



## Research Paper

## Autophagy in neonatal hypoxia ischemic brain is associated with oxidative stress

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## ABSTRACT

Autophagy is activated when the neonatal brain exposed to hypoxia ischemia (HI), but the mechanisms underlying its activation and its role in the neuronal cell death associated with HI is unclear. We have previously shown that reactive oxygen species (ROS) derived from nicotinamide adenine dinucleotide phosphate (NADPH) oxidase play an important role in HI-mediated neuronal cell death. Thus, the aim of this study was to determine if ROS is involved in the activation of autophagy in HI-mediated neonatal brain injury and to determine if this is a protective or deleterious pathway. Initial electron microscopy data demonstrated that autophagosome formation is elevated in P7 hippocampal slice cultures exposed to oxygen–glucose deprivation (OGD). This corresponded with increased levels of LC3II mRNA and protein. The autophagy inhibitor, 3-methyladenine (3-MA) effectively reduced LC3II levels and autophagosome formation in hippocampal slice cultures exposed to OGD. Neuronal cell death was significantly attenuated. Finally, we found that the pharmacologic inhibition of NADPH oxidase using apocynin or gp91ds-tat decreased autophagy in hippocampal slice cultures and the rat brain respectively. Thus, our results suggest that an activation of autophagy contributes to neonatal HI brain injury this is oxidative stress dependent.

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## 1. Introduction

Neonatal hypoxia–ischemia (HI) brain injury may occur prior to, or during, delivery. The neuronal impairment associated with HI has important clinical consequences, including acute mortality, and long-term disability such as mental retardation, epilepsy, cerebral palsy, as well as blindness [1]. Despite advances in obstetric care, the incidence of cerebral palsy from perinatal hypoxia–ischemia is still greater than 2 in every 1000 births [2]. The collapse of cellular energy production, decreased tissue glucose metabolism and the development of cell injury in the HI brain are closely related [3,4]. However, the mechanisms underlying the neuronal damage and death triggered by HI in the developing brain, remains inadequately understood. Both apoptotic and necrotic neuronal cell death have been reported in animal models of HI brain injury. Our previous studies have demonstrated that superoxide generated from NADPH oxidase plays an important role in the increase in apoptosis in neonatal rat hippocampal slice

culture exposed to OGD and the HI rat brain [5,6].

However, alternative cell death programs, such as autophagy, have been receiving increased attention. Autophagy is now regarded as an important non-apoptotic cell death pathway [4,7]. Autophagy is a cellular catabolic process that contributes to quality control and maintenance of the cellular energetic balance through the turnover of protein and organelles in lysosomes. Induction of autophagy recruits proteins and lipids from different intracellular membranes that sequester cytoplasmic material and deliver it through vesicular fusion to lysosomes for degradation [8]. Autophagy occurs constitutively at a basal level, but can also be induced by both physiological and pathological stimuli. As autophagy can be stimulated in both the adult- and neonatal-rat it has been suggested it may contribute to ischemic neuronal injury [9,10]. However, the role of autophagy as a mediator of cell death is controversial. Some studies have shown that autophagy contributes to ischemic neuronal injury [11–13], whereas others have shown that the induction of autophagy plays a protective role and prevents neuronal cell death [14].

Thus, the purpose of our study was to investigate the role of autophagy in neonatal HI injury using both hippocampal slice

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cultures, prepared from neonatal rats, and the Vannucci model of HI in the P7 neonatal rat. Our data indicate that the activation of autophagy plays a role in the neuronal cell loss associated with OGD in slice cultures and the neonatal brain exposed to HI. Further, the activation of autophagy appears to be dependent on the generation of ROS.

## 2. Methods

### 2.1. Hippocampal slice culture and OGD exposure

Neonatal rats (Sprague-Dawley, Charles River, Wilmington, MA, USA) at postnatal Day 7 (P7) were decapitated and the hippocampi dissected under sterile conditions. Each hippocampus was sliced into 400  $\mu\text{m}$  slices using a Mcllwain tissue chopper (Science Products GmbH, Switzerland). Slices were then cultured on permeable membrane Millicell inserts (Millipore, Billerica, MA, USA) (0.4  $\mu\text{m}$  pore size) in six well plates for 6 days at 37 °C in 5%  $\text{CO}_2$  as previously described [5,6]. Twenty-four hours before exposure to OGD the culture medium was changed to neurobasal-A and B27 supplement minus antioxidants. Just prior to OGD, a sucrose balanced salt solution (SBSS) (120 mM NaCl, 5 mM KCl, 1.25 mM  $\text{NaH}_2\text{PO}_4$ , 2 mM  $\text{MgSO}_4$ , 2 mM  $\text{CaCl}_2$ , 25 mM  $\text{NaHCO}_3$ , 20 mM HEPES, 25 mM sucrose, pH of 7.3) was infused for 1 h with 5%  $\text{CO}_2$  and 10 L/h nitrogen gas. The inserts were then transferred into deoxygenated SBSS and placed in a ProOxC system chamber with oxygen controller (BioSpherix, NY, USA) and exposed to 0.1%  $\text{O}_2$ , 5%  $\text{CO}_2$ , 94.4% nitrogen for 90 min at 37 °C. The slices were then returned to oxygenated serum-free neurobasal medium with B27 supplement. The autophagy inhibitor, 3-MA (EMD Biochemicals, San Diego, CA, USA) or the autophagy activator rapamycin (EMD Biochemicals, San Diego, CA, USA) were dissolved in dimethyl sulfoxide (DMSO) and added to the medium 2 h before OGD. Control experiments contained the equivalent amount of DMSO that did not exceed 0.2% (v/v). All protocols were approved by the Institutional Animal Care and Committee at Georgia Regents University.

### 2.2. Rat model of neonatal hypoxia–ischemia

Using the well established Levine procedure for hypoxia–ischemia [15] modified by Rice et al. [16], postnatal day 7 rat pups were anesthetized with isoflurane (4% for induction; 3% for maintenance), and 20% oxygen at 1 L/min flow rate. For the duration of induction and surgery, a heated induction chamber and surgical bed, (Microflex EZ Anesthesia System, Euthanex, Palmer, PA, USA), maintained core body temperature between 35 and 36 °C. Rectal temperature was monitored continuously using a sensitive microprobe thermometer (Physitemp Instruments, Clifton, NJ, USA). The right common carotid artery was exposed and permanently occluded by electrical coagulation and the incision sutured. Sham operated pups received vessel manipulation without occlusion. Immediately after surgery, pups were placed in a temperature controlled recovery chamber for 15 min before being returned to the dam for 1–2 h. For induction of hypoxia–ischemia, pups were placed in a custom made, plexiglass, multi-chambered hypoxia device (Jarroll Manufacturing, St. Louis, MO, USA). The chamber water bath temperature was maintained at 37 °C and each chamber was infused with a calibrated mixture of warm, humidified 8% oxygen/balance nitrogen for 2.5 h at a flow rate of 100 ml/min. Oxygen concentration was monitored (Mini-Ox3000 oxygen analyzer, MSA Medical Products, Pittsburgh, PA, USA) and core body temperature of pups maintained between 35 and 36 °C throughout duration of hypoxia. Sham pups were also placed in chamber with exposure to room air only. Immediately after

hypoxia, pups were placed in a temperature regulated recovery chamber (36 °C), and allowed 1–2 h of recovery before being returned to the dam. Pups were euthanized and decapitated after 2- or 24-h reperfusion. The brains were quickly removed for further experiments. All protocols were approved by the Institutional Animal Care and Committee at Georgia Regents University.

### 2.3. In vivo gp91 inhibitor treatment

An in vivo NAPPH oxidase inhibitor [17], gp91 ds-tat and its inactive analog, scrambled gp91 ds-tat were commercially synthesized with a nine amino acid sequence coupled to an HIV viral coat “TAT” moiety (Biosynthesis Inc., Lewisville, Texas) to allow cell penetration. When internalized, gp91 ds-tat antagonizes the docking of the cytosolic NADPH oxidase subunit to its transmembrane component gp91<sup>phox</sup>. Day one of dam delivery was designated postnatal day 0 (PNO). On postnatal day 6 (PN6), 24 h before surgery, randomly sexed littermates (13–16 g body weight) were randomly assigned to receive vehicle, gp91 ds-tat or scrambled gp91 ds-tat peptide. The peptides were diluted in physiological saline and intraperitoneally injected at a dose of 10 mg/kg. Vehicle groups received an equivalent injection volume of saline. The following day of surgery, PN7, intraperitoneal injections, as described above, were also administered during the post surgical recovery period, 30 min before the induction of hypoxia–ischemia.

### 2.4. Transmission electron microscopy (TEM) evaluation

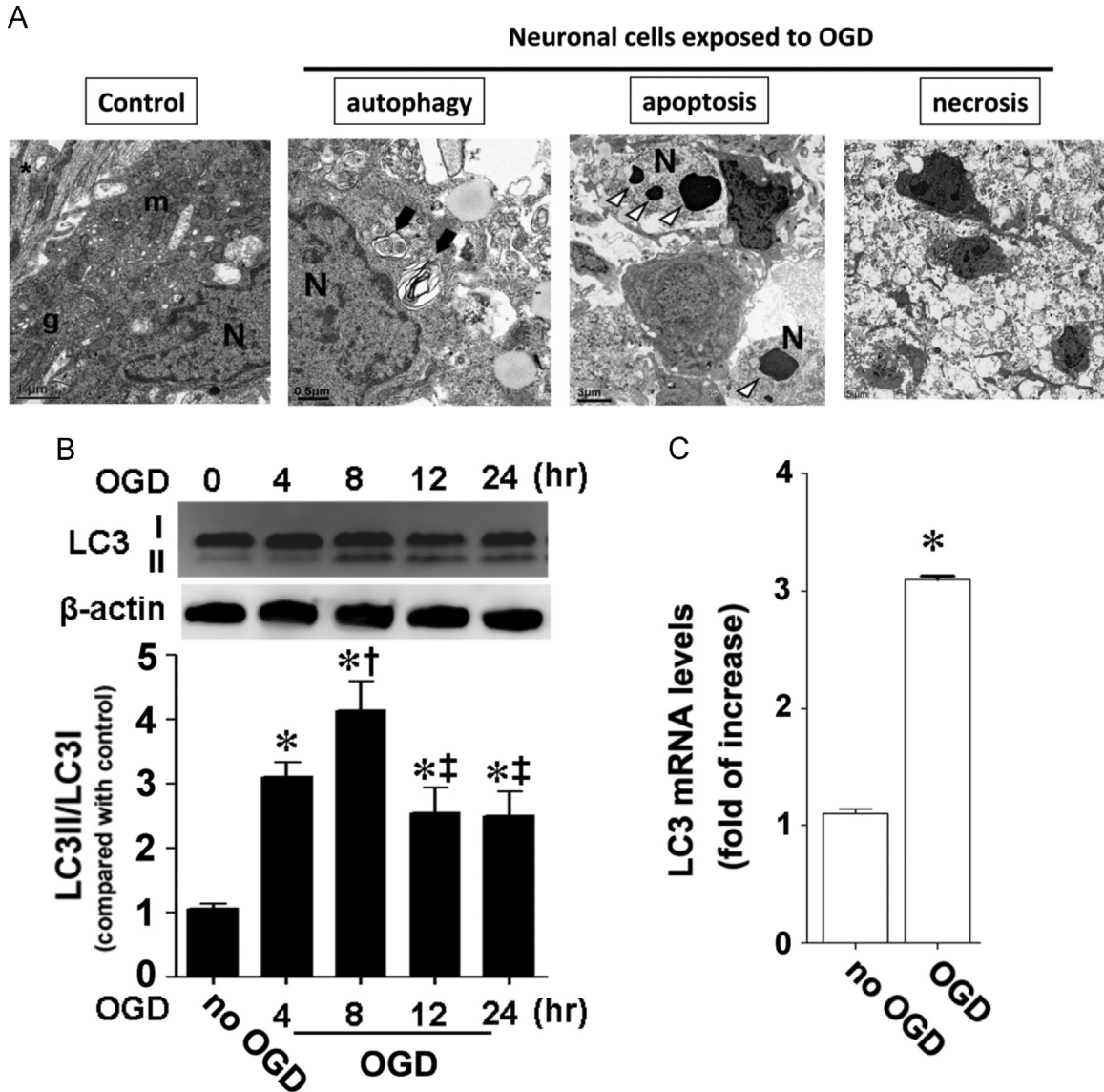
Tissue fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate (NaCac) buffer, pH 7.4, postfixed in 2% osmium tetroxide in sodium cacodylate (NaCac) buffer, stained with 2% uranyl acetate, dehydrated with a graded ethanol series and embedded in Epon–Araldite resin. Thin sections were cut with a diamond knife on a Leica EM UC6 ultramicrotome (Leica Microsystems, Inc., Bannockburn, IL), collected on copper grids and stained with uranyl acetate and lead citrate. Tissue was observed in a JEM 1230 transmission electron microscope (JEOL USA Inc., Peabody, MA) at 110 kV and imaged with an UltraScan 4000 CCD camera and First Light Digital Camera Controller (Gatan Inc., Pleasanton, CA).

### 2.5. Histologic evaluations

Slice cultures were washed in PBS, fixed in 4% paraformaldehyde (RT, 1 h), then in 30% sucrose (RT, 1 h), embedded in O.C.T embedding medium (Tissue-Tek, Sakura Fine technical, Tokyo, Japan) and stored at –80 °C overnight. Embedded tissues were sectioned (15  $\mu\text{m}$ ), mounted on glass slides and stored at –80 °C until used. In the rat HI brain model, pups were anesthetized with 75 mg ketamine/10 mg xylazine cocktail i.p., followed by transcardial saline extravasation and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were post-fixed for 24 h at 4 °C and cryoprotected in 30% sucrose before sectioning. Permeabilized sections were analyzed for the presence of LC3 antibody overnight at 4 °C, nuclei were stained with propidium iodide (PI) or DAPI. Quantification of autophagosomes was carried out by counting at least 50 cells in each slide. Quantification of slice culture cell death was carried out using PI staining 8 h after OGD and cell death was determined using fluorescent microscopy to measure PI uptake, as described [5,6].

### 2.6. Immunoblot analyses

Slice cultures were washed with ice-cold phosphate-buffered saline, homogenized in lysis buffer containing 1% Triton X-100, 20 mM Tris, pH 7.4, 100 mM NaCl, with 1  $\times$  protease inhibitor cocktail, and 1  $\times$  phosphatase inhibitor cocktail (Sigma, St. Louis,



**Fig. 1.** Autophagy is induced in rat hippocampal slice cultures exposed to oxygen glucose deprivation. Transmission electron microscopy identifies autophagosome formation (black arrow) in the neuronal cell as well as markers of apoptosis (white triangle) and necrosis, in hippocampal slice cultures 8 h after OGD. m, Mitochondria; N, nuclei; g, golgi apparatus. (A) Rat hippocampal slice cultures were harvested 4-, 8-, 12- and 24 h after OGD to determine LC3II protein (B) and mRNA levels (C). OGD increases both LC3II protein (B) and mRNA (C). Data are presented as mean  $\pm$  S.E from 4 independent experiments using 24 pooled slices per experiment. \* $P < 0.05$  vs. control, † $P < 0.05$  vs. 4 h OGD, ‡ $P < 0.05$  vs. 8 h OGD.

MO, USA). Lysates were centrifuged at 13,000g for 10 min at 4 °C to precipitate the debris, and the protein content in the supernatant determined using the Bio-Rad protein assay (Bio-Rad Laboratories, CA USA). Lysate protein (20  $\mu$ g/lane) was separated using 4–20% gradient gels (Thermo Scientific, Rockford, IL, USA) and transferred to PVDF membranes. The blots were then probed with the appropriate antibody overnight at 4 °C. Primary antibodies used were anti-LC3 (ab8982, Abcam Inc., Cambridge, MA, USA). Blots were washed in 1  $\times$  TBST (3  $\times$  15 min) and the appropriate secondary antibodies conjugated to HRP were then added for 1 h at RT (Thermo Scientific, Rockford, IL, USA). After further washing in TBST (3  $\times$  15 min) bands were visualized by chemiluminescence (West-Femto, Pierce, Rockford, IL, USA) and quantified using a Kodak Molecular Imaging System (Kodak, Rochester, NY, USA).

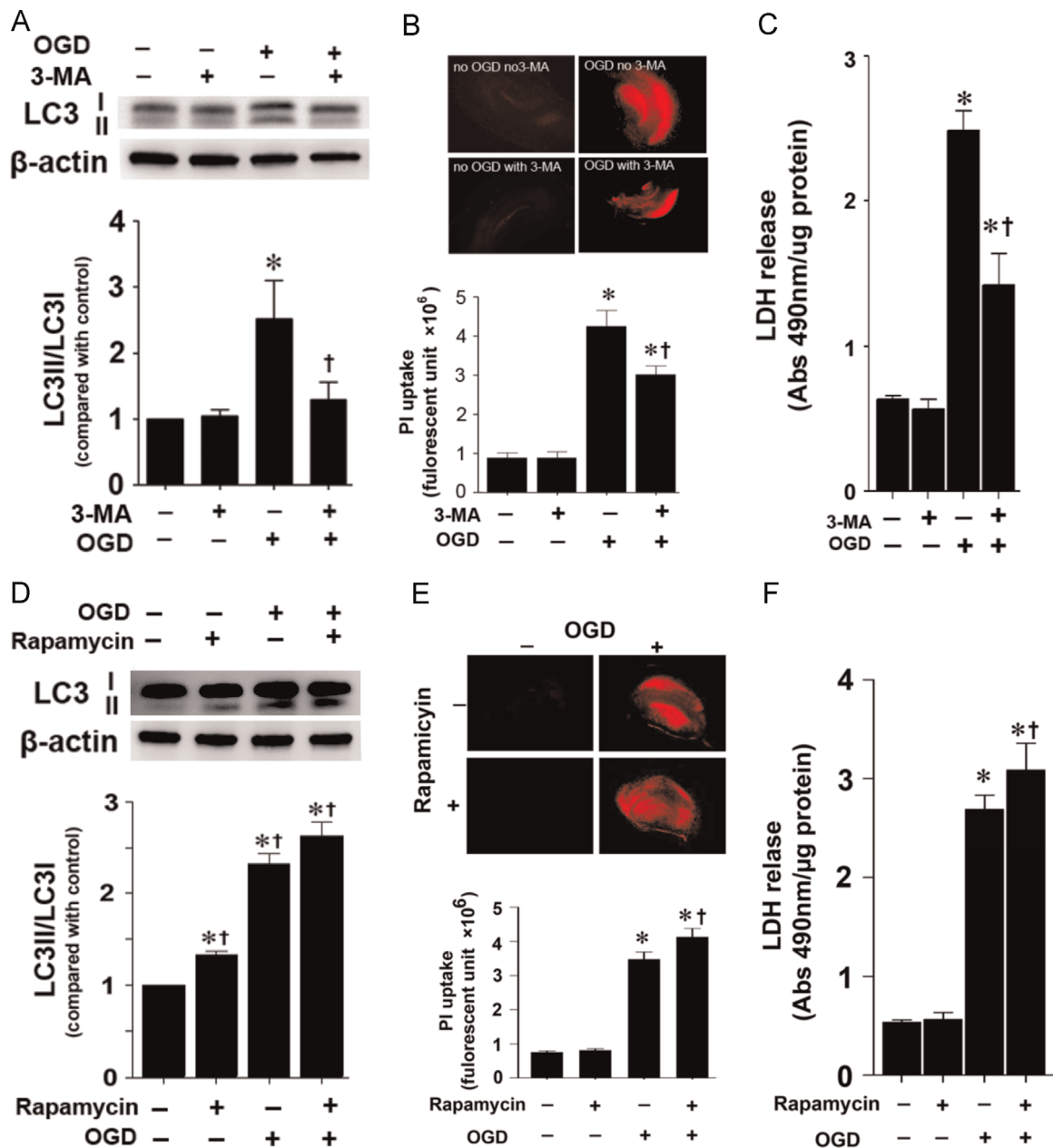
### 2.7. LDH cytotoxicity assay

Cytotoxicity was evaluated by quantification of lactate dehydrogenase (LDH) using a Cytotoxicity Detection Kit (Roche Applied

Science, Mannheim, Germany) in the slice culture medium as described [5,6]. Samples were analyzed 8 h after OGD. All LDH measurements were normalized using total protein levels (Bradford protein assay, Bio-Rad Laboratories, CA, USA).

### 2.8. Real-time RT-PCR analysis

Real-time RT-PCR was employed to verify the regulation of a list of genes of interest. Primers were designed by Primer 3. The primer sets utilized were LC3-left 5'-GG AGA TCT CGC AGG CCT AT-3'; LC3-right 5'-GGC CAG ATG TTC ATC CAC TT-3'; beta actin-left 5'-CCA CAG CTG AGA GGG AAA TC-3'; beta actin-right 5'-TGC CGA TAG TAG TAG CCT GA-3'. Real time RT-PCR was carried out in two steps. First, total RNA was extracted from cells (or tissues) using the RNeasy kit (Qiagen), and 1  $\mu$ g total RNA was reverse-transcribed using QuantiTect Reverse Transcription Kit (Qiagen, Hilden) in a total volume of 20  $\mu$ l. Quantitative real-time PCR was conducted on Mx4000 (Stratagene), using 2  $\mu$ l of RT product, 12.5  $\mu$ l of QuantiTect SYBR Green PCR Master Mix (Qiagen, Hilden),



**Fig. 2.** Modulation of autophagy alters neuronal cell death in rat hippocampal slice cultures exposed to oxygen glucose deprivation. Rat hippocampal slice cultures were exposed to OGD in the presence of the autophagy inhibitor 3-MA (5 mM, 2 h prior to OGD), or the autophagy activator, rapamycin (200 nM), both given 2 h prior to OGD. Slices were harvested 8 h after OGD and subjected to Western blot analysis to determine effects on LC3II (A and D), whole slice PI uptake (B and E) and LDH release levels (C and F). 3-MA attenuated LC3II protein levels (A), PI uptake (B) and LDH release (C). Rapamycin induced the opposite effects (D–F). Data are presented as mean  $\pm$  S.E from 4 independent experiments using 24 pooled slices per experiment. \* $P < 0.05$  vs. no OGD, † $P < 0.05$  vs. OGD only.

and primers (400 nM) in a total volume of 25  $\mu$ l. The following thermocycling conditions were employed: 95  $^{\circ}$ C for 10 min, followed by 95  $^{\circ}$ C for 30 s, 55  $^{\circ}$ C for 60 s, and 72  $^{\circ}$ C 30 s for 45 cycles. The threshold cycles (Ct) of a serially diluted control sample were plotted to generate a standard curve. Concentration of each sample was calculated by interpolating its Ct on the standard curve and then normalized to  $\beta$ -actin (housekeeping gene) mRNA levels.

### 2.9. Statistical analysis

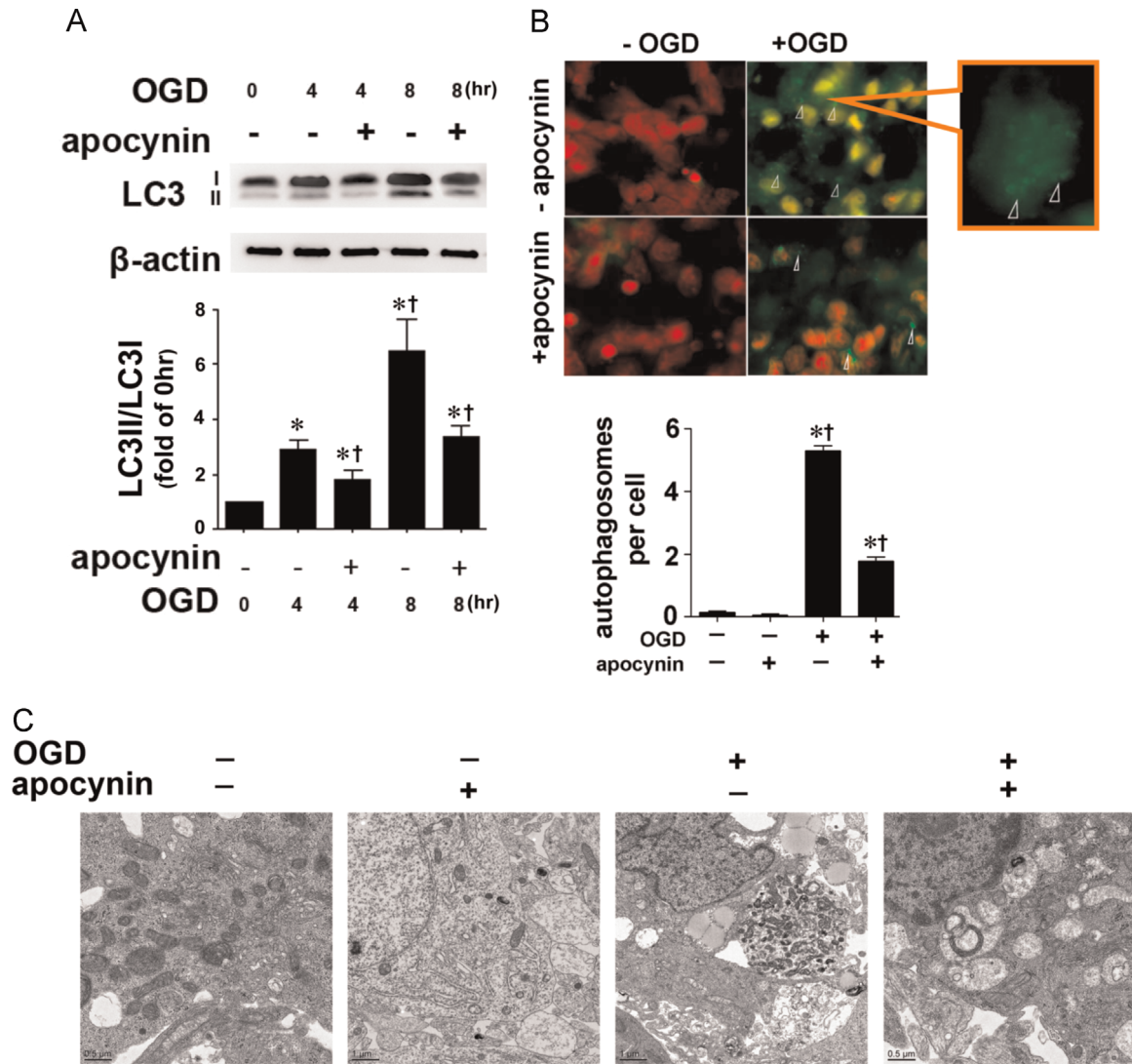
Statistical calculations were performed using the GraphPad Prism V. 4.01 software (GraphPad Software, Inc. La Jolla, CA). The mean  $\pm$  SD or SE were calculated for all samples, and significance was determined by either the Student's *t*-test or ANOVA with the Newman-Keuls or Bonferroni post hoc test. A value of  $P < 0.05$  was

considered significant.

## 3. Results

### 3.1. Autophagy is increased in neonatal brain hippocampal slice culture exposed to OGD

Hippocampal slice cultures were exposed to OGD and transmission electron microscopy (TEM) was initially used to evaluate markers of autophagy. Our data indicate that OGD induces autophagosomes, the vacuole of membrane structure in the cytosol (black arrow), as well as condensed chromatin in the nuclei indicative of apoptosis (white arrow). Evidence of necrotic cell death was also evident (Fig. 1A). Induction of autophagy was also verified



**Fig. 3.** NADPH oxidase inhibition reduces autophagy in OGD exposed hippocampal slice cultures. Rat hippocampal slice cultures were exposed to OGD in the presence of the NADPH oxidase inhibitor, apocynin (100  $\mu$ M), 2 h prior to OGD, then harvested at 8 h after OGD. Western blot analysis was used to determine the effect on the LC3II levels (A). Values are presented as mean  $\pm$  S.E from 4 independent experiments using 24 pooled slices per experiment. \* $P < 0.05$  vs. no OGD,  $\dagger P < 0.05$  vs. no treatment under OGD. Apocynin also reduced autophagosomes formation as determined using both fluorescent- (B) and transmission electron-microscopy (C). Values are presented as mean  $\pm$  S.E from 4 independent experiments. \* $P < 0.05$  vs. no OGD no treatment,  $\dagger P < 0.05$  vs. no OGD with treatment.

by Western blot analysis that identified a significant increase in the LC3II/LC3I ratio in hippocampal slice culture 8 h after exposure to OGD (Fig. 1B). This time point was then used for subsequent studies. OGD was also found to induce a significant increase in LC3 mRNA levels (Fig. 1C).

### 3.2. Inhibition of autophagy attenuates neuronal death in hippocampal slice culture exposed to OGD

The pre-treatment of hippocampal slice cultures, with the autophagy inhibitor, 3-MA, effectively reduced the LC3II/LC3I ratio (Fig. 2A). Neuronal cell death, estimated by PI fluorescence intensity, and LDH release (Fig. 2C) were also significantly reduced by 3-MA (B). Conversely, the autophagy inducer, rapamycin, increased both the LC3II/LC3I protein ratio (Fig. 2D) and neuronal cell death (E and F).

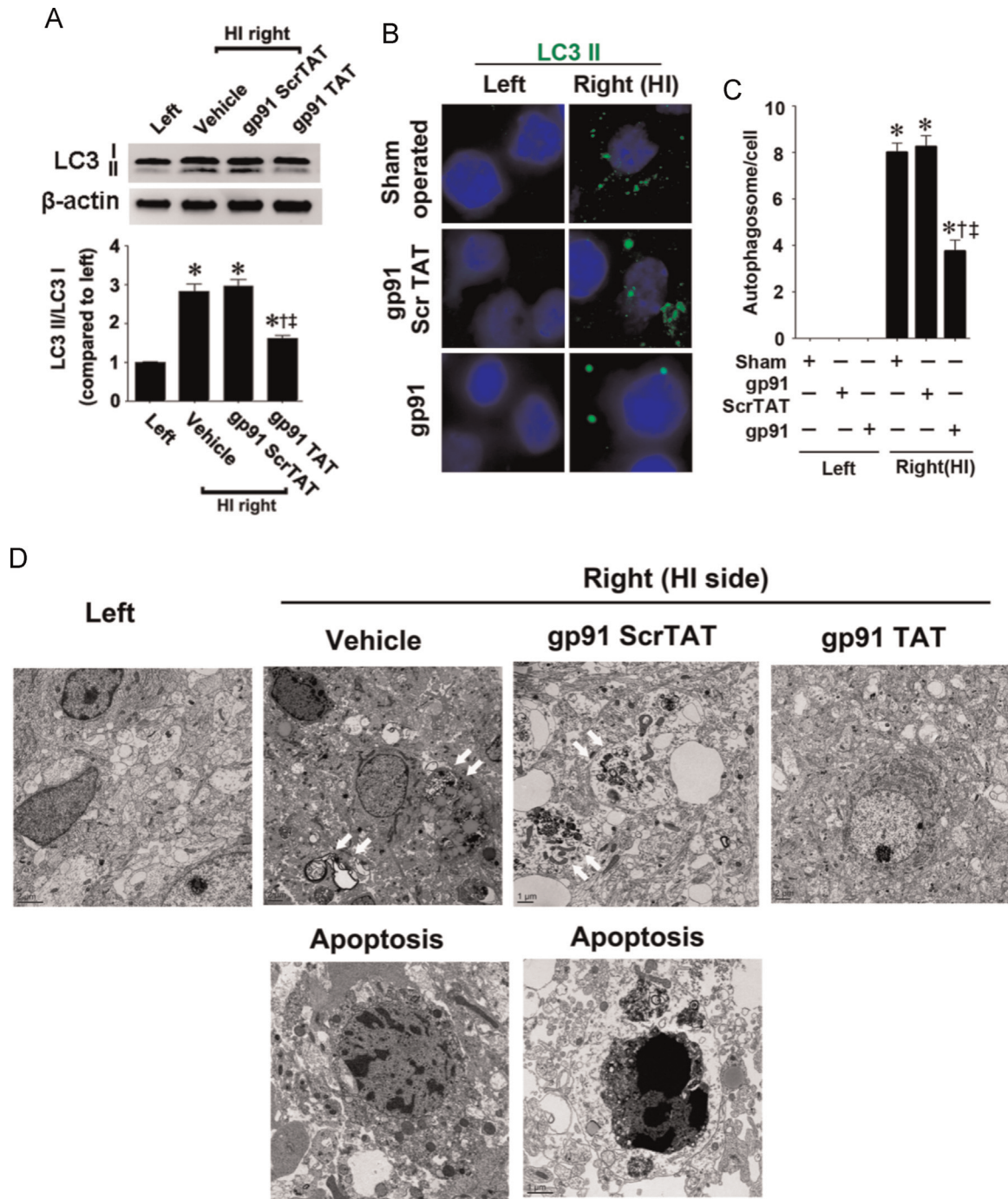
### 3.3. Inhibition of NADPH oxidase attenuates autophagy in hippocampal slice cultures exposed to OGD and the neonatal rat brain

exposed to HI

Next, we determined whether NADPH oxidase generated superoxide was involved in the activation of autophagy. The pre-treatment of hippocampal slice cultures, with the NADPH oxidase inhibitor, apocynin, effectively reduced the LC3II/LC3I ratio (Fig. 3A) and the number of autophagosomes per cell (B). Electron microscopy also confirmed that apocynin decreased the number of autophagosomes (Fig. 3C). Finally, we found that the administration of the Gp91<sup>phox</sup> docking sequence (ds) peptide, that attenuates the interaction of gp91<sup>phox</sup> (NOX2) with p47<sup>phox</sup>, reduced the LC3II/LC3I protein ratio (Fig. 4A). The number of autophagosomes was also reduced, as determined using both fluorescent microscopy (Fig. 4B and C) and TEM (D).

## 4. Discussion

Autophagy has been found to play a role in many fundamental biological process, such as aging [18], immunity [19], development [20,21], tumorigenesis [22] and cell death [23]. Autophagy



**Fig. 4.** NADPH oxidase inhibition attenuates autophagy in the neonatal rat brain exposed to hypoxia-ischemia. P7 neonatal rats were pre-treated with gp91ds-tat, the scrambled control peptide, or vehicle then exposed to HI. Two hours after HI, LC3II protein levels and tissue autophagosome formation were analyzed. There is a significant increase in the LC3II/LC3I protein ratio in the right hemisphere of the neonatal brain and this is attenuated by gp91ds-tat. The increase in autophagosome formation in the right hemisphere of the neonatal brain, as determined using fluorescent- (B and C) and transmission electron-microscopy (D), is also attenuated by gp91ds-tat. Values are presented as mean ± S.E from 6 animals per group. \**P* < 0.05 vs. left hemisphere, †*P* < 0.05 vs. HI + vehicle, ‡*P* < 0.05 vs. HI + ScrTAT.

maintains cellular homeostasis under basal conditions. It can also be activated under conditions such as excitotoxicity, starvation and hypoxia, as well as neurodegeneration. It has been suggested that autophagy is an adaptive mechanism that helps maintain cellular homeostasis during the early stage of disease in response to cellular stress. However, in certain experimental disease settings, the self-cannibalistic or, paradoxically, even the pro-survival functions of autophagy may become deleterious [24]. Because of their extreme polarization, size and post-mitotic nature, neurons may be particularly sensitive to the accumulation of aggregated or

damaged cytosolic compounds, or membranes, and thus depend on autophagy for their survival [25].

A growing number of studies focused on neurodegenerative diseases suggest that there may be a link between autophagy and brain injury. Early reports demonstrated that autophagosomes accumulate in the brains of patients with diverse neurodegenerative diseases [26]. In Huntington's disease, the autophagic pathway has been found to be upregulated while macroautophagy is impaired, at least in the early stages of the disease [27]. Recent studies suggest that the loss of basal autophagy or an imbalance in

autophagic flux can lead to neurodegeneration [28]. Unlike the chronic pathological process of neurodegenerative diseases, the pathophysiological changes associated with neonatal HI brain injury are counted in hours and days, rather than years. Glucose and oxygen deprivation, as well as ATP reduction occur within only a few minutes and induce a cascade of cellular reactions which last for hours to days and result in neuronal cell death. This has led to the idea that neuronal death occurs when the autophagic response becomes overwhelmed by the extent of the cellular damage, and as a consequence, loses its neuroprotective role [29]. Indeed, it has been shown that HI can activate autophagy in brain tissue and neuronal cells. Early TEM studies revealed distinct increases in the volume densities of both cathepsin B-immunopositive lysosomes and autophagic vacuole-like structures 3d after ischemic insult in hippocampus CA1 neurons [30]. The presence of apoptotic bodies in the nuclei suggested a link between autophagy and apoptotic neuronal death [30]. Autophagosomes have also been found in HI models in the adult rat [10,31], neonatal mouse [11] and neonatal rat [12,32]. LC3 is another important autophagy marker, as it is a constituent of autophagosome membrane [33]. Our data identified both increased autophagosome formation and LC3II protein levels in hippocampal slice culture exposed to OGD and the neonatal rat brain exposed to HI. This is in agreement with prior studies in which LC3 fluorescent intensity and LC3II protein levels were found to be increased in both neonatal and adult rodents after HI injury [11,32,34].

It should be noted that whether increased autophagic activity plays a protective or harmful role is controversial. The autophagy inhibitor 3-methyladenine (3-MA) has been shown to protect the neuron cell cultures from apoptosis [35] and prevent H<sub>2</sub>O<sub>2</sub> induced neuronal cell death [13]. While in the MCAO rat model, LC3 mRNA and LC3-II protein levels are increased, the intra-cerebral ventricle injection of 3-MA significantly reduced infarct volume [10]. 3-MA also reduces lesion volume in neonatal rats exposed to even when given > 4 h after ischemia induction [36]. Further, mice deficient in Atg7, a gene essential for the induction of autophagy, exhibit nearly complete protection from both HI induced caspase-3 activation and neuronal cell death [11]. While reducing Beclin1 expression, effectively inhibited autophagy and the reduced infarct volume in MCAO exposed rats [37]. Conversely, the autophagy inducer, rapamycin reduces necrotic cell death and protects the brain from HI insult [38]. Our data show that using 3-MA treatment significantly reduced neuronal cell death in hippocampal slice culture exposed with OGD. But using the autophagy inducer, rapamycin did not exhibit a protective effect. Thus, we propose that, at last in neonatal HI brain injury, the upregulation of autophagy likely plays a role in inducing neuronal cell death.

It is also interesting that our data demonstrate that oxidative stress appears to be required to induce autophagy in both OGD exposed hippocampal slice culture and the neonatal rat brain exposed to HI. Reactive oxygen species (ROS) serve as common upstream mediators of the activation of the type III PI3 kinase, which is critical for the initiation of autophagy [33,39]. Thus, it has been suggested that ROS might be also be involved in the regulation of autophagy [40]. Indeed in Parkinson's disease, oxidative stress increases the level of the lysosomal-associated membrane protein 2A (LAMP2A) [41]. Oxidative stress has also been linked to mitochondrial dysfunction that may increase the number of misfolded proteins and therefore lead to aggregation of  $\alpha$ -syn and subsequent death of dopaminergic neurons [42]. Previous studies have demonstrated that ROS, especially H<sub>2</sub>O<sub>2</sub>, are essential for autophagy [43]. Atg4 may be the target of this redox regulation [43]. 3-MA also reduces glutamate-induced HT22 cell death through the retardation of ROS accumulation [44]. Also in this study, the MCAO rat treated with 3-MA and lysosomal inhibitor significantly reduced the volume of ischemic damage in the

cerebral hemisphere [44]. A recent study also supported a role for Nox2 activity and ROS generation in TLR-activated autophagy in phagosomes [45]. We have previously reported that NADPH oxidase derived superoxide plays an important role in neonatal neuronal cell death [6]. While we demonstrate here that blocking NADPH oxidase activity attenuates the induction of autophagy in both OGD exposed hippocampal slice cultures and the neonatal rat brain exposed to HI, indicating its dependence on oxidative stress.

## 5. Conclusions

In summary our data show that autophagy is induced in neonatal rat brain exposed to HI and that it is intimately involved in the resulting neuronal cell death. Furthermore, we demonstrate that the induction of autophagy is oxidative stress dependent and requires the activation of NADPH oxidase. We suggest that targeting the autophagy pathway using an autophagy inhibitor combined with the inhibition of oxidative stress may be a therapeutic strategy to alleviate the brain injury associated with neonatal HI.

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