Pergamon

Neuropharmacology 42 (2002) 1109–1118

NEURO PHARMACOLOGY

www.elsevier.com/locate/neuropharm

Cytoskeleton disruption causes apoptotic degeneration of dentate granule cells in hippocampal slice cultures

Jeong-Ah Kim, Kayo Mitsukawa, Maki K. Yamada, Nobuyoshi Nishiyama, Norio Matsuki, Yuji Ikegaya *

Laboratory of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

Received 26 December 2001; received in revised form 18 March 2002; accepted 28 March 2002

Abstract

Colchicine, a potent microtubule-depolymerizing agent, is well known to selectively kill dentate granule cells in the hippocampal formation in vivo. Using organotypic cultures of rat entorhino-hippocampal slices, we confirmed that in vitro exposure to 1 μM and 10 μM of colchicine reproduced a specific degeneration of the granule cells after 24 h. Similar results were obtained with other types of microtubule-disrupting agents, i.e., nocodazole, vinblastine, and Taxol. Interestingly, the actin-depolymerizing agents cytochalasin D and latrunculin A also elicited selective neurotoxicity in the dentate gyrus without affecting survival of hippocampal pyramidal cells. The selective pattern of degeneration was observable 24 h after a brief treatment with the toxins as short as 5 min, but this delayed neuronal death was unlikely to be a result of excitotoxicity because it was virtually unaffected by glutamate receptor antagonists, tetrodotoxin, or extracellular Ca^{2+}-free conditions. The damaged tissues contained a large number of TUNEL-positive neurons and exhibited an increased level in caspase-3-like activity, suggesting that cytoskeleton disruption triggers an apoptosis-like process in dentate granule cells. Thus, this study may provide a basis for understanding the distinctive mechanism that supports granule cell survival. © 2002 Published by Elsevier Science Ltd.

Keywords: Dentate gyrus; Hippocampus; Apoptosis; Actin; Microtubule

1. Introduction

The neuronal cytoskeleton is a complex meshwork consisting of microtubules, actin microfilaments, intermediate filaments, and other associated proteins. This intracellular system is responsible for determining neuronal morphology and for regulating transport and anchoring of cellular constituents (Hirokawa, 1994).

The granule cells of the dentate gyrus (DG) are the most abundant neurons in the hippocampal formation and possess the unusual properties of prolonged postnatal neurogenesis and limited lifetime (Altman and Das, 1965; Kaplan and Hinds, 1977; Eriksson et al., 1998). As a result, they undergo a continuous turnover over the period of weeks even in the adult brains (Gould et al., 1999). To date, very little is known about how these neurons survive for such a short lifetime and die thereafter.

The microtubule-disrupting agent colchicine is a well-known neurotoxin specific for DG granule cells in the hippocampal formation and hence has been widely used as an experimental tool for selective cell ablation in neurobiology and neurotoxicology (Goldschmidt and Steward, 1980; Heale et al., 1995). The toxin binds tightly to the β-tubulin subunit of the α/β-tubulin heterodimer, thereby decreasing the soluble tubulin pool and inhibiting microtubule assembly (Uppuluri et al., 1993). In spite of ubiquitous expression of tubulin proteins, however, it is puzzling why colchicine exerts such a cell-specific toxicity. Therefore, elucidating the action of colchicine on DG granule cells would help to clarify the cellular mechanisms responsible for their unique property.

The aim of this study is to determine whether other microtubule-disrupting agents also destroy DG neurons, whether actin-disrupting agents produce a similar pattern

* Corresponding author. Tel./Fax: +81 3 5841 4784.
E-mail address: ikegaya@tk.airnet.ne.jp (Y. Ikegaya).

0028-3908/02/$ - see front matter © 2002 Published by Elsevier Science Ltd.
PII: S0028-3908(02)00052-7

of neurotoxicity, and what is the mechanism underlying the characteristic toxicity. Here we report that all tested cytoskeleton disruptors cause highly selective, apoptosis-like death of DG granule cells in organotypic cultures of hippocampal slices, suggesting a critical role of cytoskeleton dynamics in the survival of DG neurons.

2. Methods

2.1. Materials

Pharmacological agents used in this study were as follows: ascorbic acid (Wako, Osaka, Japan), catalase (Sigma, St. Louis, MO, USA) 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (RBI, Natick, MA, USA), cytochalasin D (Wako), latrunculin A (Wako), MK801 (Sigma), N^{ω}-nitro-L-arginine methyl ester (l-NAME) (Sigma), *N*-acetyl-L-cysteine (Sigma), nocodazole (Calbiochem, La Jolla, CA, USA), tetrodotoxin (Wako), Trolox (Sigma), and vinblastine (Sigma). Cytochalasin D, latrunculin A, and vinblastine were dissolved in dimethylsulfoxide. The remaining materials listed were dissolved in distilled water. The stock solutions were kept at −20 °C, except for vinblastine (4 °C stock). Immediately before use, they were diluted with culture medium so that the final concentration of dimethylsulfoxide was less than 0.1%. The presence of 0.1% dimethylsulfoxide had no apparent effect on cell viability (data not shown). Reduced Cl^- solution was prepared by replacing NaCl in Hanks' balanced salt solution (HBSS) with 120 mM sodium gluconate (Wako) and 25 mM glucose (Wako).

2.2. Organotypic slice cultures

Hippocampal slice cultures were prepared from eight to nine day old Wistar/ST rats (SLC, Shizuoka, Japan), as previously described (Ikegaya, 1999). Animals were deeply anesthetized by hypothermia, and their brains were aseptically removed and cut into transverse slices (300 μm thick) in aerated, ice-cold Gey's balanced salt solution supplemented with 25 mM glucose using a vibratome (DTK-1500; Dosaka, Kyoto, Japan). The hippocampi were dissected out under stereomicroscopic control. Selected slices were placed on 30-mm sterile membranes (Millicell-CM; Millipore, Bedford, MA, USA), and transferred into six-well tissue culture trays. Cultures were fed with 1 ml of culture medium consisting of 50% minimal essential medium (Life Technologies, Grand Island, NY, USA), 25% horse serum (Cell Culture Lab, Cleveland, OH, USA) and 25% HBSS, containing 25 mM glucose, 50 U/ml penicillin G, and 100 μg/ml streptomycin. The cultures were maintained in a humidified incubator at 37 °C in 5% CO_2. The medium was changed every 3.5 days. Experiments were performed after 10–12 days in vitro.

2.3. Assessment of cell death

Cell death was assessed by massive fluorescent intensity of propidium iodide (PI) (Molecular Probes, Eugene, OR, USA) (Ikegaya et al., 2001). PI was added to culture medium at a final concentration of 5 μg/ml, and the cultures were kept at 37 °C for 24 h. PI fluorescence images were obtained with a BioRad MRC-1000 confocal imaging system (BioRad Microscience Division, Cambridge, MA, USA) equipped with an inverted microscope (ECLIPSE TE300; Nikon, Tokyo, Japan), a Green He-Ne ion laser, and a host computer system. All imaging and processing operations were performed with Laser Sharp Acquisition (Biorad Microscience Division) and Laser Sharp Processing (Biorad Microscience Division), respectively. Pixel intensity of fluorescence (8-bit intensity levels) was measured at three different areas of the slice: the CA1 and CA3 stratum pyramidale and the stratum granulosum of the DG. Average intensity (F_t) was estimated for each slice by acquiring intensity values in ten different areas (10×400 μm^2) within each hippocampal subregion. Simultaneously, the background intensity (F_0) was obtained outside the slices. At the end of each experiment, all cells were killed by 24-h incubation at low temperature (4 °C), and the final PI fluorescence (F_{fin}) was measured. PI uptake was determined by $(F_t\text{-}F_0)/(F_{fin}\text{-}F_0)\times 100$.

2.4. Assessment of cell morphology

The carbocyanine-type membrane tracer 3,3′-dipentyloxacarbocyanine iodide (DiO) was used for labeling cells (Molecular Probes). Slices were fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde at 4 °C for 30 min, at which point a single crystal (~0.1 mm diameter) of DiO was directly inserted into the stratum granulosum of the fixed cultures under a stereomicroscope. After a 4-h incubation at room temperature, cell morphology was analyzed using a BioRad MRC-1000 confocal imaging system (BioRad) with a 60×objective (Nikon, Tokyo, Japan).

2.5. Acridine orange labeling

We used the membrane-permeable, nucleotide-binding fluorescent die acridine orange in order to observe the morphology of cell nuclei (Mizuhashi et al., 2000). Slices were fixed with 0.1 M phosphate buffer containing 4% paraformaldehyde at 4 °C for 30 min. After being washed with phosphate-buffered saline (PBS) at room temperature twice for 15 min, the cultures were exposed to 1.0 μg/ml acridine orange (Sigma) for 10 min. The slices were bathed in PBS twice for 15 min, and then fluorescence imaging was conducted with an MRC-1000 confocal microscope (BioRad).

2.6. Rhodamine phalloidin labeling

For F-actin staining, cultures were fixed with 4% paraformaldehyde at 4 °C for 30 min, permeabilized with 0.3% Triton X-100 in PBS for 30 min at room temperature, and then treated with 5 U/ml rhodamine phalloidin (Molecular Probes) at room temperature for 30 min. The fluorescence images were obtained with a laser scanning confocal system Micro Radiance (Biorad, Herculeus, CA, USA).

2.7. TUNEL staining

Terminal deoxynucleotidyl transferase-mediated biotinylated-dUTP nick-end labeling (TUNEL) was performed using an in situ cell death detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). Immediately after 30 min treatment with cytochalasin D, hippocampal slices were immersed in ice-cold 4% paraformaldehyde adjusted to pH 7.4, and fixed for 30 min. The slices were then washed three times in PBS, permeabilized with 0.3% Triton X-100 in PBS for 30 min at room temperature, and then washed again three times in PBS. After being dried, the slices were incubated in 50 μl TUNEL reaction mixture at 37 °C for 60 min in the dark. They were washed again three times in PBS. The samples were analysed with a confocal imaging system (MRC-1000; BioRad).

2.8. Assessment of caspase-3-like activity

Twenty-four h after experimental treatment, the stratum granulosum (DG) and the stratum pyramidale (Ammon's horn) were carefully dissected out by using a small, curved scalpel under stereomicroscopic control. The supernatants (20 μg of protein) were incubated with 20 μM Ac-DEVD-MCA (Peptide Institute, Osaka, Japan) (assay buffer A) or a combination of 20 μM Ac-DEVD-MCA and 5 μM Ac-DEVD-CHO (Peptide Institute) (assay buffer B) in 10 mM HEPES, and 5 mM dithiothreitol at pH 7.4 for 60 min at 37 °C in the dark. At least 10 min after the addition of stop buffer on ice, caspase-3-like activities were measured with a fluorescence microplate reader (365 nm excitation, 505 nm emission). Fluorescent intensity of blanks in assay buffer B (non-specific background) were subtracted from the values of samples in assay buffer A.

2.9. Statistical analysis

Tests of variance homogeneity, normality, and distribution were performed to ensure that the assumptions required for standard parametric ANOVA were satisfied. Statistical analysis was performed by one-way repeated-measures ANOVA and post hoc Tukey's test for multiple pairwise comparisons.

3. Results

3.1. Regionally selective toxicity of cytoskeleton-depolymerizing agents

We first tried to reproduce the distinctive toxicity of colchicine in organotypic cultures of hippocampal slices. Neuronal toxicity was assessed by quantifying the intensity of PI fluorescent staining of dying cells (Ikegaya et al., 2001). Exposure to colchicine at concentrations of 1 μM and 10 μM for 30 min caused a highly specific toxicity in the DG after 24 h without inducing apparent damages to the CA1 or CA3 region of the Ammon's horn (Fig. 1B & 2A). Cells in the DG of colchicine-treated slices were individually labeled with DiO, a fluorescent carbocyanine dye (Ikegaya et al., 2001), and confocal microscopy observation revealed that severe damage was found in granule cells but not in other types of DG neurons or glial cells (Fig. 3). Thus, we consider that this culture system is useful in investigating the selective toxicity for dentate granule cells (Newell et al., 1993; Ikegaya et al., 2001).

To determine whether DG-selective toxicity is specific for colchicine or universal in microtubule disruptors, the effects of other microtubule-disrupting agents, i.e., nocodazole and vinblastine, were examined. Treatment with either nocodazole or vinblastine for 30 min induced a selective loss of DG granule cells in a concentration-dependent manner, without affecting the viability of CA1 or CA3 pyramidale cells (Figs. 1 and 2). Likewise, the microtubule-stabilizing agent Taxol produced the same results (Fig. 2D). These results suggest that microtubule-dependent cytoskeletal organization is essential for the survival of granule cells. Although Taxol and colchicine exert pharmacologically opposite effects on microtubule dynamics (Uppuluri et al., 1993; Díaz and Andreu, 1993), pretreatment with 1 μM Taxol did not antagonize 10 μM colchicine-induced cell death (data not shown).

To evaluate the contribution of actin microfilament, another major component of neuronal cytoskeleton, to granule cell survival, the effects of the actin-depolymerizing agents cytochalasin D and latrunculin A were investigated. Interestingly, these two disruptors produced a similar pattern of selective degeneration of DG neurons in a concentration-dependent manner, sparing CA1 and CA3 neurons (Figs. 1 and 2). Therefore, actin-dependent cytoskeletal dynamics is also required for granule cell survival. Fluorescent staining of F-actin with rhodamine phalloidin revealed that cytochalasin D caused a severe disassembly of F-actin all over the hippocampus, i.e., the CA1 and CA3 regions as well as the DG (Fig. 4). This suggests that the requirement of actin microfilaments is unique to DG neuron survival.

The cytotoxicity of cytochalasin D was dependent on the duration of exposure (Fig. 5A). A significant DG

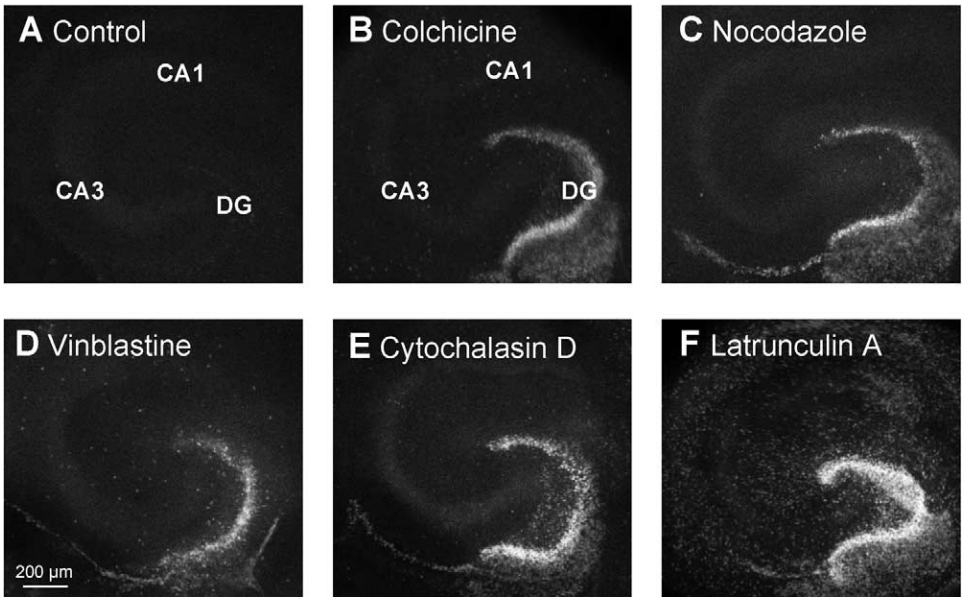

Fig. 1. PI fluorescence images of hippocampal slices 24 h after 30 min exposure to vehicle (A), 10 μM colchicine (B), 1 μM nocodazole (C), 3 μM vinblastine (D), 10 μM cytochalasin D (E), and 10 μM latrunculin A (F). All these cytoskeleton disruptors induced selective PI uptake in the DG.

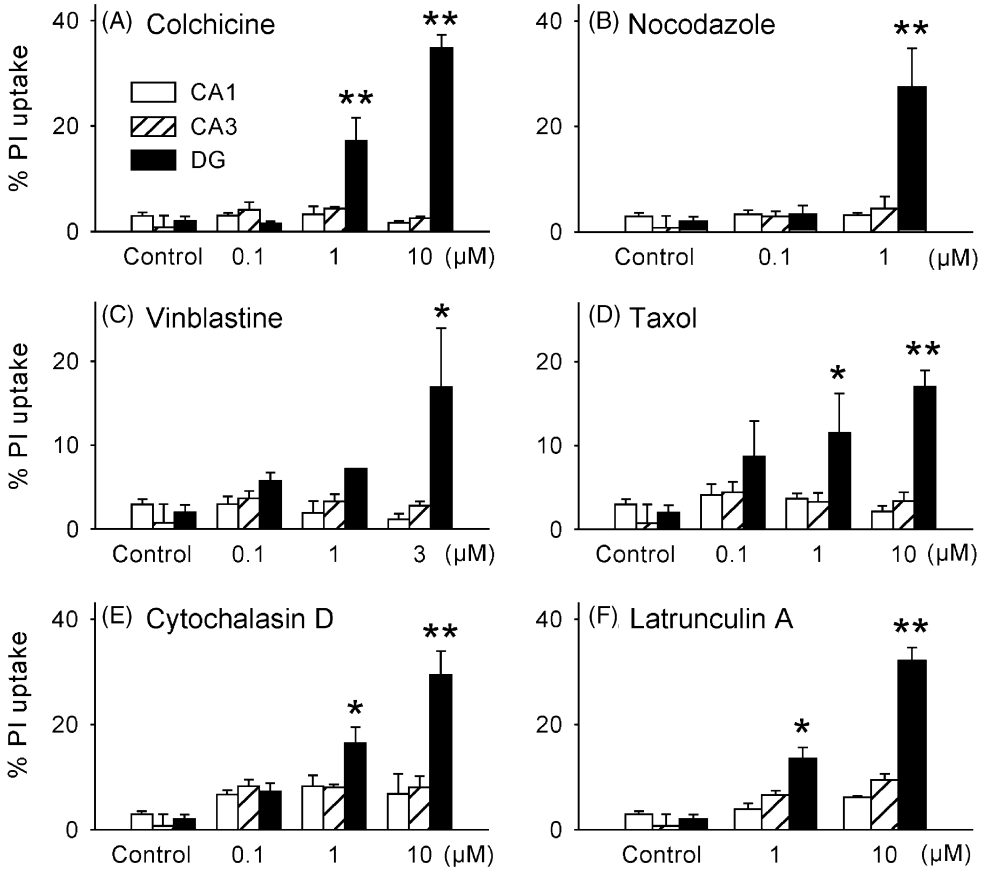

Fig. 2. Cytoskeleton-disrupting agents induced selective cell death in the DG of cultured hippocampal slices. PI fluorescence intensity in each hippocampal subregion, i.e., the CA1 region (open columns), the CA3 region (hatched columns), and the DG (solid columns), was quantitatively analyzed 24 h after 30 min treatment with colchicine (A), nocodazole (B), vinblastine (C), Taxol (D), cytochalasin D (E), and latrunculin A (F) at concentrations in the range of 0.1 to 10 μM. In all cases, massive neuronal death was observed only in the DG in a concentration-dependent manner. $*p<0.05$, $**p<0.01$ versus control. Data are the means±S.E.M. of 8–10 slices from three different experiments.

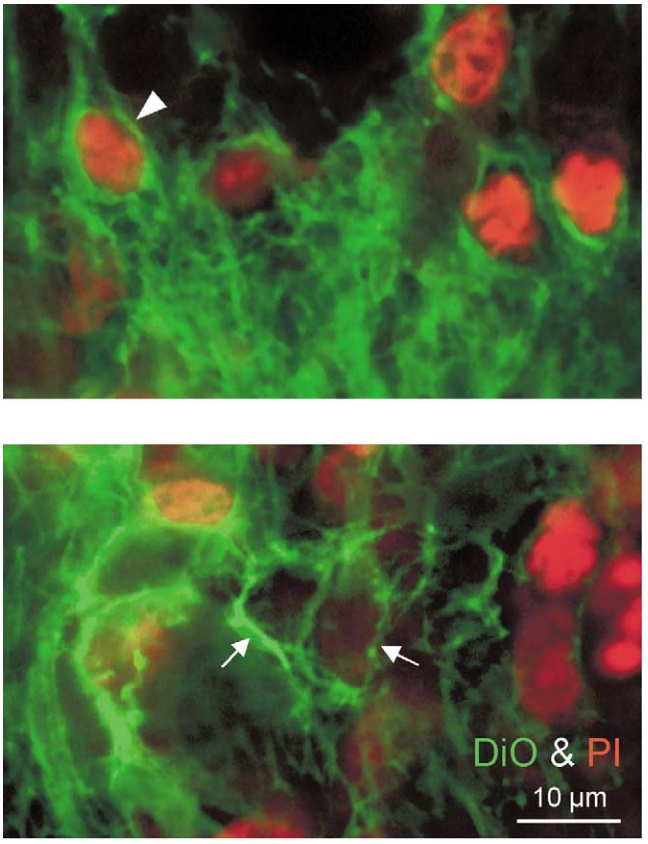

Fig. 3. Colchicine selectively kills DG granule cells in cultured hippocampal slices. Two images are representative confocal micrographs of granule cells double-labeled with DiO (green) and PI (red) 24 h after 30 min exposure to 10 μM colchicine. The plasma membrane was severely damaged in PI-positive neurons and thereby the intracellular membrane compartments were also stained with DiO (arrowheads), while only the plasma membrane was labeled with DiO in PI-negative cells (arrows). Experiments were repeated with 4 times, producing the same results.

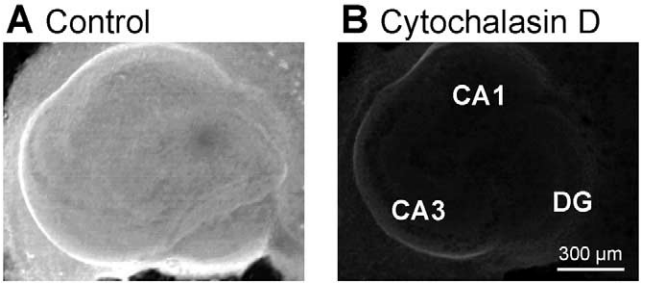

Fig. 4. Cytochalasin D induces widespread F-actin degradation. Rhodamine phalloidin fluorescence images of hippocampal slices immediately after 30 min exposure to vehicle (A) or 10 μM cytochalasin D (B). Treatment with cytochalasin D resulted in actin depolymerization not only in the DG but also in the CA1 and CA3 regions. Experiments were repeated with ten different slices, producing the same results.

damage appeared 24 h after as short as 5 min of exposure to 10 μM cytochalasin, and a 30 min exposure reached a maximal steady-state level. However, even longer exposures (up to 60 min) produced no cell death in the

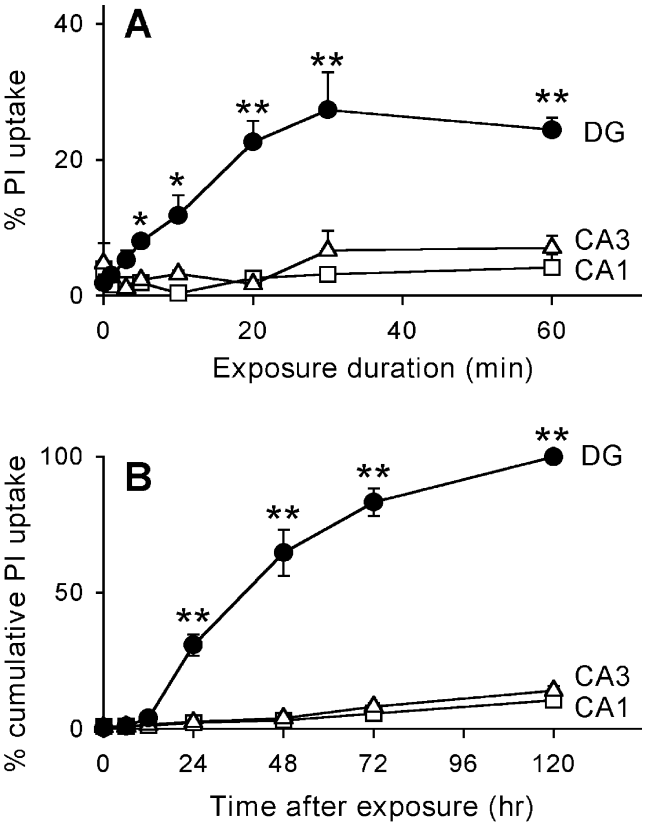

Fig. 5. Cytoskeleton disruption results in delayed neuronal death in the DG of hippocampal slice cultures. A, Cell death was assessed 24 h after 1, 3, 5, 10, 20, 30 and 60 min of exposure to 10 μM cytochalasin D. Even a brief exposure (5 min duration) significantly induced severe cell death in the DG (circles) whereas the CA1 (squares) and CA3 (triangles) regions were resistant to exposure as long as 60 min. B, Cell damage was measured 6, 12, 24, 48, 72 and 120 h after 30 min exposure to 10 μM cytochalasin D. The ordinate indicates cumulative PI fluorescence intensity relative to the DG at 120 h, i.e., accumulative values from 0 h to the indicated time points are shown as a percentage of the data of the DG at 120 h. The significant PI uptake appeared from 24 h after the treatment. $*p<0.05$, $**p<0.01$ versus control (at time 0). Data are the means±S.E.M. of 8–10 slices from three different experiments.

CA1 or CA3 region. A similar time-dependency was obtained with 1 μM nocodazole (data not shown).

3.2. Apoptotic features in cytoskeleton disruption-induced cell damage

We examined the time course of DG damages after 30 min treatment with 10 μM cytochalasin D and noticed that a significant PI uptake did not occur within 12 h but did emerge from 24 h after exposure (Fig. 5B), indicating that cytoskeleton disruption results in delayed neuronal death. The death is, therefore, suspected to be apoptotic. Consistent with this, confocal observations using acridine orange labeling revealed that 12 h after 30 min treatment with cytochalasin D, the nuclei were already degraded and highly condensed in granule cells

(Fig. 6), suggesting that PI-excluding, early dying cells already display typical apoptotic characteristics. We therefore tried to detect apoptotic damages by using the TUNEL method, a histochemical assay of DNA fragmentation (Gavrieli et al., 1992). Twenty-four h after 30 min treatment with 10 μM cytochalasin D, the cultures contained a large number of TUNEL-positive neurons in the DG; about 30% of granule cells were stained with the TUNEL method (Fig. 7A,B), whereas TUNEL-positive cells were virtually undetectable in the CA1 or CA3 region (data not shown). Colchicine (10 μM) and latrunculin A (10 μM) also produced the same results (data not shown).

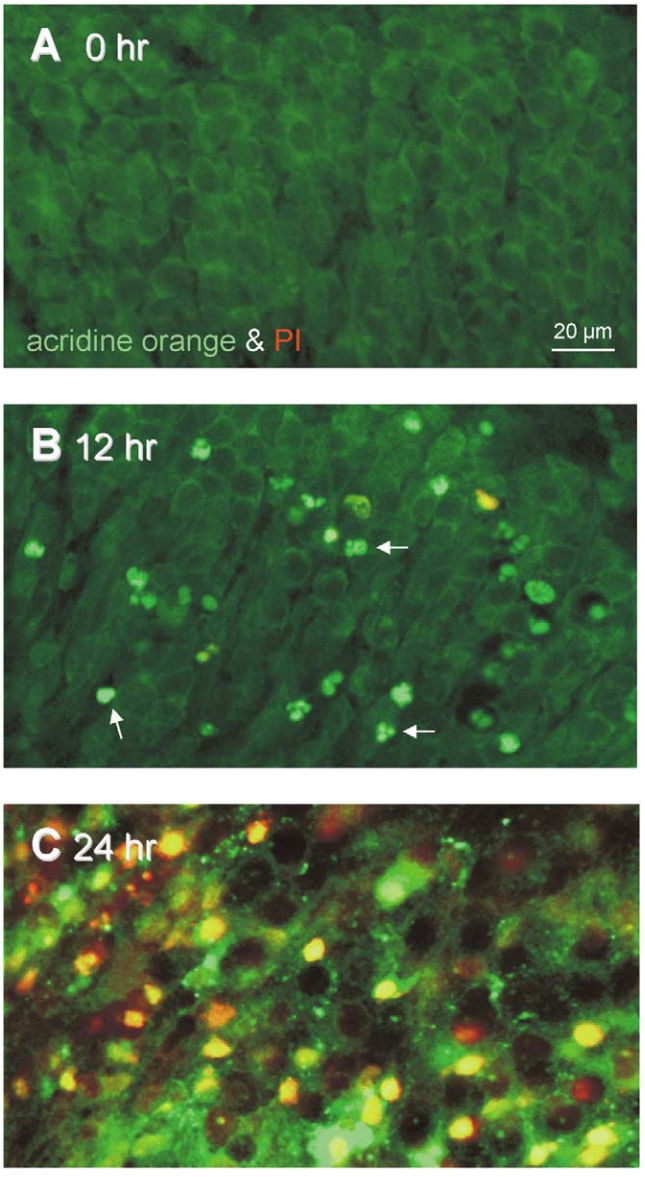

Fig. 6. Cytoskeleton disruption causes nuclear degradation preceding PI uptake. Hippocampal slice cultures were stained with acridine orange (green) and PI (red) immediately (A), 12 h (B), and 24 h (C) after 30 min exposure to 10 μM cytochalasin D. After 12 h, many degraded (but PI-negative) nuclei were detected in the DG, as indicated by arrows.

Caspase-3 is the cysteine protease that plays a pivotal role in neuronal apoptosis (Kuida et al., 1996). Caspase-3-like activity was measured by using the tetrapeptide fluorogenic substrate DEVD-MCA that mimics the cleavage site of caspase-3. When cultures were exposed to colchicine, cytochalasin D, or latrunculin A, a substantial increase in caspase-3-like protease activity was detected in the DG, while no apparent change in the activity was found in the Ammon's horn (Fig. 7C). Importantly, pretreatment with the caspase-3 inhibitor Ac-DEVD-CHO (5 μM for 12 h) prevented the elevation in caspase-3-like activity after 30 min exposure to 10 μM cytochalasin D, but it did not apparently affect PI uptake or the development of Tunnel-positive nuclei (data not shown). Taken together, we conclude that DG neurons die, at least in part, by apoptosis after cytoskeleton degradation but the death cannot be explained merely by caspase-3-dependent signaling pathway.

3.3. Pharmacological analysis on cytochalasin toxicity

A number of studies indicated the relationship between cytoskeleton and excitatory amino acid receptors. NMDA receptors and AMPA receptors are clustered in dendritic spines, which abundantly contain actin filaments (Matus, 2000). The NR1 and NR2B subunits of NMDA receptors are anchored to F-actin via the actin binding protein α-actinin-2 (Wyszynski et al., 1997). Cytoskeleton disruption is reported to a collapse of synaptic NMDA receptor clusters (Allison et al., 1998) and thereby may increase neuronal vulnerability to excitotoxicity (Sattler et al., 2000). Thus, we assessed the possibility that cytoskeleton disruption-induced cell death is mediated by excitotoxicity. Cytochalasin D was applied in the presence of a mixture of glutamate receptor antagonists (10 μM MK801 and 20 μM CNQX), which efficiently prevents NMDA-induced excitotoxicity in hippocampal slice cultures (Ikegaya et al., 2001), but the toxicity of cytochalasin D was unaffected (Fig. 8A). Although neuronal activities were blocked by 1 μM tetrodotoxin during exposure to cytochalasin D (Ikegaya et al., 2001), the cell death still occurred (Fig. 8A). In addition, although Ca^{2+} and Cl^{-} influx is one of central components of excitotoxicity (Zeevalk et al., 1989), the neurotoxicity of cytochalasin D was inhibited in neither Ca^{2+}-free solution nor reduced Cl^{-} buffer (7.6 mM Cl^{-}) (Fig. 8A). Our preliminary experiment confirmed that both the ionic conditions completely prevented NMDA-evoked excitotoxicity (data not shown, and see also Ikegaya et al., 2001). These results may be consistent with a report of cerebellar granule cell cultures, which showed that neither MK-801 nor L-type Ca^{2+} channel blockers did not prevent colchicine-induced apoptosis (Bonfoco et al., 1995). Therefore, we concluded that

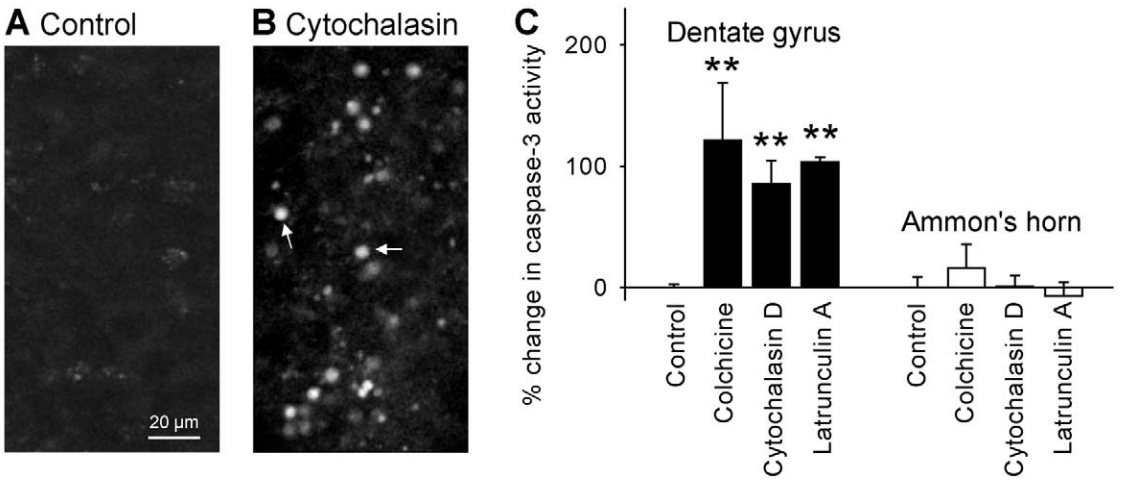

Fig. 7. Apoptotic features of granule cell damages after cytoskeleton disruption. A,B, Representative confocal TUNEL images of the stratum granulosum. Twenty-four h after 30 min treatment with vehicle (A) or 10 μM cytochalasin D (B), cultures were processed for TUNEL staining. Bright spots (arrows) indicate TUNEL-positive nuclei. Experiments were repeated with 14--16 different slices, producing similar results. C, Caspase-3-like activities in the DG (solid column) and the Ammon's horn (open column) were measured 24 h after 30 min treatment with 10 μM colchicine, 10 μM cytochalasin D, and 10 μM latrunculin A. The activity was quantified as a percentage of changes in fluorescence intensity from corresponding control values. A significant increase in the activity was seen only in the DG. $**p<0.01$ versus control. Data are the means±S.E.M. of 9–12 slices from three or four different experiments.

cytoskeleton disruption-induced cytotoxicity is not due to excitotoxicity.

The process of cell injury and death, including apoptosis, often involves free radical generation (Dubinsky et al., 1995; Chakraborti et al., 1999). We examined the effects of several antioxidants and radical scavengers. The cytochalasin D-induced toxicity was efficiently attenuated by water-soluble antioxidants (3 mM ascorbic acid or 250 μM *N*-acetylcysteine) but not by lipid-soluble antioxidants (500 μM α-tocopherol or 500 μM Trolox) (Fig. 8B). The toxicity was unaffected by the scavenger protein catalase (1000 U/ml) or the inhibitor of nitric oxide synthase l-NAME (300 μM) (Fig. 8B). Similar pharmacological data were obtained for the toxicity of 10 μM colchicine and 10 μM latrunculin A (data not shown). Therefore, hydrogen peroxide or nitric oxide is unlikely to contribute to cytoskeleton disruption-induced apoptosis, which is further supported by a previous paper showing that colchicine-induced death of cerebellar granule cells is not prevented by scavestrogen, known to efficiently block the action of hydrogen peroxide (Götz et al., 1999).

Incidentally, the validity of concentrations of the drugs used was certified by our recent study (Mizuhashi et al., 2000), and these drugs alone did not affect the survival in intact slices (data not shown). Taken together, we consider that cytoskeleton disruption causes apoptosis-like death of DG neurons through oxidative-like stress independent of hydrogen peroxide or nitric oxide.

4. Discussion

One of our main findings is that all tested disruptors of either microtubules or actin filaments equally cause a highly selective loss of DG granule cells in hippocampal slice cultures. Colchicine irreversibly binds to β-tubulin and inhibits polymerization of microtubules (Uppuluri et al., 1993), while nocodazole and vinblastine reversibly bind it at different sites and cause microtubule disassembly (Jung et al., 1992; Rai and Wolff, 1996). Taxol stabilizes microtubule by binding to assembled tubulin with an exact 1:1 stoichiometry, of which the binding site is located between protofilaments, accessible from the microtubule surface (Díaz and Andreu, 1993). Cytochalasin D, a fungal metabolite, binds to the barbed (+)-end of the actin filament and prevents its growth (Cooper, 1987). Latrunculin A, a compound isolated from the Red Sea sponge *Negombata*, inhibits actin polymerization by the formation of 1:1 molar complex with G-actin (Spector et al., 1983). Therefore, these disruptors exert their effects by different mechanisms. Although previous studies could not decide whether the toxicity of colchicine is due to cytoskeleton disruption or mediated by its side effect, our results strongly suggest that the selective granule cell death is a consequence of cytoskeleton disturbance.

Because cytoskeleton-disrupting agents induced a widespread degradation of cytoskeleton in neurons of all hippocampal subregions, i.e., the DG, the CA1 and CA3 regions (Fig. 4), the specific neurotoxicity for DG cells

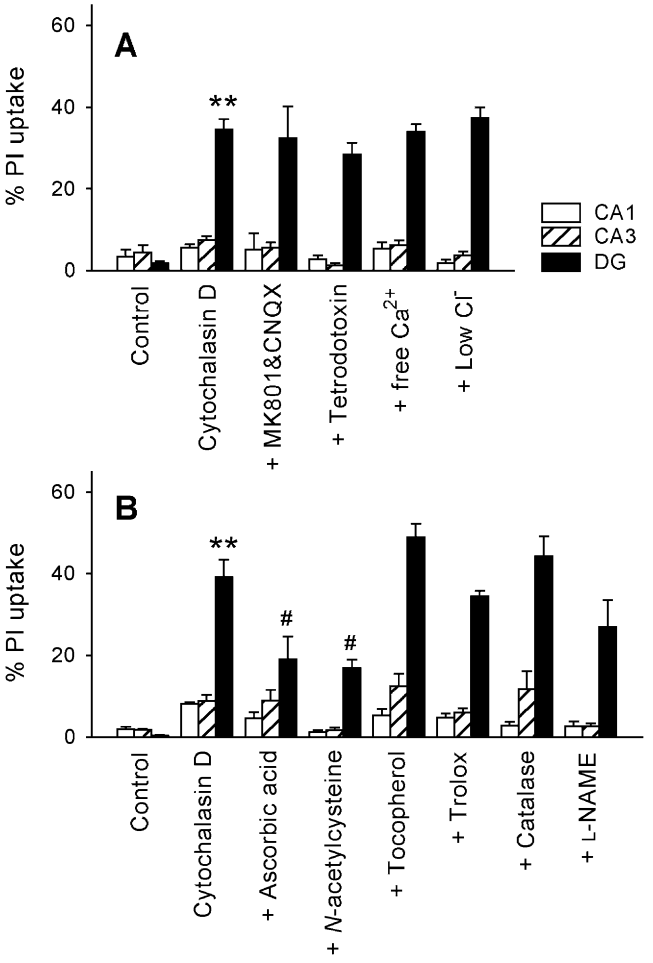

Fig. 8. Mechanisms underlying selective DG damage after cytoskeleton disruption. A, Cytochalasin D was applied in the presence of a combination of 10 μM MK801 and 20 μM CNQX or 1 μM tetrodotoxin or in Ca^{2+}-free or low-Cl^{-} buffer. B, Cytochalasin D was applied in the presence of 3 mM ascorbic acid, 250 μM N-acetylcysteine, 500 μM α-tocopherol, 500 μM Trolox, 1000 U/ml catalase, or 300 μM l-NAME. All drugs were applied 30 min before and during 30 min exposure to 10 μM cytochalasin D. PI uptake in each hippocampal subregion, i.e., the CA1 region (open columns), the CA3 region (hatched columns), and the DG (solid columns), was assessed after 24 h. $**p<0.01$ versus control. $^{\#}p<0.05$ vs cytochalasin D alone. Data are the means±S.E.M. of 8–10 slices from three independent experiments.

is quite enigmatic. Interestingly, however, the selective DG damages could be produced by toxin exposure as short as 5 min, which suggests that the survival of DG granule cells is persistently supported by a continuous cellular event that is vulnerable to a collapse of molecular dynamics of microtubules and actin microfilaments. Indeed, the finding that either stabilization or disruption of microtubules caused similar cell death implies that DG neuron survival depends on dynamic cytoskeletal organization rather than static cytoskeletal structures. But, what cellular event contributes to DG neuron survival? The neuronal cytoskeleton is believed to regulate axonal transport of cellular constituents (Hirokawa, 1994), which is sensitive to colchicine (Kreutzberg, 1969; Dahlström, 1971). One possibility is, therefore, that cytoskeleton disruption results in a depletion of retrogradely transported neurotrophic factor that is critical for granule cell survival (DiStefano et al., 1992). However, we do not believe that such a target-derived factor operates to maintain DG neuron survival, because the axotomy of mossy fibers arising from DG granule cells, which should interrupt assumed retrograde signals, causes no cell death (Ikegaya et al., 1998; Mizuhashi et al., 2001).

Some studies in vivo indicated that colchicine induces apoptosis of granule cells in the DG as well as the cerebellum (Ceccatelli et al., 1997; Ho et al., 1998). In addition, our study has shown that colchicine and other cytoskeleton disruptors induce an increase in caspase-3-like activity and cause apoptosis-like death of DG neurons in vitro. The mitochondria are well known to initiate an apoptotic signaling pathway; the opening of permeable transition pores on the inner mitochondrial membrane leads to a release of mitochondrial constituents such as cytochrome *c* and apoptosis-inducing factor, which in turn stimulates caspase-3 activity (Kroemer et al., 1998). This organelle is tightly connected with cytoskeleton elements (Heggeness et al., 1978), and the cytoskeletal degradation by colchicine or taxol is shown to extend the open state of permeability transition pores in Ehrlich ascites tumour cells (Evtodienko et al., 1996). Consistent with this, Gorman et al. (1999) demonstrated that colchicine induces a release of cytochrome *c* in primary cultures of cerebellar granule cells. Therefore, we consider that cytoskeleton disruptors may directly alter mitochondrial functions and thereby initiate the apoptosis pathway in DG neurons. Because the mitochondria are one important source of reactive intermediates, e.g., superoxide, hydrogen peroxide, and possibly hydroxyl radical (Leeuwenburgh and Heinecke, 2001), this idea is supported by our pharmacological data showing that aqueous radicals seem to participate in cytoskeleton disruption-induced damages. Thus, DG granule cells may be equipped with a unique, vulnerable cytoskeletal system for supporting mitochondrial functions.

Another possible mechanism for the neurotoxicity is the upregulation of the stress-induced protein p53, which stimulates the formation of reactive oxygen species and also activates transcription of the genes that is capable of facilitating apoptosis, e.g., *bax* and a set of redox-related *PIG* genes, through p53-responsive elements (Miyashita and Reed, 1995; Polyak et al., 1997). In fibroblasts or relevant cell lines, the expression level of p53 increases in response to pharmacological disruption of microtubules (Tishler et al., 1995) and actin filaments (Rubtsova et al., 1998). Like fibroblasts, p53 in DG neurons may also be particularly susceptible to the stressful conditions of cytoskeleton degradation.

In conclusion, this work has shown for the first time

that the disruption of actin filaments, as well as microtubules, severely causes apoptotic degeneration of DG granule cells in a time- and concentration-dependent manner in organotypic cultures of hippocampal slices. Further investigation of the relationship between cytoskeletal organization and DG neuron survival would provide a new insight into neurobiological tools for selective cell ablation, and elucidate the cellular mechanisms responsible for the unique life cycle of DG granule cells.

References

Allison, D.W., Gelfand, V.I., Spector, I., Craig, A.M., 1998. Role of actin in anchoring postsynaptic receptors in cultured hippocampal neurons: differential attachment of NMDA versus AMPA receptors. Journal of Neuroscience 18, 2423–2436.

Altman, J., Das, G.D., 1965. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. Journal of Comparative Neurology 124, 319–335.

Bonfoco, E., Ceccatelli, S., Manzo, L., Nicotera, P., 1995. Colchicine induces apoptosis in cerebellar granule cells. Experimental Cell Research 218, 189–200.

Ceccatelli, S., Ahlbom, E., Diana, A., Zhivotovsky, B., 1997. Apoptosis in rat hippocampal dentate gyrus after intraventricular colchicine. Neuroreport 8, 3779–3783.

Cooper, J.A., 1987. Effects of cytochalasin and phalloidin on actin. Journal of Cell Biology 105, 1473–1478.

Chakraborti, T., Das, S., Mondal, M., Roychoudhury, S., Chakraborti, S., 1999. Oxidant, mitochondria and calcium: an overview. Cellular Signalling 11, 77–85.

Dahlström, A., 1971. Effects of vinblastine and colchicine on monoamine containing neurons of the rat, with special regard to the axoplasmic transport of amine granules. Acta Neuropathologica Supplement 5, 226–237.

Díaz, J.F., Andreu, J.M., 1993. Assembly of purified GDP-tubulin into microtubules induced by taxol and taxotere: reversibility, ligand stoichiometry, and competition. Biochemistry 32, 2747–2755.

DiStefano, P.S., Friedman, B., Radziejewski, C., Alexander, C., Boland, P., Schick, C.M., Lindsay, R.M., Wiegand, S.J., 1992. The neurotrophins BDNF, NT-3 and NGF display distinct patterns of retrograde axonal transport in peripheral and central neurons. Neuron 8, 983–993.

Dubinsky, J.M., Kristal, B.S., Elizondo-Fournier, M., 1995. An obligate role for oxygen in the early stages of glutamate-induced, delayed neuronal death. Journal of Neuroscience 15, 7071–7078.

Eriksson, P.S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A.M., Nordborg, C., Peterson, D.A., Gage, F.H., 1998. Neurogenesis in the adult human hippocampus. Nature Medicine 4, 1313–1317.

Evtodienko, Y.V., Teplova, V.V., Sidash, S.S., Ichas, F., Mazat, J.P., 1996. Microtubule-active drugs suppress the closure of the permeability transition pore in tumour mitochondria. FEBS Letter 393, 86–88.

Gavrieli, Y., Sherman, Y., Ben-Sasson, S., 1992. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. Journal of Cell Biology 119, 493–501.

Goldschmidt, R.B., Steward, O., 1980. Preferential neurotoxicity of colchicine for granule cells of the dentate gyrus of the adult rat. Proceedings of the National Academy of Sciences of the USA 77, 3047–3051.

Gorman, A.M., Bonfoco, E., Zhivotovsky, B., Orrenius, S., Ceccatelli, S., 1999. Cytochrome c release and caspase-3 activation during colchicine-induced apoptosis of cerebellar granule cells. European Journal of Neuroscience 11, 1067–1072.

Gould, E., Beylin, A., Tanapat, P., Reeves, A., Shors, T.J., 1999. Learning enhances adult neurogenesis in the hippocampal formation. Nature Neuroscience 2, 260–265.

Götz, M.E., Ahlbom, E., Zhivotovsky, B., Blum-Degen, D., Oettel, M., Romer, W., Riederer, P., Orrenius, S., Ceccatelli, S., 1999. Radical scavenging compound J 811 inhibits hydrogen peroxide-induced death of cerebellar granule cells. Journal of Neuroscience Research 56, 420–426.

Heale, V.R., Vanderwolf, C.H., Leung, L.S., 1995. The neurotoxins colchicine and kainic acid block odor-induced fast waves and olfactory-evoked potentials in the dentate gyrus of the behaving rat. Brain Research 690, 157–166.

Heggeness, M.H., Simon, M., Singer, S.J., 1978. Association of mitochondria with microtubules in cultured cells. Proceedings of the National Academy of Sciences of the USA 75, 3863–3866.

Hirokawa, N., 1994. The neuronal cytoskeleton: roles in neuronal morphogenesis and organelle transport. Progress in clinical and biological research 390, 117–143.

Ho, L., Osaka, H., Aisen, P.S., 1998. Pasinetti GM. Induction of cyclooxygenase (COX)-2 but not COX-1 gene expression in apoptotic cell death. Journal of Neuroimmunology 89, 142–149.

Ikegaya, Y., 1999. Abnormal targeting of developing hippocampal mossy fibers after epileptiform activities via L-type Ca^{2+} channel activation in vitro. Journal of Neuroscience 19, 802–812.

Ikegaya, Y., Ikeda, Y., Saito, H., Nishiyama, N., 1998. Suppression of synaptogenesis by epileptiform discharges in hippocampal slice culture. Biology and Pharmaceutical Bulletin 2, 231–234.

Ikegaya, Y., Kim, J.-A., Baba, M., Iwatsubo, T., Nishiyama, N., Matsuki, N., 2001. Rapid and reversible changes in dendrite morphology and synaptic efficacy following NMDA receptor activation: Implication for a cellular defense against excitotoxicity. Journal of Cell Science 114, 4083–4093.

Jung, M.K., Wilder, I.B., Oakley, B.R., 1992. Amino acid alterations in the benA (β-tubulin) gene of Aspergillus nidulans that confer benomyl resistance. Cell Motility and the Cytoskeleton 22, 170–174.

Kaplan, M.S., Hinds, J.W., 1977. Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs. Science 197, 1092–1094.

Kreutzberg, G.W., 1969. Neuronal dynamics and axonal flow. IV. Blockage of intra-axonal enzyme transport by colchicine. Proceedings of the National Academy of Science USA 62, 722–728.

Kroemer, G., Dallaporta, B., Resche-Rigon, M., 1998. The mitochondrial death/life regulator in apoptosis and necrosis. Annual Review of Physiology 60, 619–642.

Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., Flavell, R.A., 1996. Decreased apoptosis in the brain and premature lethality in CPP32-deficient mice. Nature 384, 368–372.

Leeuwenburgh, C., Heinecke, J.W., 2001. Oxidative stress and antioxidants in exercise. Current Medicinal Chemistry 8, 829–838.

Matus, A., 2000. Actin-based plasticity in dendritic spines. Science 290, 754–758.

Miyashita, T., Reed, J.C., 1995. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80, 293–299.

Mizuhashi, S., Ikegaya, Y., Matsuki, N., 2000. Cytotoxicity of tributyltin in rat hippocampal slice cultures. Neuroscience Research 38, 35–42.

Mizuhashi, S., Nishiyama, N., Matsuki, N., Ikegaya, Y., 2001. Cyclic nucleotide-mediated regulation of hippocampal mossy fiber development: a target-specific guidance. Journal of Neuroscience 21, 6181–6194.

Newell, D.W., Hsu, S.S., Papermaster, V., Malouf, A.T., 1993. Colchicine is selectively neurotoxic to dentate granule cells in organotypic cultures of rat hippocampus. Neurotoxicology 14, 375–380.

Polyak, K., Xia, Y., Zweier, J.L., Kinzler, K.W., Vogelstein, B., 1997. A model for p53-induced apoptosis. Nature 389, 300–305.

Rai, S.S., Wolff, J., 1996. Localization of the vinblastine-binding site on β-tubulin. Journal of Biological Chemistry 271, 14707–14711.

Rubtsova, S.N., Kondratov, R.V., Kopnin, P.B., Chumakov, P.M., Kopnin, B.P., Vasiliev, J.M., 1998. Disruption of actin microfilaments by cytochalasin D leads to activation of p53. FEBS Letter 430, 353–357.

Sattler, R., Xiong, Z., Lu, W.Y., MacDonald, J.F., Tymianski, M., 2000. Distinct roles of synaptic and extrasynaptic NMDA receptors in excitotoxicity. Journal of Neuroscience 20, 22–33.

Spector, I., Shochet, N.R., Kashman, Y., Groweiss, A., 1983. Latrunculins: novel marine toxins that disrupt microfilament organization in cultured cells. Science 219, 493–495.

Tishler, R.B., Lamppu, D.M., Park, S., Price, B.D., 1995. Microtubule-active drugs taxol, vinblastine, and nocodazole increase the levels of transcriptionally active p53. Cancer Research 55, 6021–6025.

Uppuluri, S., Knipling, L., Sackett, D.L., Wolff, J., 1993. Localization of the colchicine-binding site of tubulin. Proceedings of the National Academy of Sciences of the USA 90, 11598–11602.

Wyszynski, M., Lin, J., Rao, A., Nigh, E., Beggs, A.H., Craig, A.M., Sheng, M., 1997. Competitive binding of alpha-actinin and calmodulin to the NMDA receptor. Nature 385, 439–442.

Zeevalk, G.D., Hyndman, A.G., Nicklas, W.J., 1989. Excitatory amino acid-induced toxicity in chick retina: amino acid release, histology, and effects of chloride channel blockers. Journal of Neurochemistry 53, 1610–1619.