## ·Original Paper·

# The calcium-binding protein Mts1/S100A4 in normal, degenerating and demyelinated spinal cord of the adult mouse[*]

FANG Zhengyu[1]   XIONG Liang[2]   HUANG Xiaolin[1,4]   ZHOU Ning[1]   Kozlova-Aldskogius Elena[3]

**Abstract  Objective:** To investigate the expression pattern of Mts1/S100A4 in mouse spinal cord；to investigate the effects of Mts1/S100A4 on glial cell responses. **Method:** The study was carried out on Mts1/S100A4 wild type and knock-out mice. The degenerative spinal cord model was established by dorsal root or sciatic nerve injury. The demyelinated spinal cord model was established by ethidium bromide injections. Then the expressions of S100A4, GFAP,NG2 and Mac1 were measured. **Result:** The expressions of Mts1/S100A4 in mice spinal cord were similar to that in rats. In WT mice this protein expressed in a thin layer of fiber bundles in the tract of Lissauer, and in white matter astrocytes. There was intracellular up-regulation of Mts1/S100A4 in white matter astrocytes of WT mice after dorsal root or sciatic nerve injury, with no difference in glial cell response between WT and KO mice. However, 7 days after ethidium bromide injection, in WT mice, the astroglial reaction was restricted on operated side, where a distinct glial scar had formed. While in KO mice, no distinct glial scar formed in demyelinated area. **Conclusion:** Mts1/S100A4 expression in mouse spinal cord is similar to the pattern as in rats; intracellular Mts1/S100A4 up-regulation does not affect glial responses in degenerative spinal cord；the presence of extracellular Mts1/S100A4, which entered the spinal cord after ethidium bromide induced demyelination, markedly affects the glial cell responses in demyelinative spinal cord, including glial scar formation.

**Author's address**  Department of Rehabilitation Medicine, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China

**Key words**  calcium-binding protein; degeneration; demyelination; Mts1/S100A4

**摘要  目的:** 研究正常、退行性病变以及脱髓鞘小鼠脊髓内 Mts1/S100A4 蛋白的表达模式,及其对胶质细胞反应的影响。**方法:** 以野生型和 Mts1/S100A4 基因敲除型小鼠为试验动物,采用背根损伤、坐骨神经损伤、溴乙啶局部微量注射的方法复制退行性病变及脱髓鞘脊髓动物模型,应用免疫荧光技术,检测 S100A4 、GFAP、NG2 、Mac1 的表达情况。**结果:** 野生型小鼠脊髓内,仅白质星型胶质细胞表达 S100A4 蛋白,且主要分布于 Lissauer 束;背根或坐骨神经损伤后, 白质星形胶质细胞内的 S100A4 及 GFAP 表达上调, 野生型与 S100A4 基因敲除小鼠 GFAP 表达量无显著差异;溴乙啶注射 7d 后,野生型小鼠脊髓脱髓鞘区域内见 S100A4 呈云雾状分布,胶质细胞反应局限于注射侧,并且形成清晰的胶质瘢痕,而 S100A4 基因敲除小鼠则未见上述病理变化。**结论:** S100A4 蛋白在小鼠脊髓内的表达模式与大鼠相似； 退行性变的脊髓内, 细胞内上调的 S100A4 蛋白并不影响胶质细胞的反应； 脱髓鞘脊髓内, 细胞外的 S100A4 蛋白明显影响胶质细胞反应,包括胶质瘢痕的形成。

**关键词**  钙结合蛋白; 退行性变; 脱髓鞘; Mts1/S100A4

Mts1/S100A4 is a member of the family of S100 calcium-binding proteins that participate in the regulation of intracellular processes such as proliferation, differentiation, motility and growth[1]. These proteins transduce calcium signals by interacting with different intracellular targets in a specific manner for different members of S100 protein family. The effects of these interactions include protein phosphorylation, regulation of enzyme activities, modification of cytoskeletal proteins and regulation of transcription factors. Some of S100 proteins are known to be secreted and exert cytokine-like or growth factors activities on target cells. The

mechanisms underlying at least some of these extracellular

effects may result from binding of S100 protein to the surface receptor RAGE (receptor for advanced glycation endproducts)[2].

Mts1/S100A4, an 11-kDa member of this family, implicated in invasive and metastatic behaviors of tumor cells, is expressed in white matter astrocytes of postnatal rat with a exception of cerebellum[3]. Following injury, Mts1/S100A4 protein up- regulated markedly in white matter astrocytes in areas of degeneration, concomitantly with activation of microglia/macrophages [3—4]. These data suggest that Mts1/S100A4 has an important function in reactive white matter astrocytes, and may be involved in regulating glial cell responses in the central nervous system (CNS) after injury in rat.

Genetically manipulated mice are prime experimental tools for exploring gene function. Mice with a germ-line inactivation of Mts1/S100A4 gene (KO)[5], provide an opportunity to explore in vivo the possible role of Mts1/S100A4 in spinal cord injury responses. However, there is currently no information on the expression of Mts1/S100A4 in mouse spinal cord. We therefore asked whether Mts1/S100A4 is expressed in adult mouse spinal cord, and, if so, whether the expression pattern is similar to rat, and regulated by injury. We subsequently use markers for astrocytes, microglia/macrophages, and oligodendrocyte precursors to examine whether the overall glial cell response to injury in the spinal cord is different between WT and KO mice[6].

## 1 Material and methods

### 1.1 Animals

**1.1.1** The study was carried out on Mts1/S100A4 wild-type mice (WT, n=17) and knock-out mice with homozygous deletions of Mts1/S100A4 gene (KO, n=15). All mice were at the age of 3–4 months. All animal experiments were approved by the regional animal research ethics committee, and carried out in accordance with the NIH guidelines for research on animals. Prior to surgery and perfusion, animals were anesthetized by intraperitoneal injection of chloral hydrate (35mg/kg body weight) or a combination of Ketamine (2mg/10g body weight) and Xylazine(0.2mg/10g body weight).

**1.1.2** Dorsal root and sciatic nerve injury：The left lumbar dorsal roots L4 and L5 were exposed via a partial laminectomy and sectioned with a pair of iridectomy scissors closing to the corresponding dorsal root ganglia in WT and KO mice (n=5 for each strain). WT and KO mice (n=4 for each strain) were subjected to section of left sciatic nerve at mid-thigh level. All animals were allowed to survive for 7 days. Three WT and KO mice were used as intact controls respectively.

**1.1.3** Ethidium bromide injections ：Ethidium bromide (Ebr)

was used to cause rapid demyelination with spared axons in white matter [7]. A partial laminectomy was made over the dorsal spinal cord at T10-11 level. Solution of 1% ethidium bromide in PBS was injected over a period of 5 ms with a glass micropipette (outer diameter 20 μm) in the dorsal funiculus on left side in 8 WT and 8 KO mice. Care was taken to keep the same angle and penetrating distance from the spinal cord surface with micropipette. Animals were allowed to survive for 2 (n=4 for each strain), or 7 days (n=4, for each strain). Intact controls were the same as above.

**1.1.4** Tissue preparation：At the time of sacrifice, the animals anesthetized and perfused via the left ventricle, first with saline (37℃), followed by solution of 4% formaldehyde (w/v) and 14% saturated picric acid solution (v/v) in 0.15M phosphate-buffered saline (PBS, pH 7.4, 4℃). Intact control animals were perfused in the same way. Spinal cord segments T 10-11 and L4-5 were removed after perfusion fixation from intact WT and KO animals. The L4-5 spinal cord segments were removed from WT and KO animals with dorsal root or sciatic nerve injury, and the T10-11 segments from WT and KO animals with EBr injection. Tissue blocks were postfixed for 4 hours, and cryoprotected overnight in a solution of 10% sucrose in PBS (4℃). Cryostat sections (14 μm) were made in the transverse plane from spinal cord of all animals. In addition, horizontal sections from the dorsal part of spinal cord were made in selected intact and dorsal root injured animals. Sections were placed on Super Frost Plus glass slides and stored at −20℃ until immunostaining.

### 1.2 Immunohistochemistry

The sections were thawed at room temperature until water condensations on the slides disappeared. A blocking solution[1% bovine serum albumin (w/v), 0.3% Triton X-100, and 0.1% sodium azide in PBS] was gently put on the top of each section for 1 hour at room temperature. After blocking, the solution was removed and then sections were incubated overnight at 4℃ in the same solution with primary antibodies. For double labelling experiments Mts1/S100A4 was combined with monoclonal glial fibrillary acidic protein (GFAP) or Mac1 antibodies. The slides were washed 3 times in PBS (7 min/wash) and then incubated with secondary antibodies. The immune complexes were visualized with FITC-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Inc., West Grove, PA) and Texas Red-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Inc., West Grove, PA) diluted 1:50 and 1:40, respectively, in PBS with 0.3% Triton X-100 and 0.1% sodium azide for 4 hours at room temperature. After another round of washing (3 times, 7 min/rinse in PBS) the slides were mounted in a mixture of PBS and glycerol (1:1; v/v) containing 0.1M propyl-gallate.

### 1.3 Microscopy

The sections were examined and photographed using a Nikon Eclipse fluorescence microscope equipped with filters for separate or combined viewing of TRITC and FITC fluorescence. For photography Nikon DXM1200F digital camera system was used.

## 2　Results

There are Fig 1—6 in the colour page.

### 2.1　Mts1/S100A4–IR in the intact mouse spinal cord

The level of Mts1/S100A4 immunoreactivity (IR) was very low in spinal cord of intact WT mice, except for intensive Mts1/S100A4–IR in a thin layer of fiber bundles in the tract of Lissauer(Fig.1 A).On horizontal sections through the surface of spinal cord, where sensory fibers entered the spinal cord, the tract of Lissauer showed as a well defined bundle of fibers (Fig.1 B). No co–localization with GFAP–IR was found (Fig.1 C) in KO mice which were completely negative for Mts1/S100A4.

### 2.2　Mts1/S100A4 immunoreactivity after injury

Following dorsal root injury in WT mice, increased Mts1/S100A4–IR was observed in astrocytes in dorsal funiculus on operated side, and to a small extent also on unoperated side. Mts1/S100A4 labelling in Lissauer's tract reduced on operated side (Fig.2 A). The Mts1/S100A4–IR extended throughout the entire rostro–caudal dorsal funiculus of lower lumbar spinal cord (Fig.2 B). Double labeling with astroglial marker GFAP, revealed no co–localization between Mts1/S100A4 and GFAP(Fig.2 C).

Following sciatic nerve injury in WT mice, Mts1/S100A4–IR decreased in Lissauer's tract. At the same time GFAP–IR increased on operated side one week after injury (Fig.3A,B). No Mts1/S100A4–IR was found in astrocytes(Fig. 3C,D).

### 2.3　Glial cell responses in spinal cord after injury: comparison between KO and WT mice

**2.3.1**　GFAP：GFAP–IR cells were distributed throughout the spinal cord in intact WT and KO mice. Following dorsal root injury there was an up–regulation of GFAP–IR in dorsal horn and dorsal funiculus ipsilateral to injury. No difference was observed between WT and KO mice(Fig.4A—D). Following sciatic nerve injury, GFAP–IR increased in dorsal horn ipsilateral to injury, with no difference between two strains of mice (Fig.4F—H).

**2.3.2**　NG2：Scattered NG2–IR cells were found in spinal cord white and gray matter in WT and KO mice. After dorsal root (Fig.5A—D) or sciatic nerve(Fig.5 E—H) injury an increase in nervous glial 2–immune reactivity (NG2–IR) was noted on operated side in WT (Fig.5 B,F) and KO (Fig.5 D, H) mice without any difference between two strains of mice.

**2.3.3**　Mac1：Immunoreactivity with antibodies Mac1 was ab-

sent in intact spinal cord (not shown). Strong induction of Mac1–IR was found after dorsal root injury (Fig.6 A—D) as well as after sciatic nerve injury (Fig.6 E—H) in both strains of mice. The increase in Mac1–IR was similar in WT and KO mice.

### 2.4　Ethidium bromide injection

Ethidium bromide (EBr) injection resulted in extensive damage in dorsal funiculus on operated side as well as minor injury on unoperated side in both WT and KO mice.

### 2.5　Mts1/S100A4

Two and seven days after EBr injection into the spinal cord of WT mice, Mts1/S100A4–IR presented in numerous small, round cells in lesioned area (Fig.7 A). At the seventh day after EBr injection, diffuse foggy extracellular Mts1/S100A4–IR appeared throughout the demyelinated area (Fig. 7B). No co–localization was observed between Mts1/S100A4– and GFAP–IR in spinal cord (not shown).

### 2.6　GFAP

Two days after EBr injection, a generalized increase in GFAP–IR was observed in Mts1/S100A WT and KO mice spinal cord (Fig.8 A, B). The increase in GFAP–IR was mainly on the side of spinal cord with less extensive demyelination. Seven days after EBr injection in KO mice, GFAP–IR cells were distributed in a similar pattern on both sides of spinal cord (Fig.8 C,D). In WT mice, the astroglial reaction was restricted on operated side, where a distinct glial scar formed (Fig. 8C). In KO mice, no distinct glial scar formed in demyelinated area (Fig. 8D).

### 2.7　NG2

Two days after EBr injection, NG2–IR cells increased in both WT and KO mice (Fig. 9A,B). In WT mice the distribution of these cells was restricted to the vicinity of demyelinated area, whereas in KO mice, NG2–IR cells were distributed over a large part of white and gray matter. Seven days after EBr injection, NG2–IR cells in WT mice accumulated along the border of demyelinated area (Fig. 9C). In KO mice, NG2–IR cells still distributed widely over a large territory and didn't concentrated along the border of demyelinated area (Fig.9D).

### 2.8　Mac1

Two days after EBr injection, scattered Mac1 positive cells were found in spinal cord of WT and KO mice, with no difference between two strains (Fig.10 A,B). Seven days after EBr injection, there was a compact accumulation of Mac 1–IR cells within the demyelinated area in WT mice (Fig.10 C), which contrasted with a much weaker Mac1 response in KO mice (Fig.10 D).

## 3　Discussion

### 3.1　Distribution of Mts1/S100A4 in astrocytes of mouse and rat CNS
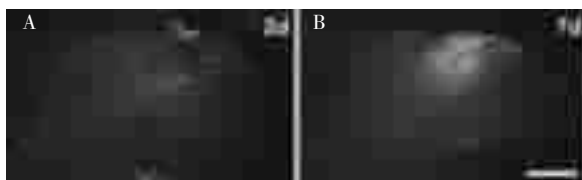
**Fig. 7　Mts1/S100A4 immunoreacivity in lower thoracic spinal cord two(A) and seven days(B) after injection of ethidium bromide in left dorsal funiculus.**

There were numerous small, round Mts1/S100A4 positive cells in the area of demyelination. At the seventh day these cells were surrounded by diffuse Mts1/S100A4−IR, which filled much of the demyelinated area.　　　　Scale bar =1μm
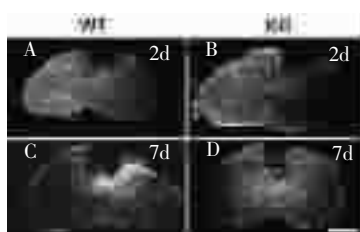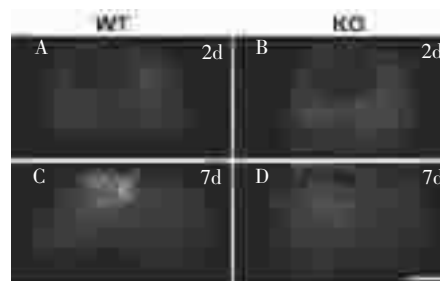


**Fig. 8　GFAP immunoreactivity (IR) in lower thoracic spinal cord 2(A, B) and 7(C,D) days after injection of ethidium bromide in left dorsal funiculus. In WT mice GFAP−IR markly increased, and concentrated closing to demy−elinated area at 7 days after injection (C). In KO mice(B, D) GFAP−IR also displayed inten−sely, and spread over most part of the spinal cord.**　　　　Scale bar=1μm
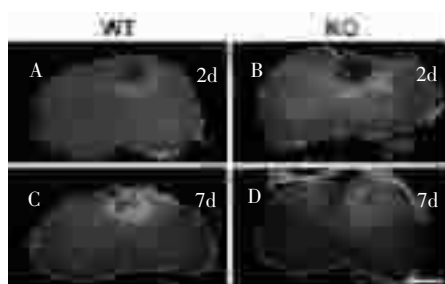


**Fig. 9　NG2 immunoreactivity (IR) in lower thoracic spinal cord two (A,B) and seven (C, D) days after injection of ethidium bromide EBr) in left dorsal funiculus. In WT mice NG2 −IR moderately increased two days after EBr injection, and a marked concentration of NG2−IR cells closed to demyelinated area at seven days after EBr injection (A, C). Two days after EBr injection, in KO mice (B) NG2 −IR displayed much more intense and widespread than that in WT mice (B). Although, in KO mice NG2−IR cells more concentrated to demyelinated area seven days after EBr injection(D), they are still more widely distributed than that in WT mice.**

Scale bar=1μm

In this study, we found low levels of expression of Mts1/S100A4 in mouse spinal cord, except for a strong ex−pression in a thin layer in Lissauer′s tract. This IR was clearly associated with fiber bundles entering from dorsal



**Fig. 10　Mac1 immunoreactivity (IR) in lower thoracic spinal cord following injection of ethidium bromide in left dorsal funiculus. Mac1−IR cells concentrated in the same area as Mts1/S100−IR cells. There was no difference in Mac1−IR at two days after injury between two strains (A, B), but at 7th days (C, D) after EBr injection, there was compact accumulation of Mac1 −IR cells within the demyelinated area in WT (C), and it was much weaker in KO mice (D).**

Scale bar =1μm

root, and most probably originated from a subpopulation of Mts1/S100A4 expressing dorsal root ganglion cells. This ex−pression started shortly after myelination began, and was maintained thereafter. These findings suggest that alternative molecule(s) exist in mouse for astroglial functions, which are regulated by Mts1/S100A4 in the spinal cord.

**3.2　Mts1/S100A4 was induced in white matter astrocytes of the mouse spinal cord following injury**

Dorsal root injury resulted in Wallerian degeneration of affected sensory fibers at segmental levels, as well as of the ascending collaterals in dorsal funiculus. In line with previ−ous findings in rat, Mts1/S100A4 was induced in astrocytes of mouse spinal cord in degenerating white matter after dor−sal root injury. Sciatic nerve injury resulted in so −called transganglionic changes in central processes of axotomized dorsal root ganglion cells. There was an induction of Mts1/ s100A4 in mouse spinal cord following sciatic nerve injury as well. However, this induction appears to be localized to fibers in Lissauer′s tract, rather than white matter astrocytes. Thus, Mts1/S100A4 can be induced in spinal cord white matter of mouse.

**3.3　The glial cell response to dorsal root and peripheral nerve injury was similar in Mts1/S100A4 WT and KO mice**

Activation of glial cells is a hallmark of degeneration and disease in CNS. This activation is characterized by pro−liferation and/or hypertrophy of GFAP expressing astrocytes, proliferation and migration of microglia/macrophages and re−cruitment of cells expressing the chondroitin sulfate proteo−glycan NG2. In order to examine the possible differences in injury −associated glial cell responses between Mts1/S100A WT and KO mice, we therefore applied markers, which i−dentify these different cellular components. KO mice have a normal postnatal phenotype, but display an abnormal sex ra−

tio in litters, with a higher proportion of male/female pups than normal. These mice develop a relatively high rate of spontaneous tumors, presumably as a result of destabilization of the p53 tumor suppressor gene, but show reduced metastatic activity.

In our previous studies, in the rat there was a strikingly close relationship between Mts1/S100A4 expressing reactive astrocytes and microglial/macrophage response, suggesting a role for Mts1/S100A4 in the activation of microglia/macrophages [4]. Our findings here show that, however, where there is a glial cell response, astrocytes, microglial cells and NG2 cells react in a similar way to dorsal root or sciatic nerve injury in WT and KO mice.  Thus, the intracellular induction of Mts1/S100A4 in mouse white matter astrocytes following these lesions does not appear to influence the response of microglia/macrophages.

**3.4**  Mts1/S100A4 influences the glial cell response to demyelination

Ethidium bromide induced demyelination results in a rapid loss of oligodendrocytes, myelin and astrocytes in lesion area [7]. These degenerative events are associated with activation of astrocytes and microglia/macrophages, and are followed by repopulation of demyelinated areas by oligodendroglial precursor cells  (OPCs)[8]. We did find a difference between WT and KO mice in glial cell response in the EBr demyelination model. In WT mice, GFAP–IR astrocytes, NG2 cells and Mac1 positive microglia/macrophages were highly concentrated to demyelinated area and its immediate surrounding. However, in KO mice, GFAP–IR astrocytes and NG2 were diffusely scattered over spinal cord and the presence Mac1–IR cells was much less prominent than that in WT mice. The NG2–IR population is probably not uniform, since the NG2 proteoglycan can be produced by different types of glial cells, including OPCs, astrocytes and microglia[6].

The possible role of Mts1/S100A4 in mediating different glial cell response in WT and KO mice is unclear, since the reactive astrocytes in WT mice were negative for this protein. This is probably a consequence of EBr mediated killing of not only oligodendrocytes but also astrocytes in dorsal funiculus [7]. However a multiple Mts1/S100A4 positive cells were present in the demyelinated area, but these cells were GFAP negative. Some of them were positive for Mac1, indicating that they were macrophages, and the overall morphology of Mts1/S100A4 –IR cells resembled hematogenous cells, in which case, they most likely entered spinal cord from vascular system. Seven days after EBr injection, these cells were embedded in a "cloud" of IR, which strongly suggested the presence of extracellular, presumably secreted Mts1/S100A4. Thus, secreted Mts1/S100A4 from invading hematogenous cells may influence the glial cell response to EBr administration, and promote formation of a distinct glial scar. Although there was an increase of GFAP–, NG2– and Mac1–IR cells in KO mice, these cells did not form the same well defined glial scar within the demyelinated area as in WT mice. These findings suggest that Mts1/S100A4 stimulates the recruitment of scar forming glial cells in demyelinated area. Intracellular Mts1/S100A4 regulates non–muscle myosin phosphorylation, and thereby influences cell motility. Extracellular Mts1/S100A4 promotes angiogenesis, migration of cultured glioma cells, and activity of metalloproteinase 13. Related mechanisms may underlie the formation of a distinct glial scar mice expressing Mts1/S100A4[9].

In summary, our findings show that Mts1/S100A4 expression is very low in astrocytes in intact adult mouse spinal cord. Mts1/S100A4 increased strongly after injury, and appears to participate in the organization of glial scar following demyelination. This role of Mts1/S100A4 may be of relevance for the subsequent attempts to remyelinate the lost myelin and restore impulse conduction.

**References**

[1]   Donato R. S100: a multigenic family of calcium –modulated proteins of the EF–hand type with intracellular and extracellular functional roles [J]. Int J Biochem Cell Biol, 2001,33 (7): 637—668.

[2]   Huttunen HJ, Kuja –Panula J, Sorci G, et al. Agneletti AL, Coregulation of neurite outgrowth and cell survival by amphoterin and S100 proteins through receptor for advanced glycation end products  (RAGE) activation [J]. J Biol Chem,2000,275(51): 40096—40105.

[3]   Kozlova EN, Lukanidin E. Metastasis–associated mts1 (S100A4) protein is selectively expressed in white matter astrocytes and is up–regulated after peripheral nerve or dorsal root injury [J]. Glia,1999,27(3):249—258.

[4]   Kozlova EN. Differentiation and migration of astrocytes in the spinal cord following dorsal root injury in the adult rat [J]. Eur J Neurosci,2003,17(4):782—790.

[5]   EL Naaman C, Grum–Schwensen B, Mansouri A, et al. Cancer predisposition in mice deficient for the metastasis –associated Mts1(S100A4) gene[J]. Oncogene,2004,23(20):3670—3680.

[6]   Stallcup WB. The NG2 proteoglycan: past insights and future prospects[J]. J Neurocytol,2002,31(6–7):423—435.

[7]   Blakemore WF. Ethidium bromide induced demyelination in the spinal cord of the cat[J]. Neuropathol Appl Neurobiol,1982,8(5): 365—375.

[8]   Talbott JF, Loy DN, Liu Y, et al. Endogenous Nkx2.2+/Olig2+ oligodendrocyte precursor cells fail to remyelinate the demyelinated adult rat spinal cord in the absence of astrocytes[J]. Exp Neurol,2005,192(1):11—24.

[9]   Fang Z, Duthoit N, Wicher G, et al. Intracellular calcium –binding protein S100A4 influences injury–induced migration of white matter astrocytes[J]. Acta Neuropathol (Berl), 2006,111(3): 213—219.