Supplementary Materials and Methods

Transgenic mice

For EAAC1 transgenic mice, the 3-kb *DINE* promoter (Kiryu-Seo et al., 2000) was used to regulate expression of EAAC1 and GFP. An IRES directing the translation of GFP was introduced into the 3' end of the EAAC1 stop codon. Transgenic animals were generated via standard microinjection techniques that used hybrid mouse embryos (C57BL/6/DBA). Founder mice were backcrossed with C57BL/6 mice. To test for transgene transmission, we isolated genomic DNA from the mouse tail; integration of the transgene was analyzed routinely by PCR using a 5'-oligonucleotide (5'-GCAGACAGAAGCAGCCTATAATCGACC-3') and a 3'-oligonucleotide (5'-TTCCTGATCAGATCCAACATGGCGTCC-3'). PCR was performed under the following conditions: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min for 30 cycles, followed by 7-min extension at 72°C.

Surgical procedures

Animals were anaesthetized with pentobarbital (45 mg/kg). For hypoglossal nerve injury, animals were positioned supine. The right hypoglossal nerve was carefully exposed under the digastric muscle. At central side of the hypoglossal nerve bifurcation, the nerve was transected with a pair of scissors and the nerve edges were placed not to touch with each other. For facial nerve injury, the right facial nerve was transected after its exit from the stylomastoid foramen. For sciatic nerve injury, the right sciatic nerve was exposed in the mid-thigh and cut with scissors.

Histology

Animals were decapitated after the operation, and the brain was removed quickly and frozen in powdered dry ice. Then, 18-μm-thick sections were cut on a cryostat, thaw-mounted onto 3-aminopropyltriethoxysilane-coated slides, and stored at -80ºC until use. For quantification of cell survival after axotomy, hypoglossal motor neurons were stained with thionine, and counted at the identical level between animals in every 5th section through population examined as previously described (Clarke & Oppenheim, 1995). Data were presented as the percentage of surviving neurons on the injured and control side. Statistical significance (*p* value) was calculated by two-tailed Student's *t*

test. All procedures for *in situ* hybridization were performed as previously described (Kiryu et al., 1995)

For immunohistochemistry using anti-HCCS antibody, the perfused section with 4% paraformaldehyde containing 0.21% picric acid in 0.1 M phosphate buffer (PB) was air dried and then rinsed in PBS. The sections were pretreated with 0.3% H_2O_2 in PBS, and incubated with PBS containing 10% normal goat serum (NGS) for 30 min at room temperature. These sections were incubated with anti-HCCS antibody overnight at 4°C. Then, the sections were incubated with biotinylated anti-rat IgG for 30 min at room temperature, and incubated in avidin-biotin horseradish peroxidase complex (Vector Labs, Inc., Burlingame, CA, USA) for 60 min at room temperature. The sections were stained in Tris-HCl containing DAB (0.2 mg/ml) and 0.003 % hydrogen peroxide and dehydrated in a graded series of ethanol to xylene. For EAAC1 immunostaining, the freshly frozen section was prepared. After fixing with methanol for 20 min, the sections were pretreated with 0.3% H_2O_2 in PBS, and incubated with PBS containing 10% normal goat serum (NGS) for 30 min at room temperature. These sections were incubated with anti-EAAC1 antibody (Chemicon) overnight at 4°C and followed by anti-mouse IgG conjugated with Alexa Fluor 488 (1:500; Molecular Probes, Eugene, OR, USA).

RT-PCR

Total RNA was isolated from the hypoglossal nuclei of twenty animals using the AGPC method. Total RNA (each 5 μg) was converted to cDNA by reverse transcription using Superscript II™ (Invitrogen, Gaithersburg, MD) and nucleotide oligo dT. Aliquots from the reverse transcription reaction mixture were used for PCR amplification. Primer pairs ubiquitously expressing GAPDH were used as a control under the following PCR conditions: 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min for 23 cycles (for GAPDH) or 28 cycles (for EAAC1, GLT-1, GLAST, ASCT1, ASCT2 and GFP), followed by 7-min extension at 72ºC. The PCR products were electrophoresed on 1.2% agarose gel.

Plasmids

For *in situ* hybridization, the fragments of EAAC1 (Accession No. D63772, 1284-1694), GLT-1 (Accession No. D43796, 1557-1886), GLAST (Accession No. NM 148938, 1487-1887) ASCT1 (Accession No. L14595, 421-1088) and ASCT2 (Accession No. D85044, 1021-1665) were amplified by PCR using cDNA isolated from rats and mice, and inserted into pGEM-T Easy Vector (Promega).

For expression analysis, wild-type and ΔN1-17 of rat EAAC1 cDNA and GLT-1 cDNA with and without the HA epitope sequence at the C-terminus were cloned into both the pME18S and pcDNA3 (Invitrogen) expression vectors. N-terminally HA-tagged EAAC1 was inserted in pcDNA3. Wild-type EAAC1 and ΔN-EAAC1 was cloned into TNT vector (Promega). N-terminally FLAG-tagged wild-type HCCS (FLAG-HCCS), FLAG-HCCS (1-152) and FLAG-HCCS (148-272) were inserted into pME18S. Wild-type HCCS, MTAV-HCCS and MSAS-HCCS without the epitope tag were cloned into pME18S. N-terminally HA-tagged HCCS (HA-HCCS), HA-HCCS (1-152) and HA-HCCS (148-272) were subcloned into pcDNA3. HCCS was also cloned into pGEX5X-1 vector to produced GST fusion protein. The plasmids encoding N-terminally FLAG-tagged hXIAP, mXIAP, c-IAP1 and c-IAP2 (pcDNA3-FLAG-XIAPs, -c-IAP1, -cIAP2), N-terminally Myc-tagged XIAP and Survivin (pcDNA3-Myc-XIAP, -Survivin) and GST-tagged BIR1 (residues 1-168), BIR2 (residues 124-242), BIR3 (residues 243-351), BIR1-3 (1-351 residues) and XIAP (pGEX4T-1-BIR1, -BIR2, -BIR3, -BIR1-3, -XIAP) were generously donated by R. Takahashi (Kyoto University, Japan).

Adenovirus Preparation

Rat EAAC1 cDNA with Kozak sequence was cloned into the Cre-inducible expression cassette pAxCALNLw, which is normally not expressed because of the neomycin resistance gene. The recombinant viruses AxCALNLEAAC1 and AxCANCre, which express Cre recombinase, and AxCALNLLacZ under the CAG promoter were generated according to a previously described method (Namikawa et al., 2000).

Cell culture

PC12 cells were grown in RPMI 1640 medium supplemented with 5% FCS and 10% horse serum with penicillin and streptomycin. For neuronal differentiation, the cells

were cultured in collagen-coated culture dishes in medium containing 100 ng/mL NGF and 0.5% FCS for 14 days. For the cell viability assay, differentiated PC12 cells were plated in 24 wells. At 24 and 48 hr after NGF withdrawal and 48 hr after p53 adenovirus infection, a biochemical method using a highly water-soluble tetrazolium salt (WST-1) was used to determine the percentage of surviving cells, according to the manufacturer's protocol (Nacalai Tesque, Kyoto, Japan). COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, penicillin, and streptomycin.

For transfection, lipofectamine 2000 was used according to the manufacturer's procedures (Invitrogen). For antisense experiment, oligonucleotides were designed as described previously (Rothstein et al., 1996). The sequence of the oligonucleotides are 5'- GCT CGG GAT GCG ACT GGC-3' (sense), 5'- GCC AGT CGC ATC CCG AGC-3' (antisense) and 5'- GCG GAT CCG TAC GCC CAG-3' (random). These were synthesized with a phosphorothioate backbone and purified to analytical grade to HPLC (Sigma Genosys). These oligonucleotides were added at a concentration of 30 μM every 24 hr. For peptide transfection, EAAC1 (1-17) peptide was synthesized and purified to analytical grade to HPLC (Sigma Genosys). The peptide (10 μM) was introduced into differentiated PC12 cells using BioPORTER according to manufacturer's protocol (Gene Therapy Systems).

Caspase Assays

PC12 cells were rinsed with PBS and harvested by scraping. The cell pellet was washed once in ice-cold PBS and resuspended in lysed in ice-cold buffer containing 50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% CHAPS, 1 mM DTT and 0.1 mM EDTA. After freezing and thawing three times, cells were centrifuged for 10 min at 10,000 g. The protein concentration of the supernatant was measured by BCA protein assay kit (Pierce) Then, the supernatant containing 50 μg of protein was incubated with 200 μM enzyme substrate, Ac-DEVD-*p*NA in assay buffer (50mM HEPES[pH7.4], 100 mM NaCl, 0.1 % CHAPS, 10 mM DTT and 0.1 mM EDTA) at 37 °C for 1 h according to the manufacturer's instruction (Calbiochem). The enzyme-catalyzed release of *p*-

nitroanilide was measured at 405 nm using Wallac1420 multilabel counter (Amersham Pharmacia Biotech).

Cell Surface Biotinylation

Cells were rinsed twice with ice-cold PBS (pH 7.5) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$. The cells were then incubated in 2 ml of biotinylation solution (1 mg/ml sulfo-NHS-biotin in PBS/Ca^{+}/Mg^{2+}) for 30 min at 4ºC with gentle shaking. The biotinylation solution was removed, rinsed twice with PBS/Ca^{+}/Mg^{2+} containing 100 mM glycine and quenched with PBS/Ca^{+}/Mg^{2+}/glycine for 30 min at 4ºC with gentle shaking. Cells were then rinsed twice with PBS/Ca^{+}/Mg^{2+}/glycine, lysed in RIPA buffer containing protease inhibitors (100 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, 1 mg/ml leupeptin, 250 mM PMSF, 1 mg/ml aprotinin) and scraped from the plates. The lysates were centrifuged for 15 min at 12,500 rpm. After removal of the cellular debris, aliquots of the lysate were taken for western analysis before the lysate was incubated with avidin-conjugated beads (the “total cell lysate” fraction). Equal volumes of avidin bead suspension (Immunopure Immobilized Monomeric Avidin, Pierce) were added to a volume of lysate and incubated for 1 hr at room temperature with occasional stirring. Then the avidin-lysate solution was centrifuged for 15 min at 16,500 g. The supernatant was removed and discarded after samples were taken for western analysis (the “intracellular” fraction). The pellet was washed four times with 1 ml of RIPA and resuspended in Laemmli buffer (62.5 M Tris-HCl, pH6.8, 2 % SDS, 20 % glycerol and 5 % 2-mercaptethanol) for 30 min with occasional shaking to elute the biotinylated proteins. The solution was centrifuged for 10 min at 16,500g and the supernantants was removed and saved for Western analysis as the “biotinylated” fraction.

Production of HCCS Monoclonal Antibody

Anti-HCCS rat monoclonal antibody was generated based on the rat lymph node method established by Kishiro et al (Kishiro et al., 1995). A ten-week-old female WKY/Crj rat was injected in the hind footpad with 300 μl of an emulsion containing 50 μg recombinant GST-fused HCCS protein and Freund’s complete adjuvant. After 3 weeks, the cells from the lymph nodes of a rat immunized with an antigen were fused

with mouse myeloma Sp2/0-Ag14 cells. At 10 days after fusion, the hybridoma supernatants were screened by means of an enzyme-linked immunoadsorbent assay (ELISA) against GST-fused HCCS. Positive clones were subcloned and rescreened by ELISA and immunoblotting.

Immunoprecipitation

For cultured cells

Cells were washed with PBS twice and lysed in following buffer. To examine EAAC1-HCCS interaction, cells were lysed in RIPA buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 5 μg/mL aporotinin, 1 mM PMSF, and 1 μg/mL leupeptin. For HCCS-XIAP interaction, COS-7 cells were transfected and the medium was changed to 10% FCS DMEM containing 5 μM MG132 at 24 hr after transfection. Then, 12 hr later, samples were lysed in Triton lysis buffer containing 20 mM HEPES (pH 7.4), 120 mM NaCl, 5 mM EDTA, 1% Triton X-100, 10% glycerol, 5 μg/mL aporotinin, 1 mM PMSF, 20 μM MG132 and 1 μg/mL leupeptin and immunoprecipitated. After centrifugation (15 min, 15,000 rpm), the supernatant was incubated with anti-FLAG (M2, Sigma), anti-HA (3F10, Roche Diagnostics) and anti-HCCS antibodies with constant agitation at 4ºC The immunocomplex was precipitated with protein G sepharose (Zymed). The pellet was washed three times and prepared for immunoblotting.

For tissue samples

Normal and injured hyopoglossal nuclei were isolated from fifteen rats and mice after axotomy. Samples were homogenized and solubilized for 1 hr at 4ºC in extraction buffer (10mM Hepes at pH7.5, 142.5 mM KCl, 5mM MgCl2, 1mM EGTA and 1% NP-40) containing protease inhibitors (5 μg/mL aporotinin, 1 mM PMSF and 20 μM MG132). The lysate was clarified by 30 min centrifugation at 15,000g and imunoprecipitaed by incubation with anti-HCCS antibody for overnight and protein G sepharose for 1hr. After washing with 500 μl of wash buffer (10mM Hepes at pH7.5, 142.5 mM KCl, 5mM $MgCl_2$, 1mM EGTA and 0.1% NP-40), the precipitates were subjected to immunoblotting.

GST pull down assay

Fusion proteins for GST alone, GST-HCCS, GST-BIR1, GST-BIR2, GST-BIR1-3 and GST-XIAP were prepared using BL21 bacteria, and purified from crude bacterial lysate according to the manufacturer's instructions (Amersham Pharmacia Biotech). Briefly, GST-fusion proteins were bound to glutathione-sepharose beads in lysis buffer (10mM Hepes at pH7.5, 142.5 mM KCl, 5mM MgCl2, 1mM EGTA and 0.5 % NP-40) and protease inhibitors ^{35}S-labelled EAAC1, ΔN-EAAC1 and XIAP proteins were synthesized by in vitro transcription and translation using a TNT T7-coupled reticulocyte lysate system (Promega). GST fusion proteins conjugated to glutathione sepharose beads were incubated with ^{35}S-labelled proteins at 4°C for 1 hr. The beads were then washed four times in lysis buffer, pelleted at 500g for 1 min, and finally taken up in SDS-PAGE sample buffer. Bound proteins were resolved by SDS-PAGE and subjected to autoradiography.

Cell lysates for pull-down analysis were prepared by lysing 100-mm dishes of HCCS-transfected COS-7 cells in lysis buffer (10mM Hepes at pH7.5, 142.5 mM KCl, 5mM MgCl2, 1mM EGTA and 0.5 % NP-40) containing protease inhibitors. Lysates were collected and cnetrifugated at 15,000g for 15 min. For pull-down analysis, 100 μg of total cell lysates and 2 μg of GST fusion proteins were added to allow for adequate mixing. After 2 hr incubation at 4 C, the protein-bead complexes were washed four times in 1 mL of ice-cold lysis buffer and resuspended in SDS sample buffer to SDS-PAGE.

References

Clarke, P.G. and Oppenheim, R.W. (1995) Neuron death in vertebrate development: in vitro methods. *Methods Cell Biol* **46**: 277-321

Kiryu, S., Yao, G.L., Morita, N., Kato, H. and Kiyama, H. (1995) Nerve injury enhances rat neuronal glutamate transporter expression: identification by differential display PCR. *J Neurosci* **15**: 7872-7878

Kiryu-Seo, S., Sasaki, M., Yokohama, H., Nakagomi, S., Hirayama, T., Aoki, S., Wada, K. and Kiyama, H. (2000) Damage-induced neuronal endopeptidase (DINE) is a unique metallopeptidase expressed in response to neuronal damage and activates superoxide scavengers. *Proc Natl Acad Sci U S A.* **97**: 4345-4350

Kishiro, Y., Kagawa, M., Naito, I. and Sado, Y. (1995) A novel method of preparing rat-monoclonal antibody-producing hybridomas by using rat medial iliac lymph node cells. *Cell Struct Funct* **20**: 151-156

Namikawa, K., Honma, M., Abe, K., Takeda, M., Mansur, K., Obata, T., Miwa, A., Okado, H. and Kiyama, H. (2000) Akt/protein kinase B prevents injury-induced motoneuron death and accelerates axonal regeneration. *J Neurosci* **20**: 2875-2886

Rothstein, J.D., Dykes-Hoberg, M., Pardo, C.A., Bristol, L.A., Jin, L., Kuncl, R.W., Kanai, Y., Hediger, M.A., Wang, Y., Schielke, J.P. and Welty, D.F. (1996) Knockout of glutamate transporters reveals a major role for astroglial transport in excitotoxicity and clearance of glutamate. *Neuron* **16**: 675-686