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Effects of Placing Micro-Implants of Melatonin in Striatum on Oxidative Stress and Neuronal Damage Mediated by N-Methyl-D-Aspartate (NMDA) and Non-NMDA Receptors

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Overstimulation of both kainate (KA) and N-methyl-D-aspartate (NMDA) receptors has been reported to induce excitatoxicity which can be characterized by neuronal damage and formation of reactive oxygen free radicals. Neuroprotective effect of melatonin against KA-induced excitotoxicity have been documented *in vitro* and *in vivo*. It is, however, not clear whether melatonin is also neuroportective against excitotoxicity mediated by NMDA receptors. In the present work, we tested the *in vivo* protective effects of striatally infused melatonin against the oxidative stress and neuronal damage induced by the injection of KA and NMDA receptors into the rat striatum. Melatonin implants consisting of 22-gauge stainless-steel cannule with melatonin fused inside the tip were placed bilaterally in the rat brain one week prior to intrastriatal injection of glutamate receptor subtype agonists. Melatonin showed protective effects against the elevation of lipid peroxidation induced by either KA or NMDA and recovered Cu,Zn-superoxide dismutase activities reduced by both KA and NMDA into the control level. Melatonin also clearly blocked both KA- and NMDA-receptor mediated neuronal damage assessed by the determination of choline acetyltransferase activity in striatal homogenages and by microscopic observation of rat brain section stained with cresyl violet. The protective effects of melatonin are comparable to those of DNQX and MK801 which are the KA- and NMDA-receptor antagonist, respectively. It is suggested that melatonin could protect against striatal oxidative damages mediated by glutamate receptors, both non-NMDA and NMDA receptors.

Key words : Melatonin, Rat striatum, Glutamate receptors, Oxidative stress, Neuronal damage, SOD, Lipid peroxidation

INTRODUCTION

Glutamate is a major excitatory neurotransmitter which is found in very high concentrations in the mammalian brain, and its action is mediated via glutamate receptors. The excessive stimulation of glutamate receptors induces excitotoxicity which may contribute to neuronal degeneration associated with a number of neurological diseases (Alzheimer disease, Huntington' disease, Parkinsons' disease)(Jhamandas *et al.*, 1994) and has also been implicated in acute brain damage associated with hypoxia-ischemia (Simon et *al.*, 1984), hypoglycemia (Weiloch, 1985), and sustained seizure activity (Clifford *et al.*, 1989). Free radical formation has been linked to excitotoxicity, that is, glutamate receptor overstimulation increases oxygen radical formation and free radicals may also enhance the glutamate release, and thus potentiate excitotoxicity.

In the mammalian brain, glutamate receptors are divided into two pharmacological classes, ionotropic and metabotropic receptors. Ionotropic glutamate receptors are ligand-gated ion channels and have been classified according to their selective agonists; N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazole-propioninc acid (AMPA), and kainate (KA) (Nakanishi, 1992). AMPA and KA receptor subtypes are also grouped as non-NMDA receptor. Stimulation of both NMDA and non-NMDA receptors have been reported to trigger neuronal damage and free radical generation (Choi *et al.*, 1988; Bondy *et al.*, 1993).

Melatonin, which is primarily synthesized and secreted from the pineal gland during the dark phase of the circadian cycle (Arendt, 1988), was recently proved to possess potent antioxidant and free radial scavenging actions. Recently, the protective roles of

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melatonin have been demonstrated in oxidative damages induced by variety of free-radical generating agents and processes both *in vitro* and *in vivo* (see reviews, Reiter *et al.*, 1997; Reiter, 1996). The neuroprotective effect of melatonin against glutamate receptor-mediated exitotoxicity was first demonstrated *in vitro* in primary neuronal cultures. This effect was selective for neurotoxicity induced by KA and was not effective in NMDA receptor-mediated excitotoxicity (Giusti *et al.*, 1995). Melatonin also reduced lipid peroxidation triggered in rat brain homogenates by KA (Melchiorri *et al.*, 1995). *In vivo,* Giusti *et al.* (1996a, 1996b) showed that intraperitoneal (i.p.) administration of melatonin prevented hippocampal neuronal death and DNA damage induced by i.p. injection of KA to rats. However, the protective effect of melatonin against NMDA receptor mediated excitotoxicities has not been documented *in vivo* as well as *in vitro*.

The striatum is located in subcortical area and receives a large and highly ordered excitatory neuronal projections mainly from the cerebral cortex (Mcgeorge and Gaull, 1989), and has been demonstrated to express high binding sites for all classes of glutamate receptors (Albin *et al.*, 1992; Wüllner *et al.*, 1994). The purpose of the present study was to examine the protective effects of melatonin on the striatal damage and oxidative stress caused by both non-NMDA and NMDA receptor agonists in rats *in vivo*. The effects of melatonin were compared with those of glutamate receptor subtype selective antagonists. In this study, melatonin implants consisting of 22-gauge stainless-steel cannule with melatonin fused inside the tip were placed bilaterally in the brain of Sprague-Dawley rats one week prior to instrastriatal injection of glutamate receptor subtype agonists. DNQX and MK801, the KA- and NMDA-receptor antagonists respectively, were co-injected with corresponding receptor agonist into the striatum. Oxidative stress was examined by measuring the level of lipid peroxidation and the activity of antioxidant enzyme, superoxide dismutase (SOD), and intrastriatal neuronal injury was evaluated both by choline acetyltransferase assay and cresyl violet staining of the brain sections.

MATERIALS AND METHODS

Animals, melatonin micro-implants and drug injection

Male Sprague-Dawley rats weighing 250~300 g were housed individually in suspended stainless steel cages in a room maintained at 22°C and under a 12-hour light/dark cycle.

The melatonin implants were made of 26-gauge stainless-steel needle with melatonin fused inside the tip, as described by Lincoln and Maeda (1992a) with slight modifications. Each needle was cut to 10 mm and preheated in hot plate to 130±5°C, and filled with molten melatonin at 130±5°C using capillary action. The needle filled with melatonin was cooled and the melatonin on the outer surface was removed with 100% ethanol. Weights of the empty needles and the melatonin-fused needles were 8.83±0.36 mg and 10.34±0.42 mg, respectively, with the amount of melatonin fused inside the needle being about 1.68 ±0.07 mg.

The stereotaxic surgeries were performed as follows. The rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.), and the head was positioned in a stereotaxic frame. The bone of the skull was exposed and about a 0.3 mm diameter hole was drilled through the skull. The micro-implants containing melatonin (melatonin bar; Mb) or empty implants (control bar; Cb), which were bent at the point of 6.5 mm from the tip of the implant, were lowered to allow the tip to be located in the striatum at the following coordinates (Paxinos and Watson, 1986): anterior 1.2 mm, lateral ± 2.5 mm, ventral -6.5 mm, and the skin was sutured over the skull. The surgery took about 30 min. The micro-implants remained in place for one week, and they removed during the operation for drug (KA, NMDA or saline for control) injection under general anaesthesia, and then a newly prepared Mb (or Cb) was replaced into same places of the brain. Glutamate receptor subtype agonists, KA (10 nmol/μl), NMDA (200 nmol/μl) were bilaterally injected (1 μl per hemisphere; 0.5 μl/min) into striatum one week after Mb or Cb treatment. All operations with Cb or Mb implants led to no fatalities. The Mb removed from the brain after treatments weighed and the weight value was subtracted from the relative Mb weight before treatments to determine the amount of melatonin released into the brain. The total mount of melatonin released into the brain for two weeks was 0.74 ± 0.03 mg (3.18 μmol).

When appropriate, 1 μl of KA antagonist, DNQX (8.9 nmol), or NMDA antagonist, MK801 (8 nmol), were co-injected with 1 μl of above concentration of corresponding receptor agonist directly into each side of the striatum.

For histological analyses, the left striatum treated with melatonin implant and glutamate receptor agonist was compared with the right side treated with empty implant and agonist, and in another groups of rats, the left striatum treated with glutamate receptor antagonist and agonist compared with the right striatum treated with agonist alone.

Rats were maintained for one week after the final surgery with food and water ad *libitum*, and then were sacrificed by decapitation for biochemical and histological analyses.

Lipid peroxidation assay

The fluorescence assay procedure for lipid peroxidation (Triggs and Willmore, 1984) was modified to suit a microassay. The freshly dissected striatum (40~60 mg) was weighted and placed in tube with 750 μl of chloroform and methanol (2:1). Each tube was capped, mixed gently, and placed on ice. The tissue was homogenized for 10 seconds and water (750 μl) was added to this. The mixture was vortexed for 1 min and left on ice for 15 min, and centrifuged at 4°C, 10,000×g for 10 min. Methanol (100 μl) was added to 400 μl of the chloroform layer. After thorough mixing, and 200 μl of the sample were scanned for fluorescence in flurospectrometer (Kontron SFM25, Switzerland). A maximum fluorescence due to oxidized products of lipid was obtained at 356 nm excitation and 426 nm emission.

Superoxide dismutase activity assay

Activities for Zn,Cu SOD was assayed by their capacity to compete with cytochrome c for superoxide (O_2^-) radicals generated by the xanthine/xanthine oxidase system (McCord and Fridovich, 1969). The dissected striatum (40~60 mg) was weighted and homogenized in 0.05 M potassium phosphate buffer (pH 7.8) and centrifuged at 4°C, 10,000×g for 15 min. The supernatant (50 μl) was added to 850 μl of 0.05 M potassium phosphate buffer (pH 7.8) containing 10^{-4} M EDTA, 0.02% (v/v) Triton X-100, 1×10^{-5} M ferricytochrome c, 5×10^{-5} M xanthine. The reactions were initiated by adding xanthine oxidase adjusted to give rates of reduction equivalent to 0.025 absorbance unit at per min at 550 nm 25°C in a total volume of 1 ml. Under these conditions, the amount of superoxide dismutase required to inhibit the rate of reduction of cytochrome c by 50% is defined as 1 unit activity. The approximately defined units of SOD activity was determined by following equation: SOD units=(v_o/v_i)-1, where the rates of ferricytochrome c reductions were determined in the absence (v_o) and in the presence (v_i) of SOD. The Mn SOD activity which was measured in the reaction mixture containing 2 mM KCN was deducted from the total activity to calculate Zn,Cu SOD activity.

Choline acetyltransferase (ChAT) assay

ChAT activity was measured by a radiochemical method according to Fonnum (1975) with some modifications. Rat striatal tissue was homogenized with Potter-type Teflon homogenizer in 50 mM sodium phosphate buffer (pH 7.4) containing 10 mM EDTA, 0.5% (v/v) Triton X-100 and centrifuged at 4°C, 10,000×g for 15 min). The pellet was reserved for protein analysis. The tissue supenatant (100 μl) was mixed with 250 μl of a solution containing 50 mM sodium phosphate, 300 mM NaCl, 10 mM choline chloride, 0.1 mM physostigmine, 10 mM EDTA (pH 7.4). Samples (25 μl) were incubated at 37°C with 5 °Cl of [^{3}H]acetyl CoA (0.645 Ci/mmol) for 15 min. The reaction was terminated by addition of cold 10 mM phosphate buffer containing with 0.15 M NaCl. Following addition of 4% Kaligonist, the contents were transferred to scintillation vials. After adding 5 ml of the counting solution (Scintilene, Fischer Scientific), the vials were capped and gently inverted several times. Radioactivity was measured using a Beckman LS 6000TA liquid scintillation counter.

Protein assay

Protein was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard. The enzyme activities were normalized on the basis of tissue protein.

Cresyl violet staining

Rats were sacrificed by decapitaion one week after the stereotaxic surgery. Brains removed quickly from the skull on ice were postfixed for about 1 hr in 10% paraformaldehyde in 0.1 M phosphate buffer, and subjected to increasing concentration of alcohol (50% →100%) in 0.1 M phosphate buffer for 1~2 days until they sank. The brain tissue blocks were embedded in paraffin and the paraffin blocks were mounted in the holder of sliding microtome, and coronal sections 35 μm thick were cut. Tissue sections were placed on slide glass, defated with xylene for 1 hr, washed with several changes of alcohol for removing xylene, and incubated at 37°C in 0.25% cresyl violet in 200 mM acetate buffer (pH 4.0) for 10 min. The stained sections were dehydrated in 95% alcohol containing 25% acetic acid and dried in air. All sections were cover-slipped with Permount and examined by light microscopy (Zeiss, Germany). Evaluation of the extent of neuronal damage was performed by independent observers blind to the experimental conditions, at the injection site and approximately 200 μm anterior and posterior to it. Three coronal sections were selected from each brain for evaluation. The damaged tissue regions are stained less intensely than normal tissue.

Chemicals

Melatonin, kainate (KA), N-methyl-D-aspartate (NMDA), cytochrome c, xanthine, xanthine oxidase, choline chloride, physostigmine, quinine, Kaligonist, Triton X-100 were obtained from Sigma (St. Louis, MO), MK-801 maleate and 6,7-Dinitroquinoxaline-2,3-dione (DNQX) from Research Biochemical International (Natick, MA), and Scintilene, gelatin and mount solution (Permount)

from Fisher Scientific Co. (New Jersey, NY). [^{3}H] acetyl CoA (10 mCi/mmol) was from New England Nuclear (Boston, MA). Pentobarbital sodium was purchased from Hanlym Inc. (Seoul, Korea), paraffin from ShinYo Chemical Co. (Tokyo, Japan), cresyl violet from Aldrich (Milwaukee, WI).

Statistics

All results are expressed as the mean±S.D. Pairwise comparisons were done by the two-tailed Student's t test.

RESULTS

Effect of melatonin implants and glutamate receptor antagonists on KA and NMDA-induced lipid peroxidation

The bilateral intrastriatal injection of KA (10 nmol/μl/hemisphere) and NMDA (200 nmol/μl/hemisphere) all significantly increased the amount of malon-dialdehyde, the lipid peroxidation product, in the striatum by 127% and 186%, respectively (Fig. 1A). To examine inhibitory effects of glutamate receptor antagonists, DNQX and MK-801 on lipid peroxidation induced by KA and NMDA, respectively, co-injection experiments were run. When DNQX (8.9 nmol/μl/hemisphere)) was bilaterally co-injected with KA, the KA-induced lipid peroxidation level was decreased by 35% relative to the control level (vehicle treatment). When NMDA (8 nmol/μl/hemisphere)) was used as a exitotoxin, co-injection of NK-801 resulted in a more notable (about 90% relative to control) inhibition against NMDA-induced lipid peroxidation (Fig. 1A).

The treatment with empty implants (Cb) enhanced lipid perxidation by about 35%, but not significantly different from the vehicle-treated control. The significant KA- and NMDA-induced increases of striatal lipid peroxidation were observed when the glutamate receptor agonists were injected one week after the Cb treatment. Striatal homogenates prepared from the rat treated with melatonin implants one week before the intrastriatal injection of KA showed a complete inhibition of KA-induced increase (by 91%) in lipid peroxidation. Likewise, the NMDA-induced increase (by 50%) in lipid peroxidation was also significantly inhibited by melatonin although the inhibition was not complete for the lipid peroxidation enhanced by NMDA at the concentration tested in this study (Fig. 1B).

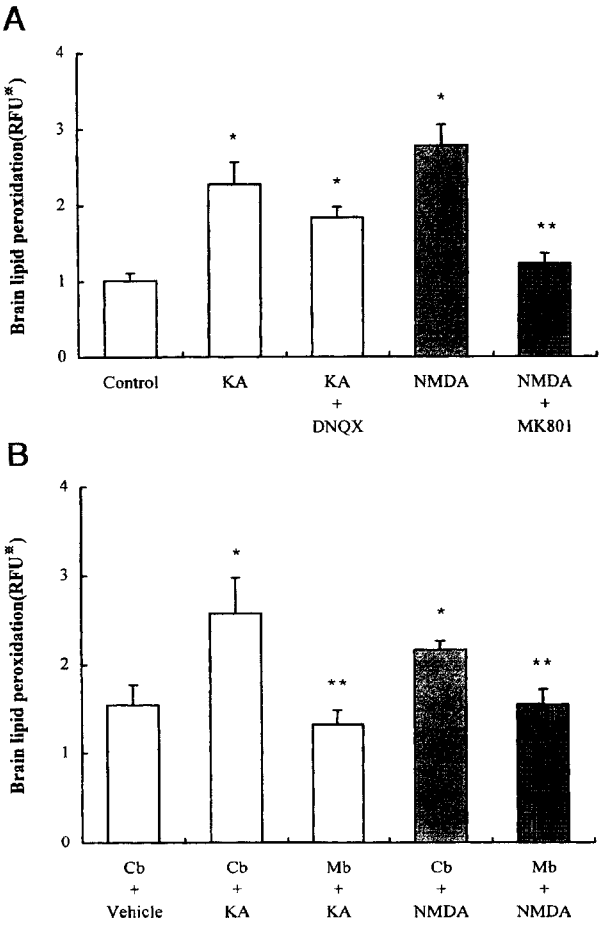

Fig. 1. Inhibitory effects of melatonin micro-implants and glutamate receptor antagonists on KA and NMDA-induced increase in lipid peroxidation in rat striatum. Each data point is the mean±S.D. of 5~14 determinations derived from 3~6 rats. (A) Rats were anesthetized and bilaterally injected into striata with saline (control), KA (10 nmol/hemisphere), NMDA (200 nmol/hemisphere) or injected with combination of KA (10 nmol) and DNQX (8 nmol) or with combination of NMDA (200 nmol) and MK801 (8.9 nmol). Rats were sacrificed by decapitation 7 days after the surgery and malonedialdehyde was determined according to the procedures given under Materials and Methods. *P<0.05 relative to control group. **P<0.05 relative to group treated only with agonist (KA or NMDA). (B) Rats were anesthetized and intrastriatally implanted with empty stainless steel bar (Cb) or melatonin-containing bar (Mb) 7 days before the intrastriatal injection of KA (10 nmol) or NMDA (200 nmol). Rats were sacrificed by decapitation 7 days after agonist treatment and assays were done in striatal homogenates. *P<0.05 relative to group implanted with Cb before vehicle treatment. **P<0.05 relative to group implanted with Cb before agonist (KA or NMDA) treatment..

Effect of melatonin implants and glutamate receptor antagonists on changes in SOD activity affected by KA and NMDA

Cytosolic superoxide dismutase (Cu,Zn SOD) level was significantly reduced by 83% in the striatum of rats treated with KA, whereas the enzyme level was reduced to a less extent (21%) following treatment with NMDA. The co-administration of either DNQX with KA, or MK-801 with NMDA enhanced the SOD level reduced by KA and NMDA, respectively, to the higher level relative to control (Fig. 2A). The treatment with empty implants (Cb) produced a 67% increase

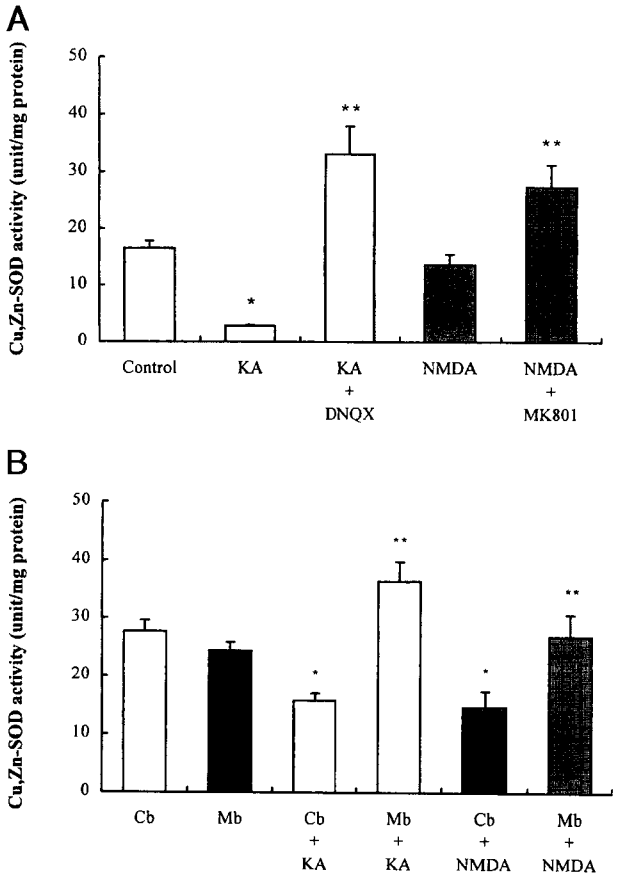

Fig. 2. Effects of melatonin micro-implants and glutamate receptor antagonists on changes in Cu,Zn SOD activity affected by KA and NMDA. Each data point is the mean±S.D. of 3~6 determinations derived from 3~4 rats. (A) Rats were anesthetized and bilaterally injected into striata with saline (control), KA (10 nmol/hemisphere), NMDA (200 nmol/hemisphere) or injected with combination of KA (10 nmol) and DNQX (8.9 nmol) or with combination of NMDA (200 nmol) and MK801 (8 nmol). Rats were sacrificed by decapitation 7 days after the surgery and Cu,Zn SOD activities were measured in striatal homogenates according to the procedures given under Materials and Methods. *P<0.05 relative to control group. **P<0.05 relative to group treated only with agonist (KA or NMDA). (B) Rats were anesthetized and intrastriatally implanted with Cb or Mb 7 days before the intrastriatal injection of KA (10 nmol) or NMDA (200 nmol). Rats were sacrificed by decapitation 7 days after agonist treatment. *P<0.05 relative to group implanted with Cb before vehicle treatment. **P<0.05 relative to group implanted with Cb before agonist (KA or NMDA) treatment..

in SOD activity relative to the vehicle-treated control. Bilateral instrastriatal injections of either KA or NMDA one week after the Cb treatment produced significant decreases in striatal SOD activities by 43% and 47%, respectively relative to sham injections with only vehicle followed by Cb treatment (Fig. 2B). Pretreatment with melatonin implants one week before intrastriatal injection of KA antagonized the KA-induced inhibition of SOD activities. Melatonin pretreatment also prevented the decrease of SOD activity induced by NMDA, but the degree of prevention was less than that against KA-induced decrease in SOD activity (Fig. 2B).

Effect of melatonin implants and glutamate receptor antagonists on KA and NMDA-induced striatal lesions

ChAT is a acetylcholine-synthesizing enzyme and used as a marker for cholinergic neurons in brain. Because striatum contains abundant cholinergic neurons, striatal tissue damage was assessed by the determination of ChAT activity in striatal homogenates. ChAT activity observed in striatal homogenates from control buffer-injected rats was 107.6±9.7 pmol/mg protein/hr (about 14.7 nmol/mg wet tissue/hr) (Fig. 3A), and this level

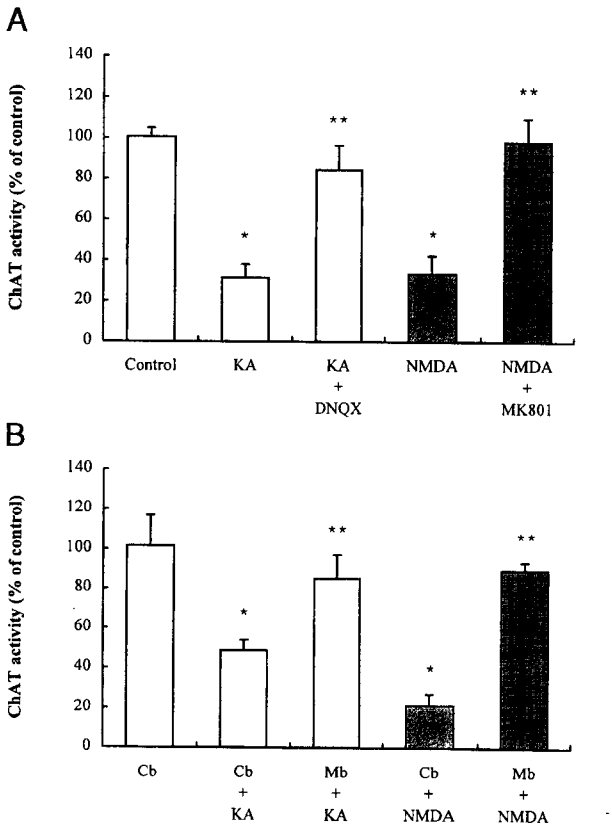

Fig. 3. Protective effects of melatonin implants and glutamate receptor antagonists against neuronal damage induced by KA and NMDA, as detected by decrease of ChAT activity. Each data point is the mean±S.D. of 3~6 determinations derived from 3~6 rats. (A) As indicated by the corresponding bar, rats were injected with saline (control), KA (10 nmol/hemisphere), or NMDA (200 nmol/hemisphere) alone, and KA- and NMDA-treated rats were co-injected with DNQX (8.9 nmol) and MK-801 (8 nmol), respectively *P<0.05 relative to control group. **P<0.05 relative to group treated only with agonist (KA or NMDA). (B) Rats were received bilateral micro-implants of melatonin (Mb) placed in the striatum (or sham operated; Cb) 7 days before the intrastriatal injection of KA (10 nmol) or NMDA (200 nmol). * P<0.05 relative to group implanted with Cb before vehicle treatment. ** P<0.05 relative to group implanted with Cb before agonist (KA or NMDA) treatment..

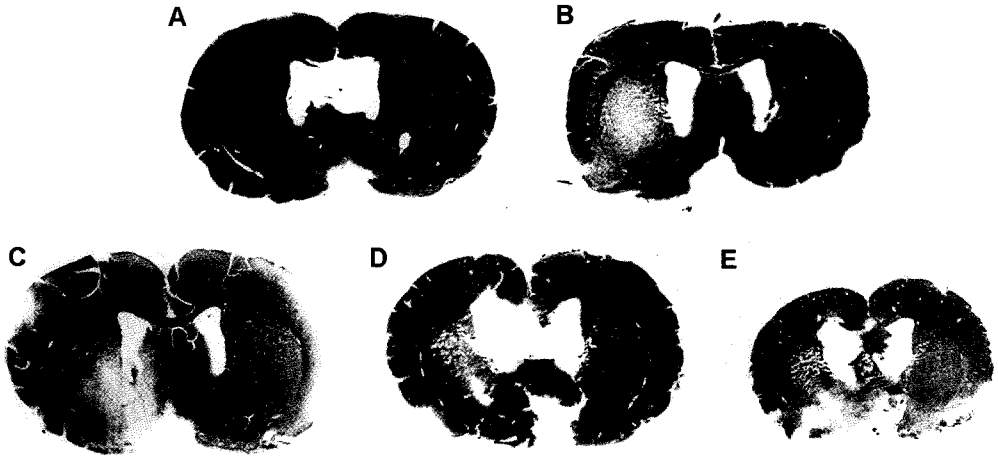

Fig. 4. Protective effect of melatonin implants and glutamate receptor antagonists against KA- and NMDA-induced neuronal damage in the striatum, as assessed by cresyl violet stainning of brain sections. (A) Rats were intrastriatally injected with saline in left side and treated with control bar in right side; (B) treated with KA (10 nmol) in left side and co-injected with KA (10 nmol)+DNQX (8.9 nmol) in right side; (C) injected with NMDA (200 nmol) in left side and co-injected with NMDA (200 nmol)+MK801 (8 nmol) in right side; (D) treated with control bar+KA (10 nmol) in left side and melatonin bar+KA (10 nmol) in right side; (E) treated control bar+NMDA (200 nmol) in left side and co-treated with melatonin bar+NMDA (200 nmol).

was in good agreement with that reported previously (Massieu and Tapia, 1994). The treatment with empty implants (Cb) did not change ChAT activity (109.7± 28.6 nmol/mg protein/hr) relative to control striatum (Fig. 3B). As shown in Fig. 3A and 3B, either administration of 10 nmol KA alone or Cb treatment with KA administration reliably produced notable decreases in ChAT activities by 69.3% and 52.8%, respectively, relative to the control striatum. ChAT activities were also significantly reduced in the striatum treated with 200 nmol NMDA in the absence and the presence of Cb pretreatment by 67.5% and 80.0%, respectively. When DNQX (8.9 nmol) was co-injected with KA, ChAT activity was diminished only by 16.2% relative to the buffer-injected control animal, indicating 78.7% protection for ChAT activity. Co-injection of MK-801 (8 nmol) with NMDA also produced 96.4% protection for ChAT activity. Similar to the protective effects of the glutamate antagonists tested, treatment with melatonin implants one week before intrastriatal injection of KA or NMDA significantly enhanced both KA- and NMDA-reduced ChAT activities, producing 68.8% and 84.9% protection, respectively.

For histological analyses animals were injected with either KA or NMDA without antagonist to the left side, and co-injected with KA or NMDA with their antagonist (DNQX or MK801) to the right side of the striatum. Another groups of rats were treated with Cb and either KA or NMDA in the left striatum, and treated with melatonin and glutamate receptor agonist in the right striatum. Expressing damage relative to the contralateral side of each brain eliminated problems associated with slight differences in staining intensity among brains. One week after instrastriatal injections of indicated agents, brain sections were processed for cresyl violet histochemistry as demonstrated in Fig. 4. The Cb-treated right striatum showed no notable damage compared to the buffer-injected left striatum although the hole generated by Cb implantation is present in the right striatum. Focal striatal injection of KA (10 nmol, n=3) resulted in marked disruption (60~90%) of striatal tissue. NMDA (200 nmol, n=3) also resulted notable damage (50~70%) in the striatum. DNQX (8.9 nmol, n=3) significantly reduced KA toxicity in the striatum from 60~90% to 10~30%, and MK-801 (8 nmol, n=3) also protected NMDA-induced striatal damage from 50~80% to 20~40%. In agreement with the ChAT measurements, cresyl-violet staining of brain sections from the rat treated with melatonin micro-implants showed similar protective effect (about 30~60% reduction) of melatonin against both KA- and NMDA-induced neuronal damage in the striatum.

DISCUSSION

Lincoln and Maeda (1992a; 1992b) have reported that the melatonin-microimplants release melatonin continuously and the constant administration of melatonin into the hypothalamus using microimplants did not cause detectable increase in the peripheral concentration of melatonin or affect the endogenous pattern of melatonin release. Therefore, it was assumed that the micro-implants of melatonin into the striatum, performed in this study, might also produce a relatively located increase in the concentration of melatonin within the striatum without effects on the endogenous melatonin signal. The total amount of melatonin infused into the brain for two weeks from the micro-implant was

about 2.28 μmol (approximately 0.16 μmol/24 hr) in this study. Physiological concentrations of melatonin at night are in the nanomolar range in the rodent and human sera (Reiter, 1991), and endogenous concentrations of melatonin in tissue are about 10^3 times higher than circulating levels (Menendez-Pelaez *et al.*, 1993). Because even the small diurnal fluctuations in tissue concentrations have a significant protective effect against DNA damage induced by the carcinogen safrole *in vivo* (Tan *et al.*, 1994), the amount of melatonin treated in this study appears to be sufficient to produce protective effect against striatal damage.

Excessive stimulation of glutamate receptors can lead to excitotoxicity, which can be characterized by triggered cell death and formation of reactive oxygen free radicals. *In vitro* treatments of rat brain synaptosomes with ionotropic glutamate receptor subtype-selective agonists (KA, AMPA and NMDA) have shown that all of those agonists elevate rates of reactive oxygen species generation (Bondy and Lee, 1993). Oxidative stress results from the biological action of oxygen free radicals. In the brain, oxygen species spread through and disrupt readily lipid membranes and free radical formation also increases the release of glutamate (Pellegrini-Giampietro, *et al.*, 1990). Glutamate may increase oxygen radical formation (Dykens *et al.*, 1987), and thus form a feedback loop that causes progressive brain damage. In the present study, striatal lipid peroxidation was significantly enhanced by the intrastriatal administration of either KA or NMDA and *in vivo* treatment with melatonin blocked these responses, indicating the protective role of melatonin against both KA- and NMDA-induced. oxidative stress. The activity of an antioxidant enzyme, Cu,Zn SOD, was depressed one week after the treatment of either KA or NMDA. Although the imbalance between the antioxidant enzymes may cause cell injury (Avraham *et al.*, 1988; Zemlan *et al.*, 1989) and overexpression of Cu,Zn SOD has been shown to exacerbates KA-induced apoptosis of cultured neurons of transgenic-Cu,Zn SOD mice (Bar Peled, *et al.*, 1996), reduced Cu,Zn SOD activity exacerbates neuronal cell injury in ischemia and familial amylotrophic lateral sclerosis (Kondo *et al.*, 1997; Hugon, 1996) and the protective role of SOD is widely accepted even when other antioxidant enzymes do not change (Freeman *et al.*, 1983; Krall *et al.*, 1976). Especially recent studies with transgenic mice or neuronal culture system demonstrates that overexression of Cu,Zn SOD protects against glutamate or KA neurotoxicity whereas reduced Cu,Zn SOD activity potentiates the neurotoxicity (Schwarts *et al.*, 1998; Schwarts and Coyle, 1998). Therefore, enhancing effect of melatonin on Cu,Zn SOD activity reduced by KA or NMDA suggests that *in vivo* treatment with melatonin protects against oxidative damage via genaration of superoxide anions ($O_2^{\cdot -}$), which may be mediated through the glutamate receptor subtypes. The balance between the antioxidant enzymes is necessary to get antioxidant protection because the increase of SOD will dismutate $O_2^{\cdot -}$, but it will increase H_2O_2, which if not efficiently removed by catalase or GSH-peroxidase, will produce · OH radicals that are highly toxic for the cell. Pharmacological levels of melatonin have been reported to stimulate the activity of GSH-peroxidase (Barlow-Walden *et al.*, 1995).

The protective effects of melatonin against KA-induced exitotoxicity have been well documented through both *in vitro* and *in vivo* studies by others (Melchiorri *et al.*, 1995; Giusti *et al.*, 1995; 1996a; 1996b), and our data correspond well with those reports. However, in contrast with the finding from the study with cultured cerebellar granule neurons which showed a inability of melatonin to protect from NMDA excitotoxicity, assessed by MTT viability assay (Giusti *et al.*, 1995), present data demonstrate that continuous melatonin treatment blocks NMDA-mediated neurotoxicity as well as KA-induced neurotoxicity in the rat striatum, assessed by the determination of ChAT activity in striatal homogenates and by microscopic observation of rat brain section stained with cresyl violet. Although exitotoxicity mediated via the non-NMDA glutamate receptors was also reported to be more susceptible to an inhibition with vitamin E analog trolox in primary neuronal cultures (Chow *et al.*, 1994), this, again, has not been demonstrated *in vivo* setting. It could be suggested from the our data, although NMDA and non-NMDA receptor mediated cellular signalling events may different (Hollman and Heinermann, 1994), melatonin exerts antioxidant effects *in vivo* by directly scavenging hydroxyl and peroxy radicals (Reiter, 1996) and interacting with antioxidant defense systems including increase in activity of SOD scavenging superoxide radicals to prevent neuronal damage associated with the formation of reactive oxygen species triggered by the activation of both NMDA and non-NMDA ionotropic glutamate receptors.

In conclusion, we have demonstrated, in vivo, that melatonin protects against striatal damages mediated by glutamate receptors, both non-NMDA and NMDA receptors, and it's protective effects are comparable to those of DNQX and MK801 which are the KA and NMDA-receptor antagonist, respectively. Whether the free radical scavenging activity of melatonin is indeed responsible for neuroprotection is needed to be further studied.

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