J Pineal Res 1999; 26:236–246
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Journal of Pineal Research
ISSN 0742-3098

# Pharmacological aspects of *N*-acetyl-5-methoxytryptamine (melatonin) and 6-methoxy-1,2,3,4-tetrahydro-β-carboline (pinoline) as antioxidants: Reduction of oxidative damage in brain region homogenates

Pless G, Frederiksen TJP, Garcia JJ, Reiter RJ. Pharmacological aspects of *N*-acetyl-5-methoxytryptamine (melatonin) and 6-methoxy-1,2,3,4-tetrahydro-β-carboline (pinoline) as antioxidants: Reduction of oxidative damage in brain region homogenates. J. Pineal Res. 1999; 26:236–246. © Munksgaard, Copenhagen

Abstract: Oxygen consumption is a necessity for all aerobic organisms, but oxygen is also a toxic molecule that leads to the generation of free radicals. The brain consumes a high percentage of the oxygen inhaled (18.5%), and it contains large amounts of unsaturated fatty acids, which makes it highly susceptible to lipid peroxidation. Melatonin (*N*-acetyl-5-methoxytryptamine), the main secretory product of the pineal gland, is a free radical scavenger that was found to protect against lipid peroxidation in many experimental models. Another compound found in the pineal gland is pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline). Pinoline is structurally related to melatonin. Evidence suggests that pinoline may have an antioxidant capacity similar to that of melatonin. In this study, the ability of pinoline to protect against $H_2O_2$-induced lipid peroxidation of different rat brain homogenates (frontal cortex, striatum, cerebellum, hippocampus, and hypothalamus) was investigated. The degree of lipid peroxidation was assessed by estimating the levels of thiobarbituric acid reactive substances, malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA). Pinoline's antioxidant capacity was compared with that of melatonin. Both melatonin and pinoline reduced the level of MDA and 4-HDA in a dose-dependent manner in all brain regions tested. To compare the antioxidant capacities, percent-inhibition curves were created, and the $IC_{50}$ values were calculated. The $IC_{50}$ values for melatonin were higher in all brain regions than were those for pinoline. The $IC_{50}$ values for melatonin in the five different brain regions ranged from 0.16mM–0.66 mM, and for pinoline, they ranged from 0.04 mM–0.13 mM. The possibility of synergistic interactions between melatonin and pinoline were also determined using the method of Berenbaum. Little evidence for either synergistic, additive, or antagonistic interactions between melatonin and pinoline was found.

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Key words: antioxidants – cerebellum – cerebral cortex – hippocampus – $H_2O_2$ – hypothalamus – lipid peroxidation – melatonin – oxidative brain damage – pinoline – striatum

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Received July 10, 1998;
accepted November 11, 1998.

## Introduction

Oxygen ($O_2$) utilization is a necessity for all aerobic organisms. Humans have an $O_2$ consumption of approximately 250 mL/min [Ganong, 1993]. It is estimated that approximately 2%–4% of all oxygen taken in by the organism is converted to free radicals [Reiter et al., 1995]. The toxic nature of free radicals relates to the fact that they possess an unpaired electron in their outer orbital, making them highly reactive and likely to interact with other radicals, as well as non-radical compounds.

The brain uses approximately 18.4% of all the oxygen consumed by the human [Ganong, 1993]. Oxidative stress occurs when there is a disparity in the natural balance between free radical generation and the various enzymatic and non-enzymatic antioxidative defense systems leading to accumulation of excessive free radical damage [Lezoualc'h et al., 1996]. The brain contains large amounts of polyunsaturated fatty acids, which are highly susceptible to degradation by free radicals. These features, combined with the fact that the brain has low activity of antioxidative enzyme systems and low concentrations of free radical scavengers, make it particularly susceptible to free radical damage.

The progression of several diseases, such as Alzheimer's disease [Pappolla et al., 1997], Parkinson's disease [Götz et al., 1994], and the mental deterioration associated with Down's syndrome [Kedziora and Bartosz, 1988], was recently shown to involve free radical damage. Also, substantial evidence exists showing that oxidative stress is involved in the damage of neural tissues during ischemia-reperfusion injury [Siesjo, 1992].

It is important to find agents that protect the brain from oxidative stress. One such agent is melatonin (*N*-acetyl-5-methoxytryptamine), which proved to be a highly effective free radical scavenger in several studies that involved neural tissue [Reiter, 1995a; Reiter et al., 1997; Reiter, 1998]. Melatonin is a naturally occurring chemical mediator derived primarily from the pineal gland, and after its secretion, it is found in all bodily fluids. Melatonin possesses both hydrophilic and lipophilic characteristics [Shida et al., 1994; Costa et al., 1995] and easily penetrates all biological membranes, including the blood–brain barrier [Menendez-Pelaez et al., 1993]. Recently, melatonin was shown to be an efficient scavenger of the hydroxyl radical (•OH), which is generated from hydrogen peroxide ($H_2O_2$) via the Fenton reaction [Tan et al., 1993; Stasica et al., 1998]. Other reactive oxygen species involved in damage of neural tissue that melatonin is believed to scavenge include the peroxyl radical [Pieri et al., 1994], singlet oxygen ($^1O_2$) [Cagnoli et al., 1995], and the peroxynitrite anion ($ONOO^-$) [Cuzzocrea et al., 1997; Gilad et al., 1997; Cuzzocrea et al., 1998]. In reference to the brain, melatonin was also found to reduce lipid peroxidation in the brain following the administration of several different free-radical-generating toxins [Melchiorri et al., 1995; Giusti et al., 1996; Yamamoto and Tang, 1996; Princ et al., 1997].

Table 1. $IC_{50}$ values for melatonin and pinoline in inhibiting $H_2O_2$-induced lipid peroxidation in brain homogenates

| | $IC_{50}$ (mM) | |
|---|---|---|
| | Melatonin | Pinoline |
| Cerebellum | 0.66 | 0.13 |
| Hypothalamus | 0.51 | 0.13 |
| Cortex | 0.50 | 0.10 |
| Hippocampus | 0.35 | 0.06 |
| Striatum | 0.16 | 0.04 |

The $IC_{50}$ values represent the observed mean of 50% inhibition of lipid peroxidation in the different brain homogenates. Lipid peroxidation was induced with 5 mM $H_2O_2$ for 60 min.

Another compound that is produced in the pineal gland is pinoline (6-methoxy-1,2,3,4-tetrahydro-β-carboline) [Langer et al., 1984; Leino, 1984]. It is structurally similar to melatonin and is found in the same physiological concentration as melatonin [Pähkla et al., 1998]. Studies showed that pinoline is a weak monoamine oxidase (MAO) inhibitor [Rimón et al., 1984], and it was reported that pinoline inhibits the uptake of serotonin (5-HT) into brain synaptosomes and into platelets [Araksinen et al., 1978; Komulainen et al., 1980]. Studies by Kawashima et al. [1995] suggest that 1,2,3,4-tetrahydro-β-carboline derivatives protect against in vitro lipid peroxidation of rat brain tissue.

Recently, pinoline was found to be an effective inhibitor of lipid peroxidation and a potent scavenger of the hydroxyl radical (•OH) in a cell-free mixture [Pähkla et al., 1998]. In their study, Pähkla and colleagues (1998) evaluated the efficacies of pinoline and melatonin as free radical scavengers and found that melatonin was significantly more effective in this regard. This seems contrary to some of our own findings, where pinoline was found to be a better inhibitor of lipid peroxidation than melatonin [Frederiksen et al., 1998].

The purpose of the present study was to evaluate pinoline's antioxidant capacity in homogenates of different rat brain regions, to compare it with melatonin, and to investigate possible interactions between melatonin and pinoline.

## Materials and methods

### Reagents

A lipid peroxidation kit from Calbiochem (La Jolla, CA) was used to measure the amount of thiobarbituric acid reactive substances, malondialdehyde (MDA) and 4-hydroxyalkenals (4-HDA). These compounds are end products of lipid peroxidation chain reactions and can be used

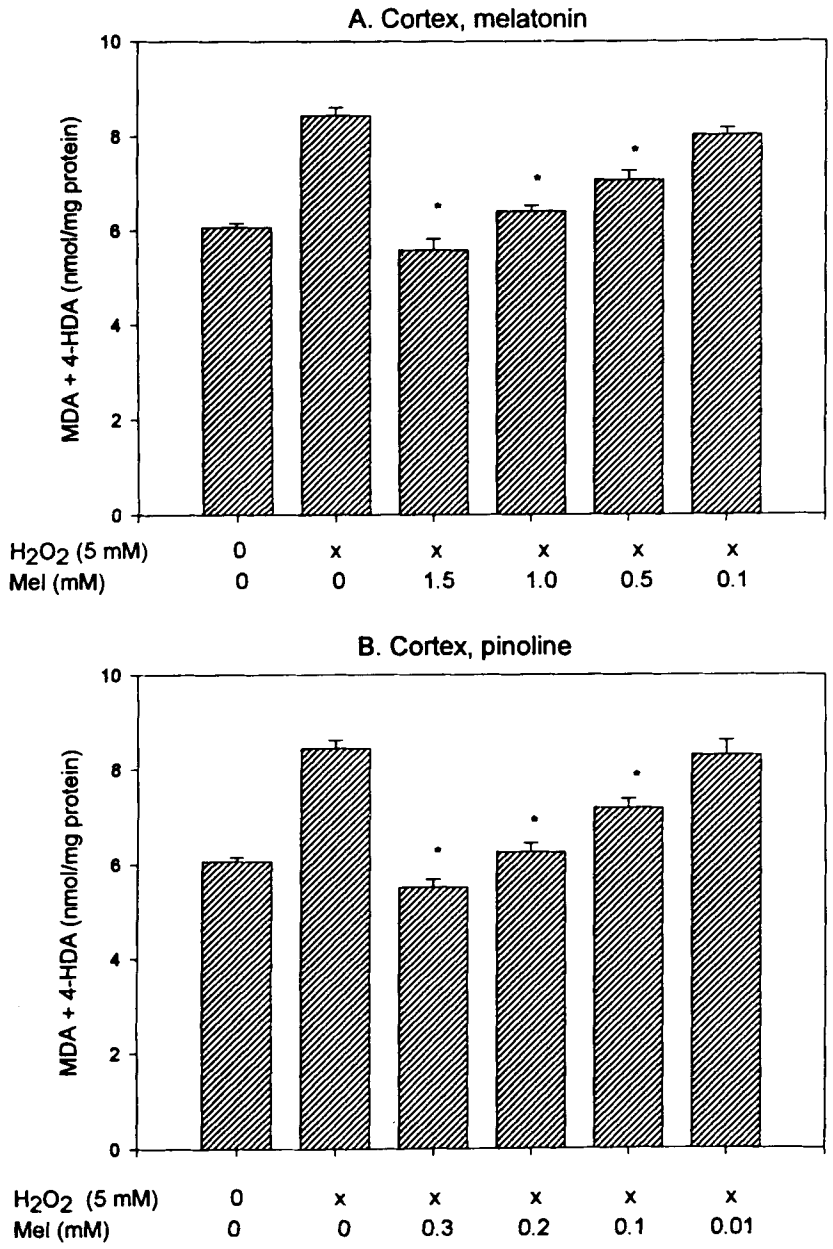


*Fig. 1.* The effect of different concentrations of melatonin (**A**) and pinoline (**B**) on $H_2O_2$-induced lipid peroxidation products in cortical homogenates. Incubation time was 60 min. Values are means ± S.E.M. (N = 6). $^*P < 0.05$ versus $H_2O_2$ only.

as an estimate of the degree of lipid peroxidation. Melatonin, pinoline, and $H_2O_2$ were purchased from Sigma (St. Louis, IL). All other chemicals were of the highest quality available.

## Methods

Male Sprague–Dawley (SD) rats were purchased from Harlan (Houston, TX). In order to obtain enough brain tissue for the homogenates, 40 rats were used; they were housed in Plexiglas cages with three rats per cage. They received standard rat chow and water ad libitum. All rats were anesthetized with rodent cocktail and perfused through the heart with 0.85% w/w saline solution. The brain was then dissected so as to obtain hypothalamus, striatum, hippocampus, cortex, and cerebellum. The brain parts were frozen on solid $CO_2$ and stored at −80°C until the time of assay. Melatonin and pinoline were dissolved in methanol. An equal amount of methanol (2%) was added to all samples.

### Tissue preparation and assay

The tissues from each brain region were pooled and homogenized in ice-cold 50 mM Tris buffer (25°C) using a Euro Turrax T20b homogenizer, and five different homogenates were thereby obtained. Protein concentration was measured by the

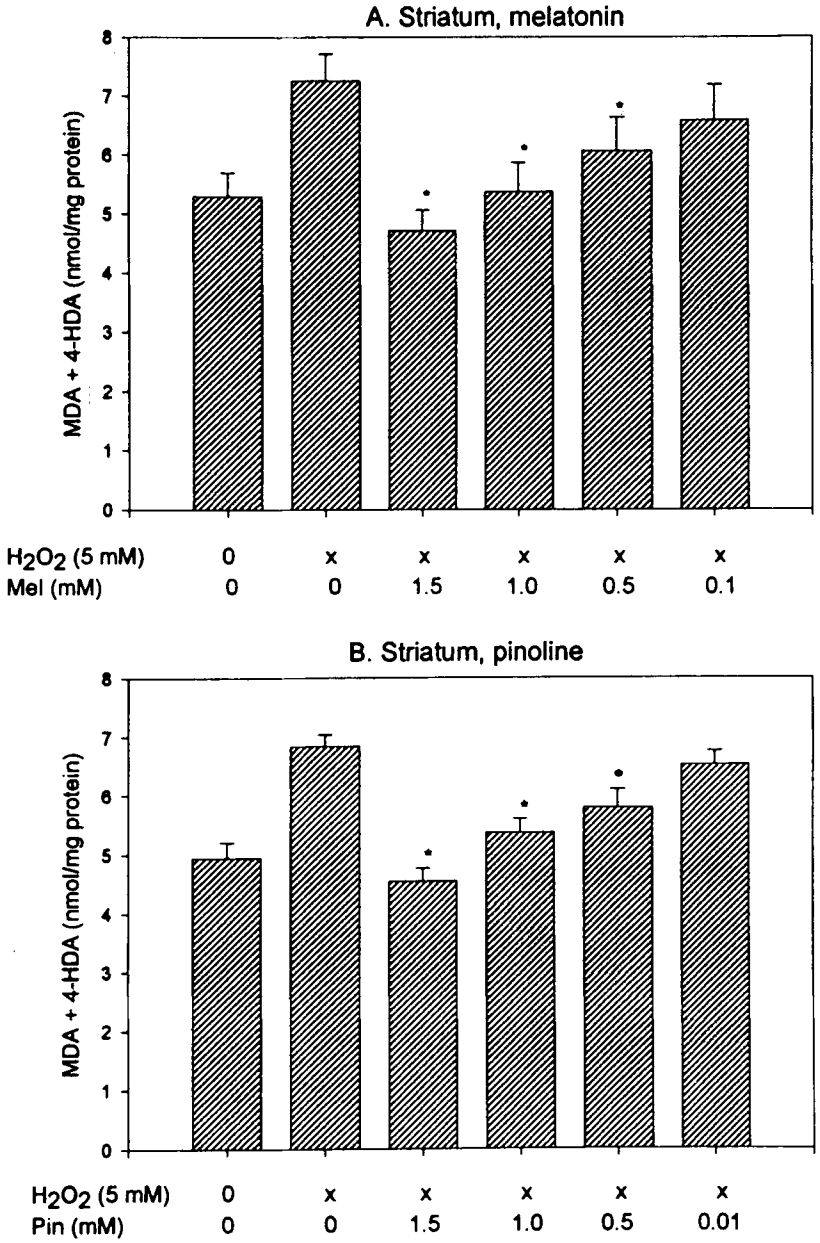


*Fig. 2.* The effect of different concentrations of melatonin (**A**) and pinoline (**B**) on $H_2O_2$-induced lipid peroxidation products in striatal homogenates. Incubation time was 60 min. Values are means ± S.E.M. (N = 5). $^*P < 0.05$ versus $H_2O_2$ only.

method of Bradford [1976] using bovine serum albumin as standard. The amount of homogenate used was calculated so the final protein concentration in the test tubes was about 3 mg/mL. Four different concentrations of melatonin and pinoline were used for the experiment. Melatonin concentrations were 0.1 mM, 0.5 mM, 1.0 mM, and 1.5 mM, while pinoline was used at concentrations of 0.01 mM, 0.1 mM, 0.2 mM, and 0.3 mM. The conditions for inducing lipid peroxidation in brain homogenates with $H_2O_2$ were based on previous work from this laboratory [Frederiksen et al., 1998]. In those studies, it was found that the optimal experimental conditions were incubation with 5 mM $H_2O_2$ for 1 hr at 37°C. The reaction was stopped by putting the tubes on ice for 10 min. The tubes were then centrifuged at 3,000g for 10 min at 4°C. The supernatant was used to measure MDA + 4-HDA levels.

The method of Berenbaum [1977] was used to determine the possibility of interactions between pinoline and melatonin; the following equation was employed:

$$\frac{[\text{melatonin}]_{\text{combin.}}}{[\text{melatonin}]_{\text{equi-effective}}} + \frac{[\text{pinoline}]_{\text{combin.}}}{[\text{pinoline}]_{\text{equi-effective}}} = \text{IF}$$

In this formula: IF, interaction factor (IF < 1, synergism; IF = 1, additivism; IF > 1, antagonism); $[\text{melatonin}]_{\text{combin.}}$, melatonin concentration in the combination mixture (melatonin + pino-

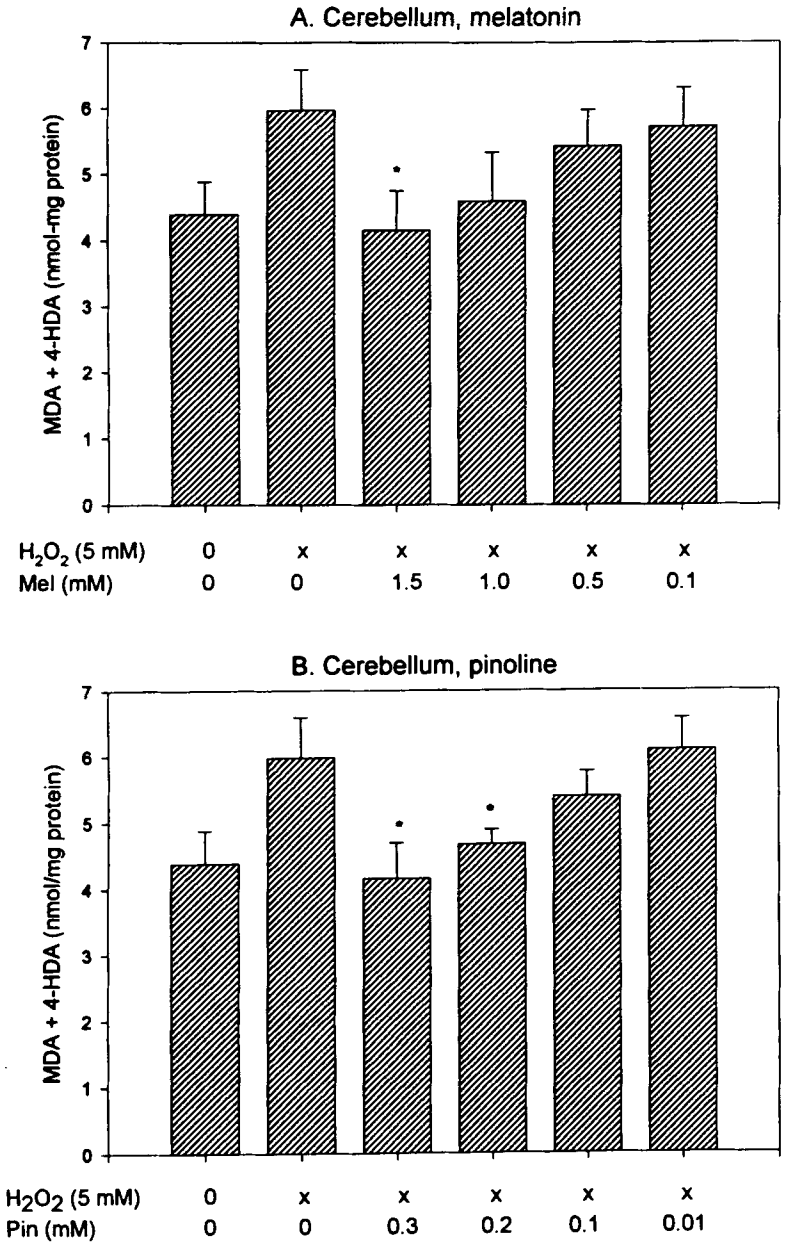


*Fig. 3.* The effect of different concentrations of melatonin (**A**) and pinoline (**B**) on $H_2O_2$-induced lipid peroxidation products in cerebellar homogenates. Incubation time was 60 min. Values are means ± S.E.M. (N + 3). $^*P < 0.05$ versus $H_2O_2$ only.

line); $[\text{melatonin}]_{\text{equi-effective}}$, melatonin concentration which, when used alone, produces the observed effect of the combination of drugs; $[\text{pinoline}]_{\text{combin.}}$, pinoline concentration in the combination mixture; $[\text{pinoline}]_{\text{equi-effective}}$, pinoline concentration which, when used alone, will produce the observed effect of the combination.

The synergy experiment was done on cortical homogenates. The equi-effective concentrations were estimated from the respective percent-inhibition curves for melatonin and pinoline (see Results). The concentrations in the combinations were obtained in an experiment with three different concentrations of pinoline (0.2 mM, 0.1 mM, and 0.01 mM) and melatonin (1.0 mM, 0.5 mM, and 0.1 mM), used in nine different combinations (Table 2).

Whenever possible, each experiment was repeated five times, but due to the limited amount of homogenates, this was not always possible.

### Statistical analyses

All data were analyzed using a one-way analysis of variance (ANOVA), followed by the Student–Newman–Keuls $t$-test. The significance level was accepted at $P < 0.05$.

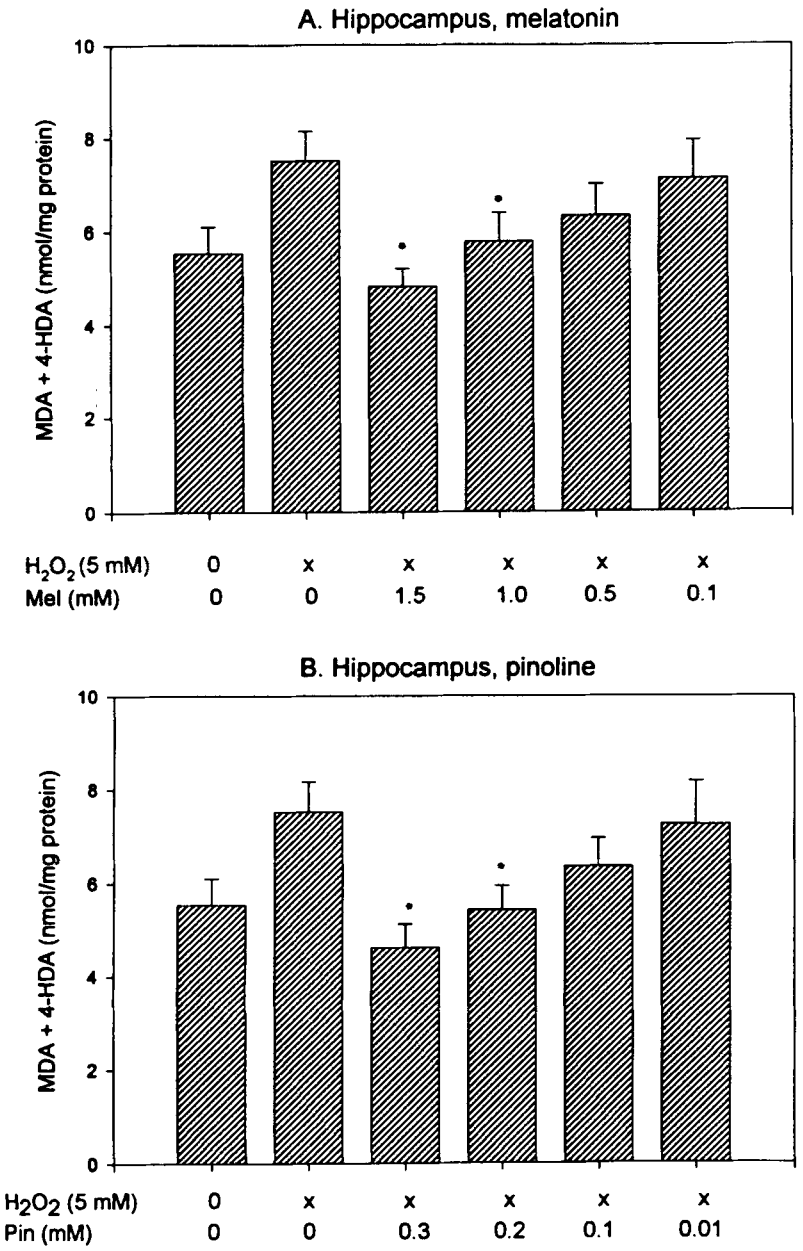


*Fig. 4.* The effect of different concentrations of melatonin (**A**) and pinoline (**B**) on $H_2O_2$-induced lipid peroxidation products in hippocampal homogenates. Incubation time was 60 min. Values are means ± S.E.M. (N = 5). $^*P < 0.05$ versus $H_2O_2$ only.

## Results

Both pinoline and melatonin were found to reduce the amount of lipid peroxidation produced by $H_2O_2$ in all brain regions in a dose-dependent manner (Figs. 1–5). However, a statistically significant difference in the level of MDA and 4-HDA produced by $H_2O_2$ compared with that in control samples was only found in cortical (Fig. 1) and striatal homogenates (Fig. 2). In spite the lack of statistical verification of the induced changes in cerebellar (Fig. 3), hippocampal (Fig. 4), and hypothalamic (Fig. 5) homogenates, it is obvious that both melatonin and pinoline reduced the level of lipid peroxidation end products, differences that presumably would have become statistically significant with the use of larger numbers of homogenates. For concentrations of melatonin greater than 1.0 mM and pinoline greater than 0.2 mM, the MDA + 4-HDA levels were, on average, below the values of the control sample in all brain regions.

From the levels of MDA + 4-HDA produced in the homogenates, the percent-inhibition of lipid peroxidation was calculated, and data was plotted as percent-inhibition curves (Fig. 6A–E). For each agent, the $IC_{50}$ was calculated as the concentration that inhibited lipid peroxidation by 50%. The $IC_{50}$ value for pinoline was less than that for melatonin in all brain regions. The $IC_{50}$ values for melatonin

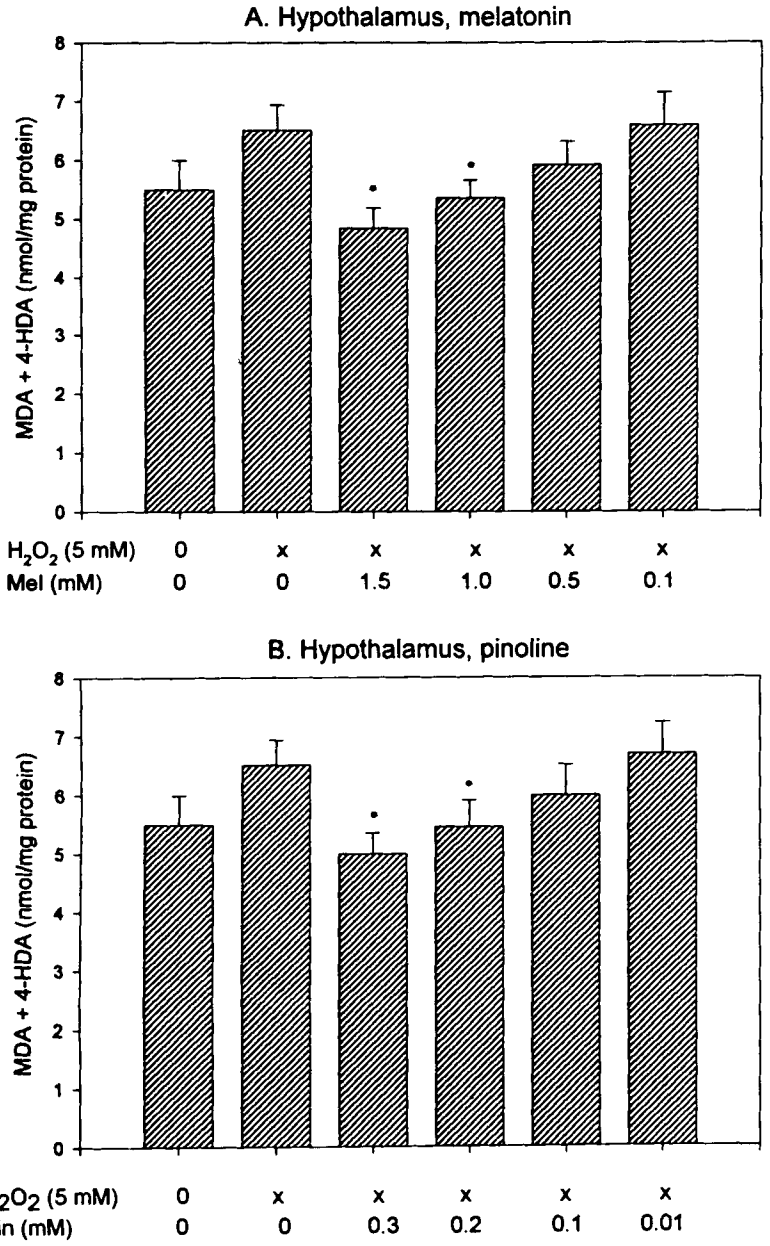


*Fig. 5.* The effect of different concentrations of melatonin (**A**) and pinoline (**B**) on $H_2O_2$-induced lipid peroxidation products in hypothalamic homogenates. Incubation time was 60 min. Values are means ± S.E.M. (N = 5). $^*P < 0.05$ versus $H_2O_2$ only.

were 0.66 mM (cerebellum), 0.51 mM (hypothalamus), 0.50 mM (cortex), 0.35 mM (hippocampus), and 0.16 mM (striatum); and the $IC_{50}$ values for pinoline were 0.13 mM (cerebellum), 0.13 mM (hypothalamus), 0.10 mM (cortex), 0.06 mM (hippocampus), and 0.04 mM (striatum) (Table 1).

The synergy calculations did not provide strong evidence for widespread synergy between melatonin and pinoline in the inhibition of lipid peroxidation. For the lowest concentration of melatonin (0.1 mM) in combination with pinoline concentrations of 0.01 mM and 0.1 mM, the IF was found to be slightly lower than 1, suggesting synergy. For higher concentrations of pinoline (0.2 mM) in all combinations with melatonin tested, the IF was higher than 1, which indicates that there may be an antagonistic interaction. For high concentrations of melatonin (0.5 mM and 1.0 mM) in combination with pinoline (either 0.01 mM or 0.1 mM), the IF was 1 or very close to 1, which indicates additivism (Table 2).

## Discussion

Numerous studies documented the antioxidant capacity of melatonin [Reiter, 1995b; Reiter et al., 1997; Reiter, 1998], but only a few investigations reported on the antioxidative effects of pinoline. Kawashima et al. [1995] tested the ability of 12

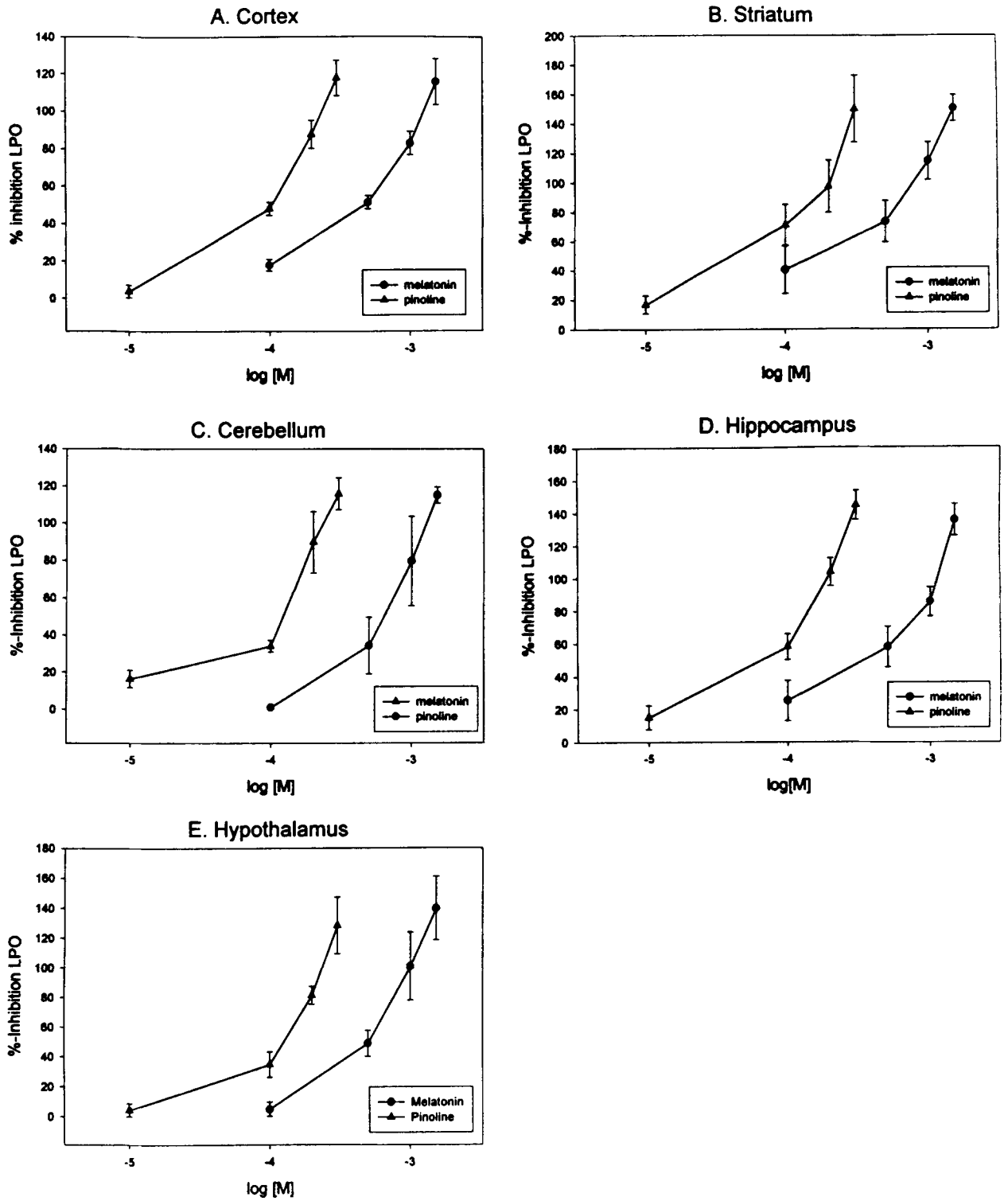


*Fig. 6.* The effects on different concentrations of pinoline and melatonin in inhibiting lipid peroxidation (LPO) in homogenates of different brain regions. (**A**) cortex; (**B**) striatum; (**C**) cerebellum; (**D**) hippocampus; and (**E**) hypothalamus. The incubation time was 60 min, and the concentration of $H_2O_2$ was 5 mM. The values are mean ± S.E.M. (N = 5).

compounds, structurally similar to pinoline, to protect against the oxidation of lipids in rats and found that 1-aryl-substituted 1,2,3,4-tetrahydro-β-carbolines were, in fact, protective against lipid peroxidation. Their findings are consistent with the observations of the present study, where pinoline was found to reduce lipid peroxidation in neural homogenates.

Table 2. Interactions of various concentrations of melatonin and pinoline in the inhibition of $H_2O_2$-induced lipid peroxidation in rat brain homogenates

| Melatonin (mM) → | | 0 | 0.1 | 0.5 | 1.0 |
|---|---|---|---|---|---|
| Pinoline (mM)↓ | 0 | 0 | 17.4 | 51.1 | 82.8 |
| | 0.01 | 3.4 | 29.8 (0.78)S | 51.5 (1.09)AD | 86.9 (1.01)AD |
| | 0.1 | 47.6 | 64.0 (0.91)S | 84.6 (1.00)AD | 105.0 (1.19)A |
| | 0.2 | 87.6 | 84.3 (1.15)A | 100.2 (1.20)A | 120.5 (1.25)A |

The number in each column represents the observed mean of percent-inhibition of lipid peroxidation (N = 4). For pinoline and melatonin combinations, the value in parentheses is the interaction factor (IF), as determined from the equation given in the Materials and methods section, according to the method of Berenbaum (1977). The letters represent synergism (S), additivism (AD), and antagonism (A). The S.E.M. of percent-inhibition of the lipid peroxidation was less than 9.0 at all concentrations of melatonin and pinoline alone, as well as in combination.

Pähkla et al. [1998] studied the ability of pinoline to protect against lipid peroxidation and compared the protective effect with that of melatonin. They found melatonin to be significantly more potent than pinoline in inhibiting the amount of $FeSO_4$-induced lipid peroxidation of linolenic acid in a cell-free system. These findings contrast with those reported here, where we found pinoline to be about five times more potent than melatonin in inhibiting $H_2O_2$-induced lipid peroxidation in brain homogenates. The apparent discrepancy between the two studies may be related to the fact that Pähkla and his coworkers used a cell-free system, while we used homogenized tissues. The bioavailability and interactions of the compounds is not a factor when using a cell-free system, and the possibility that pinoline may have an indirect antioxidative effect that is only manifested in the presence of tissue may be a reason for the variant results reported in the two studies.

In an earlier study [Frederiksen et al., 1998], $H_2O_2$ at a concentration of 5 mM was found to significantly increase the level of lipid peroxidation in homogenates of whole brain. In the present study, an $H_2O_2$ concentration of 5 mM failed to induce a significant difference in the level of MDA + 4-HDA produced in homogenates of cerebellum, hippocampus, and hypothalamus, although the values were clearly elevated. Only in frontal cortex and striatal homogenates was the difference between control and $H_2O_2$-induced values significant. Sewerynek et al. [1995] conducted experiments with the same five brain homogenates, and they found that 5 mM $H_2O_2$ significantly induced lipid peroxidation in all the homogenates and that the effect of $H_2O_2$ on lipid peroxidation was most obvious in the cortex and striatum, suggesting that the sensitivity to $H_2O_2$ is somewhat region-specific. This is confirmed by the present study, where cortex and striatum were found to be the only brain parts where $H_2O_2$ induced a statistically significant rise in lipid peroxidation products. Furthermore, Sewerynek et al. [1995] reported that melatonin treatment similarly reduced lipid peroxidation in a dose-dependent manner in all of the brain homogenates tested. In the present study, melatonin seemed to be most effective in striatal homogenates, where the lowest $IC_{50}$ value (0.16 mM) was found; for the hippocampus the $IC_{50}$ value for melatonin was 0.35 mM. The cortex, hypothalamus, and cerebellum have rather similar $IC_{50}$ values (0.50 mM, 0.51 mM, and 0.66 mM, respectively). We found the same pattern for pinoline. Earlier experiments in this laboratory showed that pinoline was seven times more efficient than melatonin in reducing $H_2O_2$-induced lipid peroxidation, with an $IC_{50}$ of 0.1 mM for pinoline and an $IC_{50}$ of 0.7 mM for melatonin in whole brain homogenates. This is similar to the $IC_{50}$ values reported in the present study, where the mean $IC_{50}$ value for the five brain parts is 0.44 mM for melatonin and 0.09 mM for pinoline.

The findings in this study show that in vitro pinoline effectively reduces $H_2O_2$-induced lipid peroxidation and thereby reaffirm our earlier findings that pinoline is an efficient antioxidant. Tsuchiya et al. [1995] reported that pinoline given as an intraperitoneal injection is quickly absorbed and readily penetrates the blood–brain barrier. These findings, along with those reported here, suggest that pinoline may be a potential neuroprotective agent in the prevention of oxidative brain damage. In evaluating the antioxidant potential of pinoline, the possible side-effects and toxicity must be considered. There is limited existing data concerning the toxicity of pinoline. The $LD_{50}$ for pinoline after intraperitoneal injection in mice was reported to be 235 mg/kg, and doses exceeding the $LD_{50}$ induce predeath convulsions [Araksinen et al., 1978]. Pinoline's MAO-inhibiting character may also lead to unwanted side-effects in vivo. Pähkla et al. [1996], 1997] reported that pinoline increases brain levels of 5-HT, thereby resembling the effects of serotonergic (antidepressant) drugs.

In contrast to this, melatonin was, so far, proven to be minimally toxic, and despite attempts to do so, no $LD_{50}$ was established and no serious acute or chronic toxicity was reported [Barchas et al., 1967]. How pinoline and melatonin will compare as antioxidants in vivo still remains to be investigated.

Melatonin was frequently tested in vivo as a protector against oxidative stress. These in vivo studies proved melatonin to be highly effective in reducing free radical damage in the brain [Melchiorri et al., 1995; Giusti et al., 1996; Yamamoto and Tang, 1996; Princ et al., 1997], as well as in other organs [de la Lastra et al., 1997; Li et al., 1997]. To date, pinoline was not tested in vivo, and the pharmacology and biochemistry of pinoline may differ from melatonin in a way that can not be predicted from in vitro studies, making in vivo studies an important aspect of the research to understand pinoline's potential as an antioxidant.

Acknowledgments

The authors would like to thank Hørslev Fonden and Pharma Nord for their financial support.

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