Exacerbation of Methamphetamine-Induced Neurochemical Deficits by Melatonin[1]

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ABSTRACT

Methamphetamine (METH), administered in large, repeated doses, compromises the dopaminergic and serotonergic systems as indicated by prolonged suppression of tyrosine hydroxylase and tryptophan hydroxylase activity and concurrent decreases in the content of dopamine and 5-hydroxytryptamine. Because dopamine is necessary for these dopaminergic and serotonergic deficits we postulated that dopamine and/or its reactive metabolites are responsible for these degenerative alterations. Because we previously demonstrated that *in vitro* reducing conditions reverse the decrease in tryptophan hydroxylase activity, we reasoned that melatonin, a purported endogenous antioxidant, may alter this response. Rats were treated with METH and/or melatonin and trytophan hydroxylase activity and 5-hydroxytryptamine content were assessed; tyrosine hydroxylase activity and dopamine content were also measured. Not only did melatonin not prevent METH-induced deficits in serotonergic and dopaminergic parameters, but coadministration of melatonin with METH actually enhanced most of the monoaminergic effects of METH. This enhancing effect could not be attributed to alteration of body temperature. Because METH abuse causes insomnia and melatonin is promoted in some countries for insomnia, the implications of the interaction between these two drugs could be clinically important.

When administered in large, repeated doses, METH and its congeners, cause marked neurochemical deficits in certain dopaminergic and serotonergic nerve terminals of the brain; these include severely compromised activity of both TH (Koda and Gibb, 1973) and TPH (Hotchkiss and Gibb, 1980; Sanders-Bush *et al.*, 1972). A corresponding decline is also observed in the content of DA and its metabolites (Koda and Gibb, 1973; Seiden *et al.*, 1975/76). The concentration of 5HT and its metabolite is also decreased in serotonergic terminal fields (Bakhit *et al.*, 1981; Ricaurte *et al.*, 1980).

DA is essential for the METH-induced neurotoxicity, because the dopaminergic deficits are prevented when synthesis of DA is inhibited by α-methyl-*p*-tyrosine; the deficits return when DA synthesis is reinstated by concurrently administering L-DOPA (Gibb and Kogan, 1979; Hotchkiss and Gibb, 1980; Schmidt *et al.*, 1985). Not only are the dopaminergic deficits dependent on the presence of DA and/or its reactive metabolites, but METH-induced serotonergic alterations are also prevented when DA is depleted by treating rats with α-methyl-*p*-tyrosine or 6-hydroxydopamine prior to administering METH (Hotchkiss and Gibb, 1980; Johnson *et al.*, 1987). Because DA is oxidatively reactive and because the

METH-induced decrease in TPH activity is attributed to oxidative stress (Stone *et al.*, 1989), it is suggested that the neurotoxicity of this drug is linked to an increase in free radical formation (Kondo *et al.*, 1994; Cadet *et al.*, 1994; Giovanni *et al.*, 1995; Fleckenstein *et al.*, 1996).

Because we hypothesize that the neurochemical deficits induced by METH are attributed to oxidative stress associated with DA and/or its reactive metabolites and because melatonin is characterized as an endogenous antioxidant (Tan *et al.*, 1993; Reiter, 1995), we administered melatonin in combination with METH to determine whether melatonin would protect against the METH-induced neurochemical deficits. Rather than protecting, melatonin exacerbated the neurochemical alterations observed with METH.

Materials and Methods

Animals and treatments. Male Sprague-Dawley rats (Simonsen Laboratories Inc., Gilford, CA) weighing 200 to 240 g were housed (three to four per cage) in a temperature-controlled room (23°C) with a 12-hr light-dark cycle. Access to food and water was *ad libitum*.

In the first experiment rats received injections with METH (5 or 15 mg/kg, s.c.) or vehicle (0.9% NaCl) at 6-hr intervals for a total of five administrations. Fifteen min before, and 2 hr after the METH injections, the rats received injections with melatonin (25 mg/kg, i.p., Sigma Chemical Co., St. Louis, MO) or vehicle (30% ethanol in

ABBREVIATIONS: DA, dopamine; 5HT, 5-hydroxytryptamine; 5-HTP, 5-hydroxytryptophan; 5-HIAA, 5-hydroxyindoleacetic acid; METH, methamphetamine; TPH, tryptophan hydroxylase; TH, tyrosine hydroxylase.

saline). Animals were killed by decapitation 18 hr after the last METH injection. The striatum, hippocampus and frontal cortex were quickly removed, frozen on dry ice and stored at -80°C until assayed. In a separate experiment, rats were injected as above with METH (15 mg/kg) and melatonin (2, 5, 10 or 25 mg/kg) and then killed. In a third experiment, rats were dosed as in the first experiment, but killed 1 wk after the last injection of METH. In a fourth experiment, the animals received only one injection of METH (15 mg/kg, s.c.) or vehicle and two injections of melatonin (25 mg/kg; i.p.) or vehicle 15 min before and 2 hr after METH. The animals were killed 3 hr after administering METH.

TPH assay. TPH activity was determined by measuring the formation of 5-HTP using high performance liquid chromatography with electrochemical detection as previously reported (Johnson *et al.*, 1992). Briefly, frozen tissues were weighed, homogenized using a Potter-Elvejhem homogenizer in ice-cold 50 mM 4-(2-hydroxyethyl)-1-piperazine-2-ethane-sulfonic acid buffer (Sigma Chemical Co.), pH 7.4 containing 0.2% Triton X-100 and 5 mM dithiothreitol (Calbiochem Corp., San Diego, CA). The homogenates were centrifuged at $40,000 \times g$ for 15 min at 4°C and duplicate 7.5-μl aliquots of the supernatant were assayed. Boiled supernatant was used for blanks. Five μl of a reagent mixture were added to each sample. Each aliquot of the reagent mixture contained the following nanomolar concentrations: 4-(2-hydroxyethyl)-1-piperazine-2-ethane-sulfonic acid buffer, 240; tryptophan, 10; m-hydroxybenzylhydrazine (NSD 1015, Sigma Chemical Co.), 5.8 and *dl*-6-methyl-5,6,7,8-tetrahydropterin (Sigma Chemical Co.), 17.5. After incubating for 30 min at 37°C, the reaction was terminated by transferring the tubes to an ice bath and

Fig. 1. Effect of melatonin on METH-induced deficit of TPH activity. METH (5 or 15 mg/kg, s.c.) was administered five times at 6-hr intervals. Melatonin (25 mg/kg, i.p.) was administered 15 min before and 2 hr after each administration of METH. Rats were killed 18 hr after the final administration of METH. Columns represent means ± S.E.M. of determinations in six rats (except in the combined METH (15 mg/kg) + melatonin where four rats were used); values are expressed as percent of control. *P ≤ .05 *vs.* control.

adding 100 μl of 0.2 N perchloric acid containing 32 ng of 5-HIAA as an internal standard. Tubes were then centrifuged at $1000 \times g$ for 15 min at 4°C and 5 μl of the supernatant were injected onto a 12.5-cm Partisphere C_{18} reverse-phase (RP) column (Whatman Inc., Clifton, NJ) equipped with a 1-cm RP guard column (Whatman Inc., Clifton, NJ). The mobile phase consisted of 0.15 M monochloroacetic acid buffer (pH 2.9) containing 2 mM disodium EDTA, 0.1 mM 1-octanesulfonic acid sodium salt (Eastman Kodak Co., Rochester, NY) and 12.5% methanol. 5-HTP and 5-HIAA were detected with a model LC-4B electrochemical detector from Bioanalytical Systems, Inc. (West Lafayette, IN) equipped with a glassy carbon electrode which was set at a potential of +0.6 V *vs.* an Ag/AgCl reference electrode. The concentrations were quantified by comparing the peak heights with those of known standards. The average TPH activity in control animals was 90, 138 and 111 nmol/g/h in the neostriatum, hippocampus and frontal cortex, respectively.

TH assay. TH activity was determined using a modification of the method described by Nagatsu *et al.* (1964). Tissue was weighed and homogenized in the same homogenization buffer used for the TPH assay. After centrifugation at $40,000 \times g$ for 15 min at 4°C, duplicate 10-μl aliquots of the supernatant were added to 40 μl of double-distilled water. After adding 50 μl of a reaction mixture, each sample contained 550,000 dpm of [3,5-^{3}H]tyrosine (54.2 Ci/mmol, New England Nuclear Research Products, Boston, MA), 10 nmol of tyrosine, 100 nmol of ferrous ammonium sulfate, 320 nmol of *dl*-6-methyl-5,6,7,8-tetrahydropterin (Sigma Chemical Co.), 10 nmol of β-mercaptoethanol and 20 μmol of sodium acetate. The radiolabeled tyrosine was previously purified on a column containing Dowex-50 resin (Sigma Chemical Co.) and stored in absolute ethanol at -20°C. The samples and reaction medium were incubated together for 15 min at 37°C after which the reaction was terminated by adding 1 ml of a 7.5% (w/v) charcoal suspension in 1 N HCl. The mixture was centrifuged for 30 min at $2000 \times g$ and an aliquot of the supernatant was counted in a liquid scintillation detector (Packard, 2000CA tri-carb, Downers Grove, IL). Activity was quantified by comparison with standards as described by Nagatsu *et al.*, (1964). In each of the figures, results are expressed as the mean ± S.E.M. percent of control. The average TH activity in control animals was 87 nmol tyrosine oxidized/g/h.

5-HT and DA assay. Concentrations of 5-HT were measured using high performance liquid chromatography with electrochemical detection. As described in the TPH assay, frozen tissues were weighed and homogenized using a Potter-Elvejhem homogenizer in ice-cold mobile phase (0.15 M monochloroacetic acid buffer (pH 2.9) containing 2 mM disodium ethylenediamine-tetraacetate, 0.1 mM 1-octanesulfonic acid sodium salt (Eastman Kodak Co.) and 12.5% methanol). The homogenates were centrifuged at $40,000 \times g$ for 15 min at 4°C and the supernatants were filtered through a 0.2-μm filter system (Bioanalytical Systems, Inc., West Lafayette, IN); 50 μl of the filtrate were injected onto a 12.5-cm Partisphere C_{18} reverse-phase column (Whatman Inc., Clifton, NJ) equipped with a 1-cm RP guard column (Whatman Inc.). DA and 5-HTP were detected with a model LC-4B electrochemical detector (Bioanalytical Systems, Inc., West Lafayette, IN), equipped with a glassy carbon electrode which was set at a potential of +0.73 V *vs.* an Ag/AgCl reference electrode. The concentrations were quantified by comparing the peak heights with those of known standards. In each of the figures, results are expressed as the mean ± S.E.M. percent of control. The average 5HT content in control animals (in ng/g tissue) was: neostriatum-523, hippocampus-709 and frontal cortex-758. The average DA content in the neostriatum of control animals was 6650 ng/g tissue.

Statistics. Data were statistically analyzed using analysis of variance and comparisons between means were performed by Fisher's protected least squares difference test. The unpaired two-tailed Student's *t* test was used to analyze differences between two groups. The differences were considered statistically significant when P < .05.
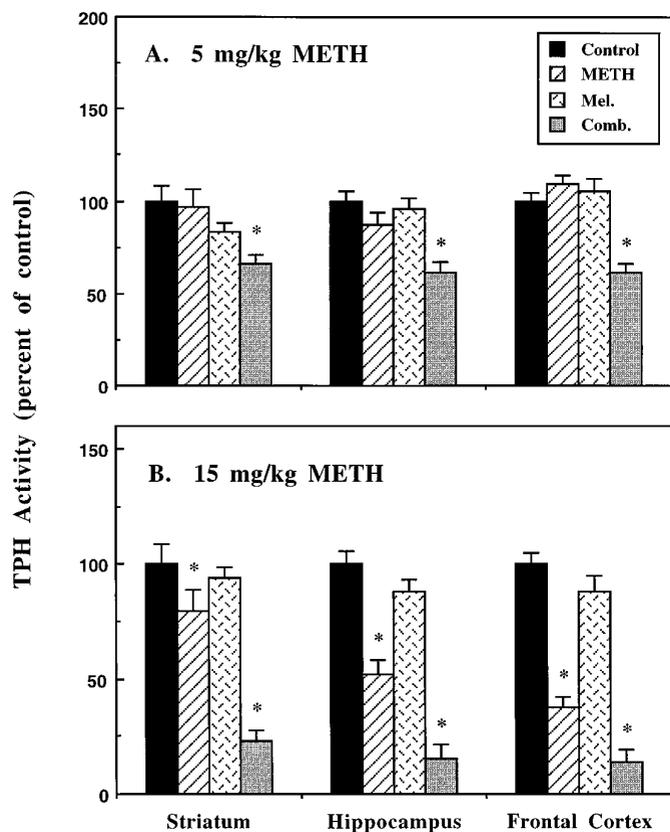
Results

The effect of melatonin on the TPH response to METH in three brain structures is depicted in figure 1. METH was administered subcutaneously at 5 (fig. 1A) or 15 (fig. 1B) mg/kg every 6 hr for five doses. Melatonin (25 mg/kg) was injected i.p. 15 min before and 2 hr after each of the 5 METH administrations; animals were killed 18 hr after the fifth administration of METH. At the lower dose of METH, TPH activity was not altered; nor did melatonin alone have an effect on enzyme activity. However, when melatonin was administered in combination with the lower dose of METH, TPH activity was decreased in the neostriatum, hippocampus and the frontal cortex. When the dose of METH was increased to 15 mg/kg, TPH activity was significantly compromised in all three brain regions and was further depressed when melatonin was combined with METH.

The content of 5HT in the neostriatum, hippocampus and frontal cortex after METH and/or melatonin is portrayed in figure 2. The experimental conditions were the same as those described above for figure 1B. The decline in the concentration of 5HT followed a similar pattern to that observed for TPH activity; the decrease in 5HT content observed after METH alone was dramatically exacerbated when melatonin was administered in combination with METH.

In figure 3, the experimental design was the same as for that described for figure 1B, except the animals were killed 1 wk after receiving the fifth dose of METH. Administration of METH alone decreased TPH activity in only the hippocampus although the combination of METH and melatonin significantly decreased TPH activity in all three brain structures. Melatonin alone decreased enzyme activity in the neostriatum and the hippocampus. The content of 5HT was depressed by METH alone only in the neostriatum although melatonin alone decreased the indoleamine content in the striatum and frontal cortex. The combination of both METH and melatonin decreased 5HT content in all three structures.

The acute response to METH and melatonin was then examined (fig. 4). A single dose of METH (15 mg/kg) was administered; melatonin (25 mg/kg) was given 15 min before
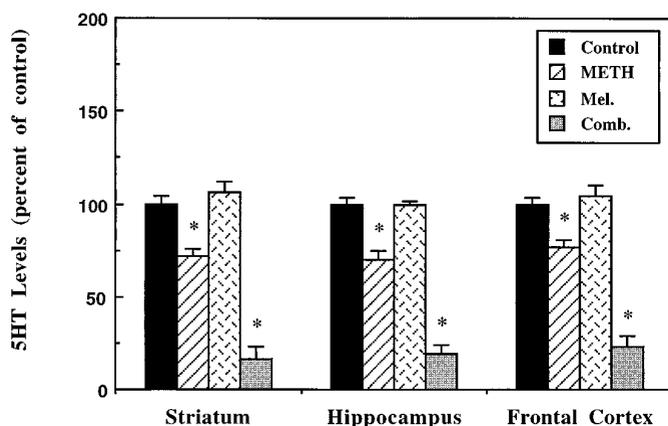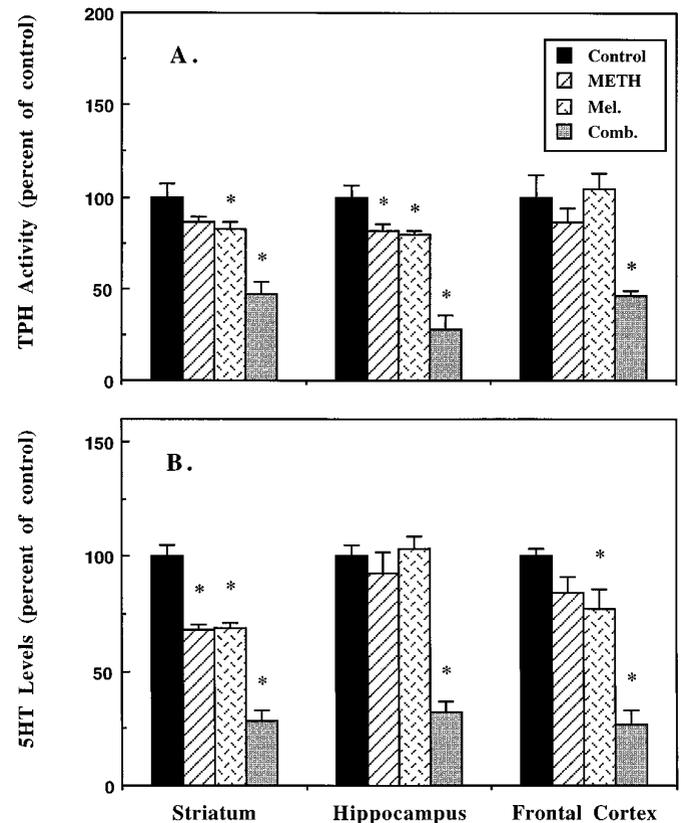
Fig. 3. Effect of melatonin on METH-induced deficit of TPH activity and 5HT content after 1 wk. Rats were treated in the same manner as described in figure 2, except the animals were killed 1 wk after the final administration of METH. Columns represent means ± S.E.M. of determinations in six rats; values are expressed as percent of control. *P ≤ .05 *vs.* control.

and 2 hr after the METH. Three hours after injecting METH, TPH activity was significantly depressed in all 3 brain areas; melatonin decreased enzyme activity only in the frontal cortex (fig. 4A). When melatonin was combined with METH, the decrease in TPH activity was enhanced. The content of 5HT was decreased only in the hippocampus after a single administration of METH (fig. 4B). However, METH in combination with melatonin significantly depressed the concentration of 5HT in all three structures.

A dose-response relationship for melatonin is depicted in figure 5. Multiple administrations of METH and melatonin as outlined above for figure 1B were used. TPH activity was decreased in all three brain regions 18 hr after the last administration of METH alone. When melatonin was administered alone, enzyme activity was decreased in the frontal cortex at a dose of 2 or 5 mg/kg; the 5-mg/kg dose also decreased hippocampal TPH activity. When melatonin was combined with METH, the depression of TPH activity was enhanced in all tissues by all doses of melatonin, except at 2 mg/kg.

The influence of melatonin on the METH-induced dopaminergic deficits in the neostriatum was also investigated. The same experimental regimen as outlined in figure 5 was used, except the 2-mg/kg dose was eliminated. Eighteen hours after the last dose of METH, TH activity was not altered (fig. 6A). However, when METH treatment was combined with melatonin, TH activity was depressed at the two higher doses of melatonin. The DA content in the neostriatum was not
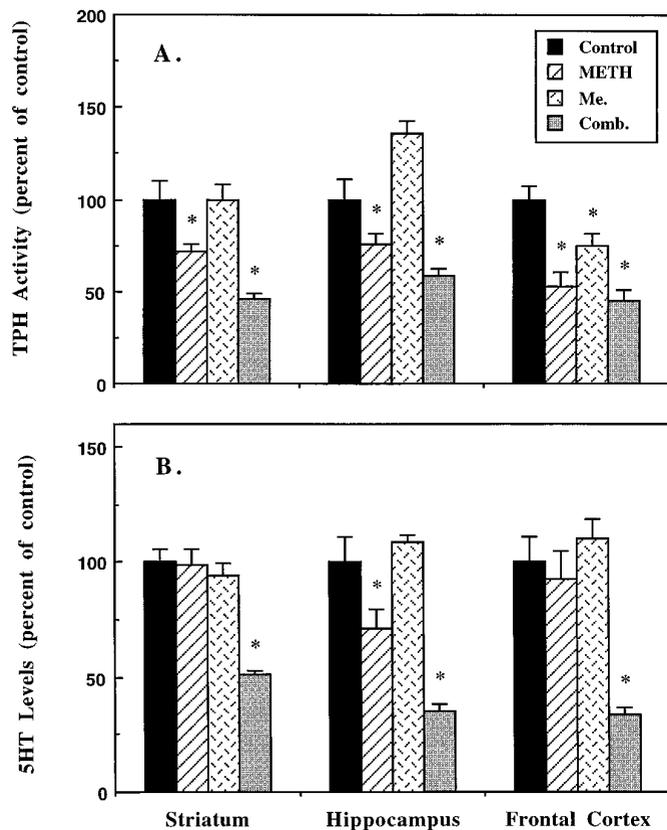
Fig. 2. Effect of melatonin on METH-induced deficit of 5HT content. METH (15 mg/kg, s.c.) was administered five times at 6-hr intervals. Rats received melatonin (25 mg/kg, i.p.) 15 min before, and 2 hr after, each administration of METH. Animals were killed 18 hr after the final administration of METH. Columns represent means ± S.E.M. of determinations in six rats; values are expressed as percent of control. *P ≤ .05 *vs.* control.

Fig. 4. Acute effect of melatonin and METH on TPH activity and 5HT content. Rats received a single administration of METH (15 mg/kg, s.c.). Melatonin (25 mg/kg, s.c.) was given 15 min before and 2 hr after METH. Rats were killed 3 hr after receiving METH. Columns represent means ± S.E.M. of determinations in six rats; values are expressed as percent of control.*P ≤ .05 *vs.* control.

Fig. 5. Effect of varying doses of melatonin on METH-induced deficit of TPH activity in the neostriatum, hippocampus and frontal cortex. Rats received five administrations of METH (15 mg/kg, s.c.) at 6-hr intervals. Melatonin, at the doses indicated, was administered 15 min before and 2 hr after each administration of METH. Rats were killed 18 hr after the last administration of METH. Columns represent means ± S.E.M. of determinations in six rats; values are expressed as percent of control. *P ≤ .05 *vs.* control.

changed by either METH or melatonin alone, but was significantly depressed when METH treatment was combined with any of the three doses of melatonin (fig.6B).

Discussion

We (Stone *et al.*, 1988) previously reported that the deficit in TPH activity in the rat brain associated with administering a toxic dose of METH is reversed when the enzyme is incubated under anaerobic conditions with certain reducing agents such as dithiothreitol. Enzyme activity can be restored only at the early stages (*i.e.,* up to 6 hr) after administering METH; thereafter the enzyme is irreversibly altered. These observations lead us to postulate that METH-released endogenous DA and/or its oxidized metabolites initiate a series of oxidative events that compromise serotonergic function, as indicated by a decrease in TPH activity and accompanying deficits in content of 5HT and its metabolite.

Because melatonin is characterized as an endogenous antioxidant (Tan *et al.*, 1993; Reiter, 1995), in our study melatonin was administered in combination with large doses of METH to determine whether this hormone would protect against the monoaminergic changes by METH. Surprisingly, rather than protect against the neurochemical deficits observed with METH alone, the deficits in the serotonergic system were exacerbated when the central nervous system stimulant was combined with melatonin.

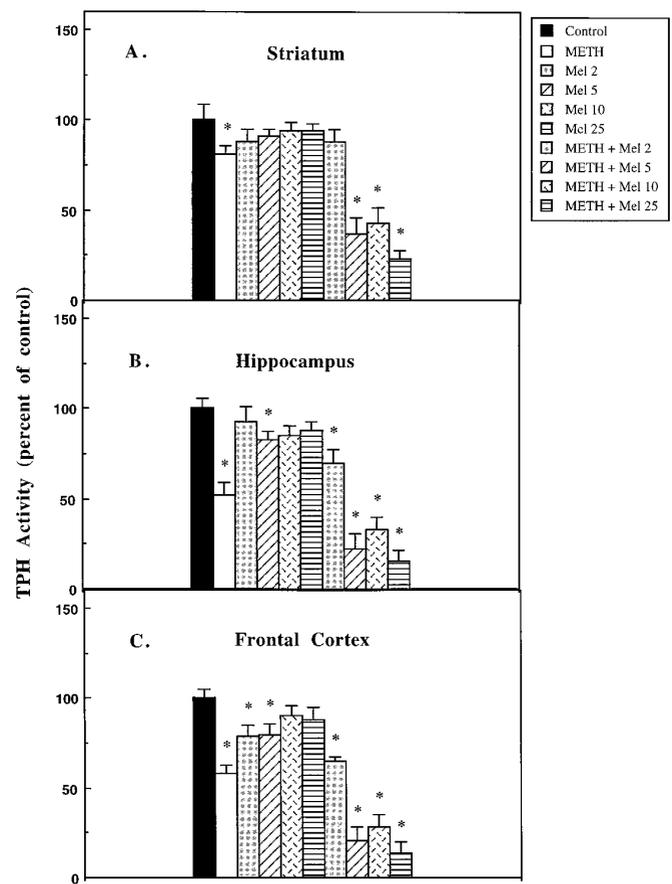The mechanism(s) responsible for these unexpected obser-

vations is not immediately apparent. Since the neurochemical deficits caused by METH are attenuated by preventing the hyperthermia associated with high doses of the drug (Bowyer *et al.*, 1992,1994; Albers and Sonsalla, 1995; Farfel and Seiden, 1995), we examined the possibility that melatonin enhanced hyperthermia. The combination of melatonin with METH did not alter the body temperature compared with that of rats treated with METH alone (data not shown).

We (Matsuda *et al.*, 1987) reported earlier that METH-induced neurochemical deficits are exacerbated when the antioxidant, ascorbic acid, is administered in combination with METH to scorbutic guinea pigs. Moreover, cysteine, an antioxidant, also enhanced the monoaminergic changes by METH (G. Hanson and J. W. Gibb, personal observation). Antioxidants can act as prooxidants as well as antioxidants, depending on dose and conditions (Li *et al.*, 1995). Ianas (1991) and Marshall *et al.* (1996) have suggested that melatonin is a prooxidant which could explain the decrease in TPH activity and 5HT content observed in some experiments after treatment with melatonin alone (*e.g.*, Fig. 3, 4 and 5); however, the melatonin effect was not consistent in all experiments.

The possibility that melatonin alters the METH effects due to changes in the cardiovascular system should also be con-
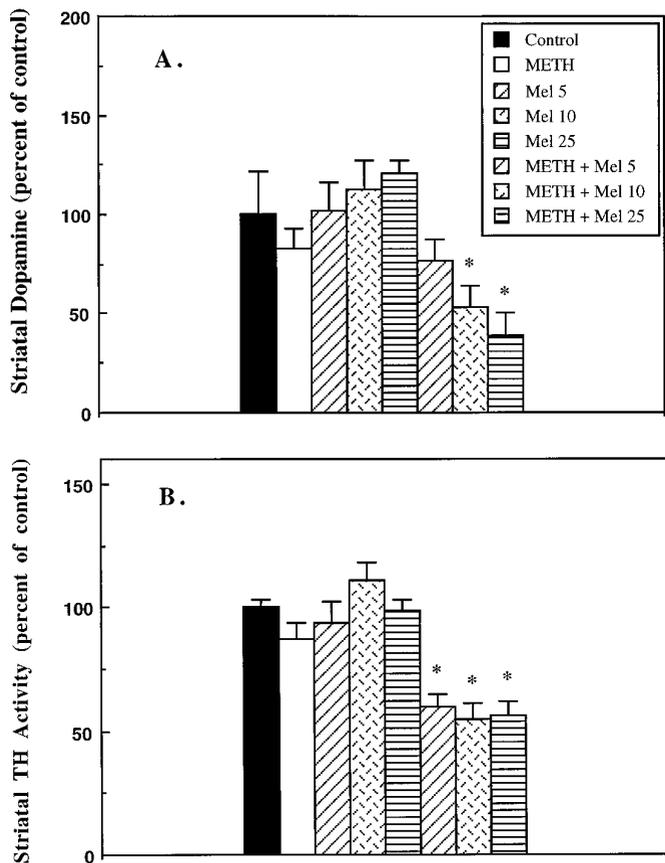
Fig. 6. Effect of melatonin on METH-induced deficit of neostriatal TH activity and DA content. Rats received five administrations of METH (15 mg/kg, s.c.) at 6-hr intervals. Melatonin, at the doses indicated, was administered 15 min before and 2 hr after each administration of METH. Rats were killed 18 hr after the last administration of METH. Columns represent means ± S.E.M. of determinations in six rats; values are expressed as percent of control. * $P \le .05$ *vs.* control.

sidered. Viswanathan *et al.* (1986) observed that melatonin decreases norepinephrine turnover in the heart. If melatonin suppresses sympathetic activity to the vasculature causing vasodilation, it is possible that as a consequence more METH is delivered to the brain and hence increases the neurochemical deficit induced by the METH. We are currently investigating other possibilities to explain why melatonin enhanced the METH-induced monaminergic effects, including possible pharmacokinetic alterations of METH by melatonin.

Melatonin is currently promoted in some countries as an over-the-counter "natural" medication for insomnia and other sleep disorders (UC Berkeley Wellness Letter, 1995). Because insomnia is one of the sequelae experienced by METH abusers, it is conceivable that they may perceive melatonin as a safe sleep-aid (UC Berkeley Wellness Letter, 1996) to overcome the insomnia associated with the "high" experienced during the METH "rush." Moreover, because those who abuse METH are often knowledgeable about the potential neurotoxic effects of METH and its congeners, it is conceivable that they might use melatonin to protect against the possible oxidative neurotoxicity associated with the use of these central nervous system stimulants. Because we report that the neurotoxicity of METH is markedly potentiated when administered with melatonin, the potential implications of this drug interaction are self-evident. It is true that

the doses of both METH and melatonin used in this investigation exceed those used by the naive abuser; however, as tolerance develops the dose of METH is markedly escalated and may approach the high doses used in these studies.

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