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EVIDENCE FOR THE RECRUITMENT OF AUTOPHAGIC VESICLES IN HUMAN BRAIN AFTER STROKE

Tony Frugier¹, Juliet M. Taylor¹, Catriona McLean², Nicole Bye³, Philip M. Beart¹, Rodney J. Devenish⁵ and Peter J. Crack¹

¹Department of Pharmacology & Therapeutics, University of Melbourne, Parkville, Australia.
²Department of Anatomical Pathology, The Alfred Hospital, Melbourne, Australia
³Department of Anatomy and Neuroscience, University of Melbourne, Parkville, Australia.
⁴Florey Institute of Neuroscience and Mental Health, University of Melbourne, Parkville, Australia
⁵Biomedicine Discovery Institute and Department of Biochemistry and Molecular Biology, Monash University, Clayton campus, Victoria, Australia

Correspondence:
Peter J. Crack
Neuropharmacology Laboratory,
Department of Pharmacology & Therapeutics,
University of Melbourne, Parkville, 3010
Melbourne, Australia
Ph: +61 3 8344 8417
Email: pcrack@unimelb.edu.au

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ABSTRACT

Autophagy is a homeostatic process for recycling proteins and organelles that is increasingly being proposed as a therapeutic target for acute and chronic neurodegenerative diseases, including stroke. Confirmation that autophagy is present in the human brain after stroke is imperative before prospective therapies can begin the translational process into clinical trials. Our current study using human post-mortem tissue observed an increase in staining in microtubule-associated protein 1 light chain 3 (LC3), sequestosome 1 (SQSTM1; also known as p62) and the increased appearance of autophagic vesicles after stroke. These data confirm that alterations in autophagy take place in the human brain after stroke and suggest that targeting autophagic processes after stroke may have clinical significance.

KEY WORDS

Autophagy, stroke, human, brain, LC3, P62
1. INTRODUCTION

Stroke is the leading cause of long-term disability in adults and a major cause of death worldwide (Lozano et al., 2012). As a disease, stroke has no effective therapy beyond a small percentage of patients who receive thrombolytics. A major reason so few therapies are available is that while the cause of stroke is known, the underlying causes of neuronal death remain poorly understood. Strokes are largely ischemic, resulting from arterial occlusion that prevents perfusion at the core of the infarct and hypoperfusion at the margin of the blood vessels’ territory (penumbra). The extent of neurological damage following stroke and the severity of the neurological sequelae depends on the viability of the hypoperfused penumbra and, if the artery occlusion is transient, the reperfusion that follows. Reperfusion triggers molecular pathways leading to programmed cell death (PCD), neuronal loss and consequent disability (Taxin et al., 2014). Additionally, neuronal function is compromised in ischemic injury by energy deficits and consequently damaged proteins, and this debris generation leads to recruitment of cellular protein degradation systems including autophagy (Luo et al., 2013).

Autophagy and the role that it plays in the progression of neuronal injury is an emerging process in the understanding of stroke (Luo et al., 2013; Puyal et al., 2013). Autophagy can be activated in cytodestructive and cytoprotective modes, dependent upon the insult, and autophagic cell death is documented in brain tissue (Higgins et al., 2011; Puyal et al., 2013). The conundrum being that autophagy can be protective in neonatal hypoxic-ischemic injury (Li et al., 2010)(Koike et al., 2008) and in models of preconditioning (Sheng et al., 2010), yet cytodestructive in models of middle cerebral artery occlusion (MCAO) (Wen et al., 2008; Shi et al., 2012). Such observations support the concept that the “load” of cellular debris and damaged proteins, as influenced by stroke severity, determine the mode of autophagy and its
recruitment, and the pattern of PCD. Despite several studies analyzing autophagy in animal models of stroke, there are no published data on human brain tissue. Therefore in the present study we used *post-mortem* brain tissue from patients with a history of stroke to document for the first time the expression and cellular localization of three autophagic markers, namely microtubule-associated protein 1 light chain 3 (LC3), sequestosome 1 (SQSTM1; also known as p62) and Beclin 1 (BECN1).
2. MATERIALS AND METHODS

2.1 Human post-mortem brain tissue

All procedures were conducted in accordance with the *National Statement on Ethical Conduct in Human Research* (2007) of the Australian National Health & Medical Research Council, the Victorian Human Tissue Act 1982, the National Code of Ethical Autopsy Practice and the Victorian Government Policies and Practices in Relation to Post-Mortem.

Brain samples from 5 individuals who suffered stroke were obtained from the National Neural Tissue Resource Centre of Australia. Cases were aged between 66 and 90 years (mean 82 years). The post-mortem intervals varied between 8.5 and 28.5 hours (mean 19.5 hours). It is important to emphasise that every patient in this study presented a variety of associated diagnosis (see Table 1 for details). The identification of the infarct regions was performed post-mortem, secondary to the main diagnosis, by a neuropathologist (Prof. Catriona McLean), with the age of infarct ranging from 24-48 hours to 2 to 4 weeks prior to death. Serial cryo-sections were used to directly compare the haematoxylin and eosin staining (serial 1) with the immuno-stained section (serial 2) to clearly identify and cross compare to the area of ischaemic damage. As the immuno-stained sections were counterstained it was also possible to determine the area containing a loss of neurons, thereby supporting the direct cross comparison of ischaemic area as seen on the haematoxylin and eosin sections and confirming the location as being in the correct area. For every patient an area without any sign of infarct was used as internal control.

Since it is challenging to determine areas of infarct in frozen brain tissue the biochemical analysis was performed on brain samples from grey matter cortical tissue in the vicinity of the areas of ischaemic damage identified by haematoxylin and eosin staining (as
described above). Four out of the five cases were suitable for this analysis (Table 1- cases S1 to S4).

Control brain samples of 4 individuals, aged between 64 and 90 (mean 80 years), without stroke or other neuropathology were also obtained from the National Neural Tissue Resource Centre of Australia. Clinical information and epidemiological details of all patients are described in Table 1.

2.2 Immunohistochemistry

Human brain paraffin embedded sections (7 µm) were examined to determine the expression and localization of LC3 and SQSTM1 using methods previously described on human postmortem brain tissue (Frugier et al., 2010). Sections were preheated for 30 minutes at 60°C and dewaxed by two successive 5-minute baths in xylene before microwave treatment for antigen retrieval. Endogenous peroxidase activity was inhibited with a phosphate-buffered saline (PBS) solution containing 3% H2O2 for 5 minutes at room temperature. Then, the sections were blocked for 1 hour at room temperature in the following solution: 5% normal serum, 1% bovine serum albumin, 0.1% Tween20 in PBS. Sections were incubated overnight at 4°C with the primary antibody (1:200) overnight at 4°C and then incubated with the appropriate secondary biotinylated-antibody (1:500) for 1 hour at room temperature. This step was followed by the addition of the ABC kit reagents (Vector Laboratories) for 30 minutes at room temperature before being visualized using 3, 3’-diaminobenzidine (Vector Laboratories).

2.3 mRNA analysis

Total RNA was extracted from fresh frozen brain cortex tissue (100 mg) using a TRIzolPlus RNA purification kit (Life technologies). The concentration and purity of the
RNA samples were assessed using a Nanodrop1000 spectrophotometer (Thermo Fisher Scientific) while the RNA integrity was assessed using the Agilent 2100 bioanalyzer (Agilent Technologies). The total RNA fractions of each sample were then converted to cDNA using SuperScript III reverse transcriptase (Life technologies) and oligo d(T)$_{20}$ as primer, and taken as template for real-time quantitative polymerase chain reaction (Q-PCR) to compare gene expression following stroke. Q-PCRs were carried out using TaqMan Universal master mix and the 7900HT Fast Real-Time PCR system (Applied Biosystems). Four control genes were used to calculate the normalization factor needed to achieve relative quantitation by applying the comparative C$_{T}$ method ($\Delta$C$_{T}$): Peptidylpropyl isomerase A (PPIA), Hydroxymethylbilane synthase (HMBS), Ubiquitin C (UBC) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The following 9 TaqMan gene expression assays (Applied Biosystems) were used in this study: Hs001777654_m1 (SQSTM1), Hs00186838_m1 (BECN1), Hs00738808_m1 (MAP1LC3 alpha), Hs00917683_m1 (MAP1LC3 beta), Hs01374916_m1 (MAP1LC3 gamma), Hs99999904_m1 (PPIA), Hs00609297_m1 (HMBS), Hs00824723_m1 (UBC) and Hs99999905_m1 (GAPDH).

2.4 Western Blot analysis

Fresh frozen cortical brain samples (100 mg) were homogenized using an ultra-turrax (Ika, Wilmington, NC) in 400 ml of lysis buffer (Tris 5 mM, NaCl 15 mM, 1% Triton X-100, 2% SDS) then sonicated for 10 seconds. Homogenates were placed on ice and on a shaker for 90 minutes then centrifuged at 2000 g for 10 minutes at 4°C. As determined by Bradford assay, 50 µg of total protein were separated on 12% polyacrylamide gels in running buffer (25 mM Tris, 200 mM glycine, 0.1% SDS) and electrophoretically transferred onto nitrocellulose membrane in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked for 2 hours in Tris buffered saline (TBS: 0.05 M Tris pH= 7.5, 0.15
M NaCl, 0.1% Tween-20) containing 5% skim milk powder and incubated with primary antibodies overnight, followed by a secondary antibody linked to horseradish peroxidase (HRP-anti-rabbit IgG (P0448, Dako), HRP-anti-mouse IgG (P0447, Dako) or HRP-anti-goat IgG (P0449, Dako): 1:2000). The blots were developed with ECL (Amersham) and images were captured with the Chemidoc MP imaging system (Bio-rad). The primary antibodies used were mouse anti-p62 lck ligand purified monoclonal antibody (1:1000, Clone 3/P62 LCK LIGAND, BD Biosciences), rabbit anti-LC3 purified polyclonal antibody (1:1000, PM036, MBL laboratories), goat anti-BECN1 purified polyclonal antibody (1:1000, SC10086, Santa Cruz Biotechnology) and mouse anti-β actin purified monoclonal antibody (1:20000; Sigma). Densitometry using ImageJ (National Institutes of Health, USA) was performed on three separate experiments. All quantitation was standardized against β-actin levels.

2.5 Statistics

Statistical analysis was performed using Prism (GraphPad) software. Two-tailed t test analysis was used and statistical significance was considered at the 5% level (p<0.05).
3. RESULTS

3.1 Strong LC3 staining and clear depiction of vacuoles in human brain tissue after stroke

In order to identify autophagy following stroke in humans, we first examined post-mortem brain tissue for the autophagosomal marker LC3 by immunohistochemistry (Fig.1). Brain regions with an infarct, including frontal cortex, occipital lobe and basal ganglia, were visualized following haematoxylin and eosin staining (Fig.1D and G), and identified by a neuropathologist (Prof McLean). As expected, LC3 was present and evenly distributed in most cortical neurons of control individuals (Fig.1B). In marked contrast, in sections where an infarct was clearly identified, numerous neurons show a strong and dense LC3 staining (Fig.1D-I). Interestingly, these strongly positive neurons were clustered along the infarct region in patients presenting acute infarction while this distribution was not observed in the cases with older infarct (Fig.1D-I).

Another interesting observation was the clear depiction in neurons of immunolabelled vacuoles, a hallmark of autophagy, in sections processed for LC3 (Fig.1J-K) from cases presenting an infarct. Neurons with vacuoles were present throughout the sections independent of their proximity to an infarct region. However, more neurons with vacuoles appear to be present in sections with an old infarct. Nevertheless, neurons with vacuoles were observed in every stroke case in this study.

3.2 Increased SQSTM1 staining in human brain tissue after stroke

To confirm the presence of autophagy following stroke in humans, we examined the same post-mortem brain samples for the autophagy receptor SQSTM1 (Fig.2). As for LC3, SQSTM1 was consistently present in most cortical neurons of control individuals (Fig.2B) as
well as in sections without an infarct from the stroke patients (Fig. 2C). In contrast, in sections where an infarct was clearly identified, numerous neurons show a strong and dense SQSTM1 immunostaining (Fig. 2E-L). In contrast to LC3, these clusters of strongly SQSTM1 immunopositive neurons were present further away from the penumbra area and direct relationship with acute and old infarction was not observed. Once again, neurons strongly positive for SQSTM1 were observed in every stroke case in this study.

3.3 SQSTM1 mRNA and protein levels are increased in human brain tissue after stroke

In order to establish whether the increased LC3 and SQSTM1 staining in stroke patients corresponded to up-regulated protein and mRNA levels, we performed western blot analysis (Fig. 3) and quantitative reverse-transcription PCR (Fig. 4), respectively.

The immunoblots showed an 85% increase in SQSTM1 protein levels in the stroke group when compared to the control group (Fig. 3A). Consistent with this result, SQSTM1 mRNA levels showed a 70% increase in the stroke group when compared to the control group (Fig. 4A).

We were also able to detect LC3-II in the stroke and control groups using western blot (Fig. 3B). A prominent 18 kDa LC3-I protein was clearly detected in both stroke and control brain samples. A 16 kDa LC3-II protein was also detected in all stroke samples and in 3 out of 4 control samples analysed. However, we were unable to detect any difference between the two groups for both protein (Fig. 3B) and mRNA levels (Fig. 4C-D).

Finally we sought to analyse the expression of Beclin 1, another autophagic marker, in post-mortem brain tissue. As expected, we were able to detect both BECN1 mRNA and protein levels in stroke and control samples, but differences were not observed between these groups (Fig. 3C and 4B).
4. DISCUSSION

Autophagy is an evolutionarily conserved pathway that involves the sequestration/packaging and delivery of cytoplasmic material to the lysosomes, where proteins and lipids are degraded and recycled (Ravikumar et al., 2010). Dysfunction of autophagy has been implicated in neuronal cell loss in both acute and chronic neurodegenerative diseases (Chen et al., 2014) (Komatsu et al., 2006), but to the best of our knowledge this is the first report of autophagy in human stroke brain. Autophagy has previously been shown to be active in animal models of stroke leading to calls that this biological process is a viable candidate for therapeutic intervention with the outcome being neuroprotection. A limiting factor in such assessments has been the lack of evidence for the existence of autophagy in the human brain after stroke. There has been very little validation of the autophagic processes occurring in human brain owing to the scarcity of human brain tissue, both as control and pathologic samples. Previous studies have established that autophagy is altered in human brain samples that expressed Alzheimer disease pathology (Nixon et al., 2005) and autophagy has been shown to be detectable in the human brain after traumatic brain injury (Clark et al., 2008).

To gather evidence for changes in autophagy after stroke in humans, we examined postmortem samples for LC3 and SQSTM1 by immunohistochemistry, and SQSTM1, BECN1 and LC3 by western blot and qPCR. Brain samples of 5 individuals who had suffered a stroke were obtained from the National Neural Tissue Resource Centre of Australia, and matching control samples from 4 individuals who had died from a non-stroke related outcome were used. Characteristic autophagic vacuoles were clearly seen in the stroke samples and intense staining of LC3 was seen in the neurons of the infarcted area, which was the penumbral zone in the cortex. This finding is consistent with the increased staining for LC3 that is found in animal models of stroke (Adhami et al., 2006) (Wen et al.,
2008) and indicative of autophagic neuronal death. Matching this strong staining for LC3 in the infarct areas of the human samples was increased SQSTM1 staining. The detection of SQSTM1, a well characterized receptor protein in autophagy which is involved in the formation of protein aggregates destined for autophagic processing (Komatsu et al., 2007) strengthens our findings from the immunohistochemical data which are indicative of an increase in autophagic vesicles. This increased level of SQSTM1 at both the protein and message level maybe indicative of inhibition of autophagy targeted to particular of aggregates of debris. Interestingly, increased oxidative stress that is known to occur in stroke is also associated with increased levels of SQSTM1 (Nezis and Stenmark, 2012).

The immunohistochemical findings were also strongly supported by the western data that showed strong expression of SQSTM1 and the presence of LC3-II in the stroke samples. Interestingly we were able to detect BECN1 at both protein and RNA levels with no difference being found between the stroke and control tissues. This finding runs counter to what has been reported concerning BECN1 expression in animal models of stroke (Zheng et al., 2009; Shi et al., 2012), although our own data indicate its role in neuronal injury is controversial (Higgins et al., 2011). The lack of change in BECN1 may be seen as an indication that induction of autophagy in the human brain after stroke is not up-regulated and that the increases in LC3 and SQSTM1 may be indicative of reduced clearance of autophagosomes owing to pathway blockage. This lack of concordance between data from the human samples and that reported in the literature regarding BECN1 may also be attributable to the timing of the human sample collection, with BECN1 expression being required in the early phases of autophagy (Li et al., 2015). As the time after death of the post-mortem samples was varied (from 8.5 to 28.5 hours), it is important to note that the presence of both LC3 and SQSTM1 staining was seen across all samples, suggesting that the
initiation and progression of autophagy is present for the duration of the stroke process. Whether the autophagic process at these various time points is beneficial or deleterious cannot be determined from this study, but the determination of its existence is of importance if autophagy is to be considered a therapeutic target in the treatment of stroke.

In stroke injury our understanding of the involvement of autophagic/UPS systems is in its infancy, since little evidence has been gathered in accepted models of hypoxic-ischemic (HI) injury. Extant data, including our own, highlight that autophagy can be activated in cytodestructive and cytoprotective modes, dependent upon the insult (Higgins et al., 2011). Moreover, the mobilization of autophagy can be protective in neonatal HI (Li et al., 2010), yet cytodestructive in models of MCAO (Wen et al., 2008; Shi et al., 2012). Such observations support the concept that the “load” of cellular debris and damaged proteins, as influenced by stroke severity, determine the mode of recruitment of autophagy, and the pattern of PCD. Observations that either activation of autophagy by rapamycin or its inhibition by 3-methyladenine can have beneficial actions in HI (Liu et al., 2013) reflect the need for strategic analyses using well defined in vitro and in vivo models. Unlike most neuropathologies where there has been initial success with drug implementation (Harris and Rubinsztein, 2012), stroke has received little such attention.

Our data have determined for the first time that alterations in autophagy takes place in the human brain after stroke and in doing so validate the literature and the experimental findings in animal models that have defined autophagy as a cellular process that is initiated during stroke. While our work has firmly established for the first time the presence of LC3-positive vacuoles and elevated SQSTM1 expression it remains to be determined whether this is due to
increased autophagy or decreased lysosomal degradation (Puyal et al., 2013). However, the data could suggest that the observed increases in LC3 and SQSTM1 are indicative of blocked autophagic vesicle processing. It will be important to further define the involvement of LC3 and GABARAP isoforms in the autophagic process after stroke as emerging evidence suggests that distinct sets of cargo are utilized by these isoforms. The identification of those isoforms expressed in the brain and possible changes in their levels after stroke will give a clearer picture of the role of autophagy. Although there is still controversy over whether autophagy is of benefit or detrimental to the neurological outcome after stroke, the confirmation of the existence of altered autophagy in human samples will ultimately aid in the translation of compounds targeting autophagy into the clinical sphere as potential therapeutics to combat the cell death associated with stroke.
FIGURE LEGENDS

Fig. 1: LC3 immuno-reactivity in neurons of the stroke penumbra region in human post-mortem brain tissue. LC3 is evenly distributed in most cortical neurons of control individuals (B). In marked contrast, in sections where an infarct is clearly identified, numerous neurons show a strong and dense LC3 staining (E&F and H&I), clustered along the infarct region. Some neurons show a clear depiction of vacuoles (J&K). Haematoxylin and eosin staining was used to identify the infarct in the sections (A, D, G); i denotes infarct region delineated by the dashed line; negative control without the primary antibody (C); F: higher magnification of E; I: higher magnification of H; K: higher magnification of J; scale bar = 5 µm (except K = 0.5 µm).

Fig. 2: Increases SQSTM1 immuno-reactivity in human post-mortem brain tissue following stroke. SQSTM1 is present in most cortical neurons of control individuals (B) and in sections without an infarct from the stroke patients (C). In marked contrast, in sections where an infarct region is clearly identified (D), clusters of neurons showing a strong and dense SQSTM1 staining (E-L) can be seen. Contrary to LC3 staining, these clusters of positive neurons are mainly found further away from the penumbra area within the same sections (G-L). Haematoxylin and eosin staining was used to identify the infarct in the sections (D); i denotes infarct region delineated by the dashed line; negative control without the primary antibody (A); F: higher magnification of E; H: higher magnification of G; K: higher magnification of J; L: higher magnification of I; Scale bar = 5 µm

Fig. 3: SQSTM1, LC3 and BECN1 protein expression in post-mortem human brain following stroke. Western-blots (left) and densitometry analysis (right) reveal a
significant increase of SQSTM1 protein levels ($p=0.0303$, two-tailed $t$ test, mean +/- SEM) in human post mortem brain homogenates following stroke (A). Detection of LC3-II in the stroke and control post-mortem tissue (B). A prominent 18 kDa LC3-I protein is clearly detected in both stroke and control brain samples; 16 kDa LC3-II protein is also detected in all stroke samples and in 3 out of 4 control samples analysed. No difference in the LC3-II/LC3-I ratio is observed between the two groups. A 60 kDa protein, corresponding to BECN1, is clearly detected but no difference is observed between stroke and control groups (C). S1-4 and C1-4 refer to stroke and control samples respectively. Details outlined in Table 1.

**Fig. 4: SQSTM1, BECN1 and LC3 mRNA levels in post-mortem human cortex following stroke.** In the human post mortem brain samples of individuals with a history of stroke there is a significant increase in SQSTM1 mRNA levels compared with controls (A; $p=0.0017$, two-tailed $t$ test, mean +/- SEM). However, BECN1 (B) and LC3 (C and D) mRNA levels in stroke samples are similar to those of the control group. LC3B: MAP1LC3 isoform beta; MAP1LC3G: LC3 isoform gamma; MAP1LC3 alpha isoform was not detectable (not shown).
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REFERENCES


<table>
<thead>
<tr>
<th>Case</th>
<th>Age (y)</th>
<th>Sex</th>
<th>PMI (h)</th>
<th>Stroke pathology of the brain regions analysed</th>
<th>Cause of death and associated diagnosis</th>
</tr>
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<tbody>
<tr>
<td>S1</td>
<td>82.5</td>
<td>M</td>
<td>8.5</td>
<td>2 to 4 week infarct in the frontal cortex</td>
<td>History of multiple cerebrovascular accident (days to years), dementia, severe debility</td>
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<td>S2</td>
<td>66.2</td>
<td>M</td>
<td>28.5</td>
<td>2 to 4 week infarct in the right parietal cortex</td>
<td>Aspiration pneumonia (11 days), multiple sclerosis (28 years), urosepsis (11 days)</td>
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<td>S3</td>
<td>85.6</td>
<td>M</td>
<td>19.5</td>
<td>Acute infarct (24 to 48 hour) in the occipital cortex</td>
<td>Spinal cerebellar ataxia (years) and aspiration pneumonia (days)</td>
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<tr>
<td>S4</td>
<td>90.2</td>
<td>F</td>
<td>22</td>
<td>Acute infarct (24 to 48 hour) in the parietal cortex</td>
<td>Right middle cerebral artery infarct (6 days), atrial fibrillation (years), subclinical hyperthyroidism (years)</td>
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<tr>
<td>S5</td>
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<td>F</td>
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<td>M</td>
<td>24</td>
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<tr>
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<td>27</td>
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<td>M</td>
<td>32.5</td>
<td>No</td>
<td>Respiratory failure (2 days), pneumonia (7 days), chronic asthma (30 years), chronic renal failure (20 years)</td>
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</tbody>
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Table 1: Details of the stroke and control cases. Cases S1-S5: stroke cases. Cases C1-C4: control cases. All brains were obtained at autopsy. PMI, post mortem interval (time between death and brain retrieval); M, male; F, female; N/A, not available.
Highlights – Frugier et al.

- The first report of the presence of autophagy in the human brain after stroke.
- Observed the increased appearance of autophagic vesicles after stroke in the human brain.
- Observed an increase in staining in microtubule-associated protein 1 light chain 3 (LC3), sequestosome 1 (SQSTM1; also known as p62) in the human brain after stroke.