Pergamon

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PII: S0306-4522(02)00217-8

Neuroscience Vol. 113, No. 2, pp. 253–256, 2002

0306-4522/02 $22.00+0.00

Letter to Neuroscience

REGIONALLY SELECTIVE NEUROTOXICITY OF NMDA AND COLCHICINE IS INDEPENDENT OF HIPPOCAMPAL NEURAL CIRCUITRY

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Key words: dentate gyrus, hippocampus, excitotoxicity, kainate, neural network.

The mechanisms by which cerebral ischemia and several neurotoxins cause regionally selective damages to the hippocampal formation are largely unknown. The CA1-selective toxicity of *N*-methyl-D-aspartate (NMDA), the CA3-selective toxicity of kainate, and the dentate gyrus (DG)-selective toxicity of colchicine were observed in organotypic entorhino-hippocampal cultures. The selective neurotoxicity of NMDA and colchicine but not kainate was present in isolated tissue cultures of each hippocampal subregion, suggesting that the regional vulnerability is irrespective of the hippocampal trisynaptic pathway. Dispersed cultures of neurons prepared from Ammon's horn and the DG still exhibited a preference for susceptibility to NMDA and colchicine, respectively. Thus, the neurons per se appear to be inherently susceptible to specific toxins independently of their original loci, intrinsic neural circuits, vascular system, or other systemic factors. © **2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.**

The hippocampal formation contains the CA1 region, the CA3 region, and the DG. These three parts constitute a categorical circuitry in order of the perforant paths onto DG granule cells, the mossy fibers onto CA3 pyramidal cells, and then the Schaffer collaterals onto CA1 pyramidal cells, generally termed entorhino-dentato-hippocampal trisynaptic pathway (Amaral and Witter, 1995). Each subfield is known to display a distinctive vulnerability to cerebral ischemia and several neurotoxins including NMDA, kainate, and colchicine. However, little is known about the mechanisms responsible for this selective neuronal susceptibility.

Early neurological studies indicated that ischemia-induced, CA1-specific neuronal death requires an intact hippocampal network because acute lesions of the trisynaptic circuitry can ameliorate postischemic cell loss in adult rats (Johansen et al., 1986; Jorgensen et al., 1987; Benveniste et al., 1989; Wieloch et al., 1985). However, there is also contradictory evidence. For example, the susceptibility of CA1 neurons to ischemia reappeared a few months after a transection of the hippocampal circuitry even in the case of no suitable network reorganization (Tonder et al., 1994). Furthermore, granule cells in DG transplants, grafted from newborn donor rats into adult rats, become susceptible to ischemia without significant host innervation (Tonder et al., 1992). To date, therefore, whether or not the regional vulnerability depends on the hippocampal trisynaptic pathway is still unclear.

We and others previously reported that the regionally selective neurotoxicity of NMDA, kainate, and colchicine is well reproducible in an *in vitro* system of organotypic hippocampal cultures (Vornov et al., 1991; Newell et al., 1993; Ikegaya et al., 2001). Using this culture system, the present work addressed the contribution of the hippocampal trisynaptic network to the vulnerability of CA1, CA3, and DG neurons.

The experimental procedures used have been described elsewhere (Ikegaya, 1999; Ikegaya et al., 2000, 2001; Mizuhashi et al., 2001).

Organotypic hippocampal cultures were prepared from 8-day-old Wistar rats, in accordance with the Japanese Pharmacological Society guide for the care and use of laboratory animals. Animals were deeply anesthetized by hypothermia, and their brains were cut into transverse slices (300 μm thick). The entorhino-hippocampi were cultured using the membrane interface techniques. In a subset of experiments, the fascia dentata with hilus and a small part of the CA3c region was isolated from Ammon's horn, which was further divided into the CA1 and CA3 regions, and then these stumps were cultivated in separate plates (Fig. 1A). Cultures were fed with 1 ml of culture medium consisting of 50% minimal essential medium, 25% horse serum, and 25% Hanks'

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Abbreviations: DG, dentate gyrus; NMDA, *N*-methyl-D-aspartate; PI, propidium iodide.

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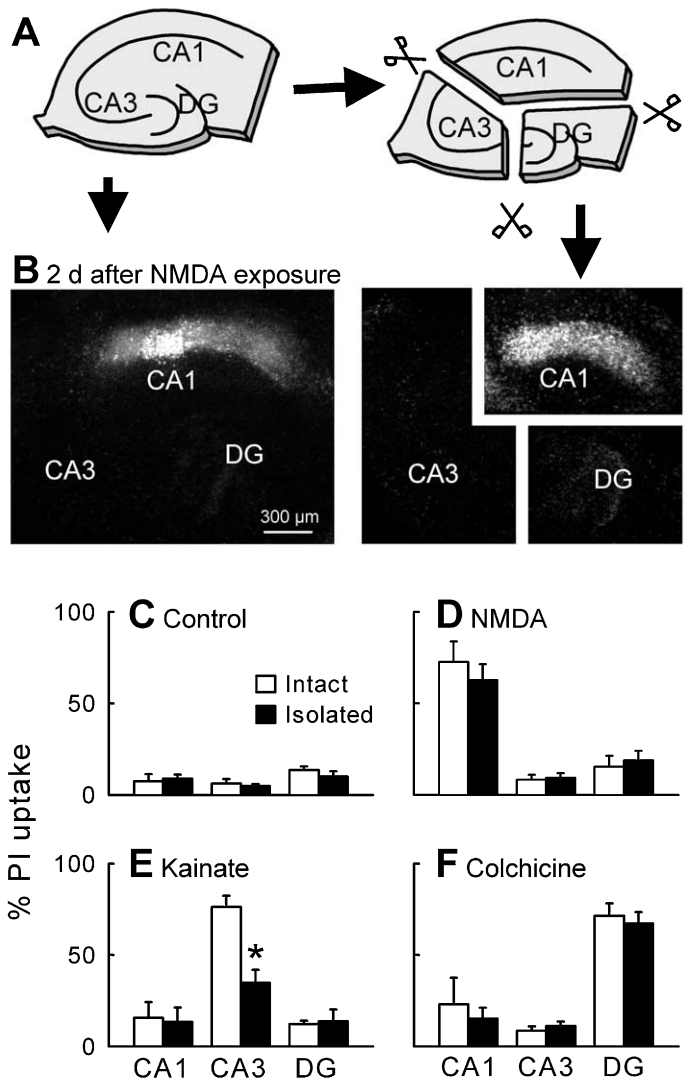

Fig. 1. The absence of intrinsic hippocampal networks does not alter the selective vulnerability to NMDA and colchicine. (A) The CA1, CA3, and DG regions were dissected out and separately cultivated for 10 days. (B) PI fluorescence images of an entorhino-hippocampal slice (left) and tissue stumps of the CA1, CA3, and DG regions (right) 2 days after 15-min exposure to 50 μM NMDA. (C–F) PI fluorescent intensities in the CA1, CA3, and DG of intact cultures (open columns) and isolated cultures (closed columns) were quantitatively analyzed 2 days after treatment with vehicle (C), NMDA (50 μM for 15 min, D), kainate (5 μM for 2 h, E), or colchicine (10 μM for 2 h, F). $*P < 0.005$ vs. Intact; Student's *t*-test. Data are the means ± S.E.M. of four different experiments (each eight to 13 slices).

balanced salt solution, and maintained at 37°C in a humidified 5% CO_2 and 95% air atmosphere. The medium was changed every 3.5 days. Experiments were performed after 10 days *in vitro*.

Cell death was assessed by quantifying massive fluorescence intensity of propidium iodide (PI) (Molecular Probes, Eugene, OR, USA). PI was added to culture medium at a final concentration of 5 μg/ml. After 24 h of incubation, PI fluorescence images were obtained with a confocal imaging system (MRC-1000; Bio-Rad Microscience Division, Cambridge, MA, USA). Pixel intensity of fluorescence (eight-bit intensity levels) was measured from the CA1 and CA3 stratum pyramidale and the DG stratum granulosum. Average intensity (F_t) was estimated by acquiring intensity values in 10 randomly selected, different areas (10×40 μm×40 μm) within each hippocampal subregion. The background intensity (F_0) was obtained outside the slices. At the end of each experiment, all cells were killed by a 24-h incubation at 4°C, and the final PI fluorescence (F_{fin}) was again measured in 10 randomly selected, different areas of each subregion. The F_{fin} values did not vary significantly from slice to slice, which suggests an equivalent content of cells and a well-randomized selection of areas. PI uptake was determined by $(F_t - F_0)/(F_{fin} - F_0) \times 100$.

Primary cultures were prepared from 3-day-old Wistar rat pups. Ammon's horn and the DG were dissected out from the formatio hippocampalis with extreme care prior to trypsinization. Because further division into CA1 and CA3 regions severely aggregated the survivability of dispersed neurons (data not shown), we could not discriminate CA1 and CA3 pyramidal cells in this culture

system. The cells were suspended in a mixture of 50% astrocyte-conditioned medium, 49% Neurobasal (Life Technologies, Gaithersburg, MD, USA), and 1% B-27 supplement (Life Technologies), and dispersed at a density of 9.0×10^4 cells/cm^2 onto 48-well plates coated with poly-D-lysine. Twenty-four hours after plating, the culture medium was changed to serum-free medium consisting of 98% Neurobasal and 2% B-27, supplemented with 2 μM cytosine β-D-arabinofuranoside. After experimental treatment, neurons were immunostained with a monoclonal antibody to microtubule-associated protein-2 (1:4000) (Pharmacia Biotech, Uppsala, Sweden), and the number of cells bearing immunopositive processes was measured as the number of surviving neurons.

In a first set of experiments, we prepared two types of organotypic cultures, i.e., whole entorhino-hippocampal slices and isolated tissues of the CA1, CA3, and DG regions (Fig. 1A). Isolation of such small stumps per se did not affect cell viability in culture, as assessed by PI uptake (Fig. 1C) and Nissl staining (data not shown, and see also Mizuhashi et al., 2001). Exposure to NMDA produced severe damage to CA1 neurons without affecting survival of CA3 or DG neurons, and this pattern of neurotoxicity was preserved in isolated cultures of the subfields (Fig. 1B, D).

Kainate induced a selective cell death in the CA3 region, sparing CA1 and DG neurons (Fig. 1E). This toxicity was significantly alleviated by tissue isolation ($t(6)=4.45$, $P=0.005$; Student's t-test), but there still remained evident damage to isolated CA3 slices, as compared with unexposed cultures ($t(6)=16.91$, $P<0.001$) (Fig. 1C, E). These results suggest that the vulnerability of CA3 neurons to kainate depends, in part, on the hippocampal network.

Our previous study showed that colchicine induced morphological aberration in almost all dendrites of CA1, CA3 and DG neurons (Ikegaya et al., 2001). But the toxin selectively killed DG neurons, and this specificity was unaltered after tissue isolation (Fig. 1F). Therefore, the regional neurotoxicity of NMDA and colchicine is likely to be irrespective of intrinsic neural connections between the subregions.

To obtain further evidence for hippocampal circuitry-independent vulnerability, neurons of Ammon's horn or the DG were cultured after complete dissociation by trypsinization and subjected to toxin exposure at 7 days *in vitro*. Even in these cultures, NMDA exhibited its neurotoxicity for Ammon's horn neurons at lower concentrations than for DG neurons ($F(1,24)=21.21$, $P<0.001$, two-way analysis of variance; $t(1,24)=4.61$, $P<0.01$, Dunnett's multiple comparison) (Fig. 2A). In contrast, colchicine-induced cell death was severe in DG neurons, as compared with that in Ammon's horn culture ($F(1,24)=17.41$, $P<0.001$; $t(1,24)=4.17$, $P<0.01$) (Fig. 2B). At higher concentrations of NMDA and colchicine, the selective vulnerability of CA1 and dentate neurons appeared to be lost (Fig. 2). In slice cultures, however, we previously confirmed no lack of the selective vulnerability at concentrations ranging from 1 to 100 μM (NMDA) (Ikegaya et al., 2001) or from 0.1 to 10 μM (colchicine) (Kim et al., 2002). Dispersed neurons may, therefore, be more vulnerable than neurons growing in organotypic cultures.

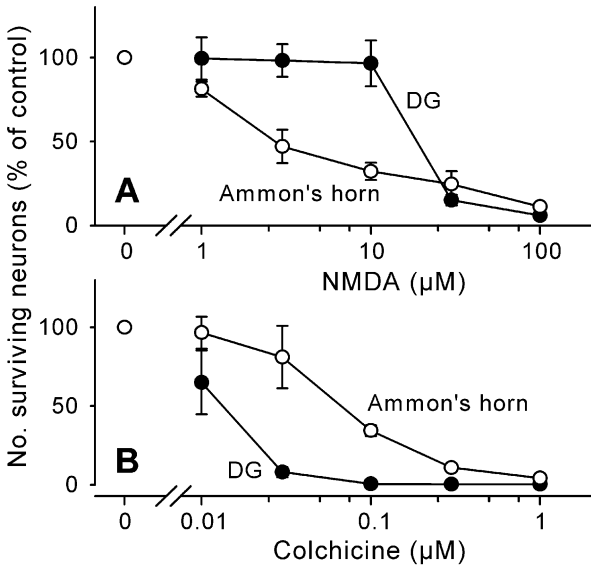

Fig. 2. Neurons of Ammon's horn (open circles) and the DG (closed circles) were dispersed by trypsinization, cultured for 7 days, and then subjected to 24-h exposure to NMDA (A) or colchicine (B). The ordinate indicates the number of surviving neurons as a percentage of the values of vehicle-treated cultures (0 μM). Data are the means ± S.E.M. of four different experiments (each four wells).

Using two different culture systems designed to characterize the regional vulnerability of the hippocampal formation in the absence of intrinsic hippocampal trisynaptic network, we have shown for the first time that the regional difference in the neurotoxicity of NMDA and colchicine is independent of the hippocampal circuitry.

Our data did not exclude the possibility that local circuits within each subregion are involved in the selective susceptibility of CA1 and DG neurons. We rather believe that especially NMDA toxicity requires network activity, because the CA1 cell death is blocked by either NMDA receptor antagonists or tetrodotoxin (Tasker et al., 1992; Ikegaya et al., 2001). None the less, the present study indicates definitively that the innervation of the Schaffer collateral onto CA1 pyramidal cells is not essential for NMDA toxicity, and also that neither the input from the perforant path nor the output via the mossy fiber is crucial for colchicine toxicity. In addition, although vascular and systemic factors have long been suspected in regional vulnerability, the result of the dispersed cultures presents conclusive evidence that selective neuronal susceptibility is observable even in the absence of such endemic factors (Mattson and Kater, 1989). Thus, we conclude that CA1 and DG neurons are intrinsically susceptible to NMDA and colchicine, respectively, independently of their original locations or inherent hippocampal circuits.

Recent evidence shows that kainate can induce CA1 cell death through NMDA receptor activation (Won et al., 1999). In our culture system, however, kainate was unlikely to stimulate NMDA receptors because its toxic effect was limited to CA3 neurons. The observation that

the toxicity was efficiently attenuated by subfield isolation is an intriguing finding. Most if not all kainate receptors in the hippocampus appear to be expressed in the mossy fibers (Represa et al., 1987), and the physiological action of exogenous kainate on CA3 neurons is mostly mediated by presynaptic mossy fiber activation (Kamiya and Ozawa, 2000; Schmitz et al., 2000). We therefore consider that the CA3-selective toxicity of kainate is dependent, at least in part, on mossy fiber innervation. This idea may be supported by an *in vivo* study demonstrating that kainate-induced seizures and subsequent CA3 degeneration are both alleviated in colchicine-pretreated rats (Okazaki and Nadler, 1988).

Several previous studies *in vivo* demonstrated that focal injections of the excitotoxins ibotenic acid and NMDA, even at very low doses, cause a loss of neurons in any hippocampal subfields, including the DG (Kohler et al., 1979; Kohler and Schwarcz, 1983; Jarrard and Meldrum, 1993). However, the present study *in vitro* has shown that treatment with NMDA and colchicine yields selective damage to CA1 and DG neurons, respectively. The different vulnerability is reproducible without intrinsic hippocampal trisynaptic network. Obviously, therefore, CA1 and DG neurons possess, at least in part, different survival properties. Thus, the difference of the *in vivo* and *in vitro* effects of excitotoxins may be accounted for by systemic factors rather than the intrinsic characteristics of neurons. Further investigation would elucidate the unique mechanisms responsible for survival of CA1 and DG neurons and might also provide a new insight into neurobiological techniques for selective cell ablation.

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(*Accepted* 23 *April* 2002)