

## The Inducible form of Heat Shock Protein 70 (Hsp70) is Expressed in the Rat Cerebellar Synapses in Normal Condition

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Heat shock protein 70 (HSP70) is a multigene family composed of constitutively expressed members (Hsc70) and stress-inducible members (Hsp70). In the mammalian nervous system, a considerable amount of HSPs is also synthesized under normal conditions suggesting that they play an important role in the metabolism of unstressed cells. In this study we examined the expression of Hsp70 in the synapses of rat cerebellar neurons. Immunohistochemistry using specific antibodies revealed that both Hsp70 and Hsc70 are expressed in the cerebellar tissue, with strongest expression in Purkinje cells followed by granule cells. Neurons in deep cerebellar nuclei were also intensely stained by Hsp70 antibody. Immunocytochemical stainings of cultured cerebellar cells showed that Hsp70 is expressed in both Purkinje and granule cells. The expression was punctate in the soma and along dendritic trees, and the punctae were colocalized with those of PSD95, a postsynaptic marker. Immunoblotting also indicates that Hsp70 is associated with the postsynaptic density fraction. Taken together, our results indicate that the Hsp70 is expressed in cerebellar neurons in normal conditions, and that some are localized in the synapses.

**Key words** – cerebellum, culture, HSP70, Purkinje cell, granule cell

Cells respond to stress by synthesizing a specific group of proteins, known as stress proteins or heat shock proteins (HSPs). The HSPs are usually classified according to their molecular weight. Among the 11 families described, attention has focused on the family of 70-kD heat shock protein (HSP70; denotes both Hsc70 and Hsp70) because it is prominently induced in a variety of models of cell stress and is also the best conserved stress protein. HSP70 is a multigene family composed of constitutively expressed members (Hsc70) and stress-inducible members (Hsp70), and both can be basally expressed in the nervous system under control conditions[2,10]. HSP70 binds and subsequently releases partially unfolded proteins in an ATP-dependent manner. More specifically, members of the HSP70 family protect nascent protein chains after synthesis, translocate proteins across membranes, and refold proteins after denaturation or during protein degradation[4,7,11].

Expression of HSP70 proteins has been demonstrated in the central nervous system (CNS). The neural tissue has a high HSP70 constitutive expression compared with other organs and tissues[19]. HSP70 expression is enhanced by

different stress conditions, and the stress response differs in different CNS regions[2,9]. In the mammalian nervous system, a considerable amount of HSPs is also synthesized under normal conditions suggesting that they play an important role in the metabolism of unstressed cells. In the rat cerebellum, intense immunostaining was evident, but was restricted in certain distinct cerebellar areas only, while no differences in the distribution of the two HSP70s were found[16]. The strongest response was detected in the Purkinje neurons but deep cerebellar nuclei were also positive. This report indicates that hsp70 has fundamental physiological functions in cerebellar neurons.

There has been controversy on the expression of inducible form of HSP70, i.e. hsp70. In postnatal day 4~5 cultures of cerebellar neurons, *in situ* hybridization analysis showed no evidence for hsp70 mRNAs in neurons[20]. Microglia were the only cell type in which hsp70 was detected in non-stressed cultures and this cell type contained the highest concentrations of hsp70 proteins in stressed cultures[20]. Hsc70 mRNA levels were increased after heat shock, but the increase was more transient. Hsc70 mRNAs and proteins were present in all cell types, again with the highest concentrations being present in microglia. Some cytoplasmic hsp70 was observed in astrocytes of the mixed neuro-as-

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troglial cultures and a delayed hsp70 immunoreactivity was observed in granule neurons in these cultures[20].

Synapses are the contact sites between neurons for communication and plasticity. The aim of the present study was to examine the expression of Hsp70 in the synapses of rat cerebellar neurons. We report here that the inducible form of HSP70, i.e. Hsp70 is expressed in Purkinje cells, granule cells, and deep cerebellar neurons in normal brain and cultures. Furthermore, its expression was punctate along dendrites and the punctae were well colocalized with PSD95, a postsynaptic marker, indicating that Hsp70 is constitutively present at synapses in certain types of cerebellar neurons.

## Materials and Methods

### *PSD preparation and detergent extraction*

'One-Triton' postsynaptic density (PSD) fractions were prepared from rat forebrain (FB) and cerebellum (CBL) by washing synaptosomes with 0.5% Triton X-100 (Triton) for 15 min at 4°C, as described previously[13,14,15]. In brief, synaptosomes were isolated from homogenates by sucrose step-gradient centrifugation using 0.85, 1.0 and 1.2 M sucrose layers, and then extracted with 0.5% Triton X-100 for 15 min at 4°C. The resulting 'One-Triton' PSD fraction was pelleted by centrifugation at  $36,800 \times g$  for 45 min. In the detergent extraction experiments, the fraction was extracted again at 4°C, either with 1.0% Triton or 1.0% n-octyl glucoside (OG) for 15 min or with 3.0% N-lauroyl sarcosine for 10 min. Thereafter, the pellet and supernatant were separated by centrifugation at  $201,800 \times g$  for 1 h at 4°C.

### *Immunoblot*

After sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE), proteins were transferred to a nitrocellulose membrane. After incubation of the blots overnight at 4°C in TTBS [0.2% Tween-20, 10 mM Tris-HCl (pH 7.5) and 0.2 M NaCl], a primary antibody was added and further incubated for 2 h at RT. Blots were rinsed in TTBS (4×20 min), and the antigen/antibody complex was visualized with alkaline phosphatase-conjugated secondary antibodies, according to the manufacturer's instructions (Roche, Germany).

### *Immunohistochemistry*

Male rats (Sprague-Dawley, 200~250 g) were allowed free access to food and water under 12 h light and dark

cycle for a week before sacrifice. Brains were fixed with 4% paraformaldehyde in 100 mM sodium phosphate buffer (pH 7.4) by perfusion through left heart ventricle for 15 min and immersed in the same solution for 12 h at 4°C followed by immersion in the same buffer containing 30% sucrose. Brains were flash-frozen on dry ice, embedded in O.C.T. compound (Tissue-Tek) and sectioned on a cryostat (35 nm). Sections were rinsed in 20 mM phosphate-buffered saline (PBS) for 3 times (15 min each) at 4°C to remove O.C.T. compound. After preincubation of the sections in 10% normal goat serum (NGS) for 1 h at 4°C to block non-specific binding, the sections were incubated with primary antibodies [rat anti-Hsc70 monoclonal (StressGen, 1:1,000) or mouse anti-Hsp70 monoclonal (StressGen, 1:100) antibodies in PBS containing 1% NGS and 1% bovine serum albumin for 48 h at 4°C. After washing in PBS, sections were incubated with secondary antibodies [biotinylated goat anti-rat IgG (Vector Laboratories, 1:500) for Hsc70 or biotinylated goat anti-mouse IgG (Vector Laboratories, 1:500) for Hsp70] in PBS for 24 h at 4°C. The sections were washed 3 times in PBS and incubated in an avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) for 1.5 h at RT. After washing twice in PBS and once in 50 mM Tris-HCl (pH 7.6), peroxidase was revealed by incubation (5~10 min) with 0.0048% H<sub>2</sub>O<sub>2</sub> in the presence of 3,3-diaminobenzidine (DAB; 0.05% in 50 mM Tris-HCl (pH7.6)). The reaction was stopped by several washes in 50 mM Tris-HCl (pH7.6).

### *Neuronal culture and immunocytochemistry*

Postnatal day 1 (P1) cerebellar cells were dissociated from P1 rat cerebellum by triturating trypsin-treated tissues, and were grown on poly-D-lysine-coated coverslips (1,000~1,500 cells/mm<sup>2</sup>) in the Neurobasal medium supplemented with B27 and 0.25 mM glutamax I (Invitrogen, Carlsbad, CA), as previously described[1,13,14,15]. Cells were fixed with methanol for 20 min at -20°C and then doublestained with rat monoclonal anti-Hsc70 (SPA-815; 1:1,000; StressGen Biotechnologies Corp., BC, Canada) or mouse monoclonal anti-Hsp70 (StressGen, SPA-810; 1:1,000) and rabbit polyclonal anti-PSD95 (Upstate Biotechnology), according to Moon *et al.*[15]. The primary antibodies were visualized with Alexa Fluor 568-conjugated goat anti-mouse or -rat IgG and Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:1,000, Molecular Probes; Leiden, The Netherlands). Images of the immunostained cells were obtained using a fluorescence microscope equipped with filter systems I3 and

N2.1 (Leica Microsystems, Wetzlar, Germany). There was no bleed-through between the two channels. Fluorescence images were processed with Adobe Photoshop 5.0 (Adobe Systems Inc.).

## Results and Discussion

### Association of HSP70 with the rat cerebellar postsynaptic density (CBL-PSD) fraction

Expression of HSP70 in the rat cerebellar synapse was investigated with the PSD fraction, which is a subfraction of synapse. Immunoblotting the PSD fraction with Hsp70 showed a signal at 70 kDa molecular size in the CBL-PSD lane (Fig. 1A, arrowhead). This signal was absent in the forebrain PSD (FB-PSD) lane. The Hsc70 blot resulted in strong signal bands at 70 kDa in both FB- and CBL-PSD lane (Fig. 1B, arrowhead). These results indicate that the inducible form of HSP70, i.e., Hsp70 is expressed only in the CBL-PSD fraction in normal condition, while the constitutive form, i.e., Hsc70 is expressed in both FB- and CBL-PSD fraction.

### Subcellular distribution of HSP70

Since HSP70 was present in the PSD fraction, subcellular

distribution was investigated. Brain homogenate (BH), synaptosome (Syn), and PSD fractions were prepared from rat cerebra and cerebella. Immunoblot analyses of the subcellular fractions revealed that Hsp70 was enriched in the PSD fraction, while Hsc70 was not (Fig. 2A). Statistical analyses using the scanned data indicated that Hsp70 was enriched by ~20% in the Syn and by ~50% in the PSD fraction from BH (Fig. 2B, open bar). In contrast, Hsc70 was reduced by ~and ~50% in the Syn and PSD fraction, respectively, from the BH (Fig. 2B, closed bar). The difference in the amount of both proteins was statistically significant in the Syn fraction ( $p > 0.05$ ), and very significant in the PSD fraction ( $p > 0.01$ ). These data suggest a fundamental functional differentiation of the two forms of HSPs in the synapse.

### Expression of HSP70 in adult rat cerebellum

Immunohistochemistry was carried out to investigate the expression of HSP70 in the adult rat cerebellum. Expression of both Hsp70 and Hsc70 was evident in the cerebellar section (Fig. 3A and 3B). At higher magnification, it was seen that highest immunoreactivity is associated with the Purkinje cell layer (Fig. 3A and 3B, P in insets). However, granule

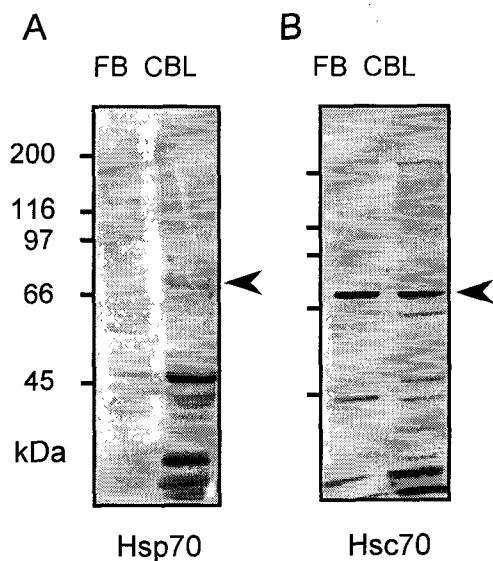


Fig. 1. Presence of HSP70 in the PSD fraction. The PSD fractions were prepared from rat forebrain (FB) and cerebellum (CBL), electrophoresed in a 8% SDS-gel. Proteins were transferred to a nitrocellulose membrane and blotted with a specific antibody against Hsp70 (A) or Hsc70 (B). The positions of HSP70 were marked by arrowheads and molecular sizes are indicated at left in kilodaltons (kDa).

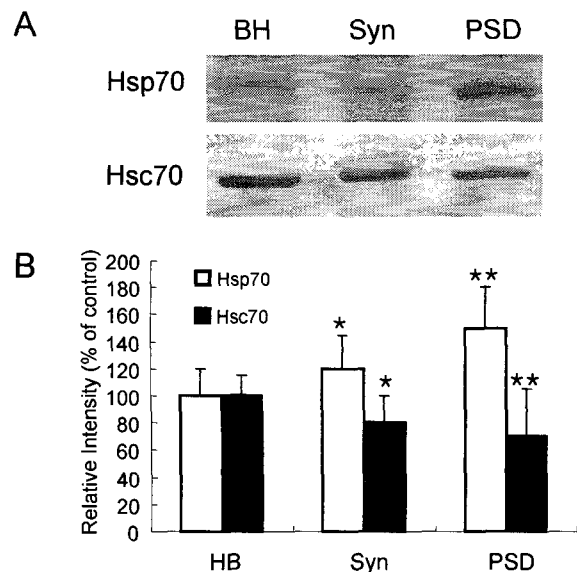


Fig. 2. Subcellular distribution of HSP70. Brain homogenate (BH), synaptosome (Syn), and PSD fractions were prepared from rat cerebellum. A. After SDS-PAGE, proteins were transferred to a nitrocellulose membrane and blotted with anti-Hsp70 or -Hsc70 antibody. B. Statistics. Digital images of the blots were acquired by scanning and densities of immunoblot signals were measured. \*,  $p < 0.05$ . \*\*,  $p < 0.01$ .

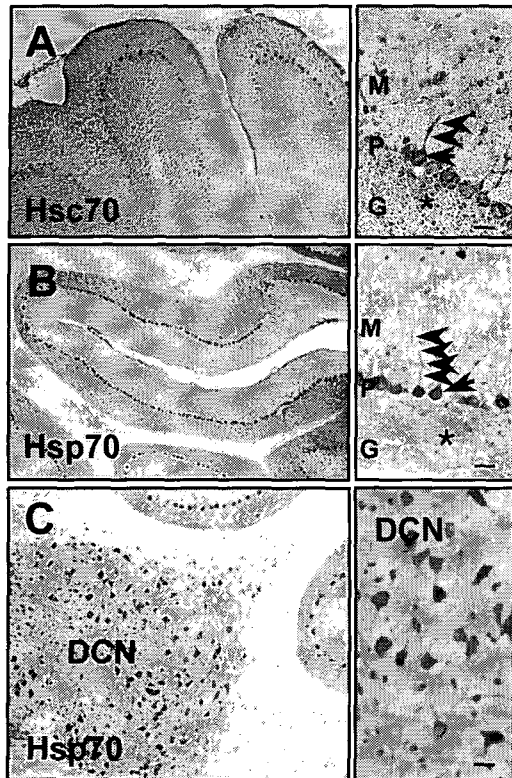


Fig. 3. Immunohistochemistry showing the expression of HSP70 in the rat cerebellum. Cerebellar sections were prepared from adult rat brains and stained with anti-Hsc70 (A), -Hsp70 (B and C). An image of deep cerebellar nucleus (DCN) is shown in C. A representative area of each stainings are shown enlarged in insets. M, Molecular layer. P, Purkinje cell layer. G, granule cell layer. Series of arrowheads indicate dendrites. A Purkinje cell and granule cells are marked by an arrow and asterisks. Scale bar, 20  $\mu$ m.

cells in granule cell layer (G) (Fig. 3A and 3B, asterisk) and the cells in molecular layer (M) were also labeled. In Purkinje cells, soma was most intensively stained (Fig. 3A and 3B, arrow) and their major dendrites were also stained (a series of arrowheads in insets). Deep cerebellar neurons (DCN) were also strongly stained with both Hsp70 (Fig. 3C) and Hsc70 antibodies (not shown). These data indicate that both Hsp70 and Hsc70 are expressed in cerebellar neurons in normal conditions.

**Expression of Hsp70 in the cultured cerebellar neurons**

Expression of Hsp70 was further investigated using cerebellar neuronal cultures. The culture was doublestained with Hsp70 and Calbindin 28Da (Calbindin), a marker for Purkinje cells. In our culture condition, Purkinje cells did not differentiate well, and dendritic arborization was very

limited. However, they could be identified by the positive staining with Calbindin and their large sizes (~40  $\mu$ m) of the soma (Fig 4. arrows). Purkinje cells were intensely stained with Hsp70 antibody. Interestingly, the staining was punctate (Fig. 4A, arrowheads in inset) (Fig. 4B, arrowheads in inset).

Next, we investigated the expression of Hsp70 in the granule cells in cultures. Immunoreactivity of the granule cells with Hsp70 was not as strong as that of Purkinje cells (Fig. 5A). However, it was evident that immunoreactivity was associated with soma and dendrites of granule cells. Immunostaining was not homogenous but punctate in soma and dendrites. A portion of dendrites was shown enlarged in the inset of Fig. 5. Many (~70%) of these punctae (Fig. 5A, inset) were colocalized with those of PSD95, indicating that Hsp70-immunoreactive punctae are dendritic spines. It is should be noted that PSD95 is expressed in cerebellar granule cells and highly enriched in spines (Fig. 5B), indicating its important role in the synaptic functions of granule cells.

Previously, we found that both Hsp70 and Hsc70 immunoreactivities are distributed throughout the soma and dendrites of dissociated hippocampal neurons[14]. In dendrites, there are many stained puncta which are mostly colocalized with PSD-95. At the electron microscopic level, both Hsp70 and Hsc70 are mainly associated with asym-

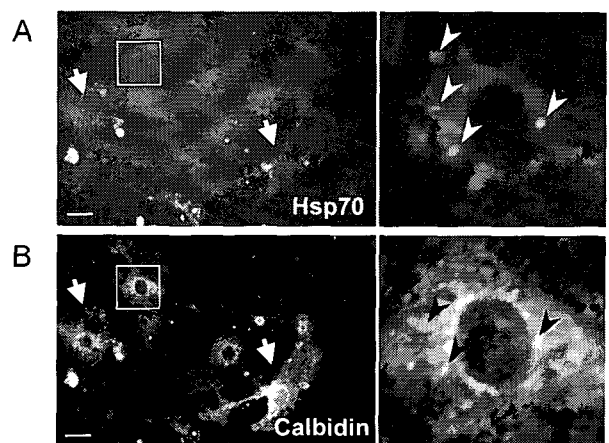


Fig. 4. Immunocytochemistry showing expression of Hsp70 in cultured Purkinje cells. P1 rat cerebellar cells were grown in Neurobasal media and doublestained with anti-Hsp70 (A) and -calbindin 28K, a marker for Purkinje cells (B) antibodies on DIV15. Purkinje cells were marked by arrows, and one representative cell from each panel was shown enlarged in insets. Examples of punctate stainings were marked by arrowheads. Scale bar, 20  $\mu$ m.

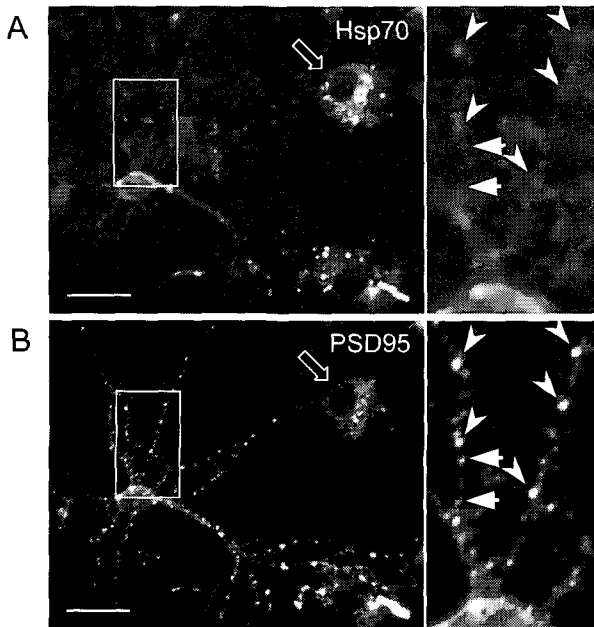


Fig. 5. Immunocytochemistry showing expression of Hsp70 in cultured granule cells. P1 rat cerebellar cells were grown in Neurobasal media and doublestained with antibodies against Hsp70 (A) and PSD95, a marker for postsynaptic spine (B). A portion of the cell (box) is shown enlarged in inset. Colocalizing and non-colocalizing punctae are marked by arrowheads and arrows, respectively. Scale bar, 20  $\mu\text{m}$ .

metrical PSDs. However, Hsc70 is also associated with amorphous subsynaptic structures and spine apparatus-like cisternae[14]. These data indicate that both Hsp70 and Hsc70 are present in the forebrain PSD but are differentially distributed at subsynaptic sites. In the present study we found that Hsp70 is expressed in the normal adult rat cerebellum and cultured cerebellar neurons. In cultured neurons, expression of Hsp70 was punctate along dendrite of granule cells, and in soma of Purkinje cells, and colocalized with PSD95 punctae. This result strongly indicate that Hsp70 is present in the spine of cerebellar granule cells.

Expression of Hsp70 in the cerebellum is not unprecedented. Using immunocytochemistry, Stacchiotti *et al.*[16] stained rat cerebellum with specific monoclonal antibodies, and showed that an intense immunostaining was evident, but was restricted in certain distinct cerebellar areas only. In accordance with our results, the strongest response was detected in the Purkinje neurons but deep cerebellar nuclei were also positive. In another study, RNA blot analysis and in situ hybridization revealed abundant expression of an 'antisense' Hsp70 transcript in several areas of adult mouse brain[15]. In situ hybridization revealed that the

sense and antisense transcripts were both predominantly neuronal and localized to the same cell types in the granular layer of the cerebellum, trapezoid nucleus of the superior olivary complex, locus coeruleus and hippocampus. These findings have revealed a distinct cellular and spatial localization of both sense and antisense transcripts, demonstrating a new level of complexity in the function of the heat shock genes.

The function of the PSD-associated HSP70 is not known. The most straightforward interpretation would be that they are involved in facilitation of folding of nascent proteins and in the repair of partially denatured proteins. Synaptic stimulation induces rapid input-specific growth of small filopodia-like protrusions[8] and formation of new spines[3,5]. Moreover, tetanic stimulation cause local protein synthesis in dendrites[6,17,18]. Therefore, HSP70 proteins may function in the process of local synthesis of new proteins required for synaptic plasticity, remodeling, neurite outgrowth and/or the stabilization of existing or nascent synapses. Further studies are necessary to elucidate the function of synaptic HSP70.

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**초록 : 흰쥐 소뇌 정상 연접에서 열충격단백질70(HSP70)의 표현**

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열충격단백질70 (HSP70)은 복수유전자족으로서 통상적으로 표현되는 Hsc70와 스트레스에 의하여 유도되는 Hsp70가 있다. 포유동물의 신경계통에서는 상당한 량의 HSP70가 정상조건에서도 표현되는 것으로 알려져 있다. 본 연구에서는 흰쥐의 소뇌 세포의 연접에서 Hsp70의 표현에 대한 연구를 하였다. 면역조직화학적으로 소뇌절편을 염색하여 관찰한 결과 Hsp70와 Hsc70 모두 표현되었는데, 소뇌 조롱박세포에서 가장 강하게 표현되었으며, 다음으로 소뇌 과립세포에서 강하게 표현되었다. 또한 깊은소뇌핵의 신경세포들도 강하게 염색되었다. 배양한 P1 소뇌신경세포를 Hsp70 항체로 염색한 결과 Hsp70는 조롱박세포와 과립세포에서 모두 표현되었으며, 세포체와 가지돌기를 따라 점박이를 형성하였다. 이들 점박이들은 PSD95 점박이와 같이 위치하였다. 그리고 PSD 분획을 이용한 면역염색에서도 PSD70이 검출되었다. 본 연구결과는 Hsp70이 정상조건에서도 소뇌신경세포의 연접에 존재함을 의미한다.