学位論文

Proteasome Inhibition Induces Selective Motor Neuron Death in Organotypic Slice Cultures

脊髄スライス培養を用いた運動ニューロンのプロテアソーム障害に対する特異的脆弱性に関する検討

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ABSTRACT

A dysfunctional ubiquitin-proteasome system has recently been proposed to play a role in the pathogenesis of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). We have previously shown that the spinal motor neurons are more vulnerable to proteasome inhibition-induced neurotoxicity, using a dissociated culture system. To further confirm this toxicity, we used organotypic slice cultures from rat neonatal spinal cords, which conserve the structure of the spinal cord in a horizontal plane, enabling us to identify motor neurons more accurately than dissociated cultures. Furthermore, such easy identifications make it possible to follow up the course of the degeneration of motor neurons. When a specific proteasome inhibitor, lactacystin (5 μM), was applied to slice cultures, proteasome activity of a whole slice was suppressed below 30% of control. Motor neurons were selectively damaged, especially in neurites, with the increase of phosphorylated neurofilaments. They were eventually lost in a dose-dependent manner (1 μM; $p<0.05$, 5 μM; $p<0.01$). The low capacity of Ca^{2+} buffering is believed to be one of the factors of selectivity for damaged motor neurons in ALS. In our system, negative staining of Ca^{2+}-binding proteins supported this notion. An intracellular Ca^{2+} chelator, BAPTA-AM (10 μM), exerted a significant protective effect when it was applied with lactacystin simultaneously ($p<0.01$). We postulate that proteasome inhibition is an excellent model for studying the mechanisms underlying selective motor neuron death and searching for new therapeutic strategies in the treatment

of ALS.

Key words: amyotrophic lateral sclerosis; motor neuron; proteasome; calcium

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease causing progressive muscular atrophy and weakness. Riruzole is the only medicine clinically approved to delay the progression, but its effect is unsatisfactory. The disease in most patients is sporadic and of unknown cause, but about 10 % of the cases are familial. In 1994, mutations of SOD1 (ALS1) were reported to cause a dominantly inherited ALS that closely resembles the sporadic form (Rosen et al. 1993). Since then, more than 110 kinds of SOD1-mutations have been reported (www.alsod.org). Because SOD1 knockout mice did not show any phenotypes of motor neuron diseases (Reaume et al. 1996) while mutant SOD1 transgenic mice reproduced clinical signs and pathologies affecting motor neurons (Gurney et al. 1994), the mechanism by which SOD1-mutations cause motor neuron degeneration is not supposed to be a loss of function but an adverse gain of function. Some attractive hypotheses, such as excitotoxicity, oxidative damage, abnormal accumulation of phosphorylated neurofilaments, mitochondrial dysfunction, and impairment of the ubiquitin-proteasome system (UPS) have been discussed (Bruijn et al. 2004).

Protein accumulation is a common and crucial pathological feature of neurodegenerative diseases that could mean impairment of protein degradation

(Ciechanover et al. 2003). In ALS, skein-like inclusions (SLIs), round hyaline inclusions (RHIs) and Bunina bodies are the specific markers of the disease in sporadic form, and Lewy body-like hyaline inclusions (LBHIs) are in ALS1 (Wood et al. 2003). SLIs, RHIs and LBHIs frequently contain ubiquitin, a signal of degradation in 26S-proteasome. UPS plays a major role in cell survival by degradation of proteins in a relatively short period of time, which regulates various functions such as signal transduction, cell cycle and synaptic modulation (Korhonen et al. 2004). The pathologies of ubiquitin-positive inclusion might suggest the involvement of UPS in ALS.

In $SOD1^{G93A}$ transgenic mice, which are hindlimb-onset, the focal decrease of proteasome activity in lumber spinal cords preceded the clinical motor symptom (Kabashi et al. 2004). It is also reported that $SOD1^{G93A}$-transfected cells fell into proteasome dysfunction and motor neurons are specifically vulnerable in primary culture systems (Kikuchi et al. 2002; Urushitani et al. 2002). On the other hand, there is a report examining protease activity in ALS patients which concluded that there were no significant changes in the cytoplasmic protease activity of the spinal cords (Shaw et al. 1996). In a study with slice cultures of mice spinal cords, no specific vulnerability of motor neurons was seen compared to neurons in the posterior horns (Vlug et al. 2004).

In the present study, to confirm whether motor neurons are vulnerable to proteasomal dysfunction, we investigated the organotypic slice cultures of neonatal rat

spinal cords exposed to the specific proteasome inhibitor, lactacystin. Proteasome inhibition induced severe degeneration of motor neurons but the minimum change in the posterior horns and the difference was considered to be due to the low buffering ability of intracellular Ca^{2+} in motor neurons.

MATERIALS AND METHODS

Materials

The following reagents were used in these experiments: SMI-31, -32 and -34 (Sternberger Monoclonals Incorporated, Lutherville, MD); anti-calretinin antibody and anti-calbindin D-28K antibody (Chemicon, Temecula, CA); Eagle's MEM and Glutamine (Nissui, Tokyo, Japan); fetal bovine serum and Gey's balanced solution (Sigma, St. Louis, MO); Hank's balanced salt solution (Gibco BRL, Grand Island, NY); lactacystin, epoxomicin and MCA-conjugated protease substrates (Peptide Institute, Osaka, Japan); 6-Cyano-7-nitroquinoxaline-2, 3-dione (CNQX) and (+)-MK-801 hydrogen maleate (Research Biochemicals International, Natick, MA); ifenprodil tartrate salt and nimodipine (Sigma); BAPTA-AM (Dojindo, Kumamoto, Japan); peroxidase anti-mouse IgG (H+L) and anti-rabbit IgG antibodies (Vector, Burlingame, CA), alexa fluor 488 goat anti-mouse IgG, alexa fluor 568 goat anti-rabbit IgG, zenon alexa fluor 488 mouse IgG1 labeling kit (Molecular Probes, Eugene, OR); rhodamine-conjugated anti-mouse Igs (Tagoimmunologicals, Camarillo, CA).

Organotypic Slice Cultures

These procedures were performed under the approval of the animal care and use committee of Hokkaido University School of Medicine. Organotypic slice cultures were prepared as previously described (Stoppini et al. 1991). Briefly, following deep anesthesia with ketamine, neonatal day 6 Sprague-Dawley rats were decapitated and their lumber spinal cords were removed. Nerve roots and excessive tissues were removed in a cooled Gey's balanced salt solution containing 6.5 mg/ml of glucose. Then the spinal cords were cut into 400-μm slices with a McIlwain tissue chopper (The Mickle Laboratory Engineering Co. Ltd., Gomshall, Surrey, UK). Four slices were put onto a membrane insert (Millicell-CM, Millipore, Bedford) and placed in a 6-well culture dish containing 1 ml of culture medium consisting of 50% Eagle's MEM, 25% Hank's balanced salt solution, 25% horse serum, 6.4 mg/ml of glucose, and 2 mM L-glutamine. The slices were incubated at 37°C, in a 5% CO_2 incubator and the culture medium was changed twice a week. All cultures were used in the experiments after 10 days in vitro.

Immunohistochemistry and Immunofluorescence

For the labeling of neurons in spinal cord slices, the cultures were fixed with 4% paraformaldehyde for 1 hour, rinsed with phosphate-buffered saline (PBS) and, after blocking, stained overnight at 4°C with SMI-32 (1:2500) or the polyclonal antibody against calretinin (1:5000), diluted in PBS containing 0.3% Triton X-100 and 0.2 % bovine albumin. After several washings with PBS containing 0.3% Triton X-100, the

cultures were incubated with a secondary antibody (1:250) for 3 hours and visualized with diaminobenzidine tetrahydrochloride (DAB).

For immunofluorescence analysis, the fixation and primary antibody procedures were the same as described for immunohistochemistry. Secondary antibodies (1:100) were incubated for 1 hour. When two kinds of mouse-derived primary antibodies are used in the same staining, Zenon mouse IgG labeling kit is useful to discriminate them. By Zenon mouse IgG labeling kit, alexa fluor 488-labeled goat Fab fragments against mouse IgG Fc fragments can form complexes with mouse-derived primary antibodies prior to their application on slices. It can simplify the procedure of multiple mouse-derived antibodies. After SMI 31 (or 34) and the secondary antibody, alexa fluor 568 tagged anti-mouse IgG, were applied, SMI 32 which had been labeled with a Zenon one kit beforehand was incubated for 90 minutes. Photographs were taken using a fluoroscope with a CCD camera (Nikon, Tokyo, Japan) and colored with imaging software, Image-Pro 2, or using confocal microscopy (MRC-1024, Bio-Rad).

Measurement of Proteasome Activity

Proteasome activity was assayed with MCA-binding substrates producing AMC when the substrates were cleaved. An increase of AMC can be monitored photometrically at 370 nm. A lysate for one experiment was made from four slices on a membrane. They were spalled in an ice-cooled buffer (20mM tris, 20mM NaCl, 1μM EDTA, 5mM 2-mercaptoethanol, pH 7.6) and divided into 50μL aliquots. Equivalent doses of a

proteolysis buffer (4mM ATP, 10mM $MgCl_2$, 8mM dithiothreitol) containing 100μM AMC-binding substrate (Boc-LRR-AMC, Suc-LLVY-AMC, Z-LLE, substrates as trypsin-like, chymotrypsin-like, peptidylglutamylpeptide hydrolytic proteolytic protease) were added. After incubation at 37°C for 30 min, a 370-nm wave-length was measured. A non-substrate-added lysate was used for the reference. Data were corrected with a protein amount and represented as a rate of the non-lactacystin-treated sample. The protein concentration was measured with a protein assay kit using Bradford's method (Bio-Rad protein assay kit, Tokyo, Japan).

Experimental Treatment of Organotypic Slices and Quantification of Cell Death

The organotypic slices were exposed to two kinds of proteasome inhibitor, lactacystin and epoxomicin, in the culture medium at various concentrations (lactacystin: 0.5-5 μM, epoxomicin: 10-50nM) for 72 hours in a 5% CO_2 incubator maintained at 37°C. To study the involvement of excitotoxicity in the proteasome inhibition-induced effects, glutamate receptor antagonists were used. These included the noncompetitive NMDA receptor blocker, MK-801 (10 μM), the specific blocker of the NMDA receptor containing an NR2B subunit, ifenprodil (10 μM), and the competitive alpha-amino-3-hydroxy-5-methylisoxasole-4-propionate (AMPA)/kainate receptor blocker, CNQX (50 μM). These inhibitors were co-incubated with lactacystin for 72 hours in the culture medium. To examine the effect of calcium activity, the intracellular calcium chelator BAPTA-AM (10 μM), and an L-type calcium channel blocker,

nimodipine (20 μM), were co-administered with lactacystin for 72 hours.

Statistical Analysis

All data presented in this study are representative of at least 4 cultured slices of every experiment, repeated three times independently. Statistical analysis was performed using ANOVA, followed by the post-hoc test (Tukey's method) with the Excel add-in software, Statcel2.

RESULTS

Early Change of Motor Neurons by Proteasome Inhibition

The organotypic spinal cord culture was established referring to past papers (Bergold et al. 1997; Stoppini et al. 1991). A horizontal structure was preserved and many motor neurons were detected in the anterior horns in SMI-32 stains (Fig. 1). Some neurons in the dorsal horns were as large as motor neurons that could be misidentified as motor neurons if their location were unavailable, as in a dissociate culture. We defined surviving motor neurons as SMI-32-positive cells located in the anterior horns, having large somas (>30 μm) and long processes. To clarify a differential effect of the proteasome inhibitor we exposed organotypic slice cultures of the spinal cords to the irreversible proteasome inhibitor, lactacystin (0.5-5 μM). Decreased proteasome activity was confirmed by measurements after a 3-hour exposure to lactacystin in a dose-dependent manner (Fig. 2). They remained suppressed at the same level of activity for 24 hours.

Thirty-six hours after exposure to 5μM lactacystin, some of the motor neurons showed morphological changes. The neurites of untreated motor neurons were thick, especially in their origins (Fig. 3a) Lactacystin-exposed neurons were exhibited as thin neurites from their origins and some neurites were fragmented (Fig. 3b). Because it is known that phosphorylated neurofilaments appear in motor neurons in ALS, we examined the double immunohistochemistry with SMI-32 and SMI-31. SMI-32 recognizes a non-phosphorylated neurofilament heavy chain, and SMI-31 recognizes a phosphorylated one. In the control slices, SMI-31-positive motor neurons were seldom observed (Fig. 4a-c). In contrast, the lactacystin-treated slices contained some motor neurons, which were double positive for SMI-32 and –31 (Fig. 4d-f). Another anti-phosphorylated neurofilament antibody, SMI-34, was also positive in lactacystin-treated cultures (Fig. 4g, h). Phosphorylation was seen in both cell bodies and neurites that occurred in both normal-shaped neurons and degenerated ones. Slice cultures are considered to have dispersion in the number of neurons initially contained in each slice, and mechanical damage by axotomy during preparation induces cell loss, even without toxic insults. However, we concluded that these changes resulted from proteasome impairment, because both severe neurite fragmentations and phosphorylation of neurofilaments were seldom observed in control cultures. In regard to statistical analysis, SMI-34-positive neurons increased significantly in 48-hour lactacystin-exposed slices (Fig. 4i). We did not find a significant difference between the 72-hour exposed

group and control, which we think was due to severe motor neuron loss after a long-term exposure.

Proteasome Dysfunction Induced Specific Death of Motor Neurons

Seventy-two hours after exposure, the number of motor neurons significantly decreased in a 5μM lactacystin treatment group in a lower-power field (Fig. 5a). The remaining cells showed atrophic, condensed somas and fragmented meandering neurites (Fig. 5b, c). Some motor neurons had lost their neurites entirely. In contrast, the SMI-32-positive neurons in the dorsal horns showed little change (Fig. 5d, e). The general structure of the dorsal horns was conserved and the thick neurites of large neurons stretched smoothly. To examine the dorsal horn more clearly, we stained it with the anti-calretinin antibody, which is considered to be a marker for dorsal horn neuron. After a 5μM lactacystin treatment for 72 hours, calretinin-positive neurons showed little morphological change (Fig. 6a, b). The number of surviving motor neurons decreased in a dose-dependent manner with a statistical significance (1 μM: $*p<0.05$, 5 μM: $**p<0.01$), but the number of calretinin-positive neurons in the dorsal horns didn't change ($p>0.05$) (Fig. 7a).

Another specific proteasome inhibitor, epoxomicin, also induced the same degeneration as lactacystin. Motor neurons were damaged in a dose-dependent manner, and showed a significant difference at 50nM, while the dorsal horn neurons didn't show any differences (Fig. 7b). We prepared 100nM epoxomicin-treated slices but could not

evaluate them because they were too fragile to be fixed by the free-floating method (only 25% of them could be fixed). Excessive proteasome inhibition might affect not only neurons but also glial cells, which are indispensable for a slice to attach to a membrane.

Motor Neurons Did Not Express Calcium-Binding Proteins with High Capability, Calretinin and Calbindin D-28K

In ALS, the nucleus of extraocular muscles and Onuf's nucleus are spared. One of the major hypotheses of a differential factor between an easily damaged group of motor neurons and a relatively resistant one is the capacity of Ca^{2+} buffering. Easily damaged groups of motor neurons were reported to lack major calcium-binding proteins (Alexianu et al. 1994), and in a comparative study of the oculomotor nerve, they had a lower capacity of Ca^{2+} buffering (Vanselow et al. 2000). We examined two Ca^{2+}-binding proteins, calretinin and calbindin D-28K, which have a high capacity for buffering. Motor neurons showed a lack of staining with both calretinin and calbindin D-28K, while many posterior horn neurons strongly expressed these proteins (Fig. 8a-e).

Intracellular Ca^{2+} Chelation Ameliorated Lactacystin-Induced Neurotoxicity

In order to confirm whether the involvement of Ca^{2+} homeostasis could be the key to the specific vulnerability of motor neurons induced by proteasome inhibition, we co-treated slice cultures with lactacystin and an intracellular Ca^{2+} chelator, BAPTA-AM. In addition, other agents affecting intracellular Ca^{2+} concentration were also examined. Co-treatment of BAPTA-AM (10 μM) with lactacystin reduced the degenerated motor

neurons significantly ($p<0.01$) (Fig. 9a). Many motor neurons preserved smooth and thick neurites in co-treated cultures (Fig. 9b). The simultaneous treatment with BAPTA-AM did not affect the proteasome inhibition by lactacystin (data not shown).

The NMDA receptor blocker, MK-801 (10 μM) and AMPA/kainate receptor blocker, CNQX (50 μM) were investigated because excessive synaptic glutamate and defective RNA Q/R-editing of GluR2 might be involved in causing ALS to occur (Kawahara et al. 2004; Rothstein et al. 1993). They had a tendency to ameliorate the lactacystin-induced toxicity but without significance. Ifenprodil (10 μM), a specific blocker of NMDA receptors containing an NR2B subunit, and the L-type Ca^{2+} (20 μM) channel blocker failed to show any protective effects.

DISCUSSION

We showed that proteasome inhibition induced severe degeneration in motor neurons compared to posterior neurons. We considered the major reason for this specific vulnerability to be the low ability of calcium buffering. We previously reported that motor neurons were more vulnerable to proteasome inhibition than other kinds of spinal cord neurons in a dissociate culture system (Kikuchi et al. 2002). There was also another report stating that motor neurons were especially vulnerable to proteasome inhibitors and the mutant SOD1-transfected cells fell into proteasomal dysfunction, which leads to motor neuron-specific cell death (Urushitani et al. 2002). However, non-specific cell death was also reported in the slice culture of neonatal mice recently (Vlug et al. 2004).

They used slice cultures applied to proteasome inhibitors and compared the effects between motor neurons and posterior calretinin-positive neurons, as we did in this study. They concluded that both groups were injured without any difference. However, there are some important differences between their study and ours. First, they used clasto-lactacystin beta-lacton, which is stronger than the lactacystin we used, and they did not mention the level of proteasome inhibition. Second, they applied the reagents for a week, which was longer than our study. Because proteasome-mediated proteolysis plays a critical role in maintaining homeostasis and regulating signal transduction for survival, excessive inhibition is fatal for any kinds of cell. In the present study, a higher concentrate application (more than 100nM epoxomicin) induced extensive cell death indiscriminately and longer-term application could also induce indiscriminate cell death, but the present study showed that motor neurons were damaged more easily than posterior neurons at an early stage.

It is uncertain how motor neurons die under proteasome inhibition. Successful protection by BAPTA-AM must indicate that intracellular Ca^{2+} was the key for motor neuron-specific vulnerability. A major change in intracellular Ca^{2+} could occur in two ways. One is the influx through calcium channels on the cellular membrane, and the other is the redistribution of organelles in the cytosol. Mitochondria and endoplasmic reticula (ER) have a large capacity to hold Ca^{2+}. Among these possibilities, we were interested in AMPA receptors, because they are considered to be involved in human

sporadic ALS (Kawahara, et al. 2002), and their surface expression is regulated by proteasome-mediated proteolysis of their anchor protein, PSD95 (Colledge et al. 2003). However, we failed to clarify the benefit of the AMPA receptor blocker, CNQX, and other agents affecting the influx of Ca^{2+}. It would be useful to examine the redistribution of Ca^{2+} inside a cell in the future.

The neurofilament phosphorylation, the important pathological mark of ALS, is observed in our system. Whether this modification is a primary event or a result of other primary events is still controversial. Some investigators claim that neurofilaments are working as sinks for excessive kinase, which could be cytotoxic (Nguyen et al. 2003). Because it is supposed to be the early event both in motor neuron degeneration in ALS patients and in our model, searching for the responsible kinase must be meaningful. For example, JNK, a major stress-activated kinase abundantly expressed in the central nervous system, was reported to phosphorylate neurofilaments and to induce apoptotic signals in N2a cells under the proteasome inhibitory condition (Sang et al. 2002). P38/MAPK could also be up-regulated by a proteasome inhibitor (Yu et al. 2004) and it can phosphorylate neurofilaments, too (Ackerley et al. 2004). In ALS pathology, JNK/SAPK and p38/MAPK were activated in glial cells and in motor neurons (Migheli et al. 1997). Clarifying the involvement of these pathways in our system might be helpful in understanding the mechanism of the disease.

There is another interesting study about proteasome inhibition using mice spinal

cord slice cultures. Puttaparthi et al. reported reversible accumulation of insoluble proteins and ubiquitinated aggregation in $SOD1^{G93A}$-transgenic mice with lactacystin (Puttaparthi et al. 2003). In the present study, no visible aggregate was seen neither in the ubiquitin- nor SOD1 immunohistochemistry (data not shown). We think it is because we used wild type rats, and because the application time was short. Despite the large amount of evidence of aggregate involvement in neuronal death, the meaning is still unclear as to whether it is pathogenic, protective or just a by-product (Arrasate et al. 2004; Lee et al. 2002). At least, in short-term application, it might not be necessary for motor neuron death.

In various neurodegenerative diseases the proteasome dysfunction is likely a critical mediator involving complex interaction of diverse mechanisms. Aging, oxidative stress, and the existence of mutant proteins could induce proteasome dysfunction (Keller et al. 2000; Urushitani et al. 2002). Proteasome impairment induces mitochondria dysfunction (Sullivan et al. 2004) and oxidative stress (Ding et al. 2004), and causes accumulation of proapoptotic signals (Lang-Rollin et al. 2004). The results of the present study indicate that, among many cascades, proteasome dysfunction is a major factor for motor neurons to decide cellular fate. We hypothesize that one reason for rapid progression of ALS compared to other neurodegenerative disorders is this vulnerability of motor neurons to proteasome dysfunction. We believe our model is useful for examining the motor neuron-specific mechanism in the down-stream of

proteasome dysfunction, which may be the key mediator in delaying the progression of ALS.

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Figure Legends

Figure 1: Immunohistochemistry of whole slice culture with SMI-32. Large, strongly SMI-32-positive motor neurons gathered in lateral anterior horns. Scale bar = 400μm.

Figure 2: Three kinds of protease activities of proteasome were significantly inhibited in a dose-dependent manner after a 3-hour exposure to lactacystin. *$p<0.05$, **$p<0.01$.

Figure 3: Immunofluorescence with SMI-32. (a) Motor neurons without lactacystin have thick and long neurites. (b) After 36 hours of exposure to lactacystin, fragmentation of neurites started.

Figure 4: Phosphorylated neurofilaments increased after lactacystin exposure. (a-f) In double immunofluorescence of SMI-31 and SMI-34, motor neurons (arrows) were not phosphorylated without lactacystin (a-c). SMI-31-positive motor neurons (arrowheads) increased after 5μM lactacystin exposure for 36 hours (d-f). (a, d) SMI-32, (b, e)

SMI-34, (c, f) merge. (g, h) In double immunofluorescence of SMI-32 (green) and -34 (red), there were few SMI-34-positive cells in control cultures (g). After a 48-hour exposure, the number of double-positive motor neurons (merge, yellow) increased (h). (i) A significant difference was seen in the number of SMI-34-positive neuron after a 48-hour exposure compared to control *$p<0.05$.

Figure 5: (a) SMI-32-immunostaining of whole slice culture treated with 5μM lactacystin for 72 hours. Severe motor neuronal loss was observed in anterior horns, while the construction of dorsal horns was preserved. Scale bar = 400μm. (b-e) In a higher-power field, compared to non-treated motor neurons (b), lactacystin-exposed motor neuron looked profoundly degenerative (c). Its neurites were fragmented and winding, the margin of the cell body became irregular. (d, e) Large neurons in dorsal horns showed no change. See the neurites, which smoothly stretched in both a non-treated slice (d) and a lactacystin-exposed slice (e). (b)-(e) All were the same magnification, scale bar = 40 μm.

Figure 6: In calretinin-immunohistochemistry, many dorsal horn neurons could be observed. There was little difference between non-treated slices (a) and 5μM lactacystin-exposed slices (b).

Figure 7: (a) After a 72-hour exposure to lactacystin, the number of surviving motor neurons decreased in a dose-dependent manner and had a statistical difference at more than 1 μM (filled bar, *$p<0.05$, **$p<0.01$). Calretinin-positive dorsal horn neurons were not damaged significantly at 5 μM (shaded bar, $p>0.05$). (b) Epoxomicin-exposed slices showed the same results as lactacystin. Motor neurons decreased in a dose-dependent manner, significantly at 50 nM (filled bar, **$p<0.01$). On the other hand, calretinin-positive dorsal neurons didn't show any remarkable change (shaded bar, $p>0.05$).

Figure 8: (a-c) Calretinin were negative in motor neurons (a) (green: calretinin, red: SMI-32, yellow: merge), while many small posterior neurons expressed it (b). Relatively large neurons in dorsal horns also expressed calretinin (c). (d, e) Calbindin

D-28K was negative in motor neurons (d) (green: SMI-32, red: calbindin D-28K, yellow: merge). There were many positive neurons in posterior horns (e).

Figure 9: Co-treated with 5μM lactacystin and candidates for protection simultaneously for 72 hours. In the BAPTA-AM-treated group motor neuron loss was significantly inhibited (a, $^{**}p<0.01$). (b) On immunohistochemistry with SMI 32, surviving motor neurons maintained smoothly stretching neurites in a BAPTA-AM-treated slice. Scale bar = 40 μm.

Fig.1

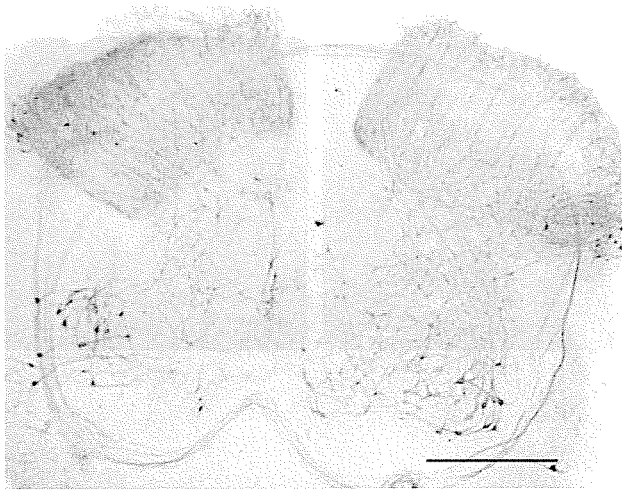


Fig. 2

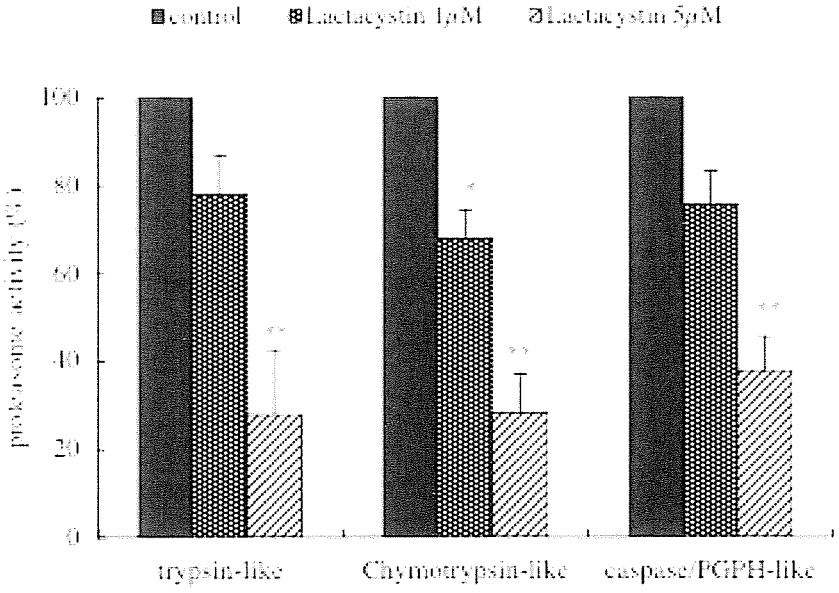

control
Lactacystin 1μM
Lactacystin 5μM
proteasome activity (%)
100
80
60
40
20
0
trypsin-like
Chymotrypsin-like
caspase/PGPH-like

Fig.3

(a) (b)

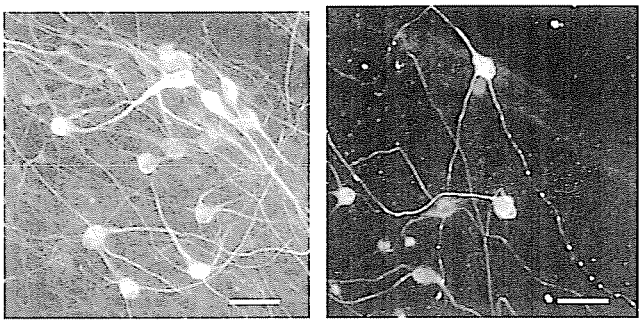


Fig.4

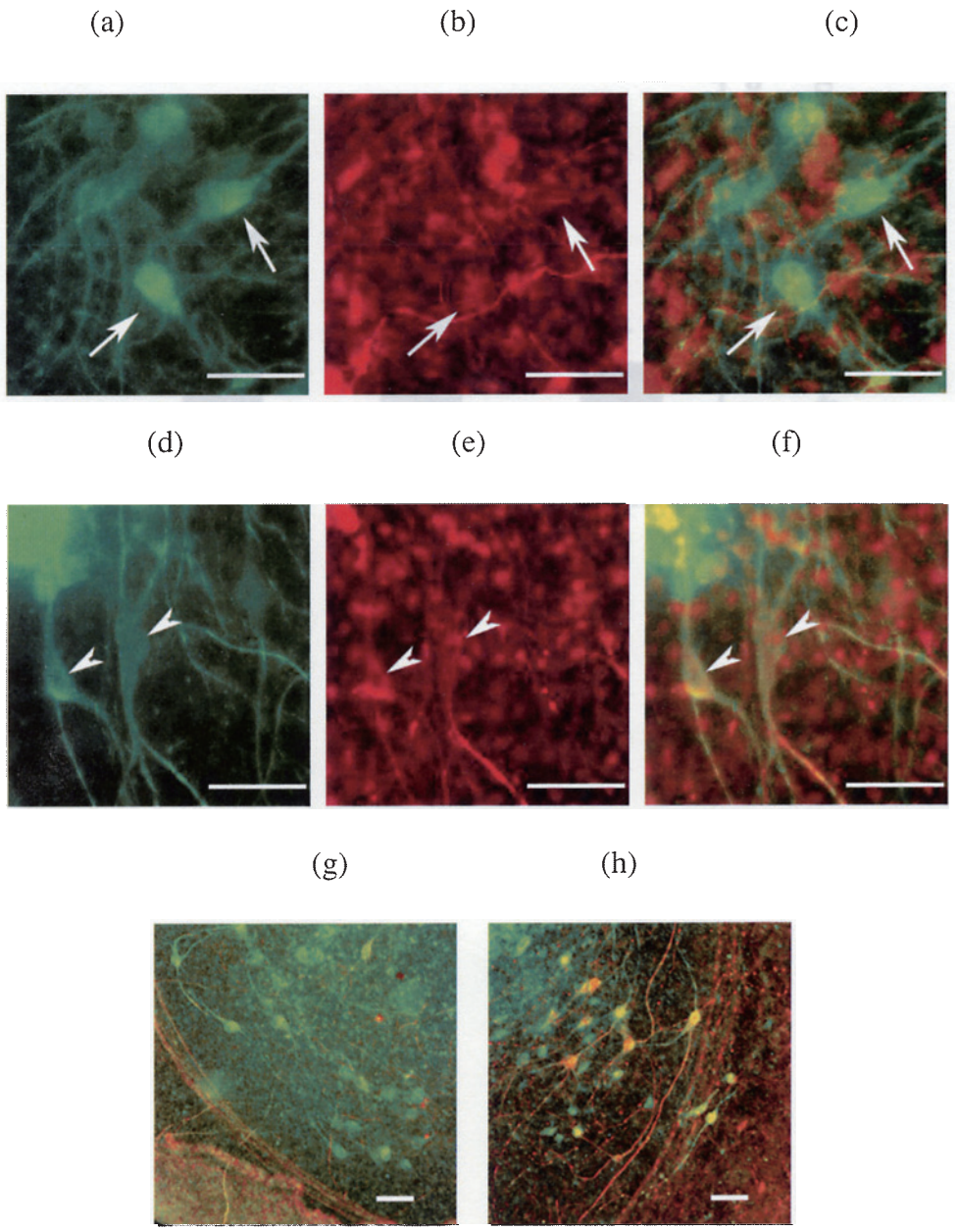


Fig. 4i

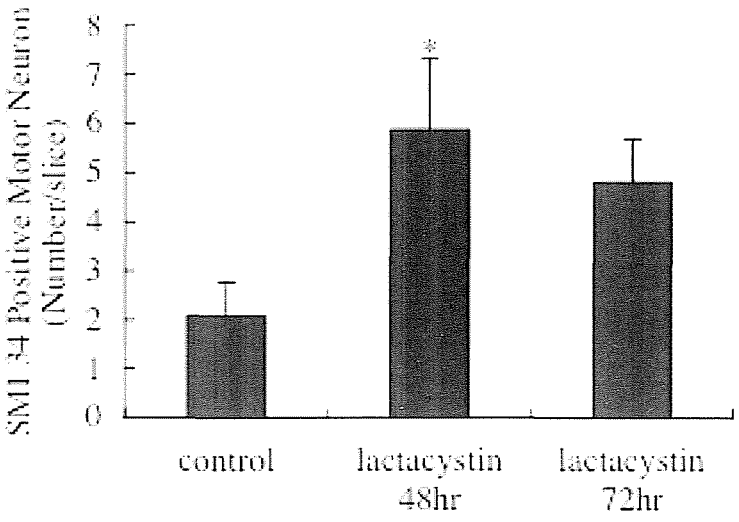
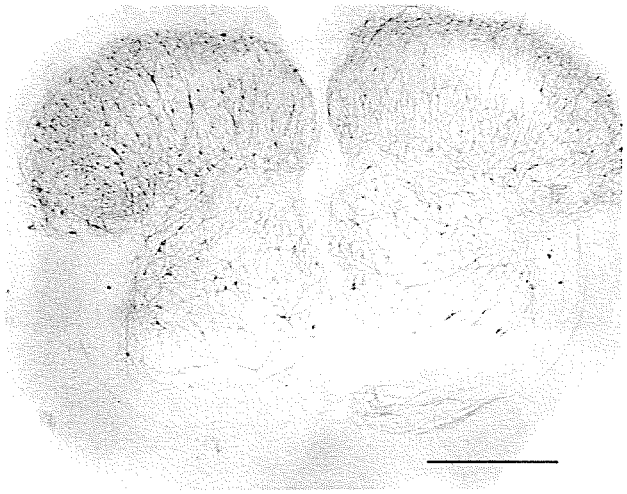
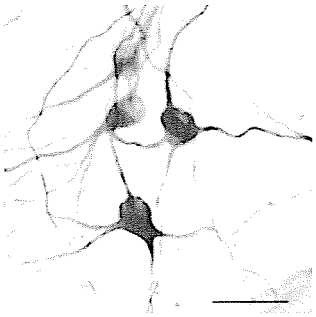

SM1 34 Positive Motor Neuron
(Number/slice)
0
1
2
3
4
5
6
7
8
*
control
lactacystin
48hr
lactacystin
72hr

Fig.5

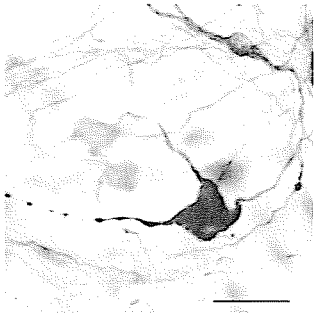
(a)



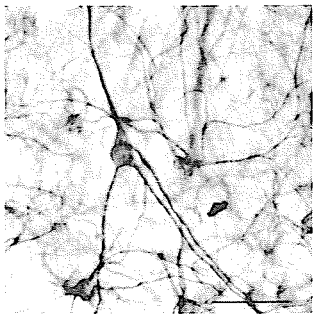
(b)



(c)



(d)



(e)

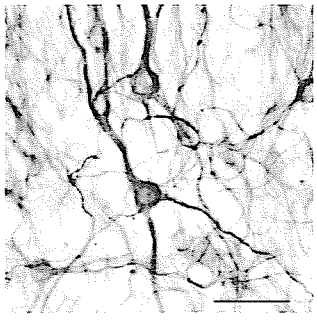


Fig.6

(a) (b)

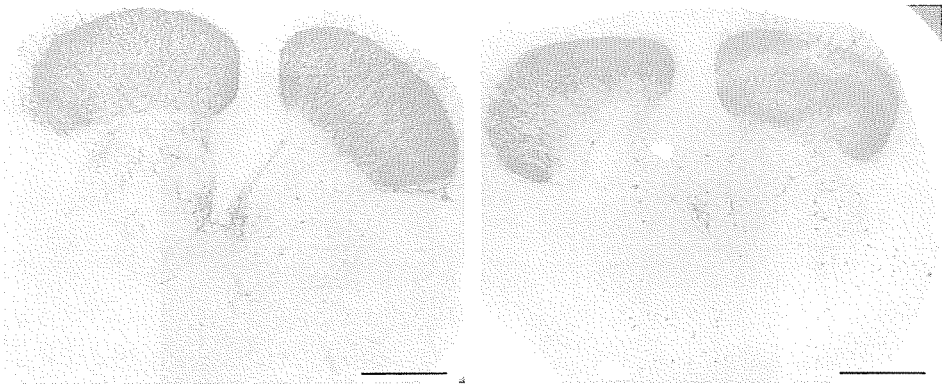


Fig. 7a

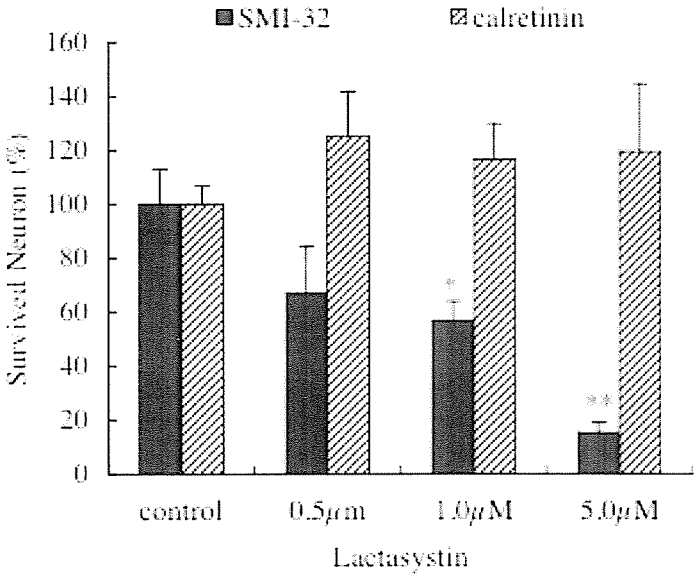

SMI-32
calretinin
Survived Neuron (%)
160
140
120
100
80
60
40
20
0
control
0.5μm
1.0μM
5.0μM
Lactasystin

Fig. 7b

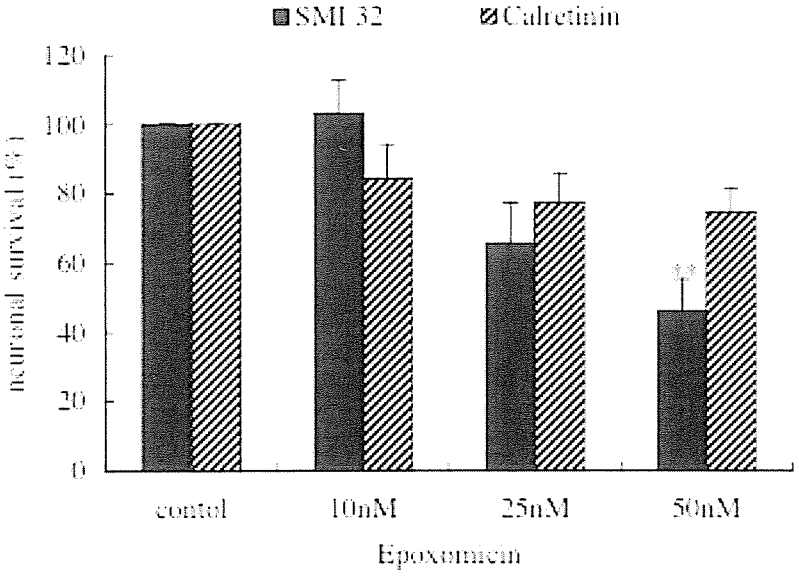

SMI 32
Calretinin
neuronal survival (%)
120
100
80
60
40
20
0
contol
10nM
25nM
50nM
Epoxomicin

Fig.8

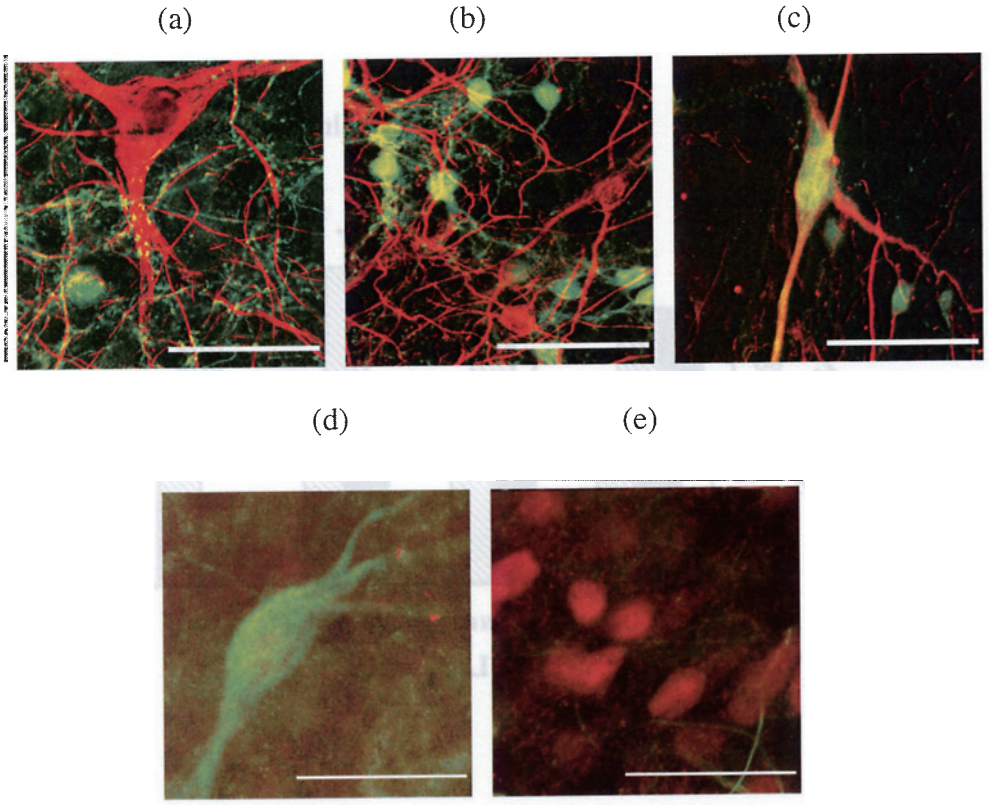
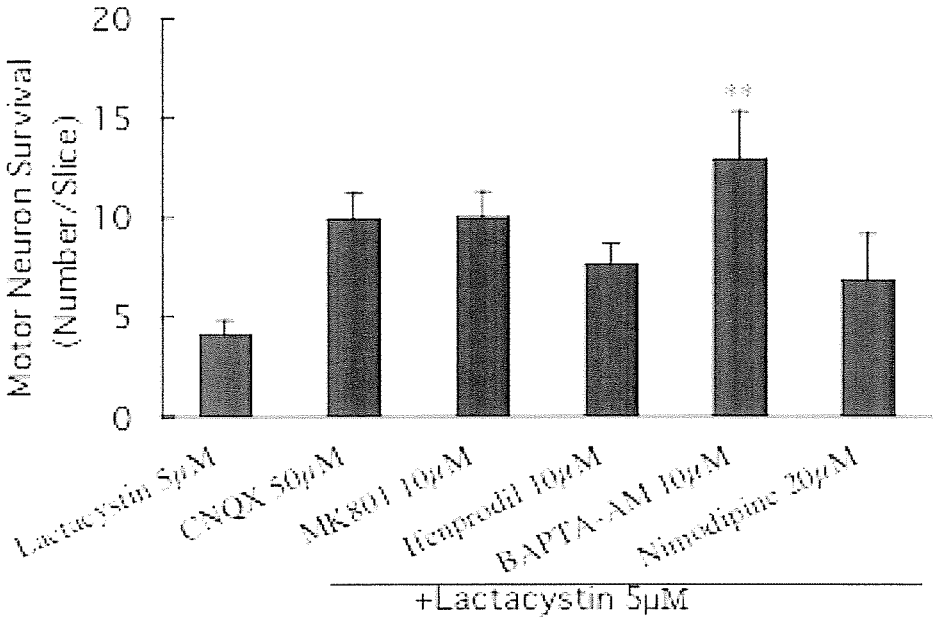


Fig. 9a


Motor Neuron Survival
(Number/Slice)
20
15
10
5
0
**
Lactacystin 5μM
CNQX 50μM
MK801 10μM
Ifenprodil 10μM
BAPTA-AM 10μM
Nimodipine 20μM
+Lactacystin 5μM

(b)