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Comparative study on Hsp25 expression in Mongolian gerbil and mouse cerebellum

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Abstract

The term 'heat shock protein (Hsps)' was derived from the fact that these proteins were initially discovered to be induced by hyperthermic conditions. In response to a range of stressful stimuli, including hyperthermia, immobilization, UV radiation, amino acid analogues, arsenite, various chemicals, and drugs the mammalian brain demonstrates a rapid and intense induction of the heat shock protein. Moreover, Hsps were expressed on the various pathological conditions including trauma, focal or global ischemia, hypoxia, infarction, infections, starvation, and anoxia. Especially, Hsp25 has a protective activity, facilitated by the ability of the protein to decrease the intracellular levels of reactive oxygen species (ROS) as well as its chaperone activity, which favors the degradation of oxidized proteins. Recently, it has clearly demonstrated that Hsp25 is constitutively expressed in the adult mouse cerebellum by parasagittal bands of purkinje cells in three distinct regions, the central zone (lobule VI-VII) and nodular zone (lobule IX-X), and paraflocculus.

The Mongolian gerbil has been introduced into stroke study model because of its unique brain vasculature. There are no significant connections between the basilar-vertebral system and the carotid system. This anatomy feature renders the mongolian gerbil susceptible to forebrain ischemia-induced seizure.

The present study is designed to examine the pattern of Hsp25 expression in the cerebellum of this animal in comparison with that in mouse.

Key words : Heat shock protein (Hsp), Mongolian gerbil, Mouse cerebellum

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Introduction

Heat shock proteins (Hsps) were discovered in 1962 when Ferruccio Ritossa and his co-worker noted that temperature shock had induced odd puffing patterns and an unusual profile of gene expression in the polytene chromosomes of salivary glands in *Drosophila melanogaster* larva[1]. However, not until 1974 were the first products of these genes identified and the term Hsps coined[2].

In response to various forms of stress, including hyperthermia and ischemia, cells from many organisms increase the expression of a class of proteins are referred to as Hsps[3-4]. They are thus also referred to as stress proteins.

Hsps are classified according to their molecular weight into general families; Hsp110 (110 kDa), Hsp90 (84-90 kDa), Hsp70 (68-78 kDa), and the small Hsps (20-30 kDa)[5].

In injured cells, Hsps contribute to cellular repair processes by refolding denatured proteins[6,7]. It is now clear that many Hsps are also constitutively expressed and are believed to act as molecular chaperones during regular protein translocation or folding[5,8-11].

The murines Hsp25 is homologous protein to the rat 27-kDa heat shock protein Hsp27[12,13]. The Hsp25 is constitutively expressed in many motor and sensory neurons of the brain stem and spinal cord[13]. In addition, the Hsp25 is highly and selectively inducible in astrocytes[12,14,15] and neurons[16]. However very little is known about the function of this low molecular weight Hsps in the central nervous system. In contrast, in non-neuronal cells a number of functions have been suggested for Hsp25/27 such as acting as a molecular chaperone[17], regulation of actin dynamics[18,19], regulating antioxidative activity[20-22], and blocking apoptosis[22,23].

Hsp25 immunoreactive neurons are abundant in various thalamic subnuclei during postnatal development, but no Hsp25 immunoreactivity is seen in the thalamus of the adult mouse[24]. Hsp25 immunoreactivity was observed in the Purkinje cells of adult mouse cerebellar vermis[1].

Hsp25 is not the only heat shock protein that is constitutively expressed in the rat cerebellum. Hsp25 immunoreactive motorneurons are seen at all levels in the ventral spinal cord in the rat, the development and adult mouse[25].

During development, Hsp25/27 is transiently expressed in Purkinje cells in the embryonic rat cerebellum, whereas in the adult mouse cerebellum, this protein is detected in a subset of Purkinje cells.

Recently, Armstrong et al[1] demonstrated a marked difference between the adult cerebellar distributions of Hsp27 in the rat and Hsp25 in the mouse. In the adult rat cerebellum, Hsp27 immunoreactivity is not expressed in Purkinje cells. On the other hand, in the adult mouse cerebellum, Hsp25 is constitutively expressed in parasagittal bands of Purkinje cells in three distinct regions.

There are no previous descriptions of Hsp25 expression in the mongolian gerbil. In this study, we explored the constitutive expression if Hsp25 in normal mouse cerebellum and gerbil cerebellum by immunohistochemistry and double

immunofluorescence to determine its distribution and whether it is associated with specific classes of neurons. These results also raised the possibility that strain difference of Hsp25 expression in cerebellum.

Materials and Methods

Animals

All mice used in this study came from a breeding colony maintained at the Korean Research Institute of Bioscience and Biotechnology (KRIBB) and housed in a constant temperature room (23℃) constant humidity (45-50%), with a 12 hrs light: dark cycle and access to food and water *ad litum.* 110 days old *Balb/C* mouse (n=10), and 110 days old gerbils (n=10) used in this study. All experimental procedures were carried out in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Tissue preparation

Both animals were deeply anesthetized with intraperitoneal injection of sodium pentobarbital (25 ㎍/10 g body weight), and perfused transcardially with 'Bouin' s solution without acetic acid. These perfused brains were removed from the skull and placed immediately in the fixative at 4℃ for 4 hrs. These post-fixed brains were transferred to 0.1M phosphate buffer saline (PBS), dehydrated, clearing (with xylene), embedded in paraffin, and sectioned serially in the coronal and sagittal plane at 5 ㎛ thickness. Cerebellar sections were irradiated with microwaves for 5 min in 10 mM citrate buffer (pH 6.0), and processed for immunohistochemistry and immunofluorescence.

Immunohistochemistry

For immunohistochemistry, cerebellar sections were rinsed for in PBS containing 0.3% Triton X-100 and were then incubated in 1% hydrogen peroxidase in 0.1 M PBS (0.9% NaCl in 0.1 M PB, pH 7.4). They were then in PBS and incubated in the primary antibody [anti-Hsp25 (1 : 1,000, Stressgen, USA) and anti-calbindin D-28kDa (1 : 5,000, Sigma, USA)] for 15-18 hrs at 4℃. Sections were then washed three times for 15 min each 0.1 M PBS and incubated in 0.1 M PBS containing biotinylated anti-mouse IgG (for calbindin D-28 kDa, 1 : 200, Vector, USA) or biotinylated anti-rabbit IgG (for Hsp25, 1 : 200, Vector, USA) for 3 hrs at 38℃. After incubation in secondary antibody, the cerebellar sections were washed 3 times for 15 min in 0.1 M PBS and the reacted with the Vectastain ABC elite Kit (1 : 200, Vector, USA) for 3 hrs at 38℃ incubator. The immunoreactive product was visualized with 0.01% 3,3′ -diaminobenzidine tetrachloride (DAB, Sigma, USA) in 0.03% hydrogen peroxide (H_2O_2) as a chromogen. Sections were dehydrated, cleared, and cover-slipped.

Double immunofluorescence

Cerebellar sections for immunofluorescence were washed for 15 min each in 0.1 M PBS (pH 7.4). The tissue was then

incubated in PBS containing 10% normal goat serum (Jackson ImmunoRes, West Grove, PA, USA) for 60 min and incubated in PBS containing a combination of the primary antibody anti-Hsp25 (1 : 1,000, Stressgen, USA) and anti-calbindin D-28 kDa (1 : 5,000, Sigma, USA)] or 16-18 hrs at 4℃. Following incubation in primary antibodies, sections were washed for 15 min in 0.1 M PBS buffer and then left in PBS containing Cy3-conjugated goat anti-rabbit and Cy2-conjugated goat anti-mouse secondary antibody (1 : 1,000, Jackson ImmunoRes, USA) for 24-48 hrs at 4℃. After incubation in secondary antibody, the sections were washed for 15 min in 0.1 M PBS, mounted onto poly-L-lysine coated slides, air-dried overnight, cleared in 0.1 M PBS, and cover-slipped with non-fluorescencing mounting medium (Fluorsae Reagent; Calbiochem, USA).

Image capture and photography

Results of immunohistochemistry and double immunofluorescence were quantified with a computer-based image analysis system. The system included a Leica digital camera (DF480, Leica, Germany) mounted on an light microscope (DMR, Leica, Germany).

The digital camera was connected to a IBM computer. The images of immunostained cerebellar sections were captured by the Leica capturing program embedded in photoshop 6.0 (Adobe, USA). The digital signals from the camera were converted into a color/gray scale digital image consisted of a 2,560 × 1,920 grid of pixels. The brightness level of each pixel ranged from 0 to 255 gray levels. To capture the images, a cerebellar section was selected and the point of focus was moved off the tissue without changing any setting and then a blank field was captured. The selected section was assembled in Adobe Photoshop 6.0 Images were cropped and adjusted for brightness and contrast but not otherwise manipulated.

Results

The cerebellar cortex is subdivided both mediolaterally and rostrocaudally into reproducible zones and strips that can be demonstrated by the presence or absence of molecular marker[37,39,42], the distribution of afferent connections[26-32], and functional analysis[33-37].

Rostrocaudal boundaries divide into four transverse zones: the anterior zone (AZ: ~ lobules Ⅰ-Ⅴ), the central zone (CZ: ~ lobules Ⅷ), and the nodular zone (NZ: ~ lobules Ⅸ-Ⅹ)[38-42].

Hsp25 expression in mouse cerebellum

Coronal sections taken through the caudal to rostral extent of the *Balb/C* mouse cerebellum demonstrated that Hsp25-immunoreactive Purkinje cells were confined to two regions of the vermis - the lobules Ⅵ~Ⅶ and Ⅸ~Ⅹ (Fig 1A, 2A, 3A), which is consistent with previous reports[1,25]. Within all two regions, Hsp25-immunoreactive Purkinje cells were arrayed as rostrocaudal distribution or parasagittal band. No Hsp25-immunopositive Purkinje cells were seen in the hemispheres or the vermis of the anterior

lobules (Fig 1A, 2A), but Hsp25-immunopositive Purkinje cells were seen in the hemispheres or the vermis of the posterior lobules (Fig 3A). In sagittal cerebellar sections, Hsp25-immunoreactive Purkinje cells was localized in two distinct regions. Two distinct regions were lobules Ⅵ/Ⅶ and lobules Ⅸ/Ⅹ (Fig 4A).

Fig 1. Coronal sections in the anterior vermis of cerebellum comparing the distribution of Hsp25-immunoreactive Purkinje cells in *Balb/c* mouse [A] and Mongolian gerbil [B] cerebellum. In *Balb/c* mouse, there no Hsp25-immunoreactive Purkinje cells. In gerbil, Hsp25-immunoreactivity was detected in entire. Scale bar = 250㎛

Fig 2. High power photography of Hsp25-immunoreactivity in anterior vermis of Balb/c mouse [A] and Mongolian gerbil [B]. In Balb/c mouse was no reaction product. Coronal sections through the gerbil cerebellar vermis immunostained by using anti Hsp25. Immunoreactive product is deposited throughout the somata, and dendrites of the Purkinje cells. Scale bar = 50㎛

Hsp25 expression in gerbil cerebellum

In gerbil sections, Hsp25-immunoreactive cells were detected in entire lobules in cerebellum (Fig 1B, 4B). In coronal sections, Hsp25-immunoreactivity were conspicuous in a significant subpopulation of cerebellar Purkinje cells in gerbils (Fig 2B). In coronal and sagittal sections, Hsp25-immunoreactive Purkinje cells were expressed in the vermis, the hemisphere, the paraflocculus, and the flocculus (Fig 1B, 2B, 3B, 4B). Due to these reasons, we could not observed any specific band like patterns in gerbil cerebellar cortex (Fig 2B). Otherwise Hsp25 was confined specially to Purkinje cells in the NZ and the CZ (Fig 4A), both of which receive visuomotor afferents from the vestibular nuclei[43] and

the pontine nuclei[44,45] in the vermis of the adult mouse.

Comparison of anti-Hsp25 and anti-calbindin D-28kDa immunohistochemistry in gerbil cerebellum

Sections through the gerbil cerebellum immunostained with anti-Hsp25 and anti-calbindin D-28kDa in adjacent sections. As demonstrated in numerous accounts, anti-Hsp25 immunohistochemistry reveals a specific subpopulation of Purkinje cells in many animal species, including the mouse. In gerbil cerebellum, Hsp25-immunoreactive Purkinje cells were expressed in the vermis, the hemisphere, the paraflocculus, and the flocculus (Fig 1B, 2B, 3B, 4B, 5A).

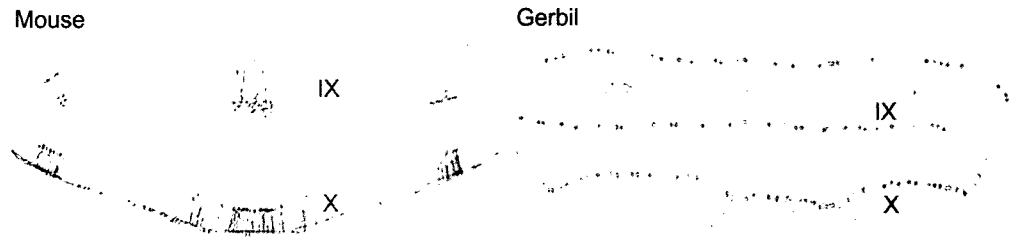
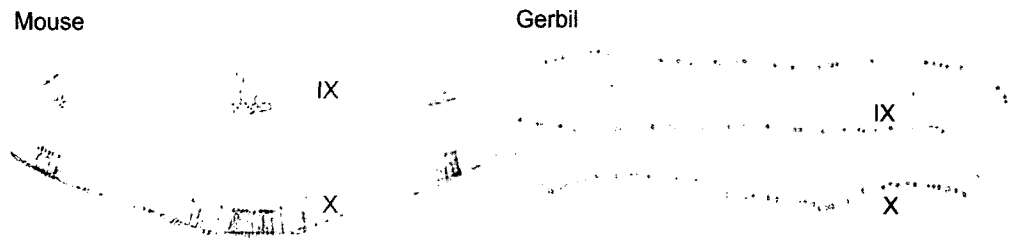


Fig 3. Coronal sections in the lobule IX and X comparing the distribution of Hsp25-immunoreactive Purkinje cells in Balb/C mouse and Mongolian gerbil. In Balb/C mouse, lobules IX/X contains parasagittal bands of Hsp25-immunoreactive Purkinje cells. Three Hsp25-positive bands were observed in the lobule IX/X. In gerbil, there is no specific band formation. Scale bar = 250㎛



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To correspond with the distribution of Purkinje cells, the sections were stained polyclonal antibody, anticalbindin D-28kDa as a Purkinje cell specific marker. When the distribution of Hsp25-immunoreactive and calbindin D-28kDa immunoreactive Purkinje cells in gerbil cerebellum were compared in adjacent sections, the two antigens appeared to reveal the same subsets of Purkinje cells (Fig. 5A, 5B). All kinds of Purkinje cells in gerbil cerebellar cortex were immuno-

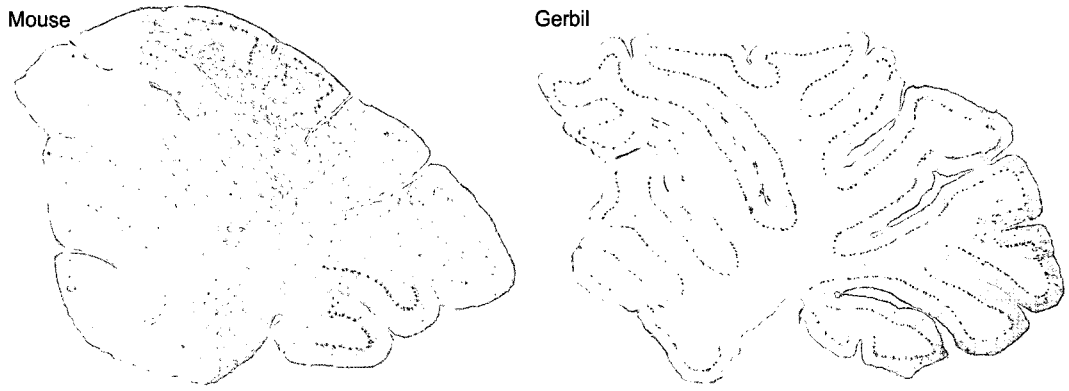

Fig 4. Sagittal sections comparing the distribution of Hsp25-immunoreactive Purkinje cells in Balb/C mouse and Mongolian gerbil cerebellum. In Balb/C, lobules VI/VII and IX/X contains Hsp25-immunoreactive Purkinje cells. In gerbil, Hsp-immunoreactivity was detected in entire lobules in cerebellum. Scale bar = 500㎛

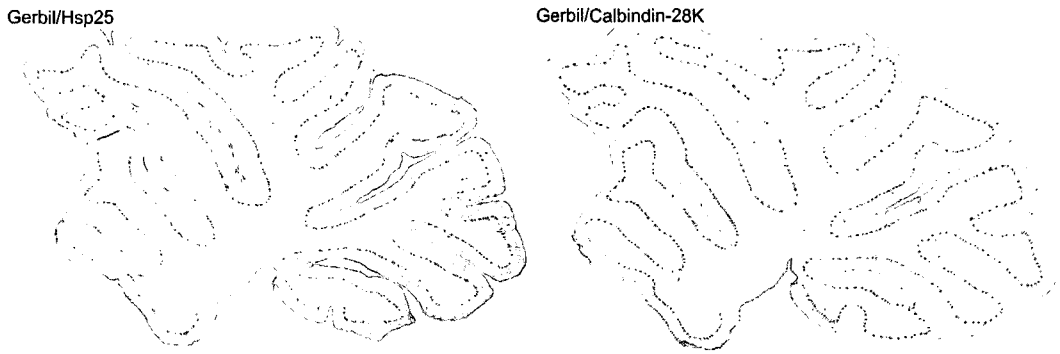

Fig 5. Sagittal sections comparing the expressive pattern of Hsp25-immunoreactive Purkinje cells and Calbindin D-28kDa immunoreactive Purkinje cells in gerbil cerebellum. There are no significant differences in expressive pattern, geographicaly and quantitatively. Scale bar = 500㎛

reactive with anti-calbindin D-28kDa and anti-Hsp25.

Anti-Hsp25 and anti-calbindin D-28kDa double immunofluorescence in Gerbil cerebellum

Double immunofluorscence demonstrated excellent correspondence between the patterns of distribution of Purkinje cell immunoreactivity for two antigens. Hsp25 and calbindin D-28kDa immunostained reaction product is confined exclusively to the Purkinje cells (Fig 5A, 5B). Double immunofluorescence in gerbil cerebellar sections revealed the characteristic pattern of calbindin D-28kDa immunoreactive Purkinje cells (Fig 6A), and the same pattern could be observed for Hsp25 immunofluoresences (Fig 6B). Double immunofluorscence revealed overlapping patterns of Hsp25-immunoreactive and

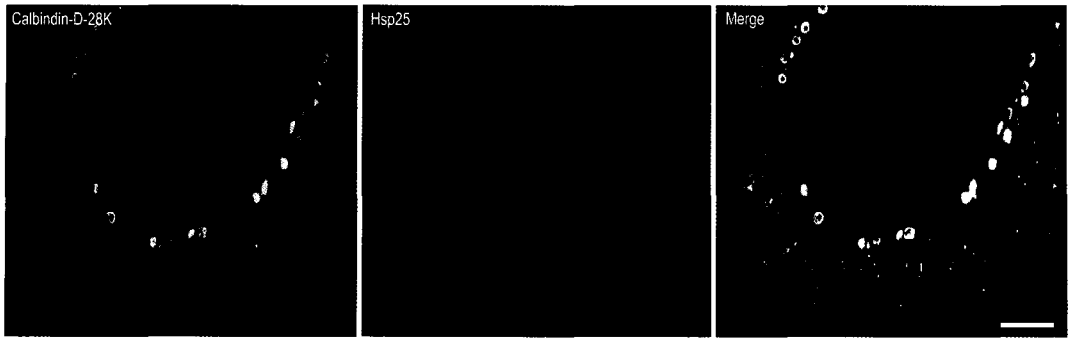

Fig 6. Coronal sections double-immunofluorescence stained for Calbindin D-28kDa (Green) and Hsp25(Red). Panel A show the calbindin D-28kDa positive cells in lobe. Panel B show the Hsp25 positive cells in lobe. In both cases, both antigens are co-expressed by the same Purkinje cell subset. Scale bar = 100㎛

calbindin D-28kDa immunoreactive Purkinje cells in all lobules of the cerebellum (Fig 6C).

Discussion

Hsps are a group of intracellular proteins that have an usually high degree of identity at the amino acid level, among diverse organisms[24,46]. As this family of proteins is induced by stressors other than heat, they are also commonly referred to as 'stress proteins' in the literature[24,46]. The term stress proteins also may refer to several other groups of proteins that respond to stressors[32].

Hsps are constitutively expressed in cells to maintain a number of critical cellular processes relating to protein folding, fidelity and translocation. These proteins also are induced in cells in response to a variety of stressors and enhance survival by protecting vital cellular functions[24]. The naming of Hsps are generally based on their molecular mass (kilodalton, kDa) as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Hsps are also grouped according to function (e.g chaperonin), DNA sequence, and antibody cross-reactivity [24,46].

Hsps are induced by many environmental stresses including exposure to trace metals[47,48] or organic pollutants[49], changed in temperature[50-52] or osmolarity[53], hypoxia/anoxia[54,55], and exposure to ultraviolet radiation[55].

Small heat shock proteins (sHsps) are more species specific than the larger stress proteins and less conserved, with significant variation occuring within the same class of organism. sHsps appear to be homologous to α-crystalline and are not synthesized under normal conditions[55]. Their synthesis is regulated during development and differentiation is modified by environmental factors. Stress proteins play a primary role in protecting cells from injuries caused by a variety of pollutants and stress protein levels have a potential use in environmental monitoring[49].

sHsps belong to the large family of heat shock proteins that fulfill essential physiological functions and that are ex-

pressed in specific tissues and organs during development[47,56]. sHsps are induced by various forms of stress, such as heat shock or oxidative injury [49]. Although little is known about the functions of Hsp25, several studies on the rat or human homology Hsp27, indicate that this protein serves a protective function against heat shock at least in cultured cells [57]. At least three functions have been suggested for Hsp25: anti-oxidative enzyme regulation, regulation of actin dynamics, and molecular chaperoning.

In vivo, several studies reported that although Hsp27 is constitutively expressed in the brainstem and spinal cord of the adult rat, Hsp27 is also induced in neurons after nerve injury [56].

Hsp25 is also induced in neurons after nerve injury. For instance, after peripheral nerve injury in adult rats, Hsp25 is up-regulated in axotomized neurons in dorsal root ganglion, and the modulation correlates with neuronal survival after axotomy[56].

Other studies indicated that Hsp27 is specifically up-regulated in surviving neurons after vagal nerve injury, suggestive also for a role of Hsp27 in neuronal survival and axonal regeneration[16].

Hsp25 is expressed in a variety if circumstances. Hsp25 is highly inducible in astrocytes after seizure activity[12], ischemic injury[14,50], and cortical spreading depression[14].

In mammals, sHsps have also been identified in developing neuronal tissues. In particular, Hsp27 (Hsp25 in mice) is expressed in large neurons of the spinal cord and in cerebellar Purkinje neurons in rat embryos[58]. In the adult, Hsp 27 is also present in a subset of sensory and motor neurons[14]. In terestingly, overexpression of Hsp27 in neuronal cells protects these cells against both apoptosis and necrosis[59,60]. Also, high levels of Hsp27 are often present in tissues from patients suffering neurodegenerative disorder[61-63]. Furthermore, elevated expression and accumulation of Hsp27 was found mainly in human brain reactive astrocytes from many neurodegenerative disorders such as Alexander' s or Alzheimer' s disease[61-63]. Although the role of Hsp25 in paralyse cell death cannot be resolved definitely at the present time.

During development, Hsp25/27 is transiently expressed in Purkinje cells in the embryonic rat cerebellum, whereas in the adult mouse cerebellum this protein is detected in a subset of Purkinje cells.

Recently, Hsp25 expression was no observed in the korean wild mice cerebellum.

Hsp25 immunoreactivity is restricted to Purkinje cells in the CZ (~lobules VI- and the NZ (~lobules IX-X) of the adult cerebellum[1]. During development this pattern is reversed, with Hsp25 first detected in the AZ (~lobules I-V) and the PZ (~lobule VIII) and then gradually spreading throughout all four zones of the cerebellum, first in the NZ and then in the CZ[25]. Finally Hsp25 expression is downregulated first in the AZ and then the PZ and is also selectively repressed in subsets of Purkinje cells in the CZ and the NZ, to leave the characteristic adult parasagittal banding pattern[24]. In the vermis, the central zone (CZ, lobule

VI-VII) and nodular zone (NZ, lobule IX-X) of the cerebellar cortex contains parasagittal bands of Hsp25-immunoreactive Purkinje cells. The paraflocculus and flocculus also contains cluster of Hsp25-immunoreactive Purkinje cells.

Mongolian gerbils, which is used as an ischemia anmal model heretically are divided into seizure-sensitive (SS) and/or seizure-resistant (SR). Many immunohistochemical data in the CNS of SR gerbils are significantly different from the data in that of SS gerbils[64]. In this study, however, we did not divided gerbils into two groups. Therfore, it is difficult to directly correlate our results with epileptogenesis, but it cannot be excluded the possibility that Hsp25 may be associated with ischemic damage or epileptogenesis in gerbils. Compared with the previous results in rat and mouse, there were several differences.

In the present study, we got the exactly same results with Armstrong et al[1] in the normal *Balb/c* mouse cerebellum. However, we observed a little different Hsp25 expression in the gerbil cerebellum. In gerbil cerebellum, we observed that all cerebellar Purkinje cells were Hsp25-immunoreactive by using calbindin D-28 kDa immunohistochemistry and double immunofluorescence. These results indicate that Hsp25 expression in cerebellum was inter-strain difference by animals. Moreover, these results also raised the possibility that strains differences for the response to the stimulus among these animals.

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