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Blockade of dopamine transporter and tyrosine hydroxylase activity loss by [D-Ala^2, D-Leu^5]enkephalin in methamphetamine-treated CD-1 mice

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Abstract

[D-Ala^2, D-Leu^5]enkephalin (DADLE) has been previously reported to prolong the survival of tissues both in the periphery and in the central nervous system. Here, we show that DADLE was able to block the protein as well as the functional loss of dopamine transporter (DAT) and tyrosine hydroxylase (TH) induced by methamphetamine. Male CD-1 mice received four injections of methamphetamine (10 mg/kg, i.p.) at 2-h intervals. DADLE (4 mg/kg, i.p.) was given 30 min before each injection of methamphetamine. Western blotting and enzymatic assays showed that DADLE blocked the protein loss and functional impairment of DAT and TH induced by methamphetamine.

Keywords: DADLE ([D-Ala^2, D-Leu^5]enkephalin); Methamphetamine; Dopamine transporter

1. Introduction

[D-Ala^2, D-Leu^5]enkephalin (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 14–46 h — the longest in the history of organ preservation (Wu et al., 1996; Oeltgen et al., 1996). Further, DADLE can promote myocardial tolerance to ischemia in isolated hearts in a fashion far superior to the standard cardioplegic procedure (Bolling et al., 1997). Recently, DADLE was demonstrated in an autoradiographic study to protect against methamphetamine-induced dopamine transporter (DAT) loss in the brain (Tsao et al., 1998). Thus, DADLE may be protective in both the periphery and the central nervous system. To further investigate the protective properties of DADLE against methamphetamine-induced neuronal damage, we examined in this study the protein levels and the functional properties of DAT and tyrosine hydroxylase (TH) after the DADLE and methamphetamine treatments in CD-1 mice.

2. Materials and methods

2.1. Drug treatment

Male CD-1 mice received four injections of methamphetamine (10 mg/kg, i.p.) or saline at 2-h intervals. DADLE (4 mg/kg, i.p.) or saline was given 30 min before each methamphetamine administration. All drugs were prepared immediately before use and injected in a volume of 1 ml/100 g body weight. Mice were killed by cervical dislocation 2 weeks later. The striata were quickly dissected and used either fresh or frozen. Cares and treatments of animals were according to procedures approved by the Institutional Animal Care and Utilization Committee.

2.2. [^{125}I]RTI-121 binding assays

The frozen striata were homogenized in Na^+ buffer (10 mM Na^+ phosphate and 120 mM NaCl) with a Teflon/Glass Homogenizer and centrifuged at $750 \times g$ for 10 min at 4°C. The supernatants were further centrifuged at $14\,000 \times g$ for 20 min at 4°C. The pellets (P2 fraction) were resuspended in Na^+ buffer. Binding assays were

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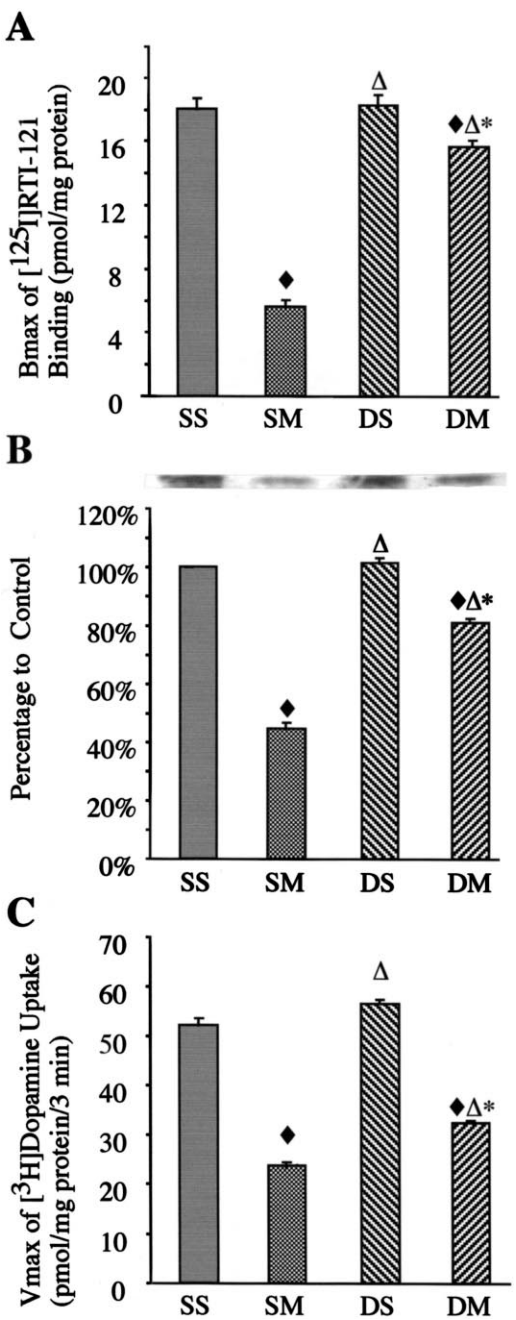
carried out at 25°C for 60 min in 0.2 ml of Na^+ buffer containing 1 mg of striatum, 125 pM of 3β-(4-[^{125}I]iodophenyl)tropane-2β-carboxylic acid isopropyl ester ([^{125}I]RTI-121; Boja et al., 1995), and various concentrations of unlabeled RTI-121 from 31 pM to 10 nM. The non-specific binding was defined by 100 μM (−)cocaine. The assays were terminated by filtering the tissue mixture through Whatman GF/B filters soaked previously in 0.05% polyethylenimine and washed three times with 5 ml of Na^+ buffer. Radioactivity was measured with a liquid scintillation spectrometry in the presence of 3.5 ml of Poly-Flour.



2.3. SDS / PAGE and Western blot analyses

The frozen striata were homogenized in 320 mM sucrose with a Teflon/Glass Homogenizer and processed as described above to obtain the P2 fraction. The pellets containing the P2 fraction were dissolved in lysis buffer (320 mM sucrose, 10 mM Tris–HCl, 1% Triton X-100, 0.2 mM DL-6-methyl-5,6,7,8-tetrahydropterine, 10 μg/ml aprotinin). Around 20 μg of protein were loaded onto 10% polyacrylamide containing sodium dodecyl sulfate for electrophoresis (SDS/PAGE), and transferred to nitrocellulose membranes (Amersham). Membranes were blocked for 1 h in Tris-buffered saline containing 5% non-fat dried milk and then probed with polyclonal anti-DAT or anti-TH antibodies (1:1000, Chemicon) at room temperature for 2 h. The proteins were detected with horseradish peroxidase-labeled secondary antibodies, followed by Enhanced Chemiluminescence Western Blotting Detection Reagent (Amersham). The protein levels were quantified by using a Macintosh computer-based analysis system (Image, NIH).

2.4. [^{3}H]dopamine uptake assays

The P2 fraction from brain was prepared as described above. Uptake assays were conducted by first mixing 50 μl of 20 mg/ml of striatal tissue, 50 μl 10 μM pargyline, 50 μl of water [or 1 mM (−)cocaine to define non-specific binding], and 300 μl of Kreb's phosphate buffer (126 mM NaCl, 4.8 mM KCl, 1.3 mM $CaCl_2$, 1.4 mM $MgCl_2$, 16 mM sodium phosphate, 2 mg/ml dextrose, and 0.2 mg/ml ascorbic acid). After preincubation at 30°C for 10 min, 50 μl of various concentrations of [^{3}H]dopamine (stock solutions from 14.2 to 4500 nM) were added into the assay tube. Thus, the final concentrations of [^{3}H]dopamine in the

Fig. 1. (A) Striatal DAT labeled by [^{125}I]RTI-121. Striatal homogenates were obtained from five mice in each group and incubated with 125 pM of [^{125}I]RTI-121 and various concentrations of unlabeled RTI-121 at room temperature for 60 min. Data were analyzed by the EBDA program using the "cold" Scatchard analysis. Values in the figure represent means ± S.E.M. of three independent determinations. (B) Western blotting showing that DADLE pretreatment inhibits the loss of DAT induced by methamphetamine in striatum. The striatal lysate from each individual mouse was separated by 10% SDS/PAGE, transferred to nitrocellulose membrane, and detected with polyclonal anti-DAT antibodies. Data are expressed as percentages of the control (saline + saline) and are means ± S.E.M. of three separate independent determinations. Note: One-way ANOVA was used to analyze the overall significance. (C) The effect of DADLE on the methamphetamine-induced decrease of [^{3}H]dopamine uptake in striatum. Dopamine uptake was assayed by incubating fresh striata from five mice in each group with [^{3}H]dopamine in final concentrations from 1.42 to 450 nM. Data were analyzed by the EBDA program. Two-way ANOVA was used to analyze the overall significance ($F = 6.473$, $P = 0.034$). Scheffe's test was employed as the post hoc test. Values are means ± S.E.M. of three independent determinations. SS, saline + saline; SM, saline + methamphetamine; DS, DADLE + saline; DM, DADLE + methamphetamine. ♦ $P < 0.05$ compared to SS; △ $P < 0.05$ compared to SM; * $P < 0.05$ compared to DS.

assay were (in nM): 1.42, 4.5, 14.2, 45, 142, 450. Determination of the trapped radioactivity on the filter was performed in the same manner as described above for [^{125}I]RTI binding. By using 0.05% polyethylenimine to presoak the filters, no specific binding of [^{3}H]dopamine to the filter was detected.

2.5. TH enzymatic assays

The measurement of TH enzymatic activity was based on the stoichiometric release of [^{3}H]H_2O from L-[3,5-^{3}H]tyrosine (Reinhard et al., 1986). The fresh striata were homogenized with a Teflon/Glass Homogenizer in 10 mM Na^+ phosphate, monobasic, containing 0.2% Triton X-100. For each reaction, 25 μl of striatal homogenates were mixed with 25 μl of reaction solution (100 μM tyrosine, 2 mM DL-6-methyl-5,6,7,8-tetrahydropterine, 4 mM DL-dithiothreitol, 5 μg/μl catalase, and 1 μCi/reaction of L-[3,5-^{3}H]tyrosine). After incubation at 37°C for 20 min, 500 μl of 7.5% charcoal in 1 M HCl was added to adsorb L-tyrosine and L-dihydrophenylalanine. The mixture was vortexed thoroughly and the charcoal was removed by centrifuging at $14\,000 \times g$ for 5 min twice. Radioactivity was counted by adding 3.5 ml of Poly-Flour to 300 μl of resulting supernatant. Blank values were obtained by performing the reaction in the absence of DL-6-methyl-5,6,7,8-tetrahydropterine, DL-dithiothreitol and catalase. The fraction of water recovered (generally 90%) was determined and was used to calculate TH activity for each reaction.

2.6. Statistical analyses

Data were analyzed by either a one-way analysis of variance (ANOVA) or a two-way ANOVA examining the overall significance. Post hoc analyses were performed by using the Scheffe's test with the significance level set at $P < 0.05$.

3. Results

In agreement with the autoradiographic studies using brain slices, [^{125}I]RTI-121 binding assays using brain homogenates indicated that the DAT level was largely reduced by the methamphetamine treatment (31% of the control; Fig. 1A). There is only one binding site found in the present study. DADLE almost completely blocked the methamphetamine-induced DAT loss (87% of the control; Fig. 1A). The K_d values of the [^{125}I]RTI-121 binding remained unaltered in all treatment groups (1.36 ± 0.07 nM for SS; 1.35 ± 0.04 nM for SM; 1.30 ± 0.07 nM for DS; 1.37 ± 0.07 nM for DM; $N = 3$). Western blotting showed a decrease of DAT protein after the methamphetamine treatment (44% of the control; Fig. 1B). DADLE significantly blocked the DAT protein loss induced by methamphetamine (81% of the control; Fig. 1B). [^{3}H]Dopamine uptake assays using fresh tissues indicated that [^{3}H]dopamine uptake (i.e., V_{max}) was compromised after the methamphetamine treatment (45% of the control; Fig. 1C). DADLE also significantly blocked the DAT functional loss induced by methamphetamine (62% of the control; Fig. 1C). The drug treatments did not alter the K_m value of the [^{3}H]dopamine uptake ($K_m = 45.51 \pm 0.39$ nM for SS; 43.98 ± 1.44 nM for SM; 44.37 ± 1.59 nM for DS; 43.89 ± 0.66 nM; $N = 3$).

The TH protein level indicated by Western blotting showed a close to 70% decrease in methamphetamine-treated animals (31% of the control; Fig. 2A). DADLE near completely blocked the TH protein loss induced by methamphetamine (85% of the control; Fig. 2A). The TH enzymatic assays using fresh tissues showed that the TH enzymatic activity was reduced by methamphetamine treat-

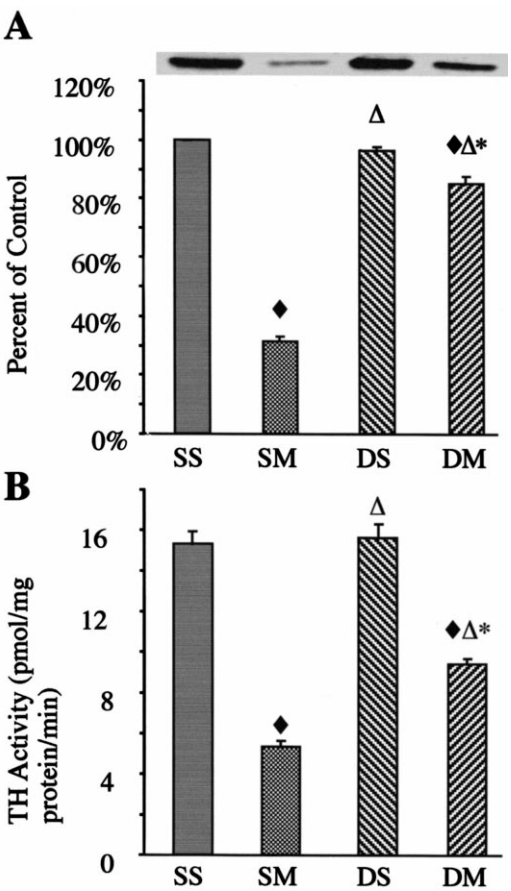


Fig. 2. (A) Western blotting showing the DADLE prevention of TH loss induced by methamphetamine. The striata from each individual mouse ($N = 5$/group) was assayed. See Fig. 1B for details of the Western blotting. The detection was performed using polyclonal anti-TH antibodies. Data are expressed as percentages of the control (saline + saline) and are means ± S.E.M. of three independent determinations. (B) The effect of DADLE on the decrease of striatal TH activity induced by methamphetamine. The fresh striata were pooled from five mice in each group and assayed according to procedures described in Section 2. Values represent means ± S.E.M. of three independent determinations. SS, saline + saline; SM, saline + methamphetamine; DS, DADLE + saline; DM, DADLE + methamphetamine. ♦ $P < 0.05$ compared to SS; △ $P < 0.05$ compared to SM; * $P < 0.05$ compared to DS.

ment (34% of the control; Fig. 2B). DADLE was able to partially block the TH activity loss induced by methamphetamine (62% of the control; Fig. 2B).

4. Discussion

DADLE is thus able to block not only the DAT and TH protein loss induced by methamphetamine, but also able to block the functional loss of those two proteins rendered by the methamphetamine treatment. Regarding the DAT protein level, the present results, either from the homogenate binding assays using [^{125}I]RTI-121 or from the Western blotting using specific antibodies, are in agreement with our previous results from autoradiographic studies (Tsao et al., 1998). All showed a dramatic loss of DAT induced by methamphetamine and an almost total blockade of the DAT level by DADLE. Our results with the TH apparently indicate that the TH protein loss induced by methamphetamine treatment could also be blocked by DADLE.

DADLE not only block the DAT and TH protein loss but can also block the functional loss of these two proteins. The present results with TH activity is however in contrast to our previous report indicating that the TH activity loss induced by methamphetamine could not be blocked by DADLE (Tsao et al., 1998). The drug treatment paradigms in the two studies are exact the same. The discrepancy may have arisen from the fact the present study used fresh brain tissue right after the animals were killed. The previous study used frozen tissues. At any rate, by using fresh tissues we now provide evidences that the functional activities of DAT and TH are protected in DADLE-treated animals against the methamphetamine-induced insult. It is noteworthy that for both the DAT and TH, the degree of protein loss blocked by DADLE is always slightly more in magnitude than the degree of functional loss blocked by DADLE. The ratio of the DAT protein level (Western blot) is 1.84 when comparing the DADLE + methamphetamine group with the saline + methamphetamine group (Fig. 1B). However, the respective functional ratio in the two groups is 1.37 (Fig. 1B). Similarly, the ratios for the TH protein level and activity level are 2.74 and 1.79, respectively in groups as indicated above. These differentials in the protein and activity levels suggest that the DADLE cannot fully block the functional loss induced by methamphetamine and that methamphetamine may exert some as yet unknown changes on the molecular properties of DAT and TH that could not be prevented by DADLE pretreatment. Further studies are needed to clarify the exact nature of these changes exerted by methamphetamine, if any.

Given the fact that both the protein levels and the activities of DAT and TH were preserved by DADLE in methamphetamine-treated animals, one would expect that the dopamine content in the DADLE-treated animals would also be preserved despite the methamphetamine insult. Our previous results from studies using exactly the same drug treatment paradigms, however, indicated that the striatal dopamine loss induced by methamphetamine was not blocked by DADLE (Tsao et al., 1998). Taken together, these results suggest that, although the DAT and TH protein levels were almost completely preserved by the DADLE pretreatment, DADLE was unable to restore the functional properties (from 45% to 62% for DAT; from 34% to 61% for TH) to such a magnitude that might significantly restore the dopamine content destructed by the repetitive high-dose insults from methamphetamine over a period of more than 6 h.

Mechanisms of the DADLE protection against methamphetamine-induced insults are not totally clear. Methamphetamine induces many changes in neurons, involving formation of free radicals (Albers and Sonsalla, 1995; Cadet et al., 1994; Cubells et al., 1994; Imam and Ali, 2000; Itzhak et al., 1998, 1999; Seiden and Vosmer, 1984; Yamamoto and Zhu, 1998), lipid peroxidation, transient biochemical alterations of DAT (Fleckenstein et al., 1997), increases in immediate early genes (Hayashi et al., 1999). DADLE at least can counteract certain actions of methamphetamine by acting as a free radical scavenger (Tsao et al., 1998) and, via an as yet unknown manner, by blocking the increase of c-*fos* expression induced by methamphetamine (Hayashi et al., 1999). DADLE not only protects against methamphetamine-induced neuronal damage, it can also protect against brain damage induced by 6-hydroxydopamine treatment in the rat (Borlongan et al., 2000). Further, DADLE can block the ischemia–reperfusion-induced cerebral damage caused by transient middle cerebral artery occlusion in the rat (Borlongan et al., 1998). Among those actions induced by DADLE, at least part of the protective effects of DADLE against methamphetamine-induced DAT loss has been shown to be sensitive to naltrexone and thus mediated via opioid receptors (Tsao et al., 1998). It is noteworthy that using the same treatment paradigm, our previous report has indicated that DADLE does not affect the basal body temperature in CD-1 mice nor does DADLE alter the increase of body temperature induced by methamphetamine (Tsao et al., 1998). Thus, the neuroprotective action of DADLE may not be attributed to an effect on body temperature. Apparently, elucidation of the exact mechanism(s) underlying the neuroprotective properties of DADLE may greatly facilitate the potential use of DADLE in combating certain neurodegenerative diseases such as Parkinsonism, Alzheimer's disease and aging.

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