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Research report

Post-insult exposure to (±) kavain potentiates *N*-methyl-D-aspartate toxicity in the developing hippocampus

Patrick J. Mulholland, Mark A. Prendergast*

Department of Psychology, University of Kentucky, 115 Kastle Hall, Lexington, KY 40506-0044, USA

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Abstract

Kavapyrone extracts of the pepper plant *Piper methysticum Forst.* have been reported to be pharmacologically active in the brain by modulating the function of several ionotropic receptor systems and voltage-sensitive ion channels. While kavapyrones have previously demonstrated neuroprotective effects against several forms of neurotoxicity, the possibility remains that perturbed function of neuronal ion transport may prove to be neurotoxic in some instances. The present studies were designed to examine the effects of the kavapyrone, (±) kavain, on viability of organotypic hippocampal explants exposed to the excitotoxin *N*-methyl-D-aspartate (NMDA). Exposure to (±) kavain (1–600 μM) for 24 h did not alter neuronal viability in the CA1, CA3, or dentate gyrus regions of hippocampal explants. However, higher concentrations of (±) kavain (≥300 μM) produced marked neurotoxicity in the lacunosum moleculare layer of the hippocampus. One hour of exposure to NMDA (20 μM) produced significant neuronal death in both the CA3 and CA1 pyramidal cell regions, effects prevented by co-exposure to MK-801 (30 μM). Co-exposure of explants to (±) kavain (1–100 μM) with NMDA did not alter the severity of NMDA-induced neurotoxicity. However, exposure of NMDA-treated explants to (±) kavain (≥10 μM) for 24 h after insult produced significant increases in neurotoxicity in the CA1 and dentate gyrus regions of explants. In conclusion, while the kavapyrone (±) kavain is neurotoxic only at high concentrations when exposed alone to the developing hippocampus, it appears to adversely affect neuronal recovery following excitotoxic insults.

Theme: Neurotransmitters, modulators, transporters, and receptors

Topic: Excitatory amino acids: excitotoxicity

Keywords: Kava kava; Neurotoxicity; *Piper methysticum*; Kavapyrone; NMDA

1. Introduction

Human consumption, by both adults and children, of beverages containing crude extracts of rhizome and roots from the pepper plant *Piper methysticum* has been documented in Polynesian and Micronesian societies for centuries [12,25]. Pharmacological studies, including double-blind, placebo-controlled studies, support anecdotal observations of sedative, euphoric, and anxiolytic effects of these extracts in humans [17,22,28]. This plant expresses several structurally similar 4-methoxy-α-pyrone derivatives that likely contribute to the sedative and anxiolytic effects of kava use [1,7,19,20]. Pharmacological analysis of these highly lipophilic compounds suggests that they modulate function of several transmitter receptor systems and voltage-sensitive ion channels. For example, *Piper methysticum* leaf and root extracts containing high concentrations of the kavapyrones 7,8-dihydrokavain and methysticin demonstrated high binding affinity for γ-amino-butyric acid type-A (GABA-A), dopamine D2, opioid (mu and delta), and histamine (H1 and H2) receptors [7,14].

Functional studies further suggest a significant effect of kavapyrones on the function of multiple transmitter systems. Kavain has been reported to produce rapid inhibition of voltage-sensitive Na^+ channel function in a synaptosomal preparation [10] and to reduce veratridine-induced striatal glutamate release in vivo [9]. Further, kavain and dihydromethisticin were reported to reduce field potential changes produced by Mg^{2+} depletion and subsequent over

*Corresponding author. Tel.: +1-859-257-6120; fax: +1-859-323-1077.

E-mail address: prender@uky.edu (M.A. Prendergast).

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activation of *N*-methyl-D-aspartate (NMDA)-type glutamate receptors in the guinea pig hippocampus [29]. Kavain and yangonin have also been reported to induce dopamine release in nucleus accumbens in awake, behaving rats [2], an effect related to the onset of sedation and muscle relaxation. Still more data suggest that some of the kavapyrones, kavain in particular, alter serotonin levels in the nucleus accumbens [2]. An additional study, however, failed to observe kavapyrone-induced alterations in dopamine or serotonin release in rat striatum or cortex [3]. As a whole, these data suggest that the anxiolytic, sedative, and euphoric effects of *Piper methysticum* extracts likely involve altered function of several neurotransmitter systems, as well as, reduced function of voltage-sensitive Na^+ and Ca^+ channels [23].

One recent report suggests that the pharmacological effects of kavapyrones may protect the CNS from different forms of excitotoxic insult. In a rodent model of Parkinson's disease, kavain injection before and after exposure to the dopamine neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) attenuated MPTP-induced degeneration of nigral neurons [24]. This protective effect of kavain was attributed to antagonism of glutamate receptor-mediated excitotoxicity subsequent to MPP^+ production and mitochondrial dysfunction. Indeed, excess glutamate release has been demonstrated to occur in dopaminergic neuronal populations following MPP^+ administration [5]. However, the effects of kavain, as well as other kavapyrones, on glutamate receptor-mediated excitotoxicity have not been specifically examined in previous studies. Thus, it remains unclear if the neuroprotective effects of kavapyrones against MPP^+ reflect antagonism of glutamatergic systems or other, uncharacterized receptor systems and ion channels. The purpose of the present studies was to evaluate the effects of the kavapyrone, (±) kavain, on hippocampal neuronal viability and to examine the effects of kavain on NMDA-induced neurotoxicity.

2. Materials and methods

2.1. Preparation of hippocampal explants

Whole brains from 8-day-old male Sprague–Dawley rat pups (Harlan) were aseptically removed and placed in dissection medium (4 °C) made of Minimum Essential Medium (MEM) plus 200 mM glutamine, 25 mM HEPES, and 50 μM penicillin/streptomycin solution [26]. Bilateral hippocampi were dissected out and placed into culture medium at room temperature. Culture medium is made of dissection medium with the addition of 36 mM glucose, 25% (v/v) Hanks' balanced salt solution (HBSS) and 25% heat-inactivated horse serum (HIHS). Each hippocampus was sectioned coronally at 200 μm using a McIllwain tissue chopper and placed into fresh culture medium. Each unilateral hippocampus yielded ~12 slices. Three slices were then transferred onto each Millicell-CM 0.4 μM biopore membrane insert in pre-incubated culture medium. Inserts were placed in 35-mm six-well culture plates and excess medium on top of the slices was aspirated so that explants remained exposed to the atmosphere of 5% CO_2/95% air at 37 °C. All culture medium solutions were supplied by Gibco BRL (Gaithersburg, MD) with the exception of HIHS (Sigma, St. Louis, MO). Care of all animals was carried out in accordance with the National Institute of Health guide for the care and use of laboratory animals (NIH publications no. 80-23).

2.2. (±) Kavain and NMDA exposure

Hippocampal explants were allowed to become attached to insert membranes and stabilized in culture medium for 5 days prior to the start of any experiments. In an initial study, explants were transferred at day 5 in vitro to six-well plates containing normal culture medium (with the addition of dimethyl sulfoxide in a dilution of 1/1000) or medium containing (±) kavain (1–600 μM; ChromaDex, Laguna Beach, CA). The culture medium of all groups also contained the non-vital fluorescent marker propidium iodide (Molecular Probes, Norwich, CT) in a concentration of 2.5 μg/ml. For all experiments, a (±) kavain stock solution was prepared in 100% dimethyl sulfoxide and a 1/1000 dilution in normal culture medium was made to prepare solutions used for hippocampal exposure. All explants were then returned to incubators for 24 h and uptake of propidium iodide was assessed in each region of explants as described below.

Additional hippocampal explants were exposed for 1 h on day 5 in vitro to 20 μM NMDA in calcium Locke's buffer (2.5 mM $CaCl_2$) without or with addition of (±) kavain (1–100 μM) or MK-801 (30 μM). Following the 1 h exposure to treatment, all cultures were washed twice in calcium Locke's buffer and placed in normal culture medium containing propidium iodide as above. Explants were then returned to the incubator for 24 h prior to examination of propidium iodide uptake. A final group of hippocampal explants was exposed to NMDA as above for 1 h in the absence of (±) kavain. Following the 1-h exposure to NMDA, explants were washed twice in calcium Locke's buffer and transferred to six-well plates containing normal medium with the addition of propidium iodide or similar medium containing (±) kavain (1–100 μM). Explants were then returned to the incubator for 24 h and uptake of propidium iodide was examined as described below.

2.3. Measurement of cytotoxicity (propidium iodide uptake)

Cell damage was detected by fluorescent microscopy of propidium iodide nucleic acid staining. Propidium iodide uptake was visualized using a 4× objective on a Nikon

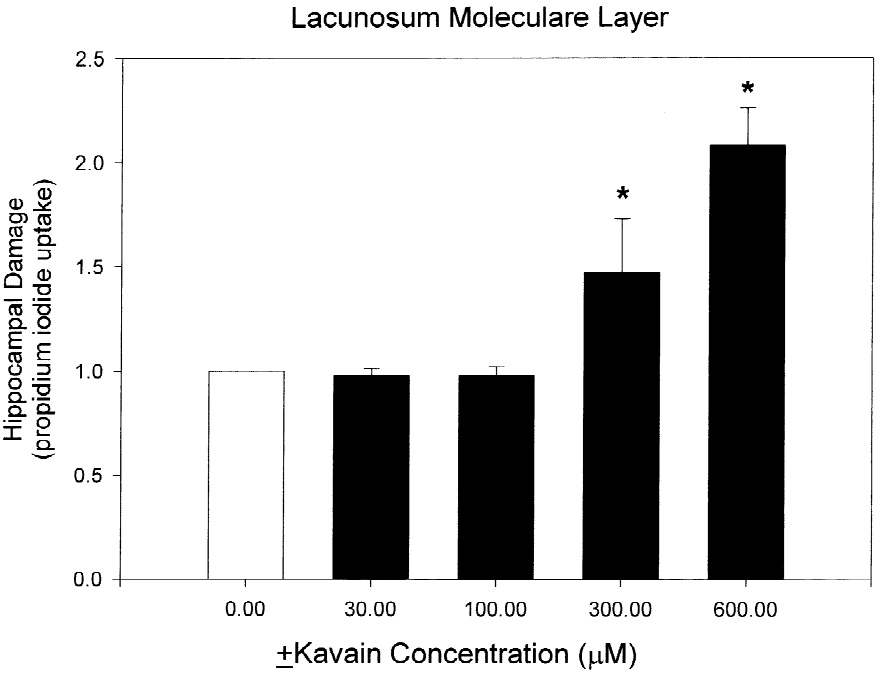

Fig. 1. Twenty-four hours of (±) kavain exposure (≥300 μM) produced significant neurotoxicity (propidium iodide uptake) in the lacunosum moleculare layer of neonatal rat hippocampal explants. Data expressed as proportion of control. *$P<0.05$ vs. control.

TE200 microscope (Nikon, Melville, NY) fitted for fluorescence detection (mercury-arc lamp) connected to a personal computer via a CCD camera. Propidium iodide has a peak excitation wavelength of 536 nm and was excited using a band-pass filter exciting the wavelengths between 510 and 560 nm. The emission wavelength of propidium iodide is 620 nm in the visual range. Intensity of propidium iodide fluorescence was analyzed by densitometry using NIH Image. Optical intensity (in arbitrary optical units) was determined in the CA1, CA3, dentate gyrus, and lacunosum moleculare layer of each individual slice culture. For each slice culture, a background optical intensity measurement was obtained from the visual field surrounding the culture and this measurement was subtracted individually from those obtained for the culture subregions prior to statistical analysis.

2.4. Statistical analyses

All fluorescence intensity measurements were recorded in arbitrary units and normalized (to daily control values) to minimize effects of potential daily variations in camera performance. All data points were normalized using the

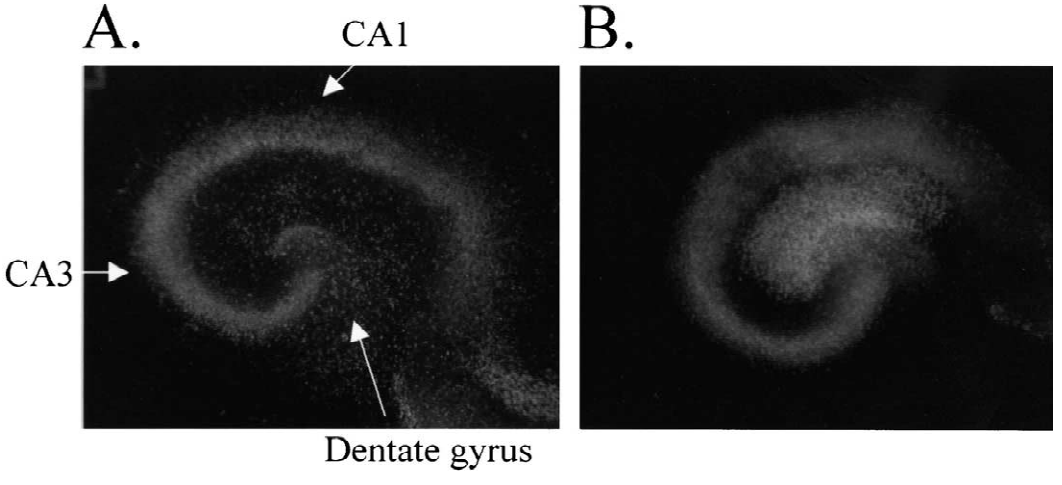

Fig. 2. Representative images of propidium iodide uptake in: (A) a control hippocampal explant and (B) an explant exposed to 300 μM (±) kavain for 24 h. Significant increases in neurotoxicity were observed only in the medial portion of the lacunosum moleculare layer, ventral to the CA1 region.

following formula: $[(S - B) - Nx]/Cx$, where S was the intensity of P.I. fluorescence for a given region in a given slice; B was background; Nx was the mean fluorescence of a particular region in controls not exposed to NMDA, and Cx was the mean fluorescence of a particular region in controls exposed to NMDA. For all measures, non-normalized data were analyzed using two-way analyses of variance to compare different treatments and different hippocampal subregions (CA1 vs. CA3 vs. dentate gyrus vs. lacunosum moleculare). Post hoc analyses, when appropriate, were conducted using the Student–Newman–Keuls method.

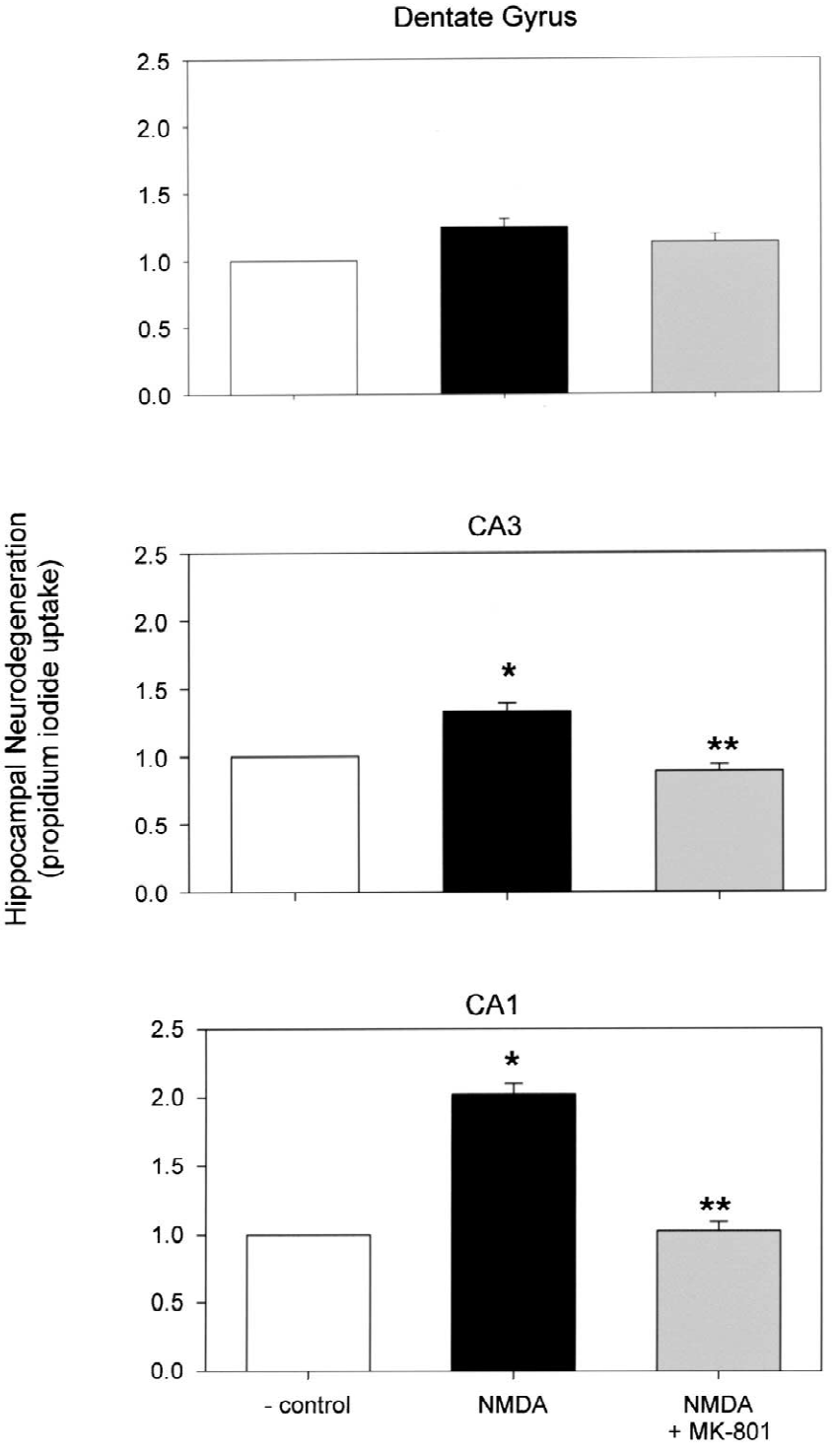

Fig. 3. One-hour of exposure to NMDA (20 μM) produced significant neurotoxicity in the CA1 and CA3 pyramidal cell layers. Co-exposure of explants to MK-801 (30 μM) markedly reduced CA1 and CA3 damage. *$P<0.05$ vs. control; **$P<0.05$ vs. NMDA.

3. Results

3.1. (±) Kavain neurotoxicity

Exposure of hippocampal explants to (±) kavain (1–600 μM) for 24 h did not significantly alter the viability (propidium iodide uptake) of cells in the CA1, CA3, or dentate gyrus regions (data not shown). However, exposure to (±) kavain produced significant damage in the lacunosum moleculare layer of hippocampal explants, proximal to the CA1 region ($F(5,43)=10.17$, $P<0.001$). Post hoc analysis indicated that propidium iodide uptake in this region was significantly greater in those explants exposed to 300 and 600 μM (±) kavain than in all other groups, representing increases of 38% and 95% above control values, respectively (Fig. 1). A representative image of propidium iodide uptake in the lacunosum moleculare layer of an explant exposed to 300 μM (±) kavain is illustrated in Fig. 2.

3.2. (±) Kavain and NMDA neurotoxicity

Hippocampal explants exposed to NMDA (20 μM) for 1 h displayed significant neurotoxicity in the CA1 and CA3 regions (two-way interaction of brain region and treatment; $F(6,81)=22.93$, $P<0.001$). Uptake of propidium iodide in the CA3 and CA1 regions was increased by 18% and 101% above control values, respectively. Damage in the CA1 region was significantly greater than that in the CA3 region. Propidium iodide uptake in the dentate gyrus and lacunosum moleculare regions was not increased by NMDA exposure. In explants co-exposed to MK-801 (30 μM) during NMDA insult, CA1 and CA3 region toxicity was significantly reduced, to control levels (Fig. 3). In contrast, co-exposure of additional explants to (±) kavain (1–100 μM) during the 1 h NMDA exposure did not result in a significant reduction in CA1 damage (data not shown). Further, no evidence of neurotoxicity associated with (±) kavain exposure was noted.

A subsequent experiment was conducted to examine the effects of (±) kavain exposure (1–100 μM) on viability of hippocampal explants following NMDA insult. Explants were exposed to NMDA as described above, explants were washed twice with calcium Locke's buffer, and then placed in wells containing normal medium with propidium iodide or similar medium containing one of three concentrations of (±) kavain for 24 h. As in previous experiments, 1 h of exposure to NMDA produced an ~100% increase in uptake of propidium iodide in the CA1 region of explants and a smaller, but significant increase in the CA3 region ($F(8,105)=4.39$, $P<0.001$). Post hoc analysis further revealed that exposure of explants to (±) kavain for 24 h after the end of NMDA insult produced a concentration-dependent increase in neurotoxicity in the CA1 region and in the dentate gyrus, a region not initially damaged by NMDA exposure (Fig. 4). This synergistic neurotoxicity

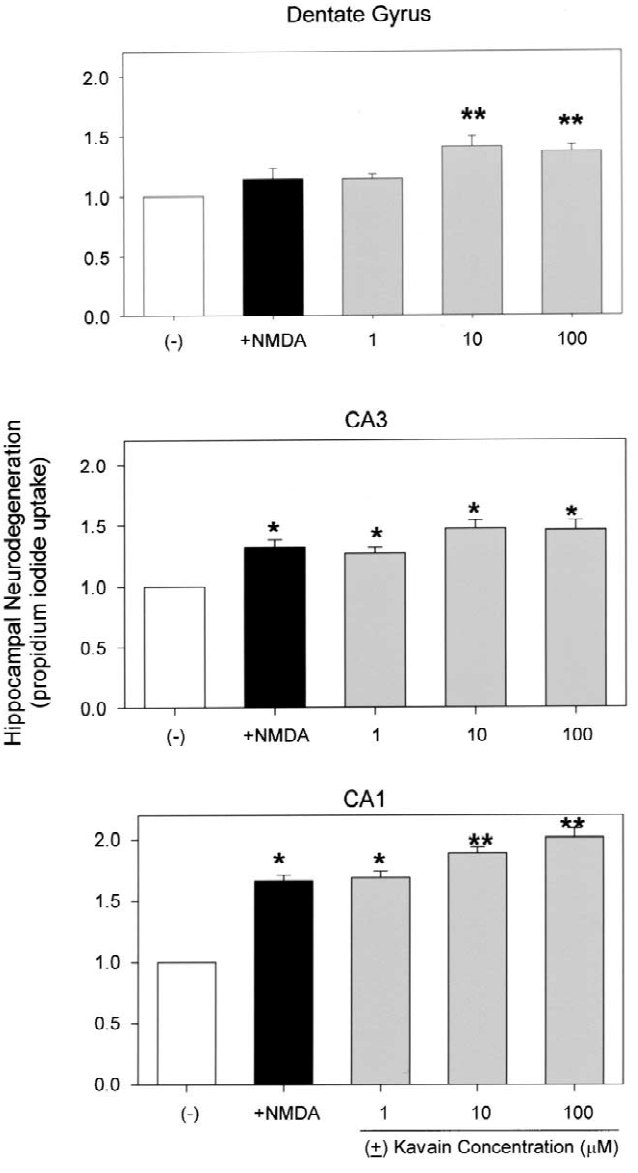

Fig. 4. (±) Kavain exposure (≥10 μM) for 24 h after NMDA insult significantly potentiated hippocampal CA1, but not CA3, damage. The dentate gyrus, a region not damaged by NMDA exposure, was damaged by (±) kavain exposure for 24 h after prior NMDA exposure. *$P<0.05$ vs. control; **$P<0.05$ vs. NMDA.

was not observed in the CA3 region. Exposure to 100 μM (±) kavain increased CA1 uptake of propidium iodide by 17%, as compared to NMDA-induced toxicity. In the dentate gyrus, neurotoxicity was increased by 19%. Representative images of hippocampal neurotoxicity in these explants are illustrated in Fig. 5.

4. Discussion

Evidence that kavapyrones reduce function of some ionotropic receptor systems and ion channels suggests a potential therapeutic use of these compounds in reducing

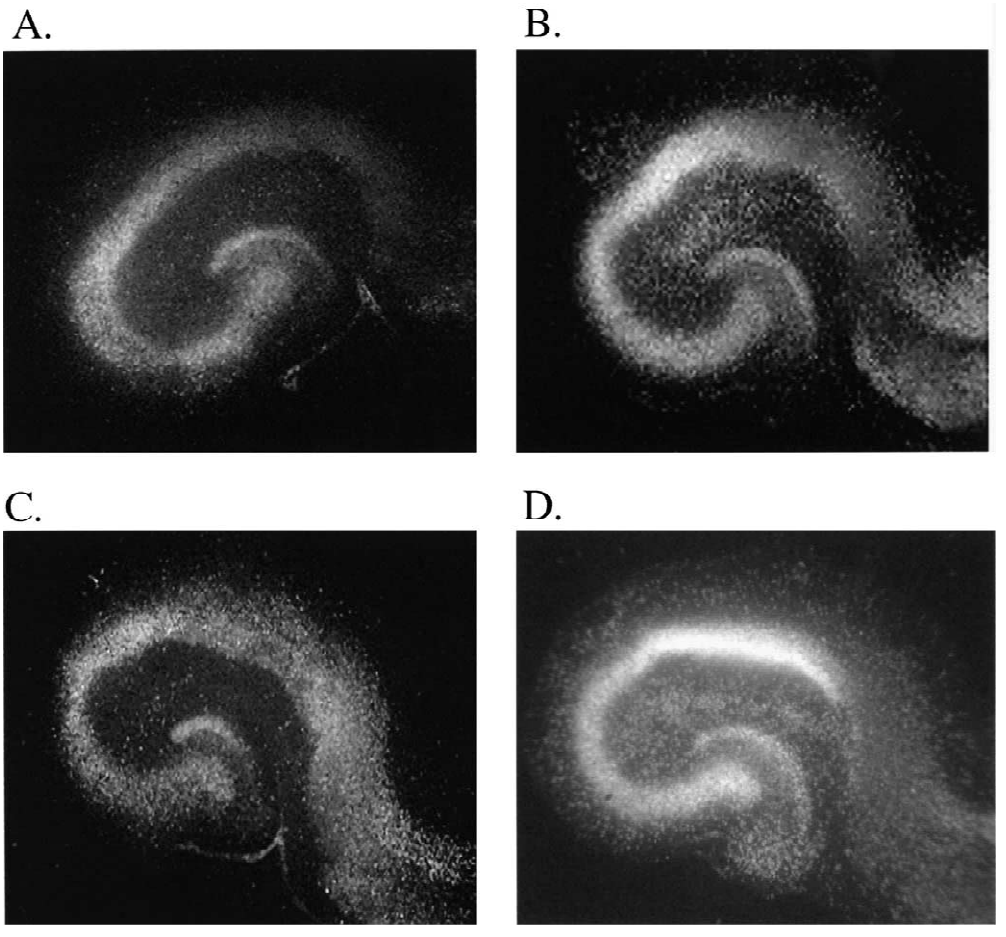

Fig. 5. Representative images of propidium iodide uptake in: (A) a control explant; (B) an explant exposed to NMDA (20 μM, 1 h); (C) an explant exposed to NMDA and MK-801 (30 μM); and an explant exposed to 10 μM (±) kavain for 24 h after NMDA insult.

neuronal damage during or following excitotoxic insult. Alternatively, perturbation of these systems may well prove to be neurotoxic in some instances, particularly in the developing brain. While one recent report has demonstrated neuroprotective effects of kavapyrones against the dopaminergic neurotoxin MPTP in adult rodent brain [24], little additional work has examined the effects of kavapyrones on neuronal viability following insult. Further, no work has examined effects of kavapyrone exposure on function of the developing CNS. The present studies represent the first examination of (±) kavain's effects on NMDA receptor-mediated excitotoxicity. Initial studies indicated that (±) kavain did not alter viability of hippocampal neurons in the dentate gyrus or pyramidal cell layers of the CA3 and CA1 region. However, high concentrations (≥300 μM) of this kavapyrone produced significant neurotoxicity in the CA1 lacunosum moleculare layer of the hippocampus. It must be noted that the lowest concentration of (±) kavain to produce lacunosum damage (300 μM) is similar to that observed in mouse brain following i.p. injection of crude kava extract at a dose of 120 mg/kg [15]. While the phenotype of those cells damaged by (±) kavain was not examined, this portion of the hippocampus does possess a small, but detectable population of NMDA receptors [18] and an extensive distribution of GABAergic interneurons [8,16]. Thus, this damage may reflect alterations in function of these systems. Indeed, both antagonism of NMDA receptor function and potentiation of GABAergic transmission are widely known to be neurotoxic in the developing brain [13,27].

Exposure of neuronal tissue to NMDA produced marked neurotoxicity in the pyramidal cell layers of the CA1 and CA3 regions of hippocampal explants, effects prevented by co-exposure to MK-801. This form of neurotoxicity is well-characterized and is associated with the Ca^{2+}-dependent activation of multiple proteases, lipases, as well as, induction of mitochondrial dysfunction [6,21]. Evidence that kavain attenuates over activation of the NMDA receptor [29] suggests that this kavapyrone may reduce NMDA-induced hippocampal degeneration. However, co-exposure of explants to (±) kavain with NMDA for 1 h did not result in a reduction in CA1 and/or CA3 damage. As in our previous study, these lower concentrations of (±) kavain (≤100 μM) did not produce neurotoxicity in the lacunosum moleculare layer of explants. In contrast, exposure of explants to (±) kavain (≥10 μM) for 24 h

beginning immediately after the end of NMDA insult produced a significant increase in CA1 and dentate gyrus neurodegeneration. The concentrations of (±) kavain that produce toxicity after NMDA exposure are well below kavain concentrations observed in rodent brain following i.p. injection of crude kava extract [15]. Though the dentate gyrus was not significantly damaged by NMDA exposure alone, these data suggest that NMDA and (±) kavain interact synergistically to produce this modest neurotoxic effect. In the CA1 region, NMDA-induced neurodegeneration was increased by 100 μM (±) kavain to 120% of control levels. However, NMDA-induced toxicity in the CA3 region was not potentiated by subsequent (±) kavain exposure. While it is unclear as to why this topographical pattern of synergistic toxicity was observed, it may be related to the unique transynaptic relationship that develops between the CA1 and dentate regions in organotypic hippocampal slice cultures in vitro [11]. It has been shown that these regions demonstrate synaptic reorganization in vitro by sprouting de novo dense excitatory efferent pathways from the CA1 pyramidal cell layer to the dentate granule cell layer [11]. Thus, synergistic toxicity in the CA1 region may have markedly potentiated dentate gyrus excitability, producing neurotoxicity. It is tempting to suggest that this synergistic neurotoxicity was related to (±) kavain enhancement of GABA-A receptor function, resulting in inhibition of recovery/repair after NMDA insult. However, the functional effects of kavapyrones on GABA-A receptor function are not known and binding studies of GABA-A receptor ligands do not clearly implicate an agonist or antagonist action of kavapyrones [4,7,14]. It will be of importance in future studies to further characterize the functional effects of kavapyrones on activity of GABA-A and other receptor systems.

In conclusion, the present data suggest that the kavapyrone (±) kavain may produce significant hippocampal neurodegeneration when exposed to CNS tissue in the absence of other toxins or following glutamate receptor-mediated insult. Alternatively, (±) kavain may well impair processes of cellular recovery and repair following CNS insult. These data are likely to be of significance, then, in suggesting that human consumption of *Piper methysticum* extracts may produce deleterious effects on the developing CNS, particularly given evidence of kava use by pregnant women [12]. Further, these data suggest that kava use may exacerbate the neurological difficulties associated with clinical conditions including epilepsy, stroke, head trauma, and alcoholism.

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