exposed to L-745,870 were distributed in the LR quadrant (early stage of apoptosis) in an early phase of incubation (60 mins) but subsequently shifted to the UR quadrant (apoptosis/necrosis) during the late phase of incubation (4 h) (data not shown). These data suggest that cell death in response to menadione is the result of apoptosis rather than necrosis. L-745,870 treatment reduced the apoptotic cell population in the UR quadrant from 45% to 20% and increased the LL and LR quadrant populations from 32% to 59% and 15% to 19%, respectively (Figure 2A). L-745,870 treatment also reduced the apoptotic cell population in the UR quadrant from 78% to 53% and increased the normal cells in the LL quadrant from 6% to 28% in the cells exposed to H_2O_2 for 40 mins (Figure 2B). These results suggest that L-745,870 specifically protects cells from apoptosis induced by oxidative stress.

L-745,870 Specifically Upregulates NAIP

To examine whether L-745,870 specifically induces NAIP expression level among the antiapoptotic proteins, expression levels of the IAP family (XIAP, cIAP-1, cIAP-2, and survivin) and Bcl-2 family (Bcl-2 and Bcl-XL) proteins were assessed in HeLa and SH-SY5Y cells by Western blotting. L-745,870 specifically elevated the endogenous level of NAIP (Figure 3A) but had no effect on the levels of other antiapoptotic proteins (Figures 3B–3G). This observation was consistent with results obtained by the DNAChip analysis (8,300 genes, including all of the known antiapoptotic proteins with the exception of the *NAIP/BIRC1* gene: the Atlas Plastic Arrays analysis; Beckton Dickinson), in which no upregulation of the antiapoptosis relating genes was observed (data not shown). Slight decreases in the levels of XIAP and cIAP-1 were noted only in differentiated SH-SY5Y cells (Figures 3B and 3C), although the physiologic significance for this small effect may not be significant. These results indicate that L-745,870 selectively enhances endogenous NAIP levels.

L-745,870 Inhibits Oxidative Stress-Induced Apoptosis Via NAIP Upregulation

To investigate whether the elevation of the endogenous NAIP level by L-745,870 is responsible for the protection against oxidative stress-induced apoptosis, *NAIP* gene expression was suppressed using NAIP-RNAi. Transfection of HeLa cells with NAIP-U6 resulted in a marked decrease in endogenous NAIP levels (Figure 4A) and a concomitant increase in susceptibility to menadione (Figure 4B). Further, L-745,870-treated/NAIP-U6 transfected HeLa cells showed higher susceptibility to menadione than cells with NAIP-U6 treatment alone (Figure 4B). These results indicate that the increased

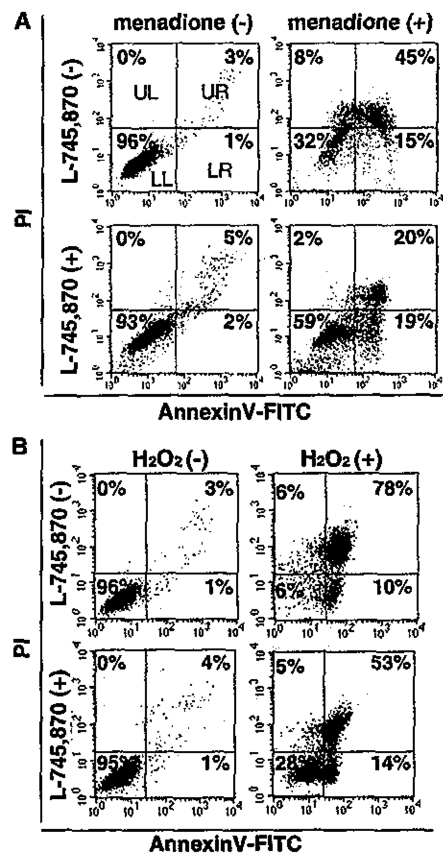

Figure 2 Effect of the neuronal apoptosis inhibitory protein (NAIP) upregulating compound (L-745,870) on the oxidative stress-induced apoptotic cell death, and a flow-cytometric analysis of the apoptotic and/or necrotic cell death in HeLa cells. HeLa cells that were precultured with vehicle or L-745,870 (10 μmol/L) for 24 h were challenged with (**A**) menadione (+: 50 μmol/L, for 4 h) or (**B**) H_2O_2 (+: 250 μmol/L, for 40 mins). Cell death was detected by staining with Annexin V-FITC and PI in conjunction with flow cytometry. FACS quadrant analysis allows the classification of the cells into four distinct categories based on the areas in the quadrant: upper left (UL), upper right (UR), low left (LL), and low right (LR). Percentage of the cell numbers in each quadrant is shown on the right.

endogenous NAIP level by L-745,870 likely mediates its ability to protect cells from oxidative stress-induced apoptosis.

Systemic Administration of L-745,870 Results in a Transient Upregulation of NAIP in Gerbil Hippocampal CA1 Neurons

L-745,870 administration had no effect on CBF (control/vehicle; 103.9 $\pm$ 13.2 mL/100 g/min, 2 h after administration; 100.3 $\pm$ 12.0 mL/100 g/min, and 24 h after administration; 98.9 $\pm$ 13.2 mL/100 g/min;

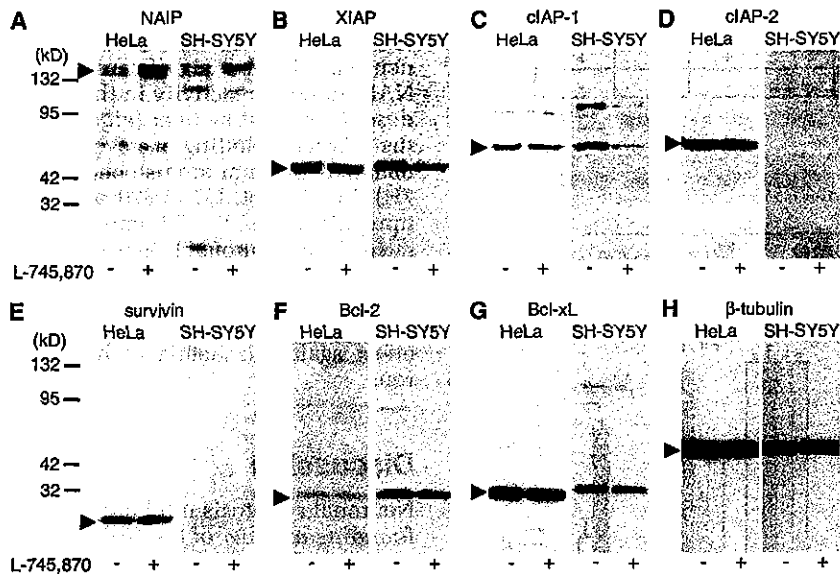

Figure 3 Western blot analysis of antiapoptotic proteins in HeLa and differentiated SH-SY5Y cells after treatment with L-745,870 (+, 10 μmol/L) or vehicle (−) for 24 h. Seven antiapoptotic proteins, including (**A**) neuronal apoptosis inhibitory protein (NAIP), (**B**) X-linked inhibitor of apoptosis (XIAP), (**C**) cIAP-1, (**D**) cIAP-2, (**E**) survivin, (**F**) Bcl-2, and (**G**) Bcl-xL, were analyzed. Each arrowhead indicates the position of each protein (NAIP: 150 kDa, XIAP: 47 kDa, cIAP-1: 65 kDa, cIAP-2: 64 kDa, survivin: 16.5 kDa, Bcl-2: 27 kDa, Bcl-xL: 28 kDa). (**H**) Expression of β-tubulin was not affected by L-745,870 treatment, showing that equal amounts of proteins were loaded in each lane of the blots.

control versus 2 h; $P>0.1$, control versus 24 h; $P>0.1$), blood pressure, and heart rate in gerbils (data not shown), which were consistent with results from a previous study (Patel *et al*, 1999). However, administration of L-745,870 (210 mg/kg) resulted in increased NAIP-immunoreactivity in the hippocampal CA1 neurons at 2 h (Figures 5A and 5B). Further, the increase in NAIP-immunoreactivity peaked at 24 h and then gradually returned to baseline at 72 h after administration (Figures 5A–5D). Results of the Western blotting were consistent with those obtained by the immunohistochemical studies (Figure 5E).

Administration of L-745,870 Attenuates Ischemia-Induced neuronal Cell Death in the Hippocampal CA1 Region

To determine whether administration of L-745,870 exerted neuroprotective against ischemic insults *in vivo*, gerbil forebrain ischemic models were established by transient BCCAO, which induces a selective loss of CA1 pyramidal cells in the hippocampus (Kirino and Sano, 1984). Because the time course of changes in NAIP expression occurred over a 72 h period after administration of L-745,870, the experimental protocol used induction of a relatively severe ischemic condition (10 min BCCAO), followed by the analysis of delayed neuronal loss in the CA1 region over the subsequent 72 h. At 72 h after the ischemic insult, vehicle treated animals showed widespread hippocampal CA1 neuron death with very few surviving neurons present in the pyramidal layer (Figures 6A and 6B). Further, a large number of glial cells were also observed in this area (Figures 6A and 6B). However, these animals showed little or no cell loss in the CA3 region or dentate gyrus (data not shown).

Administration of L-745,870 at 60 mins before the ischemic surgery exerted significant neuroprotective effects against ischemic insults in a concentration-dependent manner. A decreased dosage of L-745, 870 (7 mg/kg) showed the most modest protection in the hippocampus CA1 neurons (data not shown), whereas medium to high dosages (70 and 210 mg/kg) exhibited prominent protective effects (Figures 6C–6F). The quantitative scoring of the neuronal cell damage was consistent with these results (Figure 6I). Moreover, the highest dose of L-745,870 (210 mg/kg) was not associated with any CA1 neuron toxicity (Figures 6G and 6H). Further, these protective effects were significant even at 5 days after reperfusion in this experimental condition, despite the fact that progressive cell death in CA1 neurons was observed (data not shown). Together, these results indicate that L-745,870 inhibits progression of the ischemia-induced CA1 neuron death.

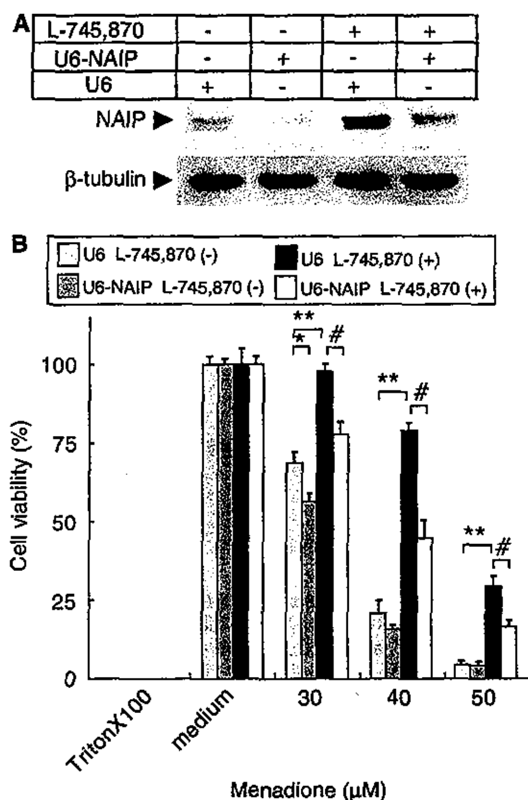

Figure 4 Effect of inhibition of neuronal apoptosis inhibitory protein (NAIP) expression on menadione-induced cell death in HeLa cells. Neuronal apoptosis inhibitory protein RNAi was achieved by ectopically expressing the small interference RNA (siRNA) corresponding to a portion of the 3′-UTR of the *NAIP* gene using the pSilencer 1.0-U6 system. (**A**) Western blot analysis of the NAIP expression in the RNAi treated HeLa cells. The lower panel represents the expression of β-tubulin and showed that equal amounts of protein were loaded in each lane in the blot. (**B**) Effect of the NAIP RNAi on menadione-induced cell death, and on the protection of cell death by L-745,870. Concentration of menadione used in this experiment was between 30 and 50 μmol/L. Cell viability is shown as percentages of the alamarBlue values in U6 or NAIP-U6-transfected cells relative to those of controls (menadione non-treated). Values represent the means ± s.e. of eight independent experiments. *$P<0.01$ and **$P<0.001$ for U6 L-745,870 (−) cells versus NAIP-U6 L-745,870 (+) cells or U6 L-745,870 (+). #$P<0.001$ for U6 L-745,870 (+) cells versus NAIP-U6 L-745,870 (+) cells. Statistical analysis was performed using Student's *t*-test.

Hippocampal CA1 Neurons Rescued by the Administration of L-745,870 Show Strong NAIP-Immunoreactivity

To investigate whether L-745,870-mediated hippocampal CA1 neuronal protection was associated with elevation of neuronal NAIP levels, immunohistochemical and Western blot analyses of endogenous NAIP were performed in CA1 neurons using an NAIP antibody. After 72 h of reperfusion, the CA1 neurons showed significant enhancement of the NAIP-immunoreactivity in the rescued neurons in a dose-dependent manner (Figures 7A–7F). Results of the Western blotting were consistent with results obtained by immunohistochemical studies (Figure 7H). In contrast, L-745,870 produced no detectable upregulation of XIAP-immunoreactivity in the rescued hippocampus (Figures 7G and 7H). These results show that L-745,870 selectively upregulates NAIP *in vivo*, which represent a potential mechanism by which L-745,870 exerts a neuroprotective effect against ischemia in hippocampal CA1 neurons.

Discussion

Neuronal apoptosis inhibitory protein (BIRC1) is the founding member of the IAP family of proteins, and has been shown to inhibit apoptosis of neurons and other types of the cell *in vitro* and *in vivo*. Increases in NAIP, by either viral-mediated NAIP gene transfer or by enhancement of endogenous levels, results in the attenuation of ischemic neuronal cell death (Xu *et al*, 1997). Further, ectopic NAIP expression enhances rescue of motor neurons from peripheral nerve axotomy (Perrelet *et al*, 2000) and leads to preservation of nigrostriatal dopaminergic neurons in the intrastriatal 6-OHDA rat Parkinson's disease model (Crocker *et al*, 2001). Neuronal apoptosis inhibitory protein also contributes to motor neuron survival through intracellular signaling of GDNF (Perrelet *et al*, 2002). Ischemic neuronal injury is associated with excessive generation of reactive oxygen species (ROS) and oxidative stress in the brain (Chan, 1994; Mattson *et al*, 2001; Friedlander, 2003), and the present study showed that NAIP suppresses neuronal cell death by exerting an antiapoptotic function against oxidative stress.

Most tissues and cells, with the exception of hematopoietic tissues, express NAIP in very low levels (Yamamoto *et al*, 1999). Thus, upregulation of endogenous NAIP in neuronal cells may represent a potent therapeutic strategy for prevention of neurodegeneration. Among the 30 NAIP upregulating compounds identified, two compounds, L-745,870 (dopamine D4 receptor antagonist) and bromocriptine (dopamine D2 receptor agonist; data not shown), exerted particularly prominent protection against neurodegeneration in an ischemia gerbil model. A previous study showed that bromocriptine protected neuronal cells from oxidative stress-induced apoptosis (Schapira, 2002), while another study showed that a number of other dopamine D2 receptor agonists attenuated neuronal cell death under ischemic conditions (Liu *et al*, 1995). L-745,870 was originally identified as a specific antagonist for the dopamine D4 receptor and as a drug candidate for antipsychotic treatment because

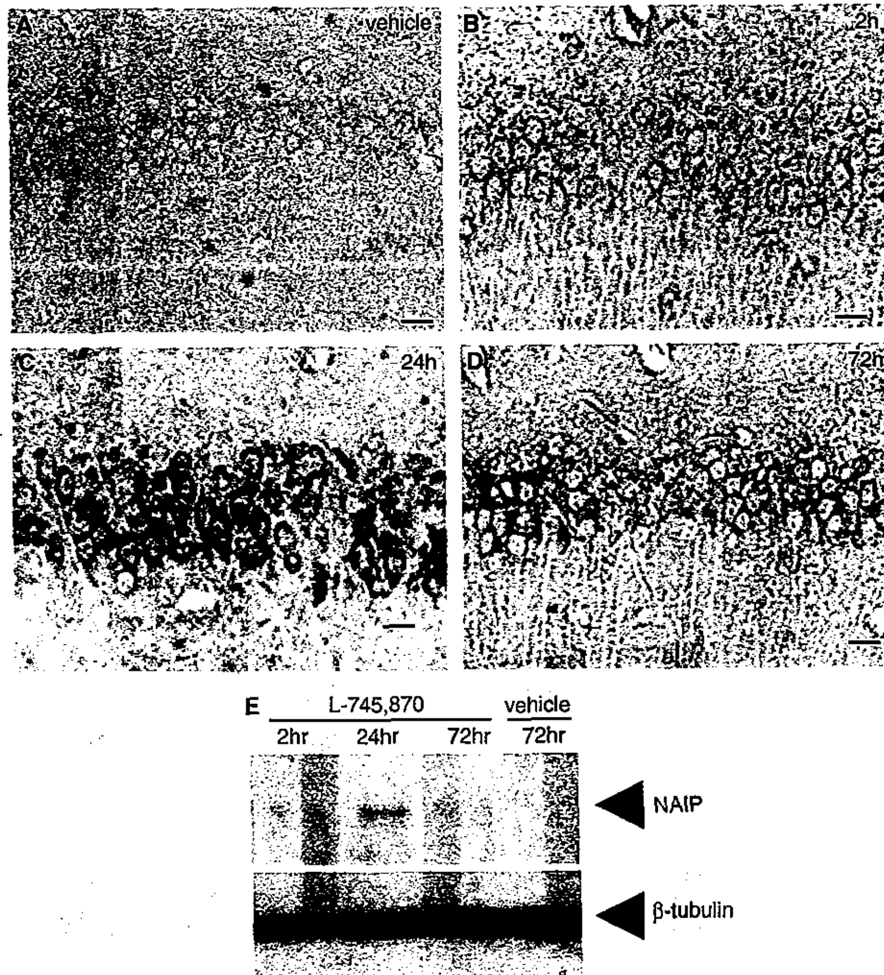

Figure 5 Upregulation of neuronal apoptosis inhibitory protein (NAIP) by L-745,870 in CA1 neurons. Adult gerbils were treated with vehicle (**A**) or L-745,870 (210 mg/kg; **B–D**). Representative coronal brain sections at the level of the dorsal hippocampus at 2, 24, and 72 h after administration of L-745,870 (210 mg/kg) are shown (**B–D**). Tissues were immunostained with the NAIP polyclonal antibody (ME1). Scale bar: 20 μm in higher magnification (× 400; **A–D**). (**E**) Western blot analysis of NAIP in hippocampus at 2, 24, and 72 h (L-745,870; 210 mg/kg) and 72 h (vehicle) after the administration of either L-745,870 or vehicle. Upper and lower panels represent the expression of NAIP and β-tubulin, respectively. Arrows indicate the proteins of interest (NAIP: 150 kDa). Expression of β-tubulin demonstrates equal loading in each lane.

of its excellent oral bioavailability and brain penetration (Patel *et al*, 1997). The present study showed the novel finding that L-745,870 specifically elevated the endogenous NAIP level and enhanced neuronal cell resistance to oxidative stress-induced apoptotic cell death and ischemic neurodegeneration.

The dopamine D4 receptor is a G-protein-coupled receptor that shares sequence homology with the D2 and D3 receptors and is classified as a member of the dopamine D2-like receptors group (Baldessarini, 1997). However, the action of L-745,870 in the present study is likely not mediated via dopaminergic receptors, because the antiapoptotic properties of L-745,870 against oxidative stress-induced cell death were observed in both differentiated dopaminergic SH-SY5Y cells, in which several types of dopamine receptors are expressed (Kamakura *et al*, 1997), and in nonneuronal cells, which do not express these receptors. Indeed, recent reports suggest that the dopamine agonists, bromocriptine and pergolide, act as free radical scavengers (Yoshikawa *et al*, 1994; Sam and Verbeke, 1995; Grünblatt *et al*, 1999) and exert their neuroprotective effects in nonreceptor-mediated fashions (Uberti *et al*, 2002). The present finding that L-745,870

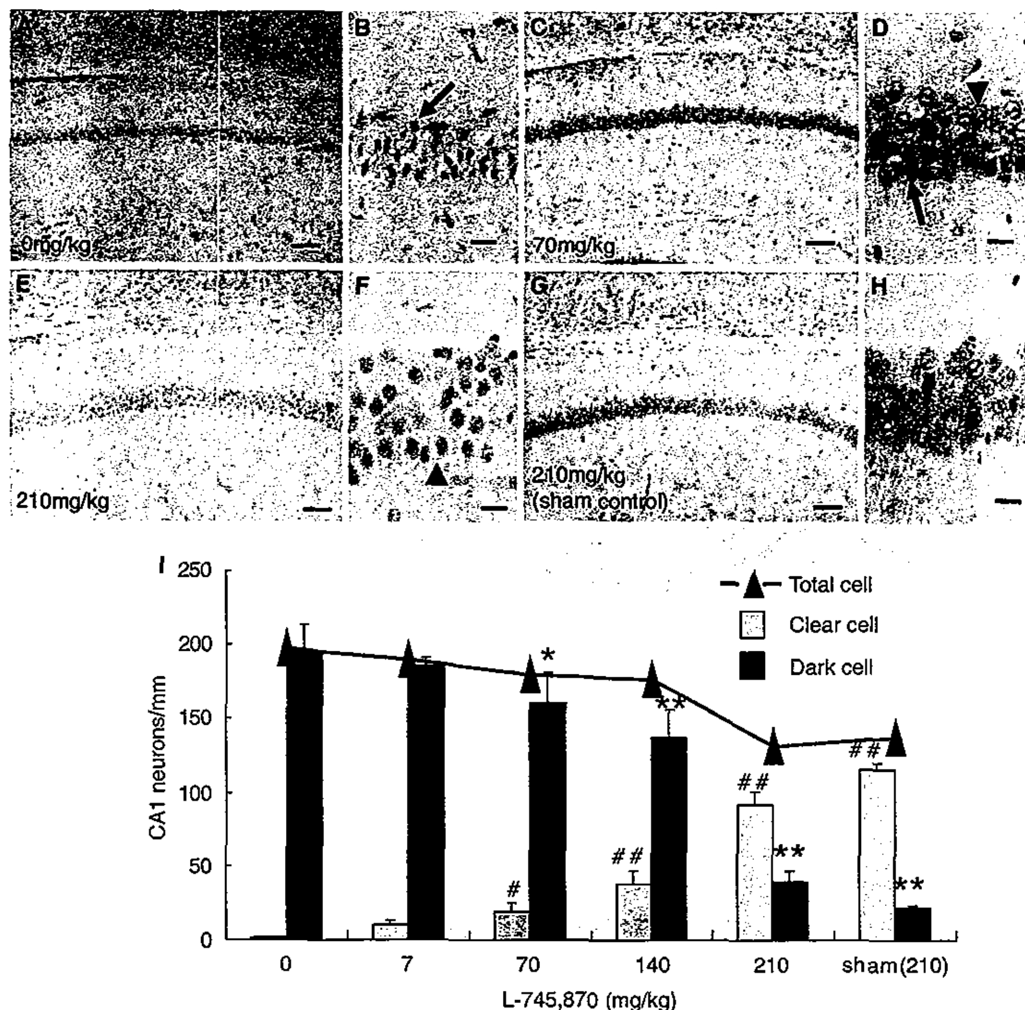

Figure 6 Effects of L-745,870 on ischemia-induced neuronal cell death in gerbils. Adult gerbils were treated with L-745,870 and then subjected to forebrain ischemia (bilateral common carotid artery occlusion (BCCAO) for 10 mins; A–F) or sham-operation (G and H). Representative coronal brain sections at the level of the dorsal hippocampus at 72 h (3 days) after reperfusion are shown. Tissues were stained with hematoxylin–eosin. Dosages of L-745,870 administered were 0 mg/kg (A and B), 70 mg/kg (C and D), and 210 mg/kg (E–H). Forebrain ischemia resulted in a significant loss of CA1 pyramidal cells. Scale bar: 100 μm in lower magnification ($\times$ 100; A, C, E, and G); 20 μm in higher magnification ($\times$ 400; B, D, F, and H). Arrowheads and arrows indicate dark (damaged) and clear (living) cells, respectively. (I) Number of pyramidal cells in the CA1 subfield of the hippocampus (cells/mm length of pyramidal cell layer). Both living (clear) and damaged (dark) CA1 neurons were count from six to seven animals in each experiment in a double-masked manner, and the values are represented as means $\pm$ s.e. Total numbers of CA1 pyramidal neurons (filled triangles; clear + dark cells) are also shown. *$P<0.01$; **$P<0.001$, compared with dark cells of vehicle-treated control (0 mg/kg). #$P<0.001$; ##$P<0.001$, compared with clear cells of vehicle-treated control (0 mg/kg). Statistical analysis was performed using ANOVA with Scheffe's *post hoc* test.

may exert its neuroprotective via increases in NAIP, either by increasing its expression or stabilization, may provide a mechanism by which all of these effector molecules exert their effects.

The present study showed that L-745,870 upregulated NAIP but not other antiapoptotic proteins. Further, L-745,870 specifically protected both neuronal and nonneuronal cultured cells from apoptosis induced by several oxidative stressors, including DMNQ, menadione, α-naphthoquinone, and H_2O_2. This increase in NAIP likely mediates the protective effect of L-745,870 because reduction of NAIP expression with RNAi inhibited the neuroprotective effect. Recent studies have shown that NAIP suppresses caspase-dependent and -independent apoptosis (Deveraux *et al*, 1997, 1998; Roy *et al*,

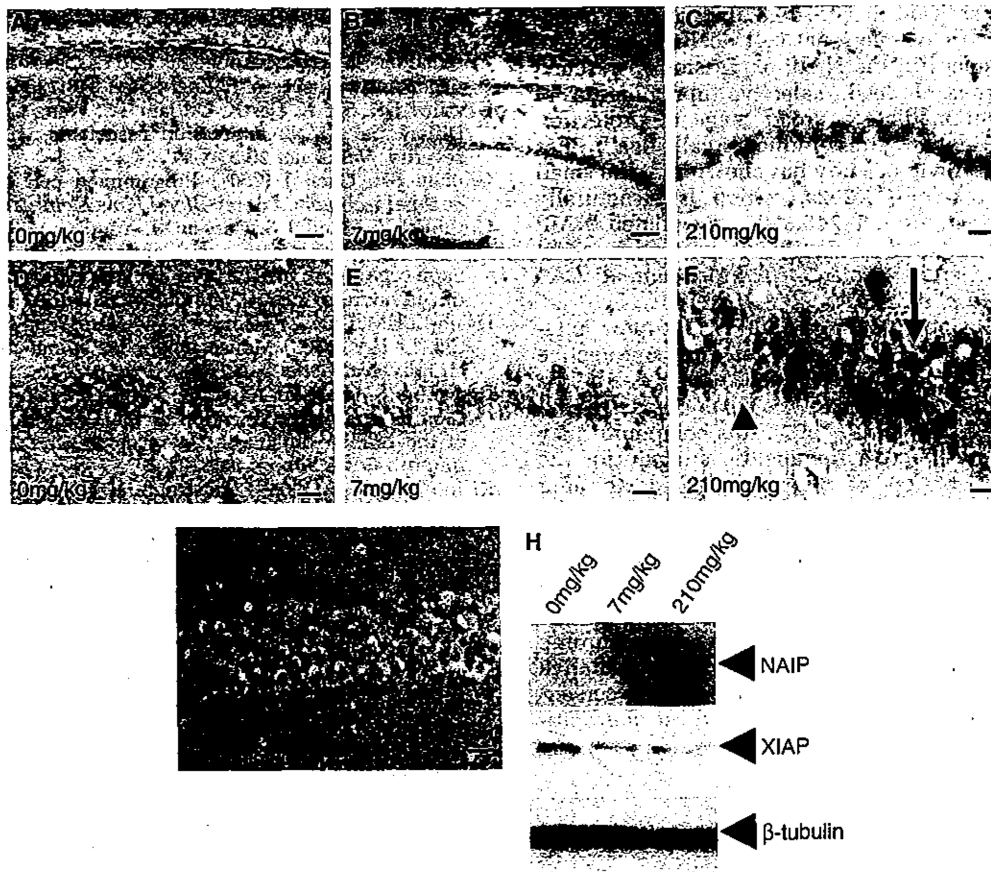

Figure 7 Effect of L-745,870 on expression of neuronal apoptosis inhibitory protein (NAIP) in CA1 neurons of ischemic gerbils. Adult gerbils were treated with L-745,870 and then subjected to forebrain ischemia (bilateral common carotid artery occlusion (BCCAO) for 10 mins; **A–G**). Representative coronal brain sections at the level of the dorsal hippocampus at 72 h (3 days) after reperfusion are shown (**A–G**). Tissues were immunostained with NAIP polyclonal antibody (ME1). Dosages of L-745,870 administered were 0 mg/kg (**A** and **D**), 7 mg/kg (**B** and **E**), and 210 mg/kg (**C**, **F**, and **G**). Scale bar: 100 μm in lower magnification ($\times$ 100; **A–C**); 20 μm in higher magnification ($\times$ 400; **D–G**). (**F**) Arrow and arrowhead indicate the NAIP-positive and negative cell, respectively. (**G**) Immunostaining of X-linked inhibitor of apoptosis (XIAP) in CA1 neurons of ischemic gerbil pretreated with L-745,870 (210 mg/kg). (**H**) Western blot analysis of NAIP and XIAP in hippocampus at 72 h (3 days) after ischemia. Upper, middle, and lower panels represent the expression of NAIP, XIAP, and β-tubulin, respectively. Each arrow indicates a position of the protein of interest (NAIP: 150 kDa, XIAP: 47 kDa). Expression of β-tubulin demonstrates equal loading in each lane.

1997; Seshagiri and Miller, 1997; Takahashi *et al*, 1998; Maier *et al*, 2002), while we previously showed that the antiapoptotic effect of NAIP was mediated by the caspase-3-independent pathway (Sakai *et al*, unpublished). Further studies to characterize the molecular mechanisms of NAIP upregulation and subsequent inhibition of oxidative stress-induced cell death would be of benefit.

It is notable that L-745,870 slightly but consistently downregulates the levels of both XIAP and cIAP-1 in differentiated SH-SY5Y cells. Recent studies have shown that a group of the ring finger-containing members of IAP family protein, such as XIAP and cIAP, can function as ubiquitin protein ligases, and regulates the levels of not only their target proteins but also themselves through ubiquitylation (Yang *et al*, 2000; Salvesen and Duckett, 2002). Thus, it is possible that L-745,870 may affect the stability of these IAP proteins via regulating the proteasome-dependent protein degradation. Equally likely is that this compound directly or indirectly modulates the expression of these genes and/or proteins in SH-SY5Y cells. Further studies will be needed to clarify the molecular mechanism underlying this inhibitory effect. Nevertheless, as the downregulation of XIAP and cIAP-1 is expected to exert the opposite effect to antiapoptosis, the decreases in XIAP and cIAP-1 observed in this study may not be a primary determinant for the antiapoptotic function associated with L-745,870.

We also showed that L-745,870 attenuated ischemia-induced CA1 neuronal cell death with concomitant increase in the NAIP expression in rescued CA1 neurons. Indeed, selective upregulation of NAIP might mediate a broad range of protection against oxidative stress-induced cell death. For example, a previous study has shown that the small molecule alkaloid, K252a, which is structurally quite different from L-745,870, upregulated NAIP levels and exerted a significant protective effect against ischemic damage in hippocampal CA1 neurons (Xu *et al*, 1997).

In conclusion, the dopamine D4 receptor antagonist, L-745,870, exerts a potent neuroprotective effect against ischemia-induced cell death via increases in NAIP. Since L-745,870 is clinically well tolerated (Bristow *et al*, 1997), this compound may represent an effective therapeutic strategy for the clinical prevention of neuronal cell death after ischemia. Future studies not only on the molecular mechanism by which L-745,870 induces the NAIP expression but also on the effectiveness of this compound in various ischemic conditions will clarify therapeutic potentials of L-745,870 in the treatment of several types of acute as well as chronic neurodegenerative diseases caused by oxidative stress. Further, our NAIP-ELISA-based drug screening may facilitate the discovery of novel neuroprotective compounds.

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ALS2タンパク質の多量体形成は，ALS2による低分子量Gタンパク質Rab5活性化および細胞内でのエンドソーム融合活性に必須である

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Homo-oligomerization of ALS2 is essential for the ALS2-associated Rab5GEF activity and its regulatory function on endosome trafficking

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*ALS2*遺伝子の機能喪失変異は，常染色体性劣性遺伝形式を示す筋萎縮性側索硬化症2型，遺伝性若年発症型原発性側索硬化症，遺伝性痙性対麻痺などの運動ニューロン疾患の原因であることが明らかにされている。このことは，ALS2タンパク質が運動ニューロンの維持に必須であることを示しており，その細胞内における分子機能に興味が持たれる。我々は，ALS2タンパク質のC末端領域（MORN-VPS9領域）にRab5に対するGEF（Guanine Nucleotide Exchange Factor）活性を同定し，この活性がALS2の制御するエンドソーム動態に深く関与していることを見出した［Otomo, A., *et al.* (2003) *Hum. Mol. Genet.*, 12, 1671-1687］。本研究では，ALS2 C末端領域によるRab5GEF活性を介したエンドソーム動態調節のメカニズムを解明するため，Y2H（Yeast Two-hybrid）法によるC末端領域結合タンパク質の同定を試みた。その結果，ALS2結合タンパク質としてALS2自体を同定した。Y2H法，免疫沈降法，およびゲルろ過法により，ALS2はC末端領域間での相互作用により8量体を形成していることが明らかになった。さらにこのALS2の8量体形成は，Rab5GEF活性および細胞内でのエンドソーム融合活性に必須であることも判明した。これらのことから，ALS2の多量体形成は，エンドソーム動態調節を担うALS2の生理的機能にとって不可欠であると考えられた［Kunita R., *et al.* (2004) *J. Biol. Chem.*, 279, 38626-38635］。

3PB-423

ALS2相同遺伝子産物ALS2CLは新規ALS2結合タンパク質である

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ALS2CL, a novel ALS2 homologous protein, interacts with ALS2

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*ALS2*遺伝子は常染色体劣性遺伝形式を示す若年発症型の筋萎縮性側索硬化症，原発性側索硬化症，および痙性対麻痺などの運動ニューロン疾患（upper motoneuron dominant ALS）の原因遺伝子の一つである。その遺伝子産物であるALS2タンパク質（ALS2）は，低分子量Gタンパク質Rab5を活性化し（ALS2rab5GEF活性），初期エンドゾームの融合を促進する (Otomo et al., Hum Mol Genet 12, 1671, 2003)。昨年度，我々は，ALS2のC末端に高い相同性を示す新規遺伝子ALS2 C-terminal like(*ALS2CL*)を同定し，その遺伝子産物であるALS2CLタンパク質（ALS2CL）が高いRab5結合能を有することを報告した。次いで，ALS2CLとALS2との機能的関連，さらには運動ニューロン疾患発症との関連を明らかにするために，ALS2CLの細胞内局在，初期エンドゾーム動態に対する作用，およびALS2との分子相互作用について検討した。その結果，ALS2CLはRab5との共発現により初期エンドゾームのtubulation phenotypeを誘起すること，ALS2との共発現によりALS2との共存を示すこと，そしてALS2の初期エンドゾーム融合促進作用を抑制することが明らかとなった。さらに，免疫沈降法およびyeast two-hybrid法により，ALS2CLはALS2と直接結合することが判明した。これらのことから，ALS2CLは新規のALS2結合タンパク質であり，細胞内においてはALS2機能の能動的調節因子として作用していると思われる。さらに，ALS2CLによるALS2rab5GEF活性の修飾効果について現在解析中である。

3PB-424

若年発症型劣性家族性ALS疾患モデル*Als2*遺伝子欠損マウスの作出と解析

○秦野 伸二[1,2], 角田 茂[3], 須藤 カツ子[3], 大友 麻子[1], 國田 竜太[2], 鈴木（宇都宮） 恭子[1], 水村 光[2], 将口（宮田） 淳子[1], 柳澤 佳子[2], 宮本 なつき[2], 古曳 英理[2], 須賀 恵津子[2], 岩倉 洋一郎[3], 池田 穰衛[1,2,4]
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Generation and characterization of the *Als2* knockout mouse, an animal model for juvenile recessive ALS

○Shinji Hadano[1,2], Shigeru Kakuta[3], Katsuko Sudo[3], Asako Otomo[1], Ryota Kunita[2], Kyoko Suzuki-Utsunomiya[1], Hikaru Mizumura[2], Junko Showguchi-Miyata[1], Yoshiko Yanagisawa[2], Natsuki Miyamoto[2], Eri Kohiki[2], Etsuko Suga[2], Yoichiro Iwakura[3], Joh-E Ikeda[1,2,4]
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*ALS2*遺伝子の機能喪失変異は，若年発症型の常染色体劣性筋萎縮性側索硬化症，原発性側索硬化症，および痙性対麻痺などの運動ニューロン疾患の原因であることが明らかにされている。我々は，これまでにALS2タンパク質が低分子量Gタンパク質Rab5の特異的活性化因子であること，細胞内においては初期エンドゾーム動態の調節機能を有することを明らかにしてきた。本研究では，*ALS2*遺伝子の生体における分子機能の解明ならびに疾患モデル動物の作製を目指して，マウス相同遺伝子破壊による*Als2*遺伝子欠損マウスの作出を試みた。ALS2タンパク質のほぼ全領域を欠損するTunisia家系型の遺伝子変異を有するマウスを作製するため，マウス*Als2*遺伝子第3エクソンにネオマイシン耐性遺伝子を挿入したターゲティングベクターを構築した。当該ベクターをマウスES細胞E14.1に導入し，得られた509個のG418耐性クローンから14クローンの相同組換え体を同定した。さらに6クローンについてキメラマウス作製を行った結果，2クローン（クローン17C6および21B5）より生殖系列キメラが得られた。そして，最終的に交配によりホモ変異マウスを作出することに成功した。また，ALS2タンパク質に対する特異的ポリクローナル抗体を用いたウエスタンブロット解析により，当該ホモ変異マウスは完全なALS2タンパク質欠失体であることも確認された。17C6 クローン由来の*Als2*遺伝子欠損マウスは，ほぼメンデルの法則に従った遺伝子型比で正常に出生し，生殖能を含めた生育は正常であった。本発表では，現在進行中の生化学的ならびに行動生理学的解析を含めた結果についても併せて報告する。

P22 SINGLE NUCLEOTIDE POLYMORPHISM ANALYSIS OF THE *ALS2* GENE AND ITS REGULATORY REGION IN JAPANESE PATIENTS WITH SPORADIC AMYOTROPHIC LATERAL SCLEROSIS

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Background: The *ALS2* gene was initially identified as a causative gene for a juvenile recessive form of amyotrophic lateral sclerosis (ALS), termed ALS2, and a rare juvenile recessive form of primary lateral sclerosis (PLSJ). Recently, six independent homozygous ALS2 mutations have been found in families segregating an infantile-onset ascending hereditary spastic paralysis (IAHSP). Thus, ALS2 mutations account for a number of juvenile recessive motor neuron diseases (MNDs), suggesting the *ALS2* gene products play an important role in maintenance and/or survival of motor neurons. Most recently, it has been reported that overexpression of the ALS2 protein protects cultured motor neuronal cells from toxicity induced by mutant SOD1, suggesting the possible neuroprotective function for the ALS2 protein. These results led us to hypothesize that expression level of the *ALS2* gene/protein could modulate the onset and/or progression of MNDs including ALS.

Objectives: To explore whether single nucleotide polymorphisms (SNPs) or mutations within the *ALS2* gene (including the 5'-upstream regulatory region) are associated with either sporadic ALS, the expression level of the *ALS2* gene, or the stability and function of the ALS2 protein.

Methods: We have designed a set of 40 primer pairs to amplify all the coding exons, intron-exon boundaries, UTR, and the 5'-upstream regulatory region of the *ALS2* gene. Genomic DNA samples extracted from 97 Japanese sporadic ALS patients were subjected to PCR-amplification using these primers, and the resulting PCR products were analyzed by direct DNA sequencing. We also analyzed genomic DNA samples obtained from 34 normal Japanese controls.

Results: We have identified a total of 61 polymorphisms in the *ALS2* gene. Within the ~1kb of the 5'-upstream promoter region, 2 frequent and 4 rare SNPs were newly identified. A number of other polymorphisms were identified in this study; 11 SNPs in coding exons, a single SNP in the 5'UTR, 5 SNPs in the 3'UTR, 35 SNPs and 3 insertion/deletions in introns. Notably, 7 of 11 exonic SNPs led to amino acid substitutions. Furthermore, 42 of the 61 polymorphisms had not been previously identified.

Conclusions: In this study, extensive DNA sequence analysis of a larger number of sporadic ALS patients as well as some normal Japanese

P23　田中一則[1,3]、宮本なつき[2]、宮田（将口）淳子[3]、池田穰衛[2,3,4]（1長寿科学振興財団、2 JST/SORST、3東海大・総医研、4オタワ大・医）

HD遺伝子プロモーター内の新規シスエレメントに結合する核－細胞質シャトルタンパク質の分子機能

ハンチントン病（HD）は遺伝性の神経変性疾患である。HD マウスモデル研究から、HD遺伝子の発現制御がHD治療技術開発の有用な手段になる可能性が提示された。HD遺伝子の発現制御機構を解明する目的で、酵母の系を利用し、我々は2つのHD遺伝子転写調節領域結合因子；HDBP1、HDBP2 を同定した。そして、HDBP1 と 2 が核－細胞質間をシャトルすること、両因子のC末領域がDNA結合活性を有し、この領域がHD遺伝子プロモーター内の7bpの新規シス調節配列を認識することを明らかにした。さらに、このシス配列が神経細胞でのHD遺伝子プロモーター活性に必須であることも示した。

P24　岡田義則[1,2]、酒井治美[1]、古曳英理[3]、須賀恵津子[3]、田中一則[1,4]、秦野伸二[1,3]、大須賀　等[1,3]、池田穰衛[1,3,5]（1東海大・総医研、2東海大・医、3JST/SORST、4長寿科学振興財団、5オタワ大・医）

NAIP発現誘導Dopamine D4 antagonistの抗アポトーシス効果

Neuronal Apoptosis Inhibitory Protein (NAIP) は、アポトーシス抑制因子である。本研究では、NAIPの発現誘導を指標にしたELISAシステムを確立し、さらにそのシステムを用いて細胞死抑制効果を有する低分子化合物のスクリーニングを行った。その結果、30個のNAIP発現誘導化合物を同定することに成功した。さらに、これらの化合物の中で最も強い *in vitro* 細胞死抑制活性を示したDopamine D4 antagonistが、*in vivo* においても脳虚血後の神経細胞死を有意に抑制することも明らかとなった。

P25 大友麻子[1,2]、秦野伸二[1,2]、岡田武也[2]、水村光[1]、國田竜太[1]、西嶋仁[3]、将口(宮田)淳子[2]、柳澤佳子[1]、古曳英理[1]、須賀恵津子[1]、安田政実[4]、大須賀等[1,2]、西本毅治[3]、成宮周[5]、池田穰衛[1,2,6]（1 JST/SORST、2 東海大・総医研、3 九大・院・医、4 東海大・医、5 京大・院・医、6 オタワ大・医）

筋萎縮性側索硬化症 2 型遺伝子産物 ALS2 の Rab5GEF 活性とエンドソーム動態調節機能

2001 年に我々は、常染色体劣性遺伝形式を示す筋萎縮性側索硬化症（ALS）2 型の原因遺伝子として *ALS2* 遺伝子を同定した。疾患発症の機構を理解するため、本研究ではその遺伝子産物(ALS2)の生化学的性質と細胞内動態を解析した。そして、ALS2 が新規の低分子量 G タンパク質 Rab5 の活性化因子(Rab5GEF)であり、細胞内においては Rab5 の活性化制御やエンドソーム融合・輸送調節に関わる因子であることを見出した。これらの結果から、2 型 ALS は細胞内物質輸送および膜輸送の異常により発症するものと考えられた。

P26　國田竜太[1]、大友麻子[2]、水村光[1]、鈴木（宇都宮）恭子[2]、将口（宮田）淳子[2]、柳澤佳子[1]、秦野伸二[1,2]、池田穣衛[1,2,3]（1 JST/SORST、2 東海大・総医研、3 オタワ大・医）

ALS2 タンパク質多量体形成の生理的意義

ALS2 タンパク質は運動ニューロンの維持に必須であり、その細胞内における分子機能に興味が持たれる。我々は、ALS2 がエンドソーム動態をその Rab5GEF 活性を介して調節することをすでに明らかにしてきた。今回、ALS2 が 8 量体として存在していること、8 量体形成が Rab5GEF 活性および細胞内でのエンドソーム融合活性に必須であることを新たに明らかにした。これらのことから、ALS2 多量体形成は、その生理的機能に不可欠であると考えられた。

P27 秦野伸二[1,2]、角田茂[3]、須藤カツ子[3]、大友麻子[2]、國田竜太[1]、水村光[1]、鈴木（宇都宮）恭子[2]、将口（宮田）淳子[2]、柳澤佳子[1]、古曳英理[1]、須賀恵津子[1]、宮本なつき[1]、岩倉洋一郎[3]、池田穰衛[1,2,4]（1 JST/SORST、2 東海大・総医研、3 東大・医科研、4 オタワ大・医）

Als2 ノックアウトマウスの作出と解析

ALS2 遺伝子の機能喪失変異は、常染色体劣性の筋萎縮性側索硬化症、原発性側索硬化症、および痙性対麻痺などの運動ニューロン疾患の原因であることが明らかにされている。本研究では、*ALS2* 遺伝子の生体における分子機能の解明ならびに疾患モデル動物の作製を目指して、マウス相同遺伝子破壊による *Als2* ノックアウトマウスの作出を行った。遺伝子ターゲッティングにより得られた6クローンの相同組換体 ES 細胞からキメラマウスの作製を行い、2クローンの生殖系列キメラが得られた。そして、交配によりヘテロ・ホモ変異マウスを得ることに成功した。

SY11-04 Apoptosis based remedy for amyotrophic lateral sclerosis (ALS) and upper motor neuron dominant ALS

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Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that manifests as selective upper and lower motor neuron degeneration, whose molecular base is not delineated yet, but apoptosis as major causative is implicated. A number of anti-apoptotic proteins, such as Bcl-2, neuronal apoptosis inhibitory protein (NAIP) and XIAP, have been found to be up-regulated in neurons under degeneration. Among these anti-apoptosis proteins, NAIP reveals an anti-apoptosis unique to oxidative stress. Ectopic- and over-expression of NAIP have shown to attenuate ischemic neuron damage, adequately rescue motoneurons in peripheral nerve axotomy, preserve nigrostriatal dopaminergic function in an intrastriatal 6-OHDA Parkinson's disease rat model, and save motor neuron through intracellular signaling of glial cell-derived neurotrophic factor. These findings favor to that NAIP plays a key role in conferring resistance to several types of apoptotic insults, and up-regulation of endogenous NAIP mitigates neurodegeneration. Thus, NAIP based exploitation of the small compounds provides new clue to delineate a molecular base and new remedy to confer for neuronal cell death in ALS and UMND-ALS.

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OB2-07 Anti-apoptotic effect of the NAIP up-regulating dopamine receptor ligands

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[1]IMS, Tokai-U, [2]Med, Tokai-U, [3]SORST, JST

Neuronal apoptosis inhibitory protein (NAIP) is an anti-apoptotic protein with therapeutic potential in neurodegenerative disorders. Exploring the compounds that up-regulate endogenous NAIP is one of the attractive steps to develop therapeutic agents for the intervention or even cure of such diseases. Here, using the NAIP-ELISA-based drug screening system, we identified several candidate compounds, and characterized one of the compounds, a dopamine receptor ligands, exhibiting most prominent effects in vitro. This compound specifically up-regulated the NAIP level and prevented the cultured cells from an oxidative stress-induced apoptosis. Moreover, administration of the compound attenuated the ischemic damage with concomitant increase in NAIP in vivo. These results suggest that NAIP upregulating dopamine receptor ligands is a potential drug in preventing disorders associating with oxidative stress -induced neuronal cell death.

Neuro 2004

P2-187 Generation and characterization of the *Als2* knockout mice

Shinji Hadano[1,2], Shigeru Kakuta[3], Katsuko Sudo[3], Asako Otomo[1], Ryota Kunita[2], Hikaru Mizumura[2], Kyoko Suzuki[1], Yoichiro Iwakura[3], Joh-E Ikeda[1,2]
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The loss-of-functional mutations in *ALS2* accounts for a number of juvenile recessive motor neuron diseases (MNDs) including a type of juvenile ALS (ALS2), juvenile-onset PLS, and infantile-onset ascending hereditary spastic paralysis. To delineate the molecular pathogenesis for these MNDs, we here generated mice homozygous for disruption of *Als2* by a standard homologous recombination strategy. Disruption of *Als2* and null expression of the ALS2 protein were confirmed by southern and western blotting, respectively. The *Als2* knockout mice were born and grew with no obvious developmental and reproductive abnormalities by 10 months of age. Behavioral and pathological analyses of these mice are currently underway. Generation of the *Als2* knockout mice will provide additional clues to the understanding of the molecular pathogenesis for MNDs caused by *ALS2* mutation.