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Heme Oxygenase-1 mediates neuro-protection conferred by argon in combination with hypothermia in neonatal hypoxia-ischemia brain injury

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Abstract:	Background: Hypoxic-ischaemic encephalopathy (HIE) is a major cause of mortality and disability in the newborn. We investigated the protective effects of argon combined with hypothermia on neonatal rat hypoxic-ischaemic brain injury. Methods: In In vitro studies, rat cortical neuronal cell cultures were challenged by oxygen and glucose deprivation (OGD) for 90 minutes and exposed to 70% argon or nitrogen with 5% CO2 balanced with O2, at 33 °C for 2 hours. Neuronal phospho-Akt , heme oxygenase-1 (HO-1) and phospho-Glycogen synthase kinase-3β (GSK-3β) expression and cell death were assessed. In In vivo studies, neonatal rats were subjected to unilateral common carotid artery ligation followed by hypoxia (8% O2 balanced with N2 and CO2) for 90 minutes. They were exposed to 70% argon or nitrogen balanced with oxygen at 33 °C, 35°C, 37 °C for 2 hours. Brain injury was assessed at 24 hours or 4 weeks after treatment. Results: In in vitro, argon-hypothermia treatment increased p-Akt and HO-1 expression, significantly reduced the expression of phospho-GSK-3β Tyr-216 expression, cytochrome C release and cell death in OGD-exposed cortical neurons. In In vivo, argon-hypothermia treatment decreased hypoxia/ischemia-induced brain infarct size (n = 10) and both caspase-3 and NF-κB activation in the cortex and hippocampus. It also reduced hippocampal astrocyte activation and proliferation. Inhibition of PI3K/Akt pathway through LY294002 attenuated cerebral protection conferred by argon-hypothermia treatment (n = 8). Conclusion: Argon combined with hypothermia provides neuroprotection against cerebral hypoxia-ischaemia damage in neonatal rats, which could serve as a new

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	therapeutic strategy against hypoxic ischemic encephalopathy.

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10th March 2016

Hugh C. Hemmings, M.D., Ph.D.
Handling Editor
Anesthesiology

Dear Professor Hemmings:

Re: MS #ALN-D-15-01436, entitled " Heme Oxygenase-1 mediates neuro-protection conferred by argon in combination with hypothermia in neonatal hypoxia-ischemia brain injury "

Thank you for offering us this opportunity to amend our manuscript. We have followed your suggestions to amend our work carefully. All changes that we have made have been highlighted in red. The answers to your questions are here.

RE: MS #ALN-D-15-01436R1 - Heme Oxygenase-1 mediates neuro-protection conferred by argon in combination with hypothermia in neonatal hypoxia-ischemia brain injury

Dear Daqing:

After careful reading of your revised manuscript and the comments of two reviewers with expertise in ischemia neuroprotection and a statistical editor, I am happy to inform you that your manuscript has been accepted for publication in Anesthesiology pending final revisions as requested by the Editorial Office (see below). You have done an excellent job in responding to the suggestions of the reviewers to improve your manuscript, which shows a significant protective effect of argon combined with hypothermia. It is now accepted for publication in Anesthesiology.

We are now providing on the first page of all original investigation articles a summary box which tells the casual reader in a few words what is known on this topic and what the current article tells us which is new. This should be simple and brief. Below is my proposed wording for this box for your article. Please let me know if you have any concerns regarding this wording. If you suggest a change, it should result in fewer and simpler words, since the goal is to provide the essence of the

article's importance in very few words to the non-expert. If you have no suggested changes, please let me know that as well.

We are happy with the wording you proposed for this paper.

For this article:

What we already know about this topic
• Therapeutic hypothermia is widely used to treat hypoxic-ischemic encephalopathy, but there is a pressing need to develop novel neuroprotective strategies.
• Xenon can enhance hypothermic neuroprotection, but the ability of the more abundant non-anesthetic nobel gas argon to do so is unknown.

What this article tells us that is new
• Argon-hypothermia treatment reduced both neuronal death in an in vitro neuronal culture model, and brain infarct size in an in vivo rat model of neonatal asphyxia.
• The protective effects of argon-hypothermia involve both inhibition of apoptosis and neuroinflammation mechanisms and activation of cell survival pathways.

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Thank you for submitting this work to us. I look forward to seeing this interesting study published in Anesthesiology.

Sincerely,

Hugh C. Hemmings, M.D., Ph.D.
Handling Editor
editorial-office@anesthesiology.org

HCH/tlc

IN ORDER FOR YOUR MANUSCRIPT TO FIT THE GUIDELINES FOR PRESENTATION AS DESCRIBED IN THE INSTRUCTIONS FOR AUTHORS (AVAILABLE AT HTTP://ANESTHESIOLOGY.PUBS.ASAHQ.ORG/PUBLIC/INSTRUCTIONSFORAUTHORS.ASPX), PLEASE ADDRESS THE FOLLOWING ISSUES:

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We have amended it accordingly.

Yours sincerely

Hailin Zhao and Daqing Ma on behalf of all authors

Manuscript (Title Page, Abstract, Body, References) OR single file with all manuscript elements, including figures (initial

Heme Oxygenase-1 mediates neuro-protection conferred by argon in combination with hypothermia in neonatal hypoxia-ischemia brain injury

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The authors declare no competing interests

Running head: Argon and hypothermia confer neuro-protection

TOC Statement: Argon combined with hypothermia treatment confers protection against neonatal hypoxic-ischaemic brain injury, which is likely mediated through Heme Oxygenase-1

What is known about the subject:

Perinatal hypoxic-ischaemic encephalopathy (HIE) is one of the largest contributors to neonatal brain injury with subsequent poor developmental outcome, there is a pressing need to develop novel neuroprotective strategies.

What this investigation documented:

Argon-hypothermia treatment significantly reduced both neuronal cell death in an *in vitro* model of rat cortical neuronal cultures , and brain infarct size in an *in vivo* rat model of neonatal asphyxia. The protective effects are likely associated with intrinsic apoptotic and neuroinflammation pathway inhibition and cellular survival signal activation.

Abstract

Background: Hypoxic-ischaemic encephalopathy (HIE) is a major cause of mortality and disability in the newborn. We investigated the protective effects of argon combined with hypothermia on neonatal rat hypoxic-ischaemic brain injury.

Methods: In *In vitro* studies, rat cortical neuronal cell cultures were challenged by oxygen and glucose deprivation (OGD) for 90 minutes and exposed to 70% argon or nitrogen with 5% CO_2 balanced with O_2, at 33 °C for 2 hours. Neuronal phospho-Akt , heme oxygenase-1 (HO-1) and phospho-Glycogen synthase kinase-3β (GSK-3β) expression and cell death were assessed. In *In vivo* studies, neonatal rats were subjected to unilateral common carotid artery ligation followed by hypoxia (8% O_2 balanced with N_2 and CO_2) for 90 minutes. They were exposed to 70% argon or nitrogen balanced with oxygen at 33 °C, 35°C, 37 °C for 2 hours. Brain injury was assessed at 24 hours or 4 weeks after treatment.

Results: In *in vitro*, argon-hypothermia treatment increased p-Akt and HO-1 expression, significantly reduced the expression of phospho-GSK-3β Tyr-216 expression, cytochrome C release and cell death in OGD-exposed cortical neurons. In *In vivo*, argon-hypothermia treatment decreased hypoxia/ischemia-induced brain infarct size (n = 10) and both caspase-3 and NF-κB activation in the cortex and hippocampus. It also reduced hippocampal astrocyte activation and proliferation. Inhibition of PI3K/Akt pathway through LY294002 attenuated cerebral protection conferred by argon-hypothermia treatment (n = 8).

Conclusion: Argon combined with hypothermia provides neuroprotection against cerebral hypoxia-ischaemia damage in neonatal rats, which could serve as a new therapeutic strategy against hypoxic ischemic encephalopathy.

Key words: Argon; Hypothermia; Hypoxic-ischaemic encephalopathy; Neuroprotection;

Introduction

Hypoxic-ischaemic encephalopathy (HIE) is a major cause of mortality and disability in the newborn and is associated with cerebral palsy, epilepsy, mental retardation and learning difficulties [1]. HIE is associated with hugely negative emotion and financial costs to the family of the affected infant and the burden to society in general. Given the severity and lifelong nature of the adverse effects of perinatal hypoxic-ischaemic encephalopathy, there is a pressing need to develop novel neuroprotective strategies.

Currently, therapeutic hypothermia, which provides modest neuroprotection in perinatal hypoxic-ischaemic encephalopathy, has been widely adopted in clinical practice [2]. Hypothermia exerts inhibitory effects at many levels within the pathological cascade of HIE that leads to delayed neuronal death. Furthermore, hypothermia has shown to delay the onset of secondary energy failure and nearly double the duration of the latent phase, the period when additional neuroprotective agents could be given, in experimental models [3]. However, despite hypothermia treatment the rate of death and disability remains high with approximately half of cooled infants dying or exhibiting neurodevelopmental disability [4,5]. There is therefore a pressing need to discover better and more effective treatment strategies to prevent or ameliorate neonatal brain damage after perinatal hypoxia-ischaemia.

The noble gas xenon has shown great promise as a neuroprotectant when administered alone [6] or in combination with therapeutic hypothermia in rats [7,8] and piglet [9] models of neonatal hypoxic-ischaemic encephalopathy. The combination of xenon and hypothermia has either a synergistic [7] or additive [10] neuroprotective effect.

However, xenon is present in very low concentrations in air and its extraction is very costly, perhaps prohibiting its widespread use. Argon, on the other hand, is the most common noble gas in the atmosphere and is emerging as a viable alternative. Unlike xenon, argon is not an anaesthetic gas and lack of anesthetic/sedative properties; hence it may be more safely administered to neonatal patients with hypoxia-ischemia brain injury [11]. The aim of this study is to investigate whether argon in combination with hypothermia is neuroprotective in our *in vitro* and *in vivo* models of HIE and to explore the underlying molecular mechanisms.

Materials and Methods

Primary cortical neuronal cell culture: The cortical neuronal cultures were derived from gestational day 16 fetal Sprague-Dawley rats. The neuronal cells were seeded into poly-L-lysine pre-coated plates and fed with neurobasal medium (Gibco) with the addition of B27 supplement and glutamine (25μM).

Oxygen-Glucose Deprivation and Gas Exposure: Oxygen-Glucose Deprivation was induced [7]. Culture medium was replaced by deoxygenated balanced salt solution and maintained in a purpose-built cell-culture chamber at 37°C for 90 minutes. Cells were then recovered in neuronal culture medium in the purpose-built chamber, which randomly filled with 75% argon or nitrogen (Air Products, Crewe, United Kingdom) and 5% carbon dioxide balanced with oxygen at 33°C for 2 hours. They were further recovered in a normal cell culture incubator for 24 hours at 37°C.

Determination of apoptosis and necrosis in vitro: Neuronal cells were stained with annexin V- propidium iodide (PI) apoptosis kit (e-Bioscience, Cambridge, UK) according to the manufacturer's guidelines. A count of 10,000 cells/sample was analyzed with flow cytometry (FACSCalibur; Becton Dickinson, Sunnyvale, CA, USA)

to determine the percentage population of apoptotic (annexin V positive, PI negative), necrotic (annexin V and PI positive), and live cells (unstained).

HO-1 siRNA Transfection and PI-3K/Akt inhibition: Neuronal cells were transfected with HO-1 siRNA (SI01522122, Qiagen, Crawley, West Sussex, UK) using lipofectamine (Invitrogen, Paisley, UK) at 20nM whilst scrambled siRNA served as negative control. Cells were incubated with siRNA for 6 hours at 37 °C in humidified air containing 5% carbon dioxide, after which it was removed and replaced with experimental medium followed by OGD treatment. For PI-3K/Akt inhibition, cultured neurons received 100 mM LY294002 [12].

Rat hypoxic-ischaemic brain injury: Seven day old Sprague-Dawley rat pups and their mother were purchased (Harlan, UK) and housed in the animal facilities in Chelsea-Westminster Hospital campus, Imperial College London. All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act of 1986. Right common carotid artery ligation was performed under surgical anaesthesia [13]. After 1 hour of recovery, the pups were exposed to hypoxia (8% oxygen balanced with nitrogen) for 90 minutes in purpose-built multi-chambers. They were exposed to 70% argon or nitrogen balanced with 30% oxygen for 2 hours through our established protocol [14]. Hypothermia (33 or 35°C) or normothermia (37°C) was achieved and sustained by the temperature-controlled water bath. Rat pups were randomly allocated to experimental conditions and their number used per group was based on the similar experimental settings established previously [7] . All the animal experiments conform to the UK ARRIVE guidelines[15]. Efforts were made to minimise the used number and/or suffering of animals throughout.

***Drug administration in vivo**:* LY294002 (Calbiochem, 0.2 mol/L in 5 µl of PBS) or the PBS vehicle (PBS) was injected intracerebroventricularly before gas treatment, as described previously [16,17].

Immunohistochemistry: For *in vitro* fluorescence staining, cells were fixed in paraformaldehyde, incubated in 10% donkey serum for 1 hour and then incubated overnight with rabbit anti-p-Akt (1:200, cell signalling, Danvers, MA, USA), or rabbit anti-HO-1 (1:200, Abcam, Cambridge, UK), or rabbit anti- phospho-GSK-3β Tyr-216 (1:200, abcam), or rabbit anti-cytochrome C (1:200, Cell Signalling), or mouse anti-α-tubulin (1:200, sigma-aldrich, Poole, UK), followed by secondary antibody for 1 hour. The mitochondria were stained with mitoRed (Sigma-Aldrich). For *in vivo* fluorescence staining, the pups were sacrificed and transcardially perfused with 4% paraformaldehyde. After dehydration, brain was cryosectioning into 25 µm slices. Coronal sections between approximately -2.5 mm and -3.7 mm from bregma (relative to the adult rat brain) were incubated with 3% donkey serum (Millipore, Massachusetts, USA) and were then incubated overnight with rabbit anti-Bcl-2 (1:200, Abcam), rabbit anti-HO-1 (1:200, Abcam), rabbit anti- phospho-GSK-3β Tyr-216 (1:200, abcam), rabbit anti-cleaved caspase-3 (1:200, Cell Signalling), rabbit anti-NF-κB p65 (1:200, Abcam) or rabbit anti-GFAP (1:200, Dako, Glostrup, Denmark) primary antibody, followed by fluorochrome conjugated secondary antibodies (Millipore, UK). For dual fluorescence labelling, cells or brain sections were incubated with the two primary antibodies overnight, followed by the two secondary antibodies. The slides were counterstained with nuclear dye DAPI and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Ten fields at ×20 view were first photographed using an AxioCam digital camera (Zeiss, Welwyn Garden City, UK) mounted on an Olympus BX60 microscope

(Olympus, Middlesex, UK). Staining was quantified using ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA). Fluorescent intensity was by one author who was blinded to the treatment and then calculated as percentages of the mean value of the naïve controls.

Western blotting: The tissue lysates from brain samples were centrifuged and the protein extracts (40μg/sample) underwent electrophoresis and then transferred to a PVDF membrane. The membrane was treated with blocking milk solution and probed with rabbit anti-Bcl2 (1:1000, abcam), rabbit anti-HO-1 (1:1000, abcam), rabbit anti-GSK-3β phospho Tyr-216 (1:1000, abcam), followed by HRP-conjugated secondary antibody. The loading control was α-tubulin (1:10000, Sigma–Aldrich). The blots were visualized with enhanced chemiluminescence (ECL) system (Santa Cruz Biotechnology) and analysed with GeneSnap (Syngene, Cambridge, UK). Protein band intensity was normalized with α-tubulin and expressed as ratio of the naive control.

Assessment of brain infarction through Caspase 3 or cresyl violet staining

Double-labelled fluorescence staining was performed on 25 μm vibratome brain sections as described previously [18]. The brain sections at the level of striatum (approximately Bregma-0.35 mm) were labelled with the rabbit anti-cleaved caspase-3 (1:200, Cell Signaling, Massachusetts, USA). The vibratome sections were also stained with nuclear staining propidium iodide and examined by the Olympus BX60 microscope (Olympus, Middlesex, UK).

For histology, 5-μm paraffin sections were stained with 0.5% cresyl violet. The coronal sections (5 mm) from rats were selected from each pup to match predefined brain regions relative to the bregma (+2 mm, +1 mm, 0 mm, -1 mm, -2 mm and 5

mm) relative to adult brain. Each slice was photographed and the size (arbitrary unit) of the healthy matter of both hemispheres was calculated with data analysis software (ImageJ version 1.31; National Institutes of Health image software, Bethesda, MD) by one author blinded to the treatment. The infarct size was calculated with a formula of (left hemisphere-right hemisphere)/ left hemisphere (%). Then the data were used to plot curves and the area under curve was calculated to indicate the infarction volume (arbitrary units)[14].

Statistical analysis: All numerical data were expressed as mean ± standard deviation (SD). To study the treatment effects on protein expression with time, two-way ANOVA and a *post hoc* Tukey's test was performed; otherwise, one-way analysis of variance followed by *post hoc* Student–Newman–Keuls test was performed for statistical comparisons (GraphPad Prism 5.0 software, San Diego, CA). A two tailed p value less than 0.05 was considered to be statistically significant.

Results

Argon exposure up-regulated heme oxygenase-1 (HO-1) in the cultured cortical neurons and neonatal rat brain

To investigate whether HO-1 was up-regulated *in vitro* and *in vivo* after argon exposure, cultured rat neuronal cells or 7 day old neonatal rats were given 70% argon for 2 hours (**Figure 1**). Four hours after gas exposure, up-regulation of HO-1 was observed in cultured neuronal cells (**Figure 1A and B**). The immunostaining of HO-1 in the cortex, CA1 and CA3 region of the hippocampus are shown in **Figure 1C**.

Argon exposure significantly increased HO-1 expression in the cortex at all three time points with the highest increase at 4 hour (**Figure 1D**), in the CA1 region at 4

and 24 hour with the highest increase at 4 hour (**Figure 1E**), and in the CA3 region at 4 hour, compared with that in the nitrogen controls (**Figure 1F**).

Argon and hypothermia up-regulated p-Akt and HO-1 in the cortical neuronal cell culture

Firstly, the effect of argon exposure on the expression of p-Akt and HO-1 was investigated in the neuronal cell culture through immunofluorescence technique (**Figure 2**). HO-1 was expressed at basal levels in the Naïve control group but was slightly increased during hypothermia treatment (**Figure 2A**). Argon significantly augmented the up-regulation of HO-1 (**Figure 2A and C**). p-Akt was detected at low level in the Naïve control group; however, expression p-Akt was moderately enhanced in neurons after being treated with hypothermia, and was also greatly enhanced by argon-hypothermia treatment (**Figure 2A and 2B**). To assess the neuro-protective effects of argon-hypothermia treatment, the neuronal cultures were challenged with oxygen-glucose deprivation (OGD) for 90 minutes and followed by nitrogen-or argon-hypothermia treatment for 2 hours. Four hours after treatment, activation of GSK-3β was found in the neurons, indicating a possible role in apoptosis. In the argon- hypothermia treated group, p-GSK-3β Tyr-216 was barely expressed in neurons (**Figure 2D** and **E**). In addition, α-tubulin staining indicated that argon-hypothermia treatment improved cellular morphology and preserved neuronal dendrites well under OGD challenge (**Figure 2D**).

Argon-hypothermia reduced ischemic neuronal injury induced by OGD in vitro

Four hours after treatment, dual labelling of mitochondria and cytochrome C demonstrated the cytochrome c release from mitochondria under OGD induced injury (**Figure 3**). Argon-hypothermia treatment significantly restrained the cytochrome c within the mitochondria, indicated by co-localization of mitochondria and cytochrome C (**Figure 3A and 3B**). Twenty-four hours later, neuronal death was assessed with PI/Annexin V staining (**Figure 3C and D**). Hypothermia alone confer a certain level of protection (p = 0.02, OGD vs OGD + hypothermia group), Argon-hypothermia increased the percentage of live neurons after OGD (69 ± 7.9% vs 38.5 ± 6.2, Ar vs N_2, P<0.01). These data indicate that argon combined with hypothermia promotes neuronal resistance against hypoxic ischemic injury induced by OGD.

Inhibition of Akt and HO-1 abolished argon-hypothermia mediated protection in cultured rat cortical neurons.

To further investigate whether p-Akt/HO-1 essentially contribute to the neuroprotective effects of argon-hypothermia treatment, the neurons were treated with either PI-3K-Akt inhibitor LY294002 or HO-1 siRNA and then subjected to the OGD challenge (**Figure 4**). Argon-hypothermia treated neurons exhibited a relative intact neuronal morphology, as demonstrated by better preserved cytoplasm and neuronal dendrites (**Figure 4A and 4C**). Furthermore, either the PI-3K-Akt inhibitor LY294002 or HO-1 siRNA blocked the protective effects of argon-hypothermia on neurons after OGD treatment. The LY294002 or HO-1 siRNA treated neurons had shrunken cell soma and fragmented neurites, in contrast to the intact cell morphology in the Argon only group. In addition, blocking either PI-3K/Akt pathway or HO-1 expression induced significant cell death at 24 hours after treatment (**Figure**

4B, D, F and H). Taken together, all this indicates that p-Akt and HO-1 mediate the neuro-protective effects of argon-hypothermia against OGD induced injury.

Argon combined with hypothermia induced the up-regulation of HO-1, Bcl-2 and the suppression of GSK-3β activation in the cortex and hippocampus

These observations suggest that HO-1 plays the central role in Argon mediated protection. Consistent with *in vitro* data, western blot analysis of *in vivo* samples showed that argon-hypothermia significant increased HO-1 (3.4 ± 0.4 vs 2.3 ± 0.5, Ar vs N_2 $p < 0.05$), and Bcl-2 (2.9±0.25 vs 2.02 ± 0.5, Ar vs N_2 $p < 0.05$), reduced p-GSK-3β Tyr 216 (0.9 ± 0.2 vs 2.5 ± 0.4, Ar vs N_2 $p < 0.05$) at 4 hour post- treatment in the cortex of neonatal rats with hypoxic-ischemic injury (**Figure 5A-C**). Furthermore, the dual immune-labelling of Bcl-2 and HO-1, HO-1 and p-GSK-3β Tyr 216 in the CA1 and CA3 regions of the hippocampus are shown in **Figure 5D and 5E** respectively. Co-localization of HO-1 and Bcl-2 were observed and HO-1 expression negatively correlated with the activation of GSK-3β after argon-hypothermia treatment (**Figure 5D-G)**.

Argon combined with hypothermia decreased cell death and tissue inflammation in the cortex and hippocampus after hypoxia-ischemia challenge

To assess the level of brain injury after hypoxia-ischemic insult, brain section was stained with caspase-3 (**Figure 6**). Argon-Hypothermia treatment caused a notable reduction of caspase-3 positive areas (**Figure 6A**). Cleaved caspase-3 expression in the cortex and the CA1 and CA3 region of the hippocampus was assessed (**Figure 6B and C**). Hypothermia alone significantly reduced the expression of cleaved

caspase-3 in these areas compared with the normothermic controls. Argon combined with hypothermia caused further significant reductions in caspase-3 expression in the cortex and hippocampus, compared to hypothermia alone (**Figure 6B and C**). NF-κB activation is an essential component of the inflammatory response in the brain. The expression and nuclear translocation of NF-κB in the cortex and the CA1 and CA3 of the hippocampus after the injury were readily detected (**Figure 6D and E**). Hypothermia alone did not significantly reduce the expression of NF-κB compared to the injured controls. Argon combined with hypothermia resulted in significant reductions in NF-κB activation (p-65 NF-κB expression and translocation) when compared with control, hypothermia alone and normothermia injury controls (**Figure 6D and E**). Astrocyte activation and proliferation (reactive gliosis) is a hallmark of neuroinflammation during hypoxia-ischemia induced neuronal injury processes. Fluorescence intensity of GFAP in the region between the pyramidal cell layer and the alveus in the CA1 and CA3 hippocampus were significantly elevated (**Figure 6F** and **G**) in the normothermia injury group. Hypothermia alone did not significantly reduce GFAP expression compared with the injury controls. Argon combined with hypothermia significantly reduced GFAP expression in the CA1 and CA3 of the hippocampus when compared with the injury controls (**Figure 6G**).

Argon combined with hypothermia reduced the infarction size

The long-term protective profile of argon-hypothermia treatment was explored (**Figure 7**). Argon combined with hypothermia (33 °C and 35 °C) significantly reduced infarction volume, when compared with nitrogen control under normothermia (reduction of infarct size by 48%, 52%, 65% respectively, when argon combined with 37 °C, 35 °C and 33 °C, compared with nitrogen group) (**Figure 7A**

and B). The reduction in pathological changes by the argon-hypothermia treatment correlated well with the body weight (**Figure 7C**), while argon exposure increased the body weight, when compared with nitrogen control.

Inhibition of PI3K/Akt pathway attenuated neuro-protection conferred by argon-hypothermia treatment.

Treatment with LY294002 dramatically decreased HO-1 expression levels in argon-hypothermia group **(Figure 8A and B)**. Caspase-3 expression was evident in LY294002 treated animals (**Figure 8C and D**). The significant reduction of infarct size afforded by argon-hypothermia was lost by this treatment on day 28 (**Figure 8E and F**). These data indicated that inhibition of PI-3K/Akt significantly attenuated HO-1 up-regulation and neuronal protection.

Discussion

The absence of a safe and effective therapy for hypoxia-ischemia brain injury in newborns has prompted the investigation of the possible protective effects of noble gases, especially xenon[19]. Argon, another noble gas, possesses similar protective properties[20]. However, the neuro-protective potential of argon combined with hypothermia has not been explored to date. The present study demonstrates that argon, when combined with mild or moderate hypothermia (35 °C and 33 °C), elicits robust and prolonged neuroprotection against ischemic brain injury in neonatal rats. PI-3K/Akt pathway activation, HO-1 up-regulation and GSK-3β inhibition were demonstrated to be the possible molecular mechanisms underlying the beneficial effects of the combined treatment both *in vivo* and *in vitro* (Figure 9). Furthermore,

inhibition of HO-1 and PI-3K/Akt pathway activation significantly attenuated argon-hypothermia-induced neuroprotection against oxygen-glucose deprivation induced injury *in vitro or in vivo.* These findings support our hypothesis that argon works synergistically with hypothermia to provide robust neuroprotection against a hypoxia-ischaemia insult in neonatal rats.

The protective effects of argon against hypoxia were only discovered recently. It was reported that argon was protective against hypoxia-induced injury in cultured neurons [21,22]. In an *in vivo* model of acute focal cerebral ischaemia in adult rats, exposure to 50% argon significantly reduced infarct volumes and neurological deficits after the occlusion [23]. Although noble gases are chemically inert, they are capable of forming induced dipole, which is attracted to the charge that induced it, or instantaneous dipole, which produces and binds to an induced dipole in a second molecule [24]. Thus, they might produce biological effects by stabilising receptors or enzymes in active or inactive forms via interactions with amino acids at the binding sites [25].

The PI3K/Akt pathway elicits a survival signal against apoptotic insults [26] and has been proposed to be involved in the well documented neuroprotective effect of IGF-1 in the immature brain[27]. Recently, it has been demonstrated to be the up-stream pathway of HO-1 [28], which acts against cellular stress, such as oxidative stress. HO-1 is an enzyme induced by oxidative stress, it catabolises free haem into labile iron, carbon monoxide and biliverdin [29]. HO-1 provides cytoprotection mainly through the catabolism of haem and several end products: HO-1 reduces oxidative stress by breaking down the pro-oxidant haem; Production of carbon monoxide leads to the degradation of pro-apoptotic p38α mitogen-activated protein kinase (MAPK) and activation of antiapoptotic p38β MAPK [30], which may induce the upregulation of Bcl-2

[31]; Production of iron limits transcription of proinflammatory genes by inhibiting phosphorylation of NF-κB p65 [32]; Biliverdin may also serve as an antioxidant [29]. *In vitro* studies using neuronal cultures have shown that HO-1 protects neurons against oxidative injury [33]. In this study, a significant upregulation of this cytoprotective protein by argon was found in both the cortex and hippocampus. We therefore postulate that argon-hypothermia increases HO-1 expression mainly in these interneurons, providing cytoprotection to them although it is plausible that multiple molecular pathways could also be involved in the protective mechanisms of argon-hypothermia against hypoxia-ischemia. Nevertheless, the evidence provided by HO-1 siRNA and Akt inhibition that the neuroprotective effect of argon-hypothermia is reversed, suggests that HO-1 is an essential component of the protective mechanism.

Glycogen synthase kinase 3 beta (GSK-3β) is a proline directed serine/ theorine kinase in mammals [34]. Dysregulation of GSK has been linked to many diseases such as cancer and neuro-degenerative disease [35]**.** The beta subunit of GSK-3 (GSK3β) is activated by phosphorylation of the tyrosine 216 residue in the kinase domain and inactivated by phosphorylation of the amino terminal serine 9 residue [36]. Activation of GSK-3β has been associated with cell death through the intrinsic pathway [37]. In this study, argon significantly decreased GSK3β activation after OGD induced injury *in vitro* and hypoxia-ischemia induced injury *in vivo*. Reduction of GSK3β correlated strongly with caspase-3 activation, this observation is consistent with the previous study by Petit-Paitel et al [38], which demonstrated that the phosphorylation of tyr216 was involved in mitochondria dependent neuronal cell death and that inactivation of GSK3β has been proposed to be important for the neuroprotection afforded by IGF-1 and hexarelin [27,39] .

During acute neuronal injury, cytochrome c causes the activation and release of apoptotic protease-activating factor-1 (Apaf-1) into apoptosome, which activates caspase-9 and subsequently caspase-3 [40,41]. An up-regulation of Bcl-2 suppresses this pathway [42] and therefore protects the neurons against apoptosis. In the current study, 70% argon significantly increased Bcl-2 expression in the cortex and the CA1 and CA3 region of the hippocampus at 4 hours after the gas exposure. Hypoxic-ischaemic injury caused marked activation of caspase-3 in the cortex and hippocampus; this was decreased by hypothermia alone and still further by the combination of hypothermia and argon.

NF-κB activation is the hallmark of neuroinflammation, which is closely associated with neuronal cell death. Upon removal of inhibitor protein IκB by IκB kinase, the NF-κB (p50/p65) heterodimer translocates into the nucleus and drives the expression of many inflammatory mediators, e.g. tumour necrosis factor-α (TNF-α), cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) [43]. Suppression of NF-κB activation has been shown to reduce neuronal damage in a rat model of global cerebral ischaemia [44]. In our study, NF-κB was highly up-regulated and nuclear translocation was evident in association with cortical and hippocampal injury. NF- κB Activation was suppressed by the combination of argon and hypothermia.

Neuronal inflammation caused by cerebral ischaemia induces astrocyte activation and proliferation (reactive astrogliosis) and an increased production of GFAP, a cytoskeletal intermediate filament protein specific to activated astrocytes [45]. Activated astrocytes also release inflammatory mediators such as iNOS and cytotoxic molecules such as ROS [43] and cause glial scar formation, which impedes axon regeneration and remyelination [46]. In our study, a large increase in GFAP

expression was found in the hippocampus of normothermic injury group and this was reversed by the combination of argon and hypothermia, reversing the potentially harmful over-activation of astrocytes.

Our study is not without limitations; first, only 70% of Argon for 2 hours duration of treatment was investigated; its optimal concentration and exposure duration were not sufficiently explored and this certainly warrants further investigation. Second, the effect of argon on the *in vitro* OGD induced neuronal injury was primarily investigated and the effects on other cell type, e.g. glia, during and after insult were not assessed.

Our study has significant clinical implementations, hypoxic-ischaemic encephalopathy is a devastating condition, which current treatments do little to reverse [1]. Our results show that treatment with the combination of argon and hypothermia result in short and long-term neuroprotection in our *in vitro* and *in vivo* models of HIE. This could serve as basis for further research with argon in combination with hypothermia as an effective strategy against hypoxia-ischemia brain injury in neonates.

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Figure legend

Figure 1. Enhanced expression of HO-1 in cultured cortical neuronal cells and brain cortex and hippocampus after argon exposure.

Rat neuronal cell culture were exposed to argon gas (70% Ar and 5% CO_2 balanced with O_2) or nitrogen gas (70% N_2 and 5% CO_2 balanced with O_2) for 2 hours and then air cell incubator at 37 °C for 24 hours. (**A**) Dual immunolabelling of α-Tubulin (Green Fluorescence) and HO-1 (Red Fluorescence); (**B**) Fluorescence intensity of HO-1 at 4 hours after gas exposure. Seven day old neonatal rats were exposed to argon gas (70% Ar balanced with 30% O_2) or nitrogen gas (70% N_2 balanced with 30% O_2) for 2 hours and then room air for 24 hours. HO-1 expression (Green Fluorescence) was assessed at 0hr, 4hr, and 24 hrs after gas exposure (**C**) The example images of the naïve control (NC), nitrogen (N_2) and argon (Ar) treated cortex, Hippocampus CA1 and CA3 at 4 hours after gas exposure. Fluorescence intensity (% of naïve control) of HO-1 after gas exposure in (**D**) cortex, (**E**) hippocampus CA1 region and (**F**) CA3 region. Cell nuclei were counterstained with DAPI (blue). Data are means ± SD; n = 8. *$p<0.05$ and **$p<0.01$ and ***$p<0.001$. Scale bar: 50μm.

Figure 2. Expression of p-Akt, HO-1 and p-GSK-3β Tyr-216 in rat cortical neuronal cell culture after argon combined with hypothermia. Rat neuronal cell culture were exposed to argon gas (70% Ar and 5% CO_2 balanced with O_2) or nitrogen gas (70% N_2 and 5% CO_2 balanced with O_2), combined with hypothermia (33 °C) for 2 hours and then room air cell incubator at 37 °C for 24 hours. (**A**) Expression of p-Akt (Green Fluorescence), HO-1 (Red Fluorescence) was assessed by immunoflorescence at 4 hour after combined treatment. Fluorescence intensity (% of naïve control) of (**B**) p-AKT and (**C**) HO-1. Rat cortical neuronal cell culture were given oxygen glucose deprivation (OGD) for 90 minutes and then exposed to argon gas (70% Ar and 5% CO_2 balanced with O_2) or nitrogen gas (70% N_2 and 5% CO_2 balanced with O_2), combined with hypothermia (33 °C) for 2 hours and then room air room temperature for 24 hours. (**D**) Duel labelling of p-GSK-3β Tyr-216 (Red Fluorescence) and α-tubulin (Green Fluorescence) in cultured neurons at 4hr after gas exposure. (**E**) Fluorescence intensity (% of naïve control) of GSK-3β, Cell

nuclei was counterstained with DAPI (blue). Data are means ± SD, (n = 8); *p<0.05 and **p<0.01 and ***p<0.001). Scale bar: 50μm. NC: Naïve control, IC: Injury control. Hy: hypothermia.

Figure 3. Effect of Argon combined with hypothermia treatment on cortical neuronal cell death after OGD challenge. Rat cortical neuronal cell culture were given oxygen glucose deprivation (OGD) for 90 minutes and then exposed to argon gas (70% Ar and 5% CO_2 balanced with O_2) or nitrogen gas (70% N_2 and 5% CO_2 balanced with O_2), combined with hypothermia (33 °C) for 2 hours and then in room air cell incubator at 33°C for 24 hours. (**A**) Dual labelling of mitochondria (Red Fluorescence) and cytochrome C (Green Fluorescence) at 4 hrs after gas exposure, Cell nuclei were counterstained with DAPI (blue). Scale bar: 10μm. (**B**) Percentage of neurons with cytochrome c release at 4 hrs after gas exposure. (**C**) Cell apoptosis and necrosis are assessed by Annexin V and Propidium Iodide (PI) staining in FACS. (**D**) Percentage of live cells (Annexin V^- and Propidium $Iodide^-$). Data is expressed as Mean ± SD (n=8); ***p<0.001). NC: naïve control, IC: injury control. Hy: Hypothermia.

Figure 4. Inhibition of p-Akt or HO-1 attenuated the cytoprotective effects conferred by argon combined with hypothermia in cortical neuronal cells after OGD challenge. Rat cortical neuronal cells transfected with scramble siRNA or HO-1 siRNA for 6 hours, prior to OGD treatment, or were treated with PI3K-Akt inhibitor LY294002, after OGD treatment. Rat cortical neuronal cell culture were given oxygen glucose deprivation (OGD) for 90 minutes and then exposed to argon gas (70% Ar and 5% CO_2 balanced with O_2) or nitrogen gas (70% N_2 and 5% CO_2 balanced with O_2), combined with hypothermia (33 °C) for 2 hours and then room air for 24 hours. (**A** and **C**) Duel labelling of p-GSK-3β Tyr-216 (Red Fluorescence), and α-tubulin (Green Fluorescence) in cultured neurons at 4hr after gas exposure, Cell nuclei were counterstained with DAPI (blue). (**B** and **D**) Cell apoptosis and necrosis are assessed by Annexin V and Propidium Iodide (PI) staining in FACS. (**E** and **G**) Fluorescent intensity (% of naïve control) of p-GSK-3β Tyr 216. (**F** and **H**) percentage of live cells (Annexin V^- and Propidium $Iodide^-$). Data is expressed as Mean ± SD. (n = 8); **p < 0.01 and ***p < 0.001). Scale bar: 50μm. NC: naïve control; IC: injury control; Ve: Vehicle;Ly: LY294002; SS: scramble siRNA; HS: HO-1 siRNA; Hy:hypothermia.

Figure 5. Expression of HO-1, Bcl-2 and GSK-3β in brain cortex and hippocampus after combined treatment of argon and hypothermia. Seven day old rat pups were subjected to unilateral carotid artery ligation and then exposed to 8% oxygen balanced with nitrogen for 90 minutes and then exposed to argon gas (70% Ar balanced with 30% O_2) or nitrogen gas (70% N_2 balanced with 30% O_2)

under hypothermia (33 °C) for 2 hours and then room air for 24 hrs. Expression of (**A**) HO-1, (**B**) Bcl-2 and (**C**) p-GSK-3β Tyr 216 in the cortex was assessed by western blot, at 4 hours after gas exposure. (**D)** Dual labelling of HO-1 (green fluorescence) and Bcl-2 (red fluorescence) in the hippocampus; (**E**) Dual labelling of HO-1 (green fluorescence) and p-GSK-3β Tyr 216 (red fluorescence) in rat hippocampus; fluorescence intensity (% of naïve control) of (**F**) Bcl-2 and (**G**) p-GSK-3β Tyr 216 in CA1 and CA3 region of rat hippocampus. Cell nuclei were counterstained with DAPI (blue). Data are means ± SD (n = 8); *p<0.05 and **p<0.01 and ***p<0.001. Scale bar: 50μm. NC: naïve control; Ar = argon; HI: hypoxic ischemic insult.

Figure 6. Effect of Argon combined with hypothermia treatment on expression of cell death, tissue inflammation and astrocyte activation in the cortex or hippocampus after hypoxia-ischemia. Seven day old rat pups were subjected to unilateral carotid artery ligation and then exposed to 8% oxygen balanced with nitrogen for 90 minutes and then exposed to argon gas (70% Ar balanced with 30% O_2) or nitrogen gas (70% N_2 balanced with 30% O_2) under hypothermia (33 °C) for 2 hours and then room air for 24 hrs. (**A**) Coronal sections of the brain 16 hours after hypoxia-ischemia are shown. The caspase-3$^+$ areas indicated initiation of caspase-3 activation which is showed with green fluorescence which intact region was counterstained with nuclear staining propidium iodide (PI, red). Expression of (**B**) caspase-3 (Green Fluorescence), (**D**) NF-κB (Red Fluorescence) was assessed by immunoflorescence at 4hr after gas exposure. Fluorescence intensity (% of naïve control) of (**C**) caspase-3 and (**E**) NF-κB. Cell nuclei were counterstained with DAPI (blue). (**F**) GFAP (green fluorescence) in the hippocampus CA1 and CA3 of rat brain of naïve control (NC), injury treated only, N_2 or Ar combined with hypothermia treated at 24 hours, scale bar: 50μm. (**G**) Fluorescence intensity (% of naïve control) of GFAP. Data are means ± SD (n = 8); *p < 0.05, **p < 0.01 and ***p<0.001; Scale bar: 50μm. NC: naïve control; HI: hypoxic ischemic insult; Hy: Hypothermia

Figure 7. Effect of Argon combined with hypothermia on infarct size and body weight of the rats with hypoxia-ischemia brain injury. Seven day old rat pups were subjected to unilateral carotid artery ligation and then exposed to 8% oxygen balanced with nitrogen for 90 minutes and then exposed to argon gas (70% Ar balanced with 30% O_2) or nitrogen gas (70% N_2 balanced with 30% O_2) under different temperature (37 °C, 35 °C and 33 °C) for 2 hours and then room air for 24 hrs. (**A**) Representative brain micrograph, stained by cresyl violet. (**B**) Infact volume. (**C**) Body weight of rats assessed at 28 days after experiments. Data are means ± SD (n = 10); *p < 0.05 and **p < 0.01 and ***p < 0.001). NC: Naïve control, HI: Hypoxic ischemic insult.

Figure 8. Inhibition of PI-3K/Akt abolished argon-hypothermia mediated neuroprotection. Seven day old rat pups were subjected to unilateral carotid artery ligation and then exposed to 8% oxygen balanced with nitrogen for 90 minutes. PI3K-Akt inhibitor LY294002 or vehicle was then administered intracerebroventricularly after hypoxic-ischemic injury before exposed to argon gas (70% Ar balanced with 30% O_2) or nitrogen gas (70% N_2 balanced with 30% O_2) under moderate hypothermia (33 °C) for 2 hours and then room air for 24 hrs. (**A**) HO-1 expression (green fluorescence) in the cortex and hippocampus at 4 hours after gas treatment. Scale bar: 10μm. (**B**) Immunofluorescence intensity of HO-1 at 4 hours after gas treatment. (**C**) Caspase-3 expression (green fluorescence) in the cortex and hippocampus at 4 hours after gas treatment. Scale bar: 50μm. (**D**) Immunofluorescence intensity of caspase-3 at 4 hours after gas treatment. (**E**) Representative brain micrograph, stained by cresyl violet, on 28 days after treatment. (**F**) Infarct volume on 28 days after treatment. Data are means ± SD (n = 8); *$p < 0.05$ and ***$p < 0.001$). NC: Naïve control; HI: Hypoxic ischemic insult. Hy: Hypothermia, Ve: vehicle, Ly: LY294002.

Figure 9. Putative molecular mechanisms of Argon combined with hypothermia mediated neuro-protection. Argon combined with hypothermia activated PI-3K/Akt pathway, enhanced HO-1 and Bcl-2 expression and reduced p-GSK-3β Tyr216 expression. This leads to reduced tissue damage and inflammation in neonatal rat after hypoxia-ischemia brain injury.

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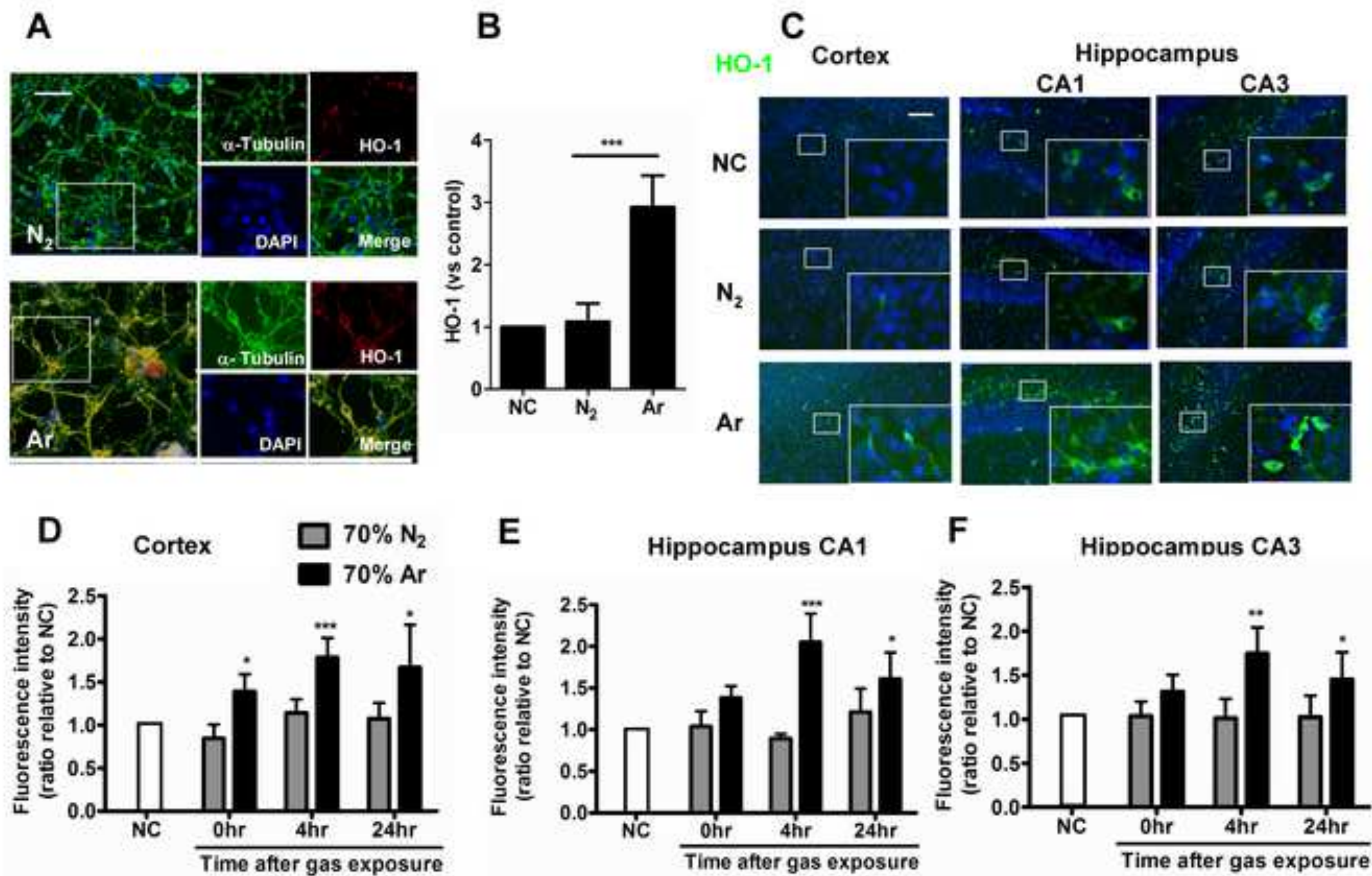

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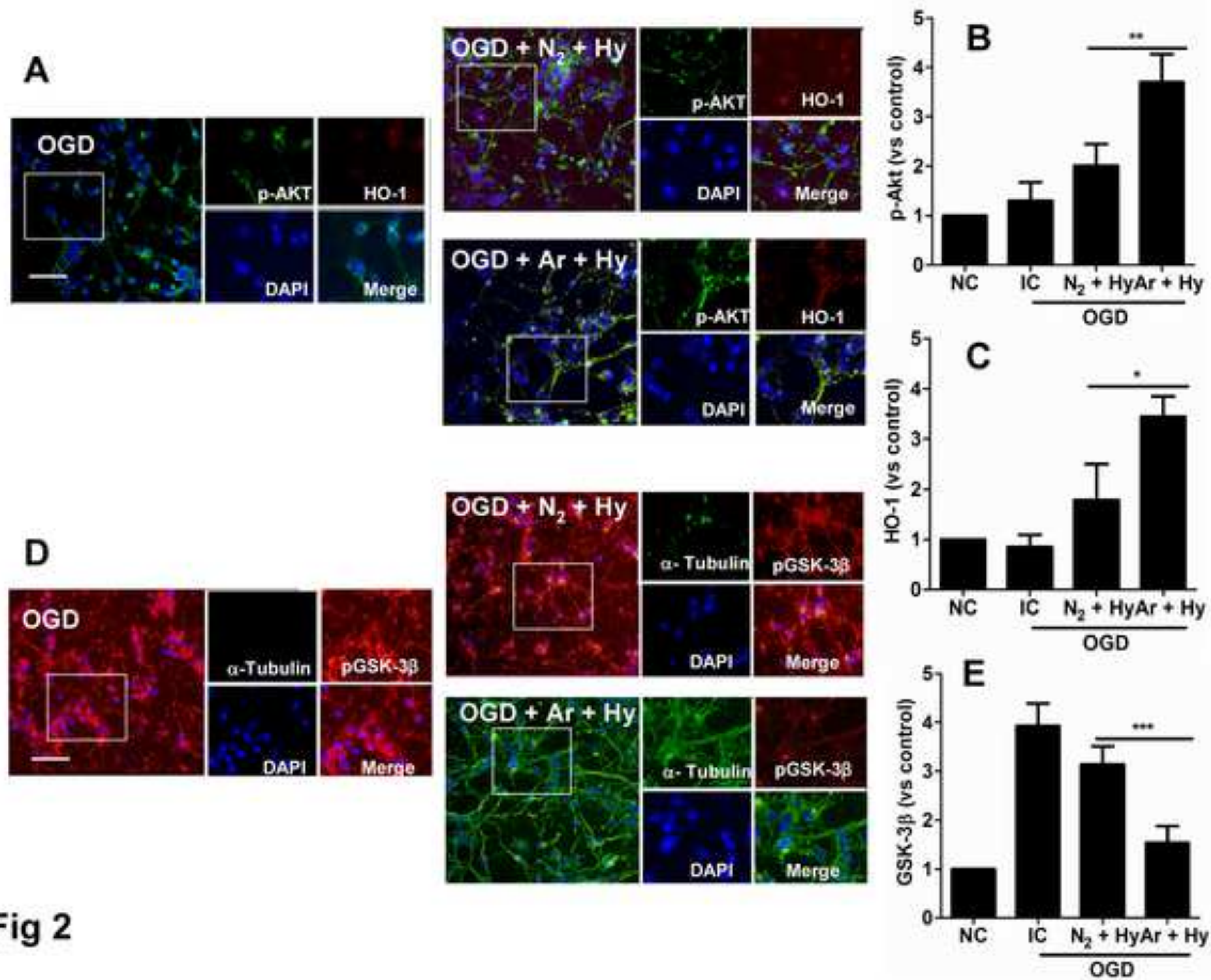

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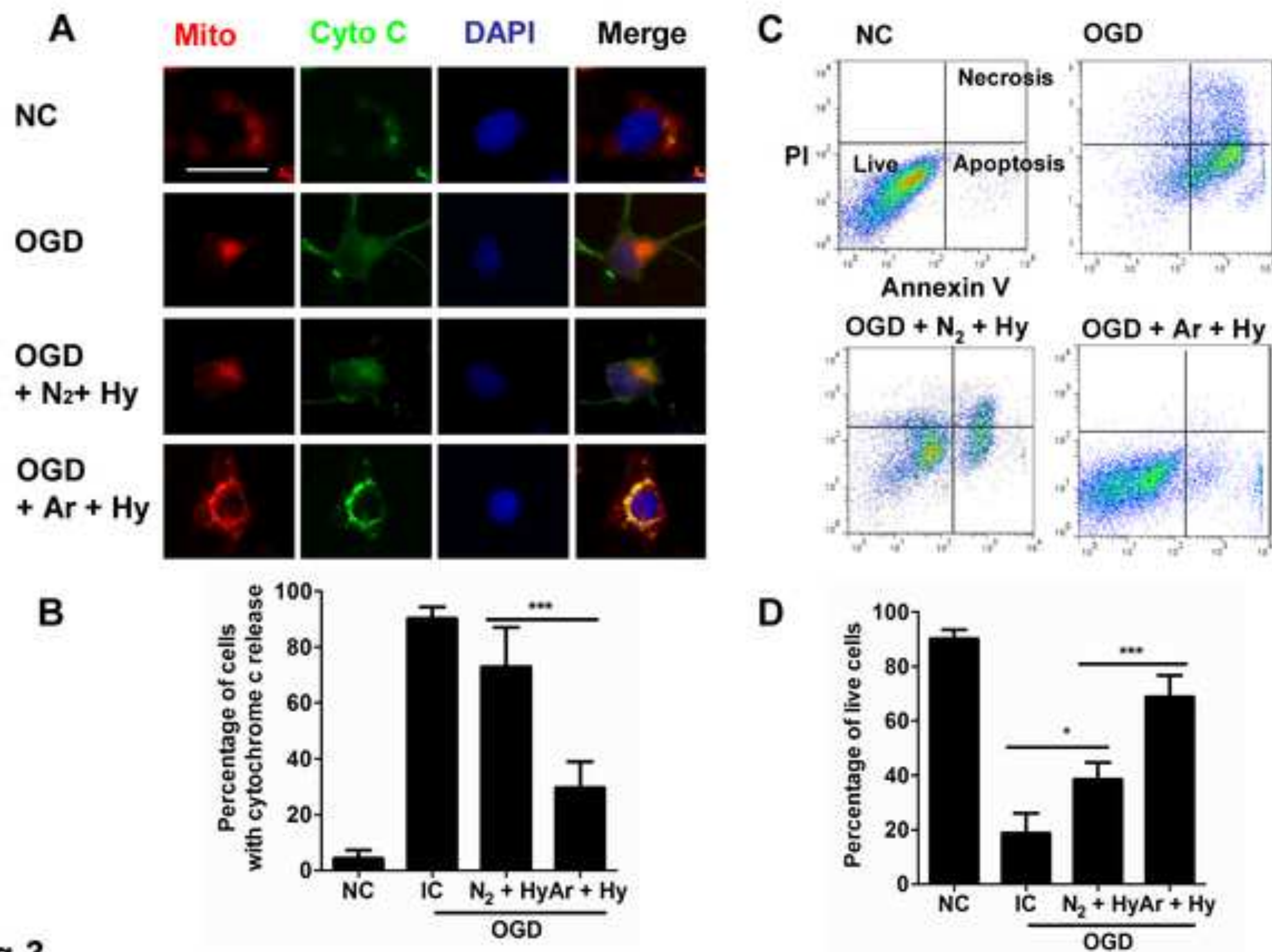

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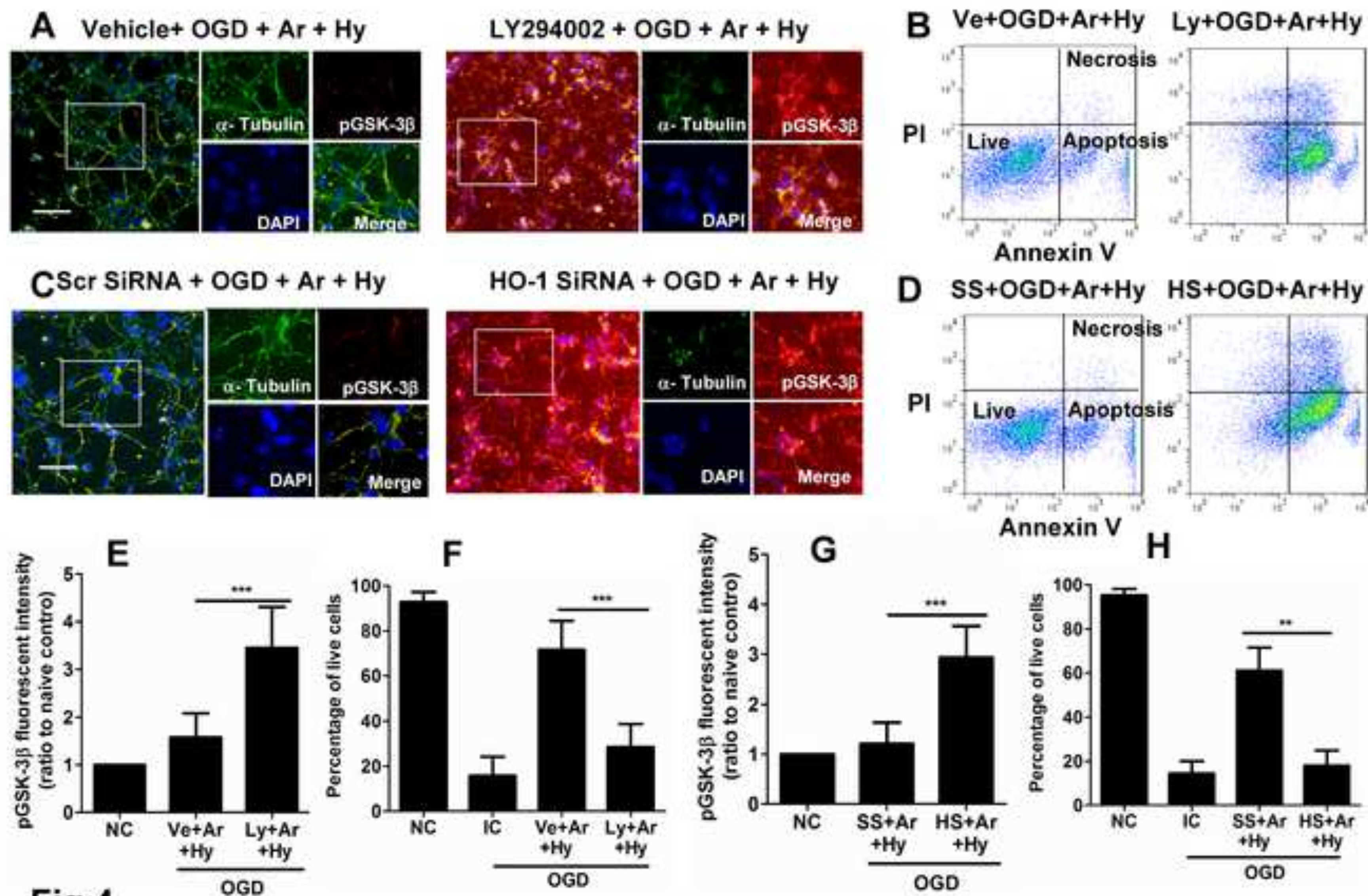

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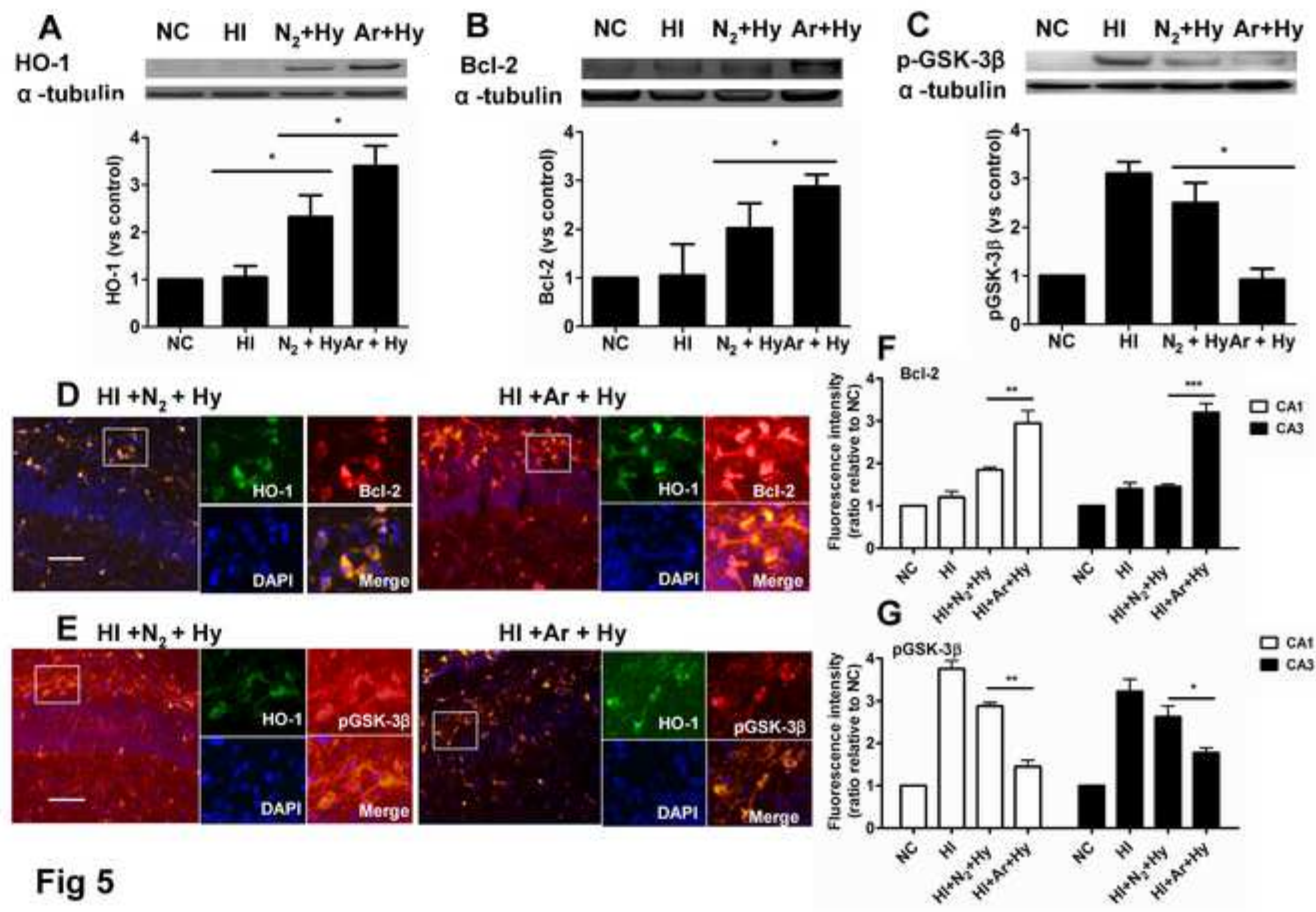

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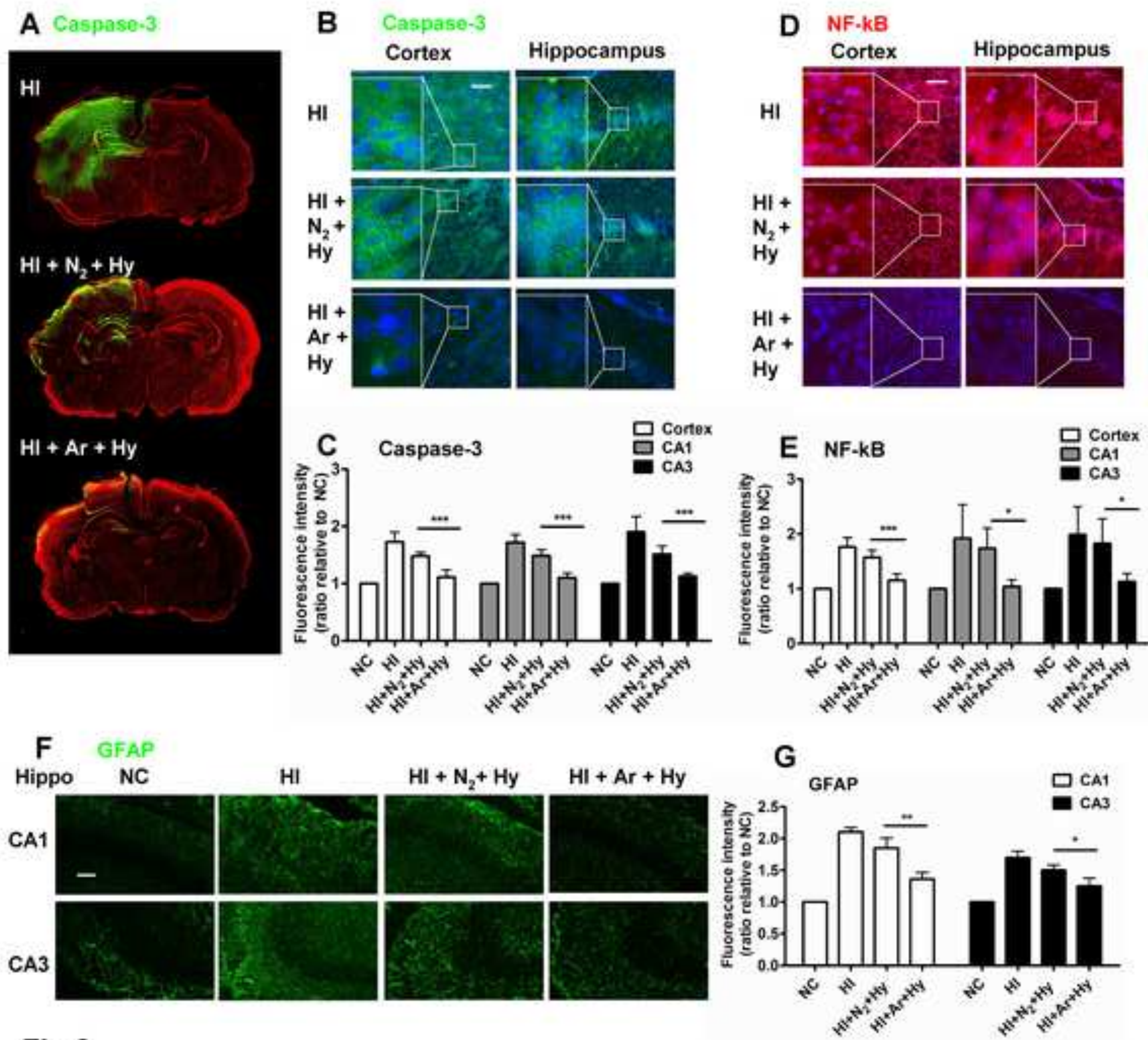

Fig 6

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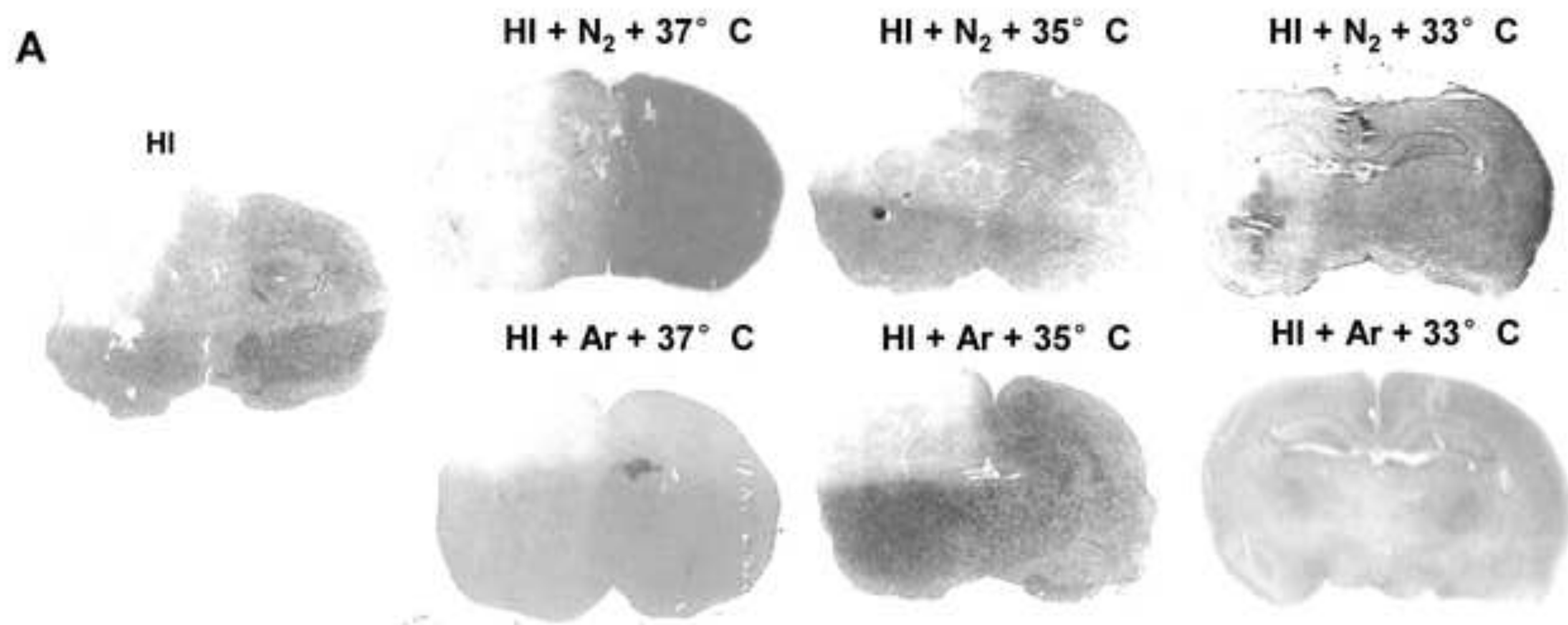

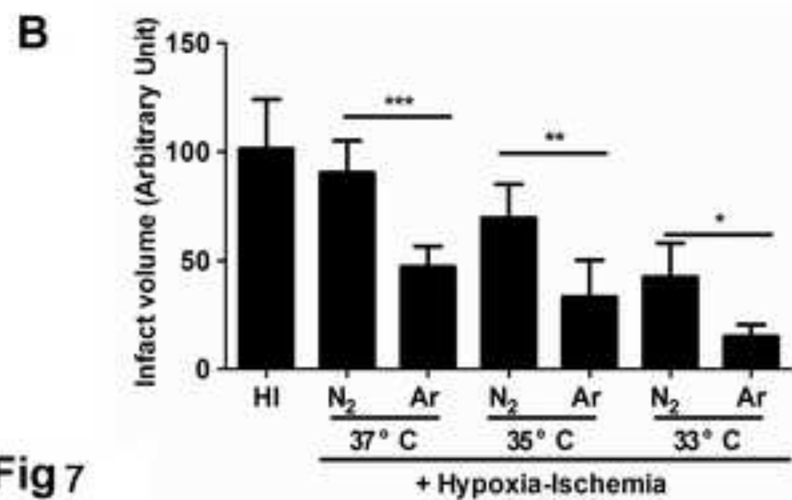

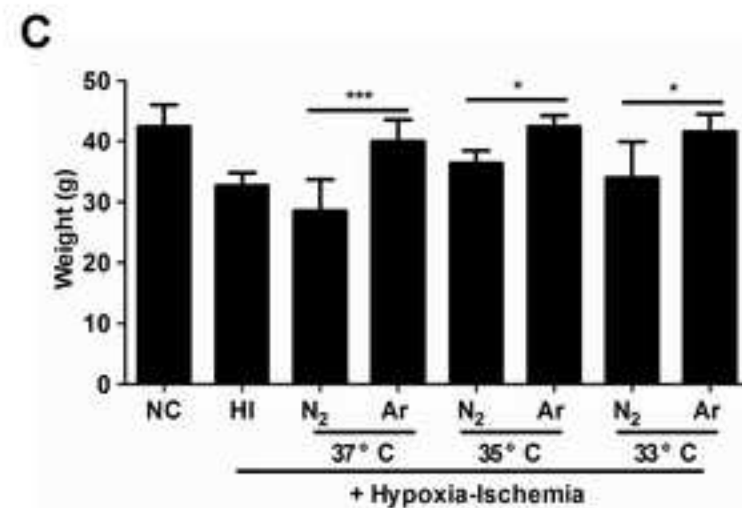

Fig 7

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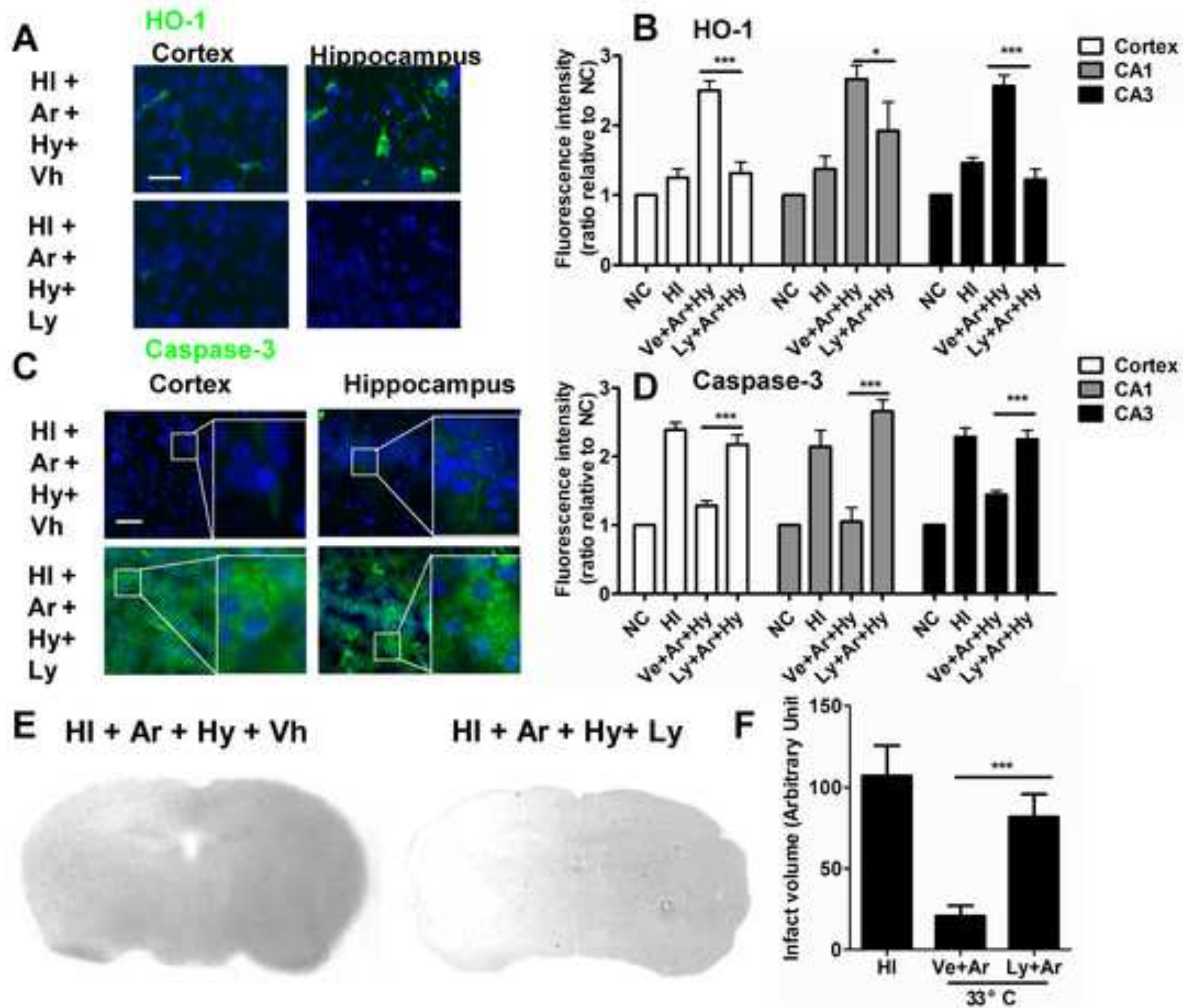

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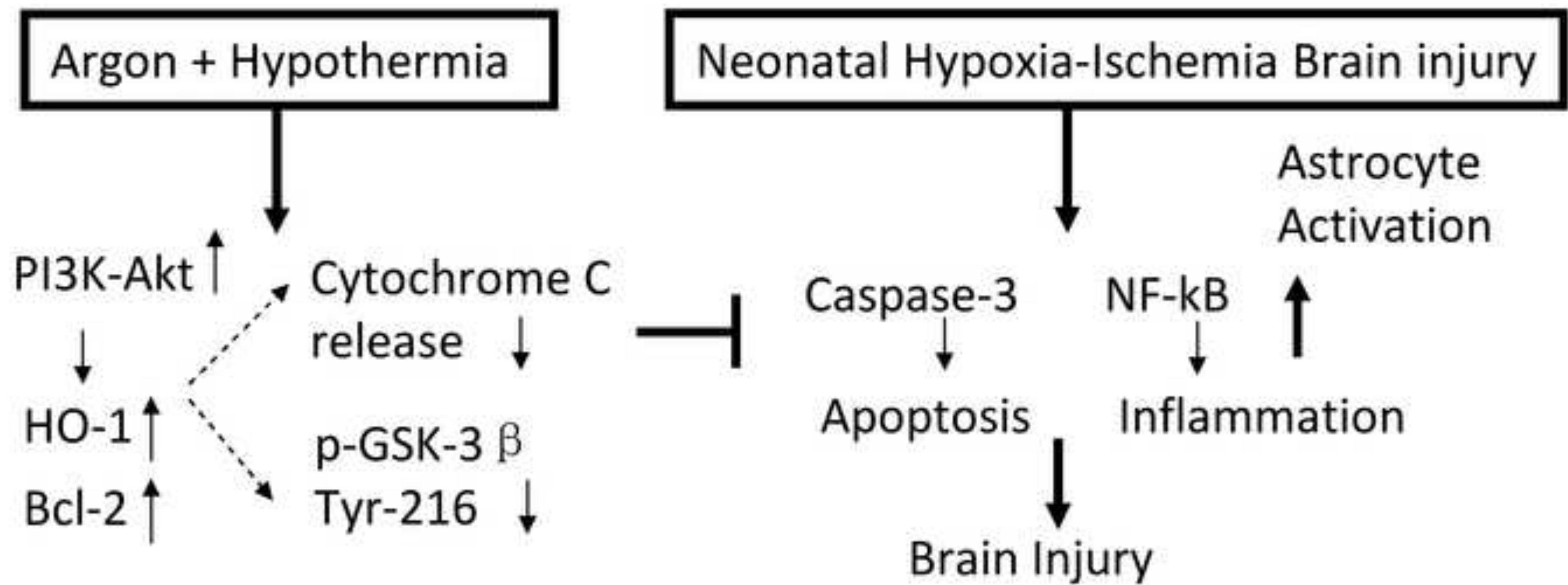

Figure 9