Changes in the Density of Nitrergic Neurons in the Hippocampus of Rats Following Kainic Acid and Melatonin Administration

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Summary

Nitric oxide (NO) may play a role in the pathophysiology of excitotoxicity. It is also possible that increase in Ca^{2+} overload and NO-mediated events are involved in neuronal loss during excitotoxicity. Using nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry, we have investigated the effects of melatonin on NADPH-d positive hippocampal neurons after kainic acid (KA) induced excitotoxicity in female rats of Wistar strain. Cytosolic Ca^{2+} (free calcium) in all the respective experimental groups was also studied. Kainic acid was administered, with a single dose of 10 mg/kg/bw (body weight) to the animals. KA treated rats were given melatonin at a dose of 20 mg/kg/bw (for 14 day). On the last day of treatment, animals were transcardially perfused with 4 % paraformaldehyde under deep thiopental anaesthesia. Cryostat sections (20 µm) were cut and stained for NADPH-d positive neurons. KA exposed animals showed a significantly increased number of NADPH-d positive neurons in the dorsal and ventral blade of the dentate gyrus (DG), hilus, CA1 and CA3 area of hippocampus, with a parallel increase in intracellular free Ca^{2+} ion concentration, as compared to the control group. KA + melatonin-treated animal groups showed reduced number of NADPH-d positive neurons in DG, hilus, CA1 and CA3 areas and a decline in cytosolic Ca^{2+} concentration, as compared to KA treated group. Our study suggests that the enhanced levels of cytosolic Ca^{2+} and nitric oxide (NO) play an important role in kainate induced excitotoxicity. Inhibition of NO production may be another means whereby melatonin can reduce oxidative damage and seems to play important role in neuroprotection.

Key words

NADPH-diaphorase • Kainic acid • Melatonin • Nitric oxide • Hippocampus

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Introduction

During excitotoxicity different pathways are activated, involving elevated free radicals and ROS, rise in intracellular calcium, loss of membrane integrity, progressive proteolysis, and neuronal loss. The alterations due to excitotoxicity lead to nitric oxide synthase (NOS) activation, resulting in enhanced nitric oxide (NO) production (Christopherson and Bredt 1997). NO takes part in the inflammatory and apoptotic pathways (Farooqui *et al.* 2001). NO has been implicated in the pathophysiology of some neurodegenerative diseases such as Parkinson's (Hantraye *et al.* 1996), in brain ischemia (Samdani *et al.* 1997) and epilepsy (Chuang *et al.* 2009).

NO acts as a neurotransmitter and is also involved in synaptic plasticity, neurite formation and dendritic branching (Bredt 1999). The production of NO in intact neurons occurs in response to excitatory stimuli that requires an intracellular Ca^{2+} ion influx (Kiss 2000). NO reacts with superoxide radical $(O_2^-)^{\bullet}$, yielding peroxynitrite radical (NOO^-) and eventually hydroxyl ion ($HO^{\bullet}$), which are highly reactive free radical species; and the intermediate products which are powerful inducers of lipid, protein, and DNA per oxidation as well (Moncada *et al.* 1991). An excessive amount of NO, a free radical which is generated by the inducible form of NO synthase, is known to cause cytotoxic changes in cells (Calabrese *et*

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al. 2007). Hence, NO synthase is considered a pro-oxidative enzyme, and any factor that reduces its activity would be considered an antioxidant.

Nitric oxide (NO) is produced from L-arginine by NOS. Three different forms of NOS are known, eNOS (endothelial nitric oxide synthase) an endothelial form is responsible for cardiovascular actions, iNOS (inducible nitric oxide synthase) an inducible form found originally in macrophages and involved mainly in immunological processes (Benešová *et al.* 2005), and nNOS (neuronal nitric oxide synthase) which is a neuronal form. Although all forms can be found in the CNS, the specific actions attributed primarily to NO production are to nNOS. nNOS (NOS type I) is related with signal transduction in peripheral and central neurons. It is expressed by only a small percentage of neurons, considered to be interneurons. nNOS activity is dependent on intracellular calcium and its production is a calmodulin-dependent process, therefore it must be preceded by elevation of intracellular Ca^{2+} concentration (Kiss 2000). Ca^{2+} influx is induced by activation of kainate receptors, in excitotoxicity (Lu *et al.* 1996). Rise in Ca^{2+} concentration in neurons may eventually lead to the increased excitability, further leading to the activation of nNOS. The enhanced release of nitric oxide due to activation of nNOS ultimately leads to cellular toxicity, which may be an important factor for massive neuronal loss.

Earlier studies have shown that nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) may correspond to nNOS, and it is therefore suggested that neurons containing NADPH-d might be capable of producing NO. The hippocampus, particularly CA3 and CA1 areas are especially sensitive to the excitatory and neurotoxic effect of KA and it is the area of its primary action (Ben-Ari *et al.* 1981). The neural events in the hippocampus have been studied intensively (Zagulska-Szymczak *et al.* 2001) due to its involvement in a variety of neurodegenerative conditions as well as its probable role in learning and memory. The marked sensitivity of hippocampus to excitotoxic and neurotoxic effects of KA is due to the great density of binding sites for excitatory amino acids in this region (Benešová *et al.* 2005, Kubova *et al.* 2001). It has been studied that KA induces neural cell death due to apoptosis as well as by necrosis (Chuang *et al.* 2009).

Melatonin, the major hormone produced by the pineal gland, is shown to have antioxidant, anticonvulsant and neuroprotective effects (Jain and Bhatnagar 2007). Recent studies have shown that melatonin inhibits the activity of NO synthase, beside it is NO and peroxynitrite scavenging activity (Tapias *et al.* 2009). Inhibition of NO production may be another means whereby melatonin can reduce oxidative damage and seems to play important role in neuroprotection.

This study is an attempt to investigate the effects of melatonin on NADPH-d positive hippocampal neurons in kainic acid (KA) induced excitotoxicity in rats. Aim of the study was to assess the cytosolic Ca^{2+} concentration, NOS activity and to investigate role of melatonin in amelioration of KA induced changes on the nitrergic neurons in hippocampus.

Methods

Adult female rats, 2 months old of Wistar strain (body weight 120±5 g) were used for the experiments. The animals were housed in polypropylene cages at controlled temperature (25±2 °C) and relative humidity (60±5 %) according to standard laboratory protocol. The animals were given food and water *ad libitum* and were housed in an animal house with a 12-h light/dark cycle. All experiments were approved by IAEC (Institutional Animal Ethical Committee) and the laboratory was approved by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India (Approval no. #973/ac/06/CPCSEA).

There were 48 animals in all the experimental groups, with 12 in each group. Animal groups used in the experiment: group I – control/saline solution (0.2 ml), group II – a single dose of kainic acid (10 mg/kg), group III – a single dose of kainic acid (10 mg/kg) then melatonin (20 mg/kg) (melatonin was given immediately after KA treatment on the first day and was given a single dose daily for 14 days), and group IV – a single dose of melatonin (20 mg/kg) for 14 days. Each injection was intraperitoneal and in the same volume (0.2 ml). Only those rats exhibiting full limbic seizures, forelimb clonus with rearing, were included in the study. Seizures occurred 45-50 min after KA injection. On average, the survival was 66 % in the KA treated group (8 rats survived) and in melatonin + KA treated group, it was 83 % (10 rats survived). KA was dissolved in phosphate-buffered saline (PBS), and the pH adjusted to 7.4 with NaOH. Melatonin was dissolved in absolute ethanol and further diluted in saline to a final ethanol concentration of 2 % (Chung and Han 2003). Melatonin injection was intraperitoneal and in the same volume (0.2 ml).

All the included animals were decapitated on the 14th day under diethyl ether anesthesia. NADPH-d histochemistry was carried out by the method of Scherer-Singler *et al.* (1983). Briefly, brain of control and experimental groups were dissected out, fixed and placed in sucrose solution overnight in each treatment (10 %, 20 % and 30 % respectively). We studied the hippocampus region between the AP plane 2.5 mm and 4.0 mm posterior to the bregma. Serial sections were obtained of 20 μm thickness on cryostat. Every third section was selected for quantification of NADPH-d positive neurons. 20-25 sections were quantified from each brain. Brain sections were incubated in 0.1 M Phosphate buffer (pH=7.4), containing 0.2 mg/ml nitro blue tetrazolium, 25 mg/ml NADPH and 0.6 % Triton. Sections were mounted in glycerine jelly. NADPH-d positive neurons were then quantified per section area, under a light microscope (Olympus Provis AX 70) in the CA1, CA3, and DG regions of hippocampal formation. The effect of KA was studied in two distinct hippocampal regions: in the dentate gyrus (DG) and cornu amonis (CA) subfields.

Cytosolic free Ca^{2+} was measured by using Fluo-3 AM fluorescent dye and by the method of Mattson *et al.* (1993). Briefly, brain homogenate was centrifuged at 1000 g and supernatant was then incubated with 5 μM Fluo-3 AM for 30 min in dark. Fluorescence was then measured spectrofluorimetrically at 506 nm excitation and 526 nm emission. Free cytosolic Ca^{2+} was expressed as AFU (arbitrary fluorescence units).

Results were expressed as the mean ± SD. For the statistical calculation data were analyzed using ANOVA (one way analysis of variance) followed by Bonferroni post test to compare all pairs of groups. Level of significance was set at $p<0.05$. Prism Software program (Graphpad Software Inc.) was used.

Results

Systemic KA administration produced the well described sequential behavioral changes (Sperk 1994). Initially the rats exhibited loss of mobility and rigid postures. After 45-50 min, they exhibited changes like staring spells, head nodding, clonic convulsion with rearing and falling. In the present study, co-treatment with melatonin did not significantly alter the clinical course of KA induced limbic seizures.

The results show that kainic acid treatment lowered the number of NADPH-d positive neurons

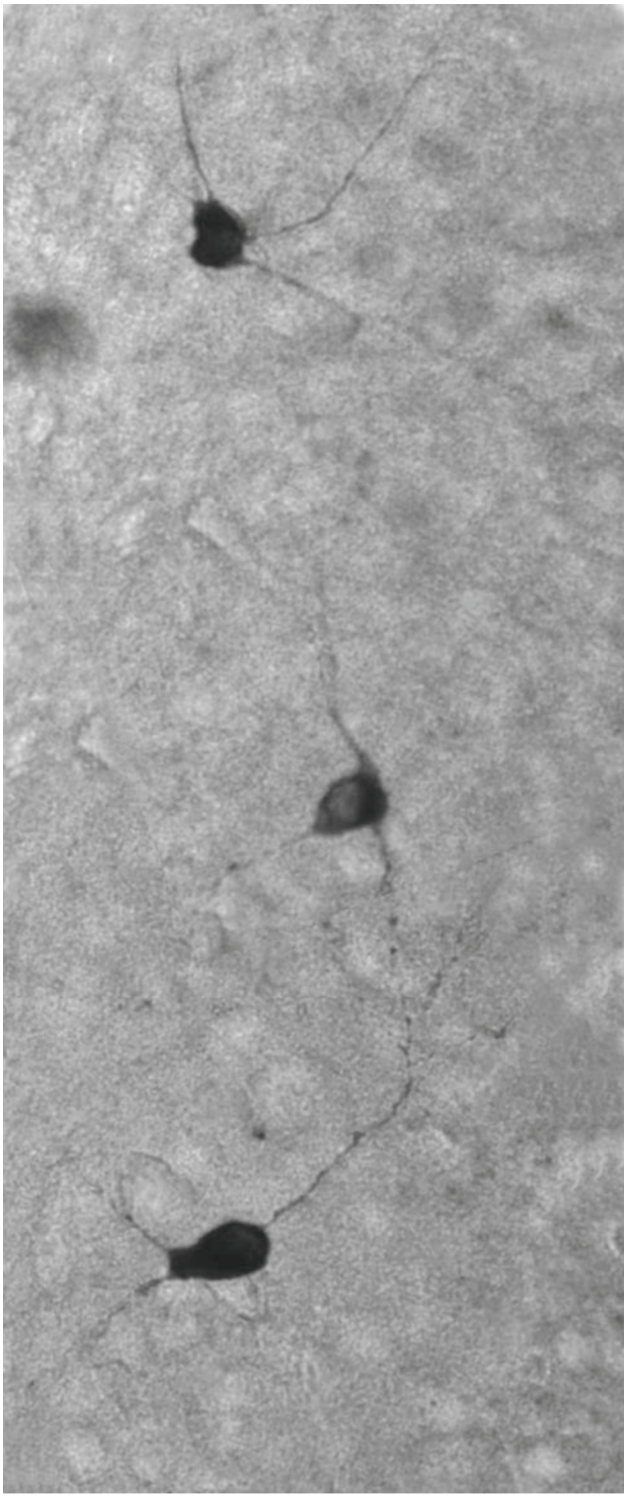

Fig. 1. NADPH-d positive interneuron in the CA1 region of hippocampus. NADPH-d staining. Direct magnification 400x.

(Fig. 1) in all the investigated areas of the hippocampus, compared to the control group which was considered as baseline (Fig. 2A-2E). A significant increase in the intracellular Ca^{2+} concentration ($p<0.05$) in KA exposed animals, was also observed when compared to the control

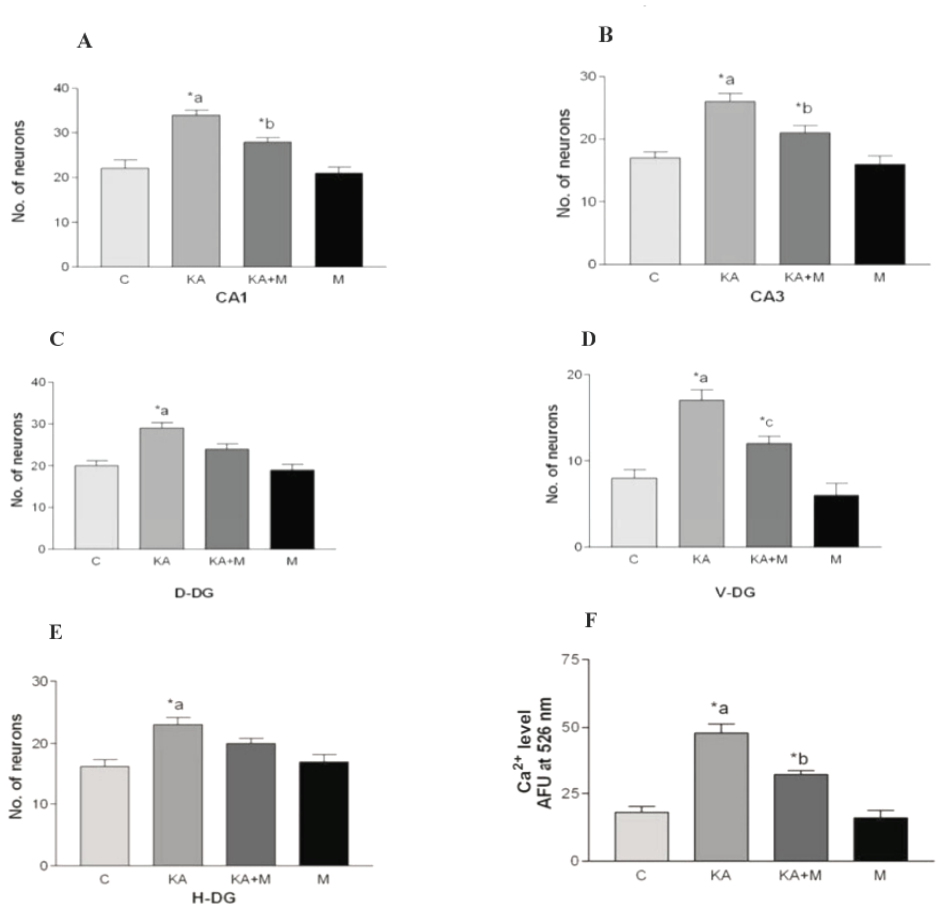

Fig. 2. NADPH-d histochemistry in rat hippocampus (C – Control, KA – kainic acid treated, KA+MEL – kainic acid + melatonin-treated and M – melatonin-treated). **A**: Number of NADPH-d positive neurons in the CA1 region of hippocampus per section area. **B**: Number of NADPH-d positive neurons in the CA3 region of hippocampus per section area. **C**: Number of NADPH-d positive neurons in the dorsal blade of dentate gyrus per section area. **D**: Number of NADPH-d positive neurons in the ventral blade of dentate gyrus per section area. **E**: Number of NADPH-d positive neurons in the hilus of dentate gyrus per section area. **F**: Free cytosolic Ca^{2+} level measured by using Fluo-3 AM, Ca^{2+} concentration is measured arbitrarily by determining fluorescence units. All values are expressed as mean ± SD, data comparisons were carried out using one way analysis of variance followed by Bonferroni post test to compare all pairs of groups, ($p<0.05$). * – difference is significant at 5 % level of probability, *a – when compared with control group, *b – when compared with KA-treated group, *c – when compared with KA+M-treated group.

group (Fig. 2F). Melatonin treatment successfully prevented the rise in number of NADPH-d positive neurons in hippocampus. Melatonin treatment in group III, significantly ($p<0.05$) decreased the number of NADPH-d positive neurons in CA1 (Fig. 2A), CA3 (Fig. 2B), and DG (Fig. 2C, 2D) regions of the hippocampus, when compared to the KA treated group II. Melatonin treatment after KA exposure in group III, showed a significant ($p<0.05$) decline in the elevated cytosolic Ca^{2+} concentration, when compared to KA treated animals indicating the possible role of melatonin in calcium homeostasis (Fig. 2F).

In the present study, treatment with melatonin did not significantly alter the number of NADPH-d positive neurons in group IV. Results show that this group treated with melatonin alone did not show any significant difference in the number of NADPH-d positive neurons in all investigated regions of the hippocampus, when compared to the control group. No significant change in free cytosolic calcium ion concentration was recorded when compared to the control group (Fig. 2F).

Discussion

In this investigation, we demonstrated that treatment with kainic acid increased the number of NADPH-d positive neurons, in all examined areas of the hippocampus. It is evident that nNOS activity in kainate treated group is not regionally homogenous, hippocampal CA1 and CA3; regions show enhanced production of NO when compared to the control group. Our data were consistent with previous reports showing that the CA1 and CA3 zones, which are most susceptible to neuronal injury following KA treatment, never recovered protein synthesis, indicating that a prolonged deficit in protein synthesis correlates with selective vulnerability of different nuclei (White *et al.* 2000, Farooqui *et al.* 2001, Benešová *et al.* 2005). These finding are consistent with our report that show hippocampal neurons to be the most vulnerable to KA toxicity. This also support the view that KA causes a rise in the level of ROS, a rapid increase in Ca^{2+} overload, enhanced NO production, generalized depolarization, progressive proteolysis, and loss of membrane integrity, which ultimately result into neurodegenration (Montecot

et al. 1997, Benešová *et al.* 2004).

While KA has been widely used as a model of temporal lobe epilepsy and selective hippocampal neurodegenration, few attempts have been made to characterize the role of calcium imbalance and nNOS activity associated with this limbic seizure model. Although the mechanism of neurotoxic effect of NO in KA-induced injury is not clearly known, it is attributed to the oxidative effects and calcium imbalance.

Our results show that melatonin co-treatment against KA toxicity, decreased the density of nitrergic elements in all the examined hippocampal sub regions. Melatonin administration decreased nNOS activity, calcium overload and kainate induced neurodegenration. The underlying mechanism of neuroprotection by melatonin is attributed to its potent free radical scavenging ability and maintenance of calcium homeostasis (Jain and Bhatnagar 2007, Tapias *et al.* 2009). Furthermore melatonin inhibits the production of NO and may contribute to its neuroprotective properties in various pathophysiological conditions (Chung and Han 2003). Many studies have shown that nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) may correspond to the neuronal NOS, and it is therefore suggested that neurons containing NADPH-d are likely to be capable of producing NO (Montecot *et al.* 1997). The method used to demonstrate nitrergic elements in the brain is based on histochemical reaction for NADPH-d. The relatively simple NADPH-d histochemical technique was widely used to identify NO producing elements in the brain of representatives of all vertebrate classes (Benešová *et al.* 2005).

It has been repeatedly proved that NADPH-d activity and NOS immunoreactivity in the nervous system is widely co-localized in the same sets of neurons (Moreno *et al.* 2002). KA administration brought about enhanced release of NO in all examined regions of the hippocampus which was significantly observed in CA1, CA3 areas of the hippocampus causing maximum injury to the neurons. The principal cell type of CA1 and CA3 regions is the pyramidal cell (Lorente de Nó 1934). CA3 pyramidal neurons are among the most responsive neurons to kainate in the brain (Ben-Ari and Cossart 2000). It is well established that prolonged seizure activity can lead to irreversible brain damage, by both necrotic and apoptotic types of cell death, which has been recently reported as a consequence of seizures (Langmeier *et al.* 2003). Intraperitoneal administration of KA results in the death of neurons in the CA1 and CA3 areas due to excitotoxicity as evident from our results. The altered cellular processes caused by KA administration include: enhanced ROS production, excessive Ca^{2+} overload, NOS activation, neuronal cell loss, and glial reactivation (Wojtal *et al.* 2003).

The number of NADPH-d positive neurons in dorsal and ventral blades of the dentate gyrus increased after KA administration but these changes were not as evident as in CA1 and CA3 regions of the hippocampus. Our results, showing a regulatory effect of melatonin on NOS activity and intracellular Ca^{2+} could be explained by melatonin's neuroprotective role. It is a potent free radical scavenger, an antioxidant that protects cells against the damage induced in different pathologic conditions (Chung and Han 2003). NADPH-d positive neurons are probably interneurons. The reason of the alteration in number of NADPH-d neurons can also be a result of changes in gene expression by melatonin (Kotler *et al.* 1998). Our findings also suggest that Ca^{2+} overload induced higher expression of nNOS, which could contributes to the sustained neuronal excitation and ultimately in enhanced activity of nNOS. In fact, NOS gene expression activated by hypoxia in central and peripheral neurons had recently been observed (Chang *et al.* 2003).

In conclusion, this study provides evidence that melatonin posses neuroprotective properties against KA-induced toxicity, and indicates its efficiency to regulate Ca^{2+} and NO levels in different regions of hippocampus. The changes in the density of nitrergic neurons in KA and melatonin exposed animals are region specific in rat brain. Based on our findings, it seems that melatonin has an important role to play and regulate neuronal loss in excitotoxicity.

Conflict of Interest

There is no conflict of interest.

Abbreviations

NADPH-d – nicotinamide adenine dinucleotide phosphate-diaphorase; NO – nitric oxide; Ca^{2+} – free calcium ion; KA – kainic acid; ROS – reactive oxygen species, mg – milligram; kg – kilogram; bw – body weight; μm – micrometer; DG – Dentate Gyrus; CA1 – cornu ammonis 1; CA3 – cornu ammonis; CNS – central nervous system; NOS – nitric oxide synthase; eNOS – endothelial nitric oxide synthase; iNOS – inducible nitric oxide synthase; nNOS – neuronal nitric oxide synthase.

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