = 3; P < .037, Student's *t* test) (fig. 6). DADLE did not affect the activity of hydrogen peroxide whereas catalase dramatically reduced the concentration of hydrogen peroxide (fig. 7).

Discussion

This study demonstrates for the first time that an opioid peptide can block METH-induced DAT loss in the brain. Because the protection of DADLE against METH-induced loss of DAT in the striata was antagonized by opioid antagonist naltrexone (fig. 2 A and B), it is likely that DADLE exerts the protective effect in the striata via an opioid receptor mediated mechanism. However, the effect of DADLE in the nucleus accumbens appeared to be unaffected by naltrexone (fig. 2C). The latter suggests that DADLE might possibly exert the protective effect in the nucleus accumbens via a nonopioid mechanism. Because our *in vitro* experiments in-

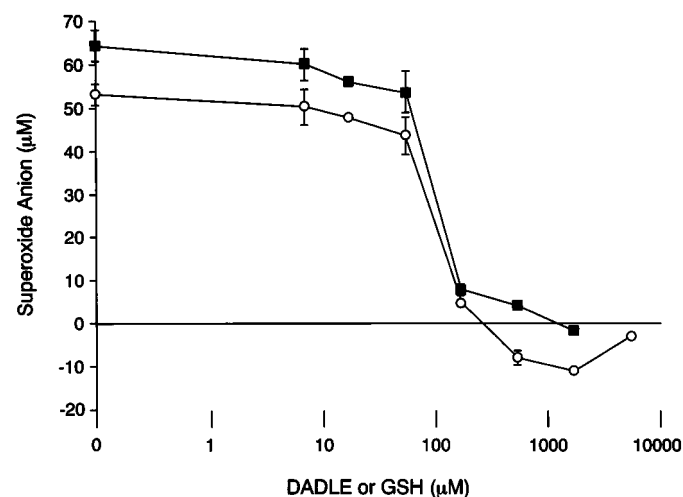


Fig. 4. DADLE inhibits superoxide anion formation *in vitro*: Comparison of potency with GSH. Superoxide anions were generated by the hypoxanthine and xanthine oxidase system. The generation of superoxide anions were followed spectrophotometrically by measuring the reduction of ferric ions using bathophenanthroline disulfonic acid as a chelator (see "Methods"). Data shown are mean ± S.E.M. from one representative experiment assayed in triplicate. The experiment was performed at least three times with similar results. Closed squares, DADLE; open circles, GSH.

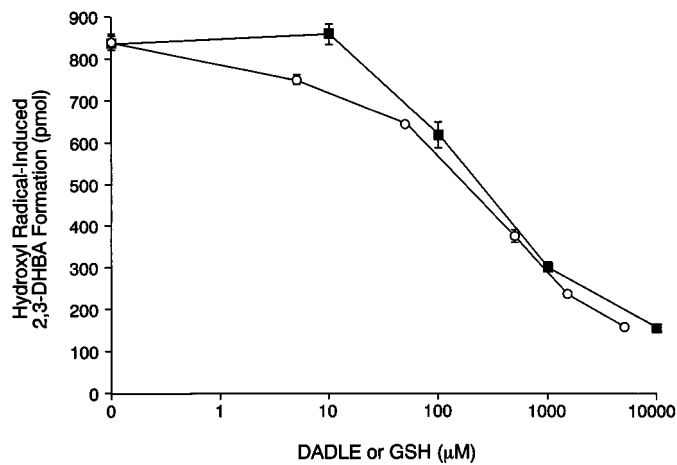


Fig. 5. Comparison of the antioxidative effects of DADLE and GSH on the generation of hydroxyl radicals caused by ferrous citrate. Hydroxyl radicals generated by 25 μM ferrous citrate were trapped by using 1 mM sodium salicylate (1 ml). Hydroxyl adducts of salicylate such as 2,3-DHBA were assayed by a HPLC-EC procedure (see "Methods"). Freshly prepared GSH (open circles) or DADLE (closed squares) were added to the hydroxyl radical generating and trapping solution and incubated at 34°C for 4 hr. Results depicts the generation of hydroxyl radicals as reflected by the production of 2,3-DHBA in a typical experiment assayed in triplicate. The experiment was performed at least three times with similar results.

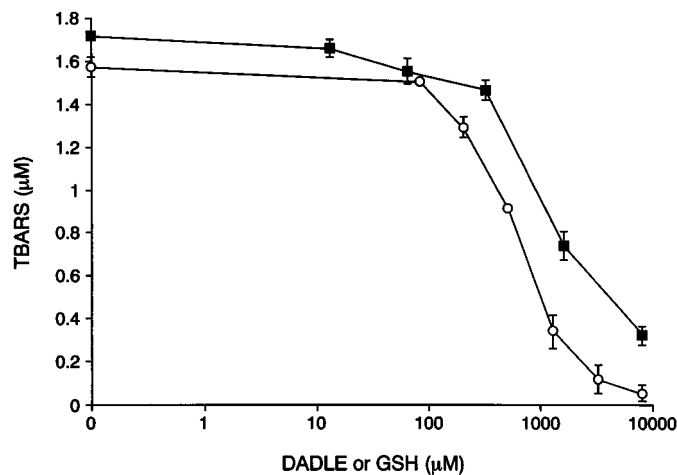


Fig. 6. Relative potency of DADLE and GSH on the inhibition of iron/ascorbate-induced brain lipid peroxidation. FeSO_4 /ascorbate-induced lipid peroxidation in brain homogenates was measured by assaying the thiobarbituric acid (TBA) reactive substances (TBARS) of the lipid peroxidation products (see "Methods"). DADLE (closed squares) and GSH (open circles) were added into the reaction mixture just before the addition of FeSO_4 /ascorbate. Results depicts the formation of TBARS in a typical experiment assayed in triplicate. The experiment was performed at least three times with similar results.

dicates that DADLE can act as a free radical scavenger and because it is known that METH-induced neurotoxicity involves ROS, it is tempting to speculate that DADLE might, at least in part, exert its tissue protective effect through the sequestration of free radicals. However, further demonstration that DADLE may sequester METH-induced free radical formation *in vivo* must be carried out to confirm this speculation.

Our results demonstrating the differential antagonism of naltrexone in the nigrostriatal *vs.* mesolimbic DA terminals are consistent with previous reports indicating a differential distribution of functional *delta* opioid receptors in the nigro-

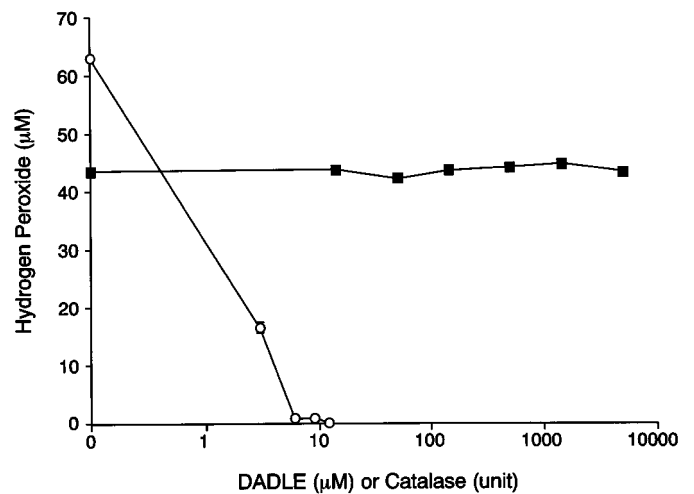


Fig. 7. The effect of DADLE on hydrogen peroxide. The concentration of hydrogen peroxide was determined spectrophotometrically by using the mixture of titanium (IV) and 4-(2-pyridylazo)resorcinol (see "Methods"). Catalase was used as a control. Data are mean \pm S.E.M. from a representative experiment assayed in triplicate. The experiment was performed three times with similar results. Closed squares, DADLE; open circles, catalase.

striatal and mesolimbic systems. For example, Petit *et al.* (1986) found that a *delta* opioid receptor modulation of [^3H]DA release is absent in the nucleus accumbens and concluded that in comparison with the striatum, mesolimbic DA terminals were devoid of a functional presynaptic *delta* opioid receptors. Jiang and North (1992) also found that activation of *delta* opioid receptors led to decreased activity of GABAergic neurons in the striatum but not in the ventral tegmental area (Johnson and North, 1992). These differential distribution of functional *delta* opioid receptors in the nigrostriatal *vs.* mesolimbic systems may explain our observation that naltrexone can antagonize the protective effects of DADLE in both aspects of striata but not in the nucleus accumbens (fig. 2). It is worth mentioning that the METH-induced DAT loss in the lateral striatum was more difficult to block by DADLE (fig. 2B) when compared to the medial striatum (fig. 2A). It is also interesting to note that *delta* opioid receptors are 25% higher in density in the dorsolateral striatum when compared to the ventromedial striatum (Benfentani *et al.*, 1991). These results, when taken together, suggest a possibility that the dorsolateral striatum is more sensitive to the administration of psychostimulant when compared to the ventromedial striatum and that the lateral aspect of striatum may be more sensitive to oxidative stress. In fact, consistent with this speculation, it has been reported that administration of cocaine or amphetamine alters the regional expression of neuropeptides within striata. Greater elevation of mRNA expression of striatal neuropeptides was found in the dorsolateral striatum than in the ventromedial striatal region (Hurd and Herkenham, 1992). In view of the differential effects that DADLE appeared to exert in the lateral and medial aspects of striata and in the nucleus accumbens, it would be useful in future experiments to correlate the DAT levels with the DA contents in those three separate regions in future experiments. The lateral and medial aspects of striata were not separated for neurochemical analyses in our study.

The exact mechanism by which DADLE, via opioid recep-

tors, protects against METH-induced damage on DA terminals is unknown. Because DA over-release is an underlying cause for the METH-induced neurotoxicity and because it is known that opioid receptors are related to the modulation of presynaptic DA release, it is tempting to speculate that DADLE might block the neurotoxicity of METH by attenuating the DA release via opioid receptors. This speculation, however, is against a conventional understanding. It is thought that the opioid receptor-mediated modulation of DA release would be an impulse-dependent event and that the METH-induced DA overflow is an impulse-independent event. As such, an opioid receptor mediated modulation should not interfere with METH-induced DA release. However, in a microdialysis study examining the striatal DA content, it was reported that opioid receptor mediated mechanism can attenuate the amphetamine-induced DA release, which is presumably quite similar to the action of METH (Schad *et al.*, 1996). A possibility exists therefore that, against conventional understanding, DADLE might attenuate the METH-induced insult by the modulation of DA release. It has to be noted that our results showing that DADLE is neuroprotective are in a way in direct contrast to the above-mentioned report (*i.e.*, Schad *et al.*, 1996) showing that it is the *delta* opioid antagonist, not the agonist, that attenuates the striatal DA increase evoked by amphetamine. However, consistent with our speculation made above, another report (Schlösser *et al.*, 1995) has shown that DADLE can decrease presynaptic DA release via opioid receptor-mediated mechanism in striatal slices (Schlösser *et al.*, 1995). Further, it was reported that naloxone, an opioid antagonist, "potentiates" (in lieu of attenuating) amphetamine-induced behavior (Feigenbaum *et al.*, 1984). Certainly, further experiments are needed to clarify the opioid receptor-mediated mechanism underlying the protective action of DADLE.

This study constitutes the first report that DADLE, an opioid, can act as a free radical scavenger *in vitro*. In fact, the authors are not aware of a free radical scavenging property of any opioid. At present, the exact chemical nature underlying the free radical scavenging property of DADLE is unknown. Although it remains to be determined whether DADLE will sequester free radicals *in vivo*, previous reports with the isolated preparation of hearts (Bolling *et al.*, 1997) and lungs (Oeltgen *et al.*, 1996) have suggested that DADLE, *ex vivo*, might affect the oxidative state of tissues. Direct monitoring of the free radical sequestration examined *in vivo* using DADLE and METH administrations may provide direct support to our speculation. Nevertheless, the demonstration of the opioid and the nonopioid nature of the protective effect of DADLE is in keeping with the complex nature of neurodegenerative processes that appear to affect different regions of the brain differentially. It is worth mentioning that our results directly demonstrating a free radical scavenging property of DADLE *in vitro* lend support to a notion that the antioxidative property of DADLE may play an important role in its action in prolonging organ survival as reported in previous studies (Chien *et al.*, 1994; Oeltgen *et al.*, 1996; Bolling *et al.*, 1997). Thus, DADLE, or by extension, the endogenous *delta* opioid peptide enkephalin may represent one of nature's tissue protective agents both in the periphery and in the CNS via perhaps a direct action at the opioid receptor or by acting as a free radical scavenger. In this regard, it is important to point out that although DADLE

sequesters the formation of superoxide anions, the opioid antagonist naltrexone does not affect this superoxide anion sequestering property of DADLE and that naltrexone by itself does not sequester superoxide anions (Tsao L-I and Su T-P, unpublished results). Because DADLE's protective effect against METH-induced DAT loss in the nucleus accumbens appeared to be resistant to naltrexone antagonism (fig. 2C) and because, as mentioned earlier, there is an apparent lack of functional delta opioid receptors in the nucleus accumbens, it is possible that METH-induced neurotoxicity in the nucleus accumbens is mediated mainly via, for example, free radical formation. Additionally, because we speculate that DADLE serves as a free radical scavenger in attenuating the METH-induced DAT loss, it is interesting to note that PTBN, a spin trapping agent, has been reported to attenuate the METH-induced DA neurotoxicity in rats (Cappon *et al.*, 1996). As with DADLE, PTBN did not affect the METH-induced hyperthermia. However, unlike DADLE, PTBN at 60 mg/kg can partially attenuate the METH-induced DA content loss in the striatum (from 55 to 22% loss). The reason for such a difference is unknown. Unfortunately, the effect of PTBN on DAT loss induced by METH was not examined in the PTBN study, making a further comparison between the effects exerted by DADLE and PTBN difficult. Whether DADLE serves as directly as a spin trapper remains to be examined. Our results with the antioxidative property of DADLE is in direct contrast with results of a study using human neutrophils. Haberstock and Marotti (1995) demonstrated that DADLE increases superoxide anion formation in human neutrophils and that the production of free radicals is mediated by *delta* opioid receptors. The results of Haberstock and Marotti's study (1995) would implicate that DADLE might damage tissues. However, we have shown that DADLE is tissue protective in both the periphery and the CNS (this study). Further examinations are needed to clarify these discrepancies.

The protection of DADLE against METH-induced DAT loss is not due to a regulation of body temperature. Decreasing animal's body temperature protects them against METH-induced toxicity (Ali *et al.*, 1994). METH increases body temperature (Bowyer *et al.*, 1994) whereas DADLE can increase or decrease body temperature depending on the route of administration and condition the animals were handled (Appelbaum and Holtzman, 1986). Nevertheless, our results indicate that METH-induced elevation in body temperature was not altered by DADLE in this study (see fig. 3).

It is possible that the protection of DADLE against the METH-induced neurotoxicity is centrally mediated. DADLE has been demonstrated to cross the blood brain barrier and has a higher brain uptake index value and a greater penetration than GABA, alanine, serine and D-tyrosine (Kastin *et al.*, 1976). DADLE has also been demonstrated to be transported across the blood-brain barrier by peptide transport systems (Banks and Kastin, 1990). Thus, DADLE might exert its protective effect against METH-induced damage via a central action.

Our results indicating that DADLE blocked the DAT loss whereas it did not affect the reduction of DA content and TH activity caused by METH are interesting and may provide new insight for the definition of METH neurotoxicity. The traditional concept of METH neurotoxicity is that the DAT loss is always accompanied by the reduction of DA content

and TH activity and vice versa. However, our results suggest that this may not always be the case. Our results indicate that protection against DAT loss does not necessarily result in protection against depletion of DA content and reduction of TH activity. Our results also tend to suggest that the DA depletion and the terminal membrane destruction caused by METH are mediated by separate biochemical events and that the depletion of DA caused by METH is not due to a breakdown, or presumably a "leak," of the terminal membrane. This study constitutes the first report indicating the dissociation of those two closely related effects caused by METH. Level of synaptic DA content is largely determined by the rates of synthesis and release or overflow of DA. Our results suggest that DADLE could not alter all or the rate-limiting step of the DA production system. It is unknown at present why DADLE can protect DAT on the plasma membrane against the METH-induced insult whereas it fails to protect the DA depletion in the striatal terminal. As such, the normalcy of DAT level in the striatal area may not suggest an integrity of the DA terminal because the terminal DA content could still be reduced even though the DAT level remains normal. We do not know at present whether the opioid receptor mediated mechanism *vs.* the free radical scavenging property exerted by DADLE might attribute to the discrepancy between the DA content and the DAT level. It is unlikely that the observed discrepancy caused by DADLE is attributed to the sampling of brain slices. The brain slices used for each autoradiographic examination in this study were chosen (see "Methods") so that they encompassed the whole rostral to caudal aspect of each region under study. Behaviorally, it has to be mentioned that animals showing normal DAT levels by receiving the DADLE+METH treatment, were sensitive to touching just like animals receiving METH alone (Tsao L-I and Su T-P, unpublished observation). The locomotor activity test using the activity chamber also did not show a dramatic difference between the METH animals and the DADLE+METH animals (Ladenheim B, Tsao L-I and Su T-P, unpublished observation). These observations suggest that DA contents is the determining factor for the observed behaviors and that the DAT level did not appear to affect the altered behavior caused by the depletion of DA.

In summary, using a metabolically stable analog of endogenous *delta* opioid peptide enkephalin, our study suggests that *delta* opioid peptide may represent a naturally occurring protective substrate against DA neuronal damage. When taken together with previous findings that DADLE is a protective agent in peripheral organs, our results suggest that perhaps one of the most important roles of endogenous opioid peptides might be tissue protection—both in the periphery and in the CNS. Because Parkinson's disease (PD) is a neurodegenerative disorder of the nigrostriatal DA system and because METH-induced DA terminal damage has been suggested as a model for PD (Walsh and Wagner, 1992), it is tempting to invoke a dysfunction of the opioid system in that disease. Because DADLE appears to maintain the integrity of DA terminals as indicated by our study, the combination of DADLE with L-dopa may serve as an interesting treatment paradigm for patients with PD.

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