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CALPAIN CLEAVED-55kDa N-TERMINAL HUNTINGTIN DELOCALIZES FROM NEURONS TO ASTROCYTES AFTER ISCHEMIC INJURY

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Abstract

The huntingtin (htt) mutation causes a polyglutamine expansion in the N-terminal region of protein. Mutant N-htt proteolytic fragments aggregate and cause cell death in Huntington's disease (HD). The normal huntingtin also can be cleaved by calpain and produce N-terminal htt fragments following ischemic injury, but the fate of cleaved fragment in dead neurons in the brain are unclear. To determine the localization of huntingtin following proteolysis, we examined htt expression after transient ischemic injury. Huntingtin immunoreactivity in mixed cultures of neuronal and astrocytes-derived clonal cells showed alteration of immunoreactivity from neurons into astrocytes. In the brain, both focal and global ischemia induced reactive astrocytes that were co-immunoreactive for huntingtin with elevated GFAP expression. The immunoreactive huntingtin was 55kDa calpain-cleaved N-terminal fragment, which appeared initially in the process, and extended into the cytoplasm of astrocytes. The results showed, after ischemic injury, huntingtin accumulated in astrocytes indicating that astrocytes may play a role in uptake of cleaved N-htt fragments.

Key words: GFAP expression, Huntington's disease, N-terminal huntingtin, ischemia, immuno-reactive astrocytes.

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Abbreviations: htt: huntingtin; **HD**: Huntington's disease

INTRODUCTION

The gene for Huntington's disease (HD) causes a polyglutamine expansion in the N-terminal region of huntingtin (N-htt) (1, 22). Huntingtin is highly enriched in neurons but the role of huntingtin in neurons is unknown. Studies point to potential involvement in membrane transport, cytoskeletal stability and nuclear functions (3). The expression of truncated mutant N-htt fragments causes the formation of nuclear and cytoplasmic aggregates, cellular dysfunction and apoptosis (6, 7, 11, 21, 25-27). Thus, it is of importance in determining how N-htt fragments are formed and accumulated in cells (12). Huntingtin is cleaved in the N-terminal region by caspases (14, 27, 31, 32) and calpains (17). Mouse brain extracts treated with calpain I or II express N-htt fragments of about 55 kDa and 65 kDa; these fragments are smaller than the caspase-cleaved fragments (17). Ischemic hypoxic injury also produced calpain-cleaved htt fragments which accumulate in the brain (15).

Since huntingtin is highly enriched in neurons, the main pathogenic process of huntingtin proteolysis and its accumulation has been known to take place in neurons. However, when the neurons undergo cell death and disappear, the fate of cleaved huntingtin fragments in dead neurons is unexplored.

In the brain, following ischemic injury, liquefaction necrosis or gliosis has been described. The reaction of astrocytes near these dying neurons with huntingtin fragment has been rarely described. Mutant huntingtin was known to suppresses the secretion and production of chemokines by astrocytes (5) or modulate the excitotoxicity (28). However, little is known about the expression of huntingtin in astrocytes. Our results is to determine the localization of calpain-cleaved huntingtin fragment following ischemic injury to show whether huntingtin fragments originated from neurons can be and accumulated in reactive astrocytes.

MATERIALS AND METHODS

Cell culture

To gain the indirect information whether huntingtin be able to transport from neuron to glial cells, a human neuroblastoma cell line, SK-SH-SY5Y and a human astrocyte-derived U393 glial cells were tested. The cultures were maintained in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% fetal bovine serum (FBS), 100U of penicillin and 0.1mg of streptomycin per ml at 37°C under 5% CO_2/95% air. The expression of huntingtin was evaluated by culturing neuronal or astroglial cells either separately or in mixed condition. Immunocytochemistries for huntingtin were performed before and after the ischemic insult.

In vitro ischemia experiment

The ischemic experiments were performed by incubating cells in a humidified hypoxic chamber. To induce hypoxia, cultures were incubated in a 95% N_2/4% CO_2/1% air atmosphere at 37°C. For glucose deprivation, serum-free culture media without glucose was used. Each or both cell lines were seeded in a 10mm culture plate with coverslip for the immunocytochemistry. The cells in the plate were maintained at 37°C in 95% air/5% CO_2 until 70% confluency was obtained. Starvation with DMEM containing 0.2% FBS were performed for the hypoxic condition and remained throughout the experimental course.

Experimental animal models

Adult male Sprague-Dawley rats were used to produce two experimental models of transient ischemic brain injury. The protocols and the use of animals in these procedures were approved by Seoul National University Animal Care Committee. In one group of rats (n=16), transient global forebrain ischemia was induced by clipping the bilateral carotid artery with hemorrhagic hypotension by withdrawing blood from the femoral vein (29). The hypotensive state was maintained for 10 minutes and the blood was reinfused. Animals were evaluated at 0, 1, 3 days and 1 week following ischemia. In the other group of rats (n=25), the transient left middle cerebral arterial occlusion model (20) was applied. After anesthesia by zolitel, the left common carotid artery was exposed and the branches from the external carotid artery (ECA) were coagulated. The pterygopalatine artery was ligated with a 5-0 silk suture. The ECA was transected, and a 5-0 nylon monofilament suture- its tip rounded by heating- was inserted into the ECA stump. Rectal temperature during surgery was maintained at 37 ± 0.5°C using a thermistor-controlled heating blanket. After 20 minutes of occlusion, the monofilament was removed. Reperfusion lasted 1-7 days, during which food and water were freely available to the animals. After the animals were sacrified, the area of ischemic infarction was confirmed by 2,3,7-triphenyltetrazolium chloride staining.

Immunohistochemistry

Rats were anesthetized and perfused through the heart with 4% paraformaldehyde in phosphate-buffered saline (PBS). The coronal sections were cut on a sliding microtome at 40 μm and incubated in a solution containing 10% normal goat serum (NGS), 1% bovine serum albumin (BSA), 0.2% Triton X-100, and 1% H_2O_2 in PBS. After washing three times in PBS, the sections were incubated in anti-huntingtin antibody Ab1 (1μg/ml) in 10% NGS and 1% BSA for 40 hours at 4°C. Ab1 is directed against the N-terminal region of huntingtin (Ab1; aa 1 – 17; a gift from Marian DiFiglia, MGH, Harvard Medical School). Labeling for huntingtin was detected by the immunoperoxidase method using D-amino-benzedine (DAB) as the substrate (Dako,Carpinteria, CA). Some sections were counterstained with hematoxylin and eosin (H and E) to determine the extent of neuronal loss. Anti-GFAP (glial fibrillary acidic protein, monoclonal, Sigma, St. Louis, MO) or anti-neurofilament (monoclonal, Sigma, St. Louis, MO) antibodies were used for double-labeling with Ab1. Cy3- or Bodipy-conjugated secondary antibody (Molecular Probe, Eugene, OR) was used for the detection of immunofluorescence staining. The labeled sections were evaluated with a laser confocal microscope (Biorad 1024, Richmond, CA).

Protein extraction from brain

Protein extracts from the brain were prepared as previously described (4, 8). Whole brain, one hemisphere (right or left), or isolated hippocampus were used. In brief, rats were deeply anesthetized, then rapidly decapitated. The hippocampus was dissected from rats with global ischemic injury of the brain. In rats with MCA occlusion of the left hemiphere, the brain was dissected into right (control) and left (ischemic) hemispheres. They were placed on ice in 10 volumes of cold homogenization buffer (50mM Tris, 120mM NaCl, pH7.4) with freshly added protease inhibitors (Complete Mini, Gibco, Grand Island, NY). The protein was aliquoted and stored at –70°C. Protein concentration was further determined by the Bradford method (Bio-Rad, Richmond, CA).

Western blot analysis

Protein extracts from brain tissue (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein separation was performed using 7.5% polyacrylamide with 1% bis-acrylamide or 10% polyacrylamide with 0.05% bis-acrylamide, according to published procedures (2, 8). The proteins were transferred to nitrocellulose, and the blots were probed with anti-huntingtin antibody Ab1 (0.5 μg/ml). Anti-γ-actin antibody (Sigma-Aldrich, 1:5000 dilution, monoclonal) was used for internal control. Immunoreactivity

was detected by using enhanced chemiluminescence (Supersignal, Pierce, Rockford, IN). Autoradiographic films were exposed from one second to 30 minutes.

Neuronal extract treatment in cultured astrocytes

Neuronal cells were incubated for two hours under hypoxic condition. They were then harvested and centrifuged at 2000g for eight minutes after washing twice with PBS. The neuronal-extracts were prepared by suspending cells in PBS, subjecting them to three cycles of rapid freeze/thawing and isolation of the supernatant after centrifugation at 15000 g. The BCA Protein Assay Kit (Rockford, USA) was used for quantification of the total protein content of the extract. U393 astrocyte cells were incubated for one hour under hypoxic conditions and then treated with neuronal-extracts. In 48 hours, U393 astrocytes were stained with MAB5942, another anti-huntingtin antibody generated against N-terminal epitope (Millipore Corp., Bedford, MA, USA).

Detecting self-expressed huntingtin mRNA in astrocytes (in-situ hybridization)

Sprague-Dawley male rats with ischemic middle cerebral artery occlusion (MCAO) models were prepared. The tissue sections were collected into final steps of immersing to cryoprotection buffer (30% sucrose, 30% ethylene glycol, 50 mM PB, 0.02% DEPC) and stored at 20 °C until processing for hybridization. Then, tissue sections were rinsed in 0.1% DEPC-activated 0.1 M phosphate-buffered saline (PBS, pH 7.4). For tagging, FISH TagTM RNA Kit (Invitrogen, Carlsbad CA, USA) was used for detection. To detect the huntingtin mRNA, the previous reference was used (Trifonov, Houtani *et al.*, 2009). N-htt detection sequence was 3'- gccgcctcaa ccccctcagc cgccgcctca ggggcagccg ccgccaccac cgccgctgcc aggtccggcc gaggagccgc tgcaccgacc aaagaaggaa ctctcagcca ccaagaagga ccgtgtgaat cactgtctaa caatatgtga aaacattgtg gcacagtctc tcagaaattc tccagaattt cagaaactct tgggcattgc tatggaactg tttctgctgt gcagcgacga tgcggagtca gacgtcagaa tggtggctga tgagtgcctc aacaaagtca tcaaagcttt gatggactct aatcttccaa ggctacagtt agaactctat aaggaaatta aaaagaatgg - 5' respectively.

Image analysis

Images obtained by immunocytochemistry were analysed by a histogram of portion of the colour stained (Adobe photoshop Histogram). Standard deviation, mean, median, and pixels were measured to see the amount of signal from huntingtin immunoreactivities in each cell and compared with control group, to see the delocalization of huntingtin fragment to glial cells.

RESULTS

The altered huntingtin immunoreactivity between neuronal and glial cells following in vitro ischemia

To determine whether huntingtin expression can be altered between neurons and glial cells, huntingtin immunocytochemistry was done by culturing each or mixed cell lines with or without hypoxia. Following ischemia, neuronal cells showed a decrease in the immunoreactivity of huntingtin, whereas glial cells alone did not (Fig. 1-A). In the mixed cultures with neuronal and glial cells, huntingtin immunoreactivities were increased in glial cells, whereas those of neurons were decreased. Distribution of huntingtin in glial cells showed a punctuate-round appearance without clustering. A few vacuole-like structures resided in the processes of astrocytes while the others appeared around nuclei (fig. 1-B). Imaging analysis of huntingtin increased in glial cells by three-folds following ischemia, with the mean signal values from 20.65 to 57.16, while those of neurons were not significant between single and mixed culture (fig. 1-C).

Huntingtin immunoreacitivy increased following transient global cerebral ischemia

Following hypoxia, a progressive neuronal loss in the CA1 area of the hippocampus began to appear at day 3, and by the 7th day, most neurons appeared to be affected. Huntingtin immunoreactivity was detected in cytoplasm and processes of cells in the CA1 area, which were widely distributed with glia-like appearance. The number of huntingtin (+) cells increased with time from day 0 to day 7 (fig. 2-A). Following transient global ischemia, the immunoblot showed a increase of 55kDa-sized N-htt fragments, whereas the expression level of 350 kDa sized-full length huntingtin reduced (Fig. 2-B).

Huntingtin expression after focal cerebral ischemic injury

Transient focal ischemic injury to the left hemisphere caused neuronal death in the affected brain at 1 to 2 days after reperfusion (fig. 3-A). In an immunohistochemistry of the ischemic brain, the increased N-terminus huntingtin immunoreactivities were detected. Cells appeared to have morphological feature of reactive glia (Fig. 3-B, C). The number of huntingtin (+) cells began to appear as early as day 1 and increased over time. Huntingtin immunoreactivity appeared mainly in the processes of these cells after one to three days of reperfusion and occurred in cell bodies at day 7 following reperfusion (Fig. 3-D). To further determine the non-specific staining of anti-huntingtin antibody in the ischemic brain, sections from ischemic brain with 7 days reperfusion were processed for without the huntingtin antibody (Ab1). In addition, preabsortion control with Ab1 in the presence of a peptide encoding 1-17 amino acids of htt was performed, which did not show any staining in cells (Fig. 3-D). Following transient focal brain ischemic injury, levels of full-length htt was reduced, while the levels of the 55 kDa N-htt

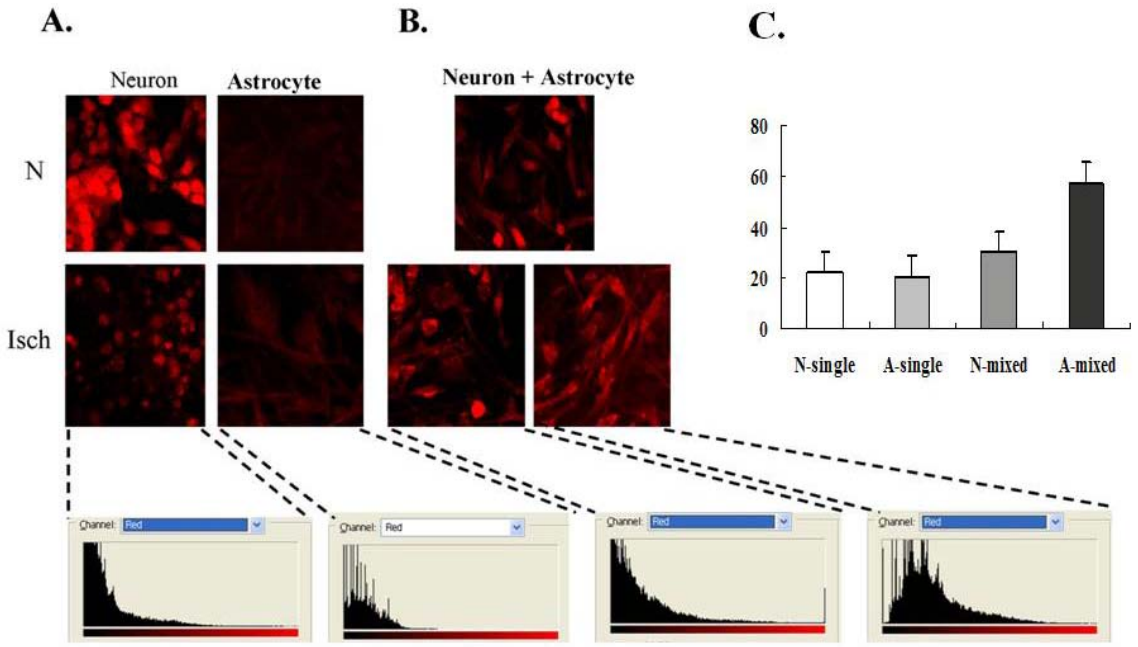

Figure 1. Delocalization of Huntingtin immunoreactivity following *in vitro* ischemia. Huntingtin immunoreactivity in human neuronal cells shows dense or fragmented appearance following hypoxic condition. Huntingtin immunoreactivities in glial cells were minimal, thus ischemic insult did not show difference (A). However, in mixed neuronal and glial cell culture, hypoxic insult increased huntingtin immunoreactivity in glial cells, whereas those of neuroblastomas did not show significant changes (B). The order of imange analysis represent neuronal cells alone (N-single) vs. glial cells alone (A-single) vs. mainly neuronal cells population in mixed culture (N-mixed) vs. mainly glial population in mixed culture (A-mixed). The huntingtin immunoreactivity of glial cells in mixed culture increased approximately by three fold, when compared to that of glial cells (C).

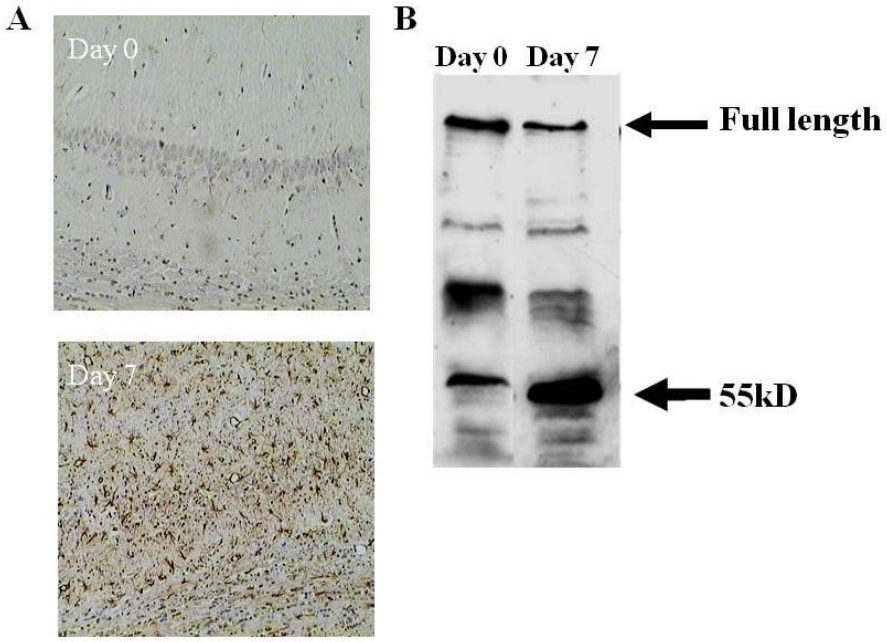

Figure 2. Transient global ischemia in hippocampus. At day 7, huntingtin immunoreactivities (Ab1) in CA1 area is increased when compared to those of day 0. These immunoreacitivities are widely distributed, rather than restricted to the pyramidal cell layer. At day 7, most neurons in CA1 area are lost or delocalized. Background is counterstained with hematoxylin (A). Western blot probed with Ab1 antibody in transient global ischemia increased signal of 55kD sized N-terminal fragment of huntingtin following ischemic insult (B).

fragment were increased compared to the control brain (Fig. 3-E). GFAP expression increased, whereas γ-actin expression for internal control was relatively unchanged (Fig. 3-E).

Huntingtin was co-localized with reactive astrocytes after ischemic injury

When double labeling of huntingtin with anti-neurofilament or anti-GFAP, most of cells with ab1 (+) showed positive for GFAP, a molecular marker for astrocytes (Fig. 4-C). The neurofilament labeling in the control brain disappeared following ischemic condition and were not able to detect neurofilament (+) cells or neural processes in the infarcted regions (Fig. 4-B). GFAP positive cells began to appear in 24 hours. The number of GFAP (+) cells increased over time following ischemic injury, both in global and focal cerebral ischemia.

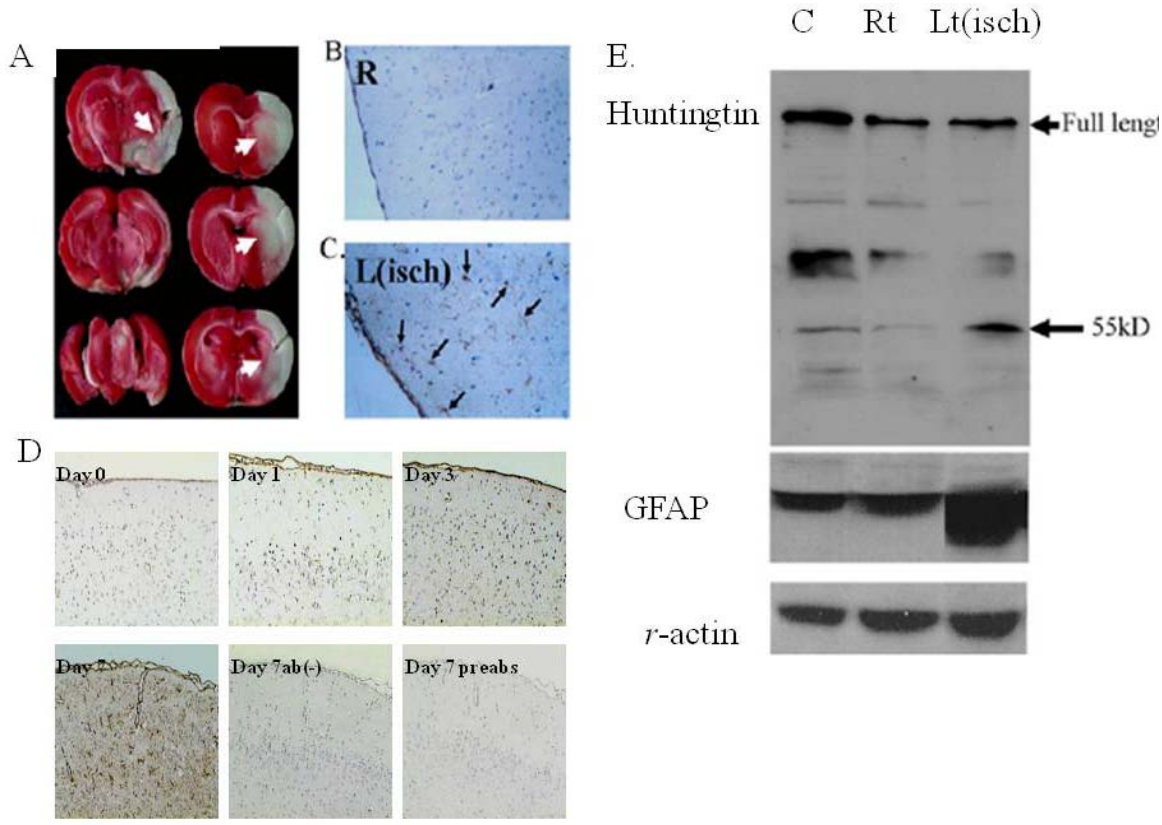

Figure 3. Coronal sections of brain stained by 2,3,7-triphenyltetrazolium chloride, demonstrating white area with ischemic infarction (white arrows, A). Transient focal ischemia (day 1) shows increased huntingtin immunoreactivities in the area with ischemia (arrows) (L) when compared with right (R) hemisphere without ischemic infarction (B, C). Huntingtin immunoreactivities increase over time. In the early stage, they began to appear in the processes (day 0, 1, 3), but extends into the cytoplasm at day 7. Controls treated 'without Ab1' or 'with Ab1 antibody in the presence of blocking peptide (preabsorption control)' do not show huntingtin labeling. Background is counterstained with hematoxylin. (D).Western blot probed with Ab1 revealed an N-terminal fragment at 55kD following ischemic insult. GFAP immunoreactivity in the ischemic side increased (E). (C: control, Rt: right (non-infarction area), Lt: left (infarction area), *r*-actin for internal control).

Huntingtin in the astrocytes is from neurons

To further determine from where the huntingtin in the reactive astrocyte is originated, *in-situ* hybridization of ischemic brain was performed to detect the expression of huntingtin mRNA in the astrocytes. In addition, incubation with huntingtin fragment in the astrocytes culture was also performed to know whether astrocytes can uptake this protein cleaved in the neurons. Neuronal extract treatment following hypoxic injury showed increased labeling of huntingtin in the processes asstocytes as well as in the perinuclear area(Fig. 5-A, B) The *in-situ* hybridization did not show any detectable htt mRNA in the ischemic brain (Fig. 5-C).

DISCUSSION

The purpose of this study is to determine the changes of localization of normal huntingtin proteolytic fragment following ischemic cerebral injury. Both in focal or global cerebral ischemia,

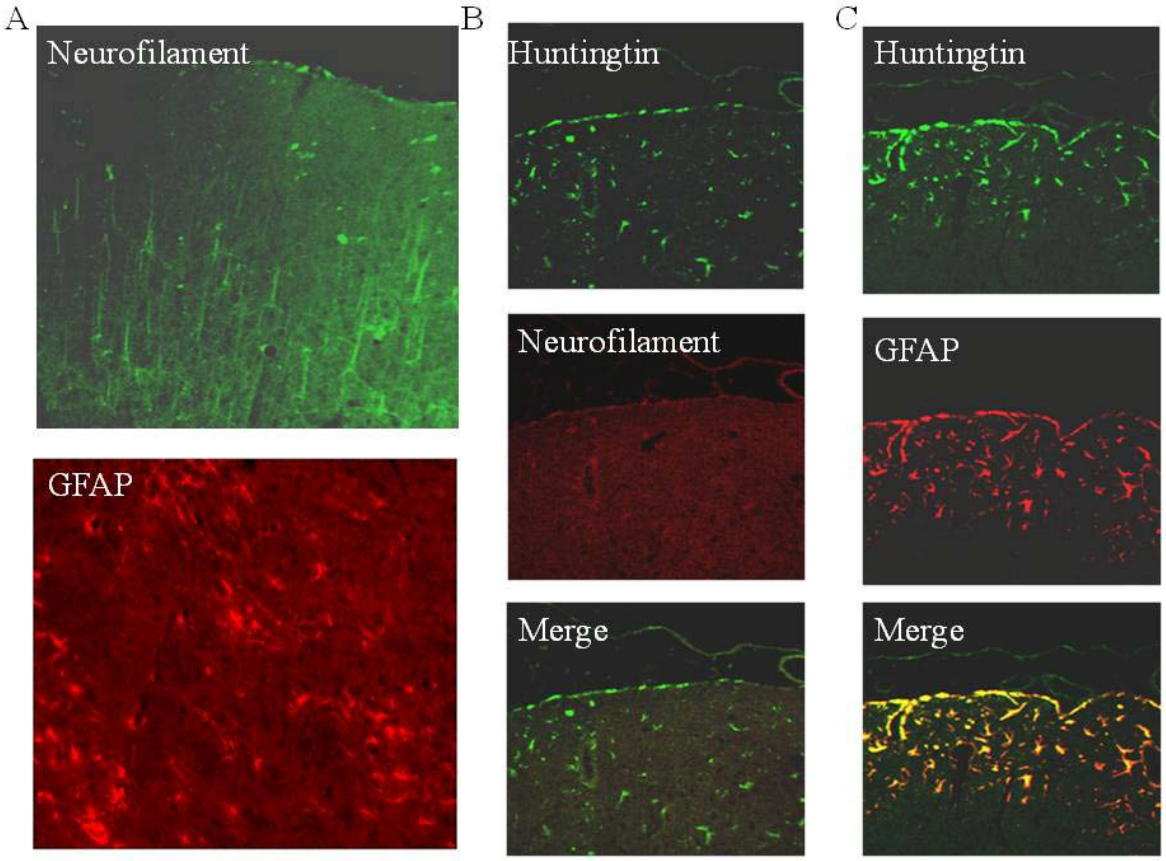

Figure 4. Double labeling with Huntingtin vs. GFAP or neurofilament. Neurofilament or GFAP staining in normal control tissue (A). Following the ischemia, the immunoreactivities for neurofilament disappeared (B) where as GFAP signal is activated (C). The cells with huntingtin immunoreactivity (Ab1) are co-localized with GFAP(+) cells (GFAP: glial fibrillary acidic protein).

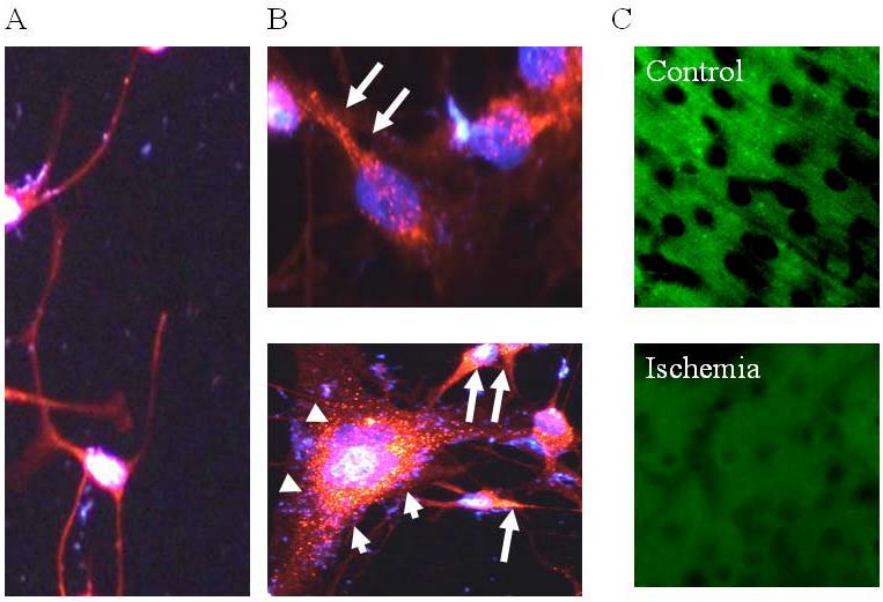

Figure 5. Huntingtin fragment absorption by glial cells and *in-situ* hybridization for huntingtin.
Following the incubation of 10 μg of neuronal extract, increased uptake of N-htt staining are observed in the processes of astrocytes (B, long arrows) and perinuclear area (B, short arrows), whereas such patterns is not detected in control condition (A). *In-situ* hybridization to detect huntingtin mRNA in the ischemic rat brain. Including astrocyte, there is no detectable signals comparing to the control condition which reveals a few punctuate huntingtin mRNA expressions (C). DAPI was used for nuclei staining.

huntingtin immunoreactivity steadily increased in astrocytes as time progressed. The level of 55 kDa-sized huntingtin fragment was also increased by ischemic injury. Huntingtin was detected in the astrocyte when damaged neuronal extract was treated. *In-situ* hybridization further suggest that these huntingtin were not self-expressed by astrocytes but rather absorbed.

Reactive astrocytes appear after ischemic injury (9) and may play multiple roles in the injured region (13). It is also becoming evident that astrocytes can contribute in neurodegenerative disorders. Amyloid-β42-positive material is reported within the activated astrocytes in Alzheimer's disease (33). In amyotrophic lateral sclerosis, accumulation of mutant superoxide dismutase 1 is also observed in astrocytes (30). In this study, we attempted to determine the expression of huntingtin in the reactive asctocytes following ischemic injury.

It is known that normal full-length htt increases in differentiating neurons *in vitro* (16) and in the brain (4). It is also known that accumulation of mutant huntingtin fragment can mediate neuronal death in Huntington's disease (6, 7, 11, 21, 25-27). However, distribution of normal huntingtin fragment or huntingtin other than neuronal cells has not been rarely described. Thus, in this study focused fragment of normal huntingtin as well as detection in the reactive astrocytes. To determine the origin of normal huntingtin fragment, *in vitro* ischemia model using the mixed culture or incubation of neural extract indicate that fragment of huntingtin can be re-absorbed into the astrocyte. Inability to detect huntingtin mRNA additionally support that, immunoreactivities in astrocytes were not the result of huntingtin expression in the astrocyte itself. No expression of huntingtin in the cultured glial cells before or after the ischemia also support this finding.

In the neurons, the immunocytochemical staining showed loss of huntingtin. In the mixed cell line cultures, there were also losses of huntingtin in degenerating neurons but increases of huntingtin staining in the glial cells. This finding also seemed to postulate a mechanism in which astrocytes might reabsorb the huntingtin cleaved from neurons. The presence of huntingtin immunoreactivity in reactive astrocytes after ischemia was unknown.

In our experiment, immunohistochemistry showed increased labeling of huntingtin over time, probably the amount of huntingtin may likely increased. The western blot showed the decreased level of the full-length huntingtin. This finding suggests that increased-huntingtin immunoreactivity cannot be the result of up-regulation of huntingtin. There was an increase in cleaved-huntingtin fragments, which seemed to accumulate in astrocyte and then caused the increase the huntingtin level in the astrocytes.

The main portion of 55 kDa huntingtin fragment was caused by calpain activation in the brain (15). Calpain activation after brain injury and during cell differentiation *in vitro* occurs mainly in neurons and not in glial cells (10, 19, 23, 24, 34). Calpain immunoreactivity was increased in neurons and not glial cells following global ischemic injury. Also, in a normal brain, glial cells have very low levels of htt mRNA and protein expression compared to neurons (4, 8, 18). Therefore, it is likely that the degradation of full-length htt and the production of the 55 kDa N-htt fragment seen in the brain after ischemic injury can be from neurons.

The function of huntingtin fragment in astrocytes is unknown. The huntingtin may be closely associated to inflammation-related chemotactic factors (5). This is assumed to activate the surrounding microglia, which may react by cleansing unwanted leftovers from hypoxic conditions, as there are reports that microglia is beneficial in improving hypoxic conditions.

In conclusion, ischemia causes the breakdown of endogenous full-length huntingtin in neurons. Also, calpain-cleaved 55 kDa N-htt fragments are additionally accumulated through peripheral sites in astrocytes, but not self-expressed, under ischemic environments. Our study is limited as the function of huntingtin and its fragments is not fully understood. Therefore, as brain is an organ that glial cells interact with neurons, it is indispensable to confirm further investigation, that clarifies the role of normal huntingtin in neuronal and glial biosystems, ahead of solidifying the mechanism of Huntington's disease.

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