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Evidence that γ-secretase-mediated Notch signaling induces neuronal cell death via the NFκB-Bim pathway in ischemic stroke

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List of nonstandard abbreviations: Aβ: amyloid beta; BIM: Bcl-2-interacting mediator of cell death; FBS: fetal bovine serum; DMEM: dulbecco's modified Eagle's medium; GD: glucose deprivation; DAPT: N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; DBZ: ((S,S)-2-[2-(3,5-difluorophenyl) acetylamino]-N-(5-methyl-6-oxo-6,7-dihydro-5H-dibenzo [b,d] azepin-7yl) propionamide); I/R: ischemia reperfusion; JAK: janus kinase; MCAO: middle cerebral artery occlusion; NICD: Notch intracellular domain; NFκB: nuclear factor kappa B; OGD: oxygen and glucose deprivation; NaCN: sodium cyanide; siRNA: small interfering RNA; TTC: 2% 2,3,5-triphenyltetrazolium chloride.

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ABSTRACT:

Notch-1 (Notch) is a cell surface receptor that regulates cell-fate decisions in the developing nervous system and it may also have roles in synaptic plasticity in the adult brain. Binding of its ligands results in the proteolytic cleavage of Notch by the γ-secretase enzyme complex, thereby causing the release of a Notch intracellular domain (NICD) that translocates to the nucleus, where it regulates transcription. Here we show that activation of Notch modulates ischemic neuronal cell death *in vitro* and *in vivo*. Specifically, our findings from the use of Notch-1 siRNA or the overexpression of NICD indicate that Notch activation contributes to cell death. Using modified NICD, we demonstrate an apoptosis-inducing function of NICD in both the nucleus and the cytosol. NICD transfection-induced cell death was reduced by blockade of calcium signaling, caspase activation and Janus kinase signaling. Inhibition of the Notch-activating enzyme, γ-secretase, protected against ischemic neuronal cell death by targeting an apoptotic protease, cleaved caspase-3, nuclear factor kappa B (NFκB), and the prodeath BH3-only protein, Bim (Bcl-2-interacting mediator of cell death). Treatment of mice with a γ-secretase inhibitor, compound E, reduced infarct size and improved functional outcome in a model of focal ischemic stroke. Furthermore, γ-secretase inhibition reduced NICD, p-P65 and Bim levels *in vivo*. These findings suggest that Notch signaling endangers neurons following ischemic stroke by modulating the NFκB, prodeath protein Bim, and caspase pathways.

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INTRODUCTION:

Gamma-Secretase (γ-secretase) is a membrane-embedded proteolytic complex which generates the amyloid beta-peptide (Aβ) in Alzheimer's disease (AD), and it plays important roles in physiology, especially in Notch-1 receptor-mediated signaling. γ-secretase belongs to a diverse family of intramembrane-cleaving proteases and is composed of four integral membrane proteins: presenilin, nicastrin, Aph-1, and Pen-2 (Krishnaswamy et al., 2009), with presenilin being an aspartyl protease that is the catalytic subunit (De Strooper et al., 1998). Notch-1 is a single-pass type 1 transmembrane receptor that regulates cell-fate decisions in the developing nervous system and it may also play a role in regulating synaptic plasticity in the adult brain (Wang et al., 2004; Lathia et al., 2008). Binding of ligands, such as Delta and Jagged, results in proteolytic cleavage of Notch. This cleavage is facilitated through the γ-secretase complex, which further leads to the release of a Notch-1 intracellular domain (NICD) that translocates to the nucleus, where it regulates transcription (Kramer, 2000, Kopan, 2002). We have recently shown that activity of γ-secretase, and subsequent Notch-1 activation, are elevated after ischemic stroke *in vivo* (Arumugam et al., 2006). Furthermore, γ-secretase activity was elevated in cultured cortical neurons subjected to ischemic-like conditions *in vitro*, consisting of 3-6 h of oxygen and glucose deprivation (OGD) (Arumugam et al., 2006). γ-secretase-mediated Notch-1 signaling endangered neuronal survival in ischemic stroke by modulating pathways that increase their vulnerability to apoptosis, and by activating microglial cells and stimulating the infiltration of proinflammatory leukocytes (Arumugam et al., 2006). These effects on cell death were reduced by intravenous treatment with γ-secretase inhibitors, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) or ((S,S)-2-[2-(3,5-difluorophenyl) acetylamino]-N-(5-methyl-6-oxo-6,7-dihydro-5H-dibenzo [b,d] azepin-7yl) propionamide) (DBZ), resulting in reduced brain damage and improved functional outcome *in vivo* (Arumugam et al., 2006). There is much evidence to suggest that γ-secretase is involved in the regulation of cellular Ca^{2+} homeostasis (Leissring et al., 2002; Smith et al., 2005; Tu et al., 2006). We have recently examined the effect of γ-secretase inhibitors on Ca^{2+}-triggered cell death in B103 rat neuroblastoma cells (Choi et al., 2010). However, a role for the Notch pathway in Ca^{2+}-mediated neuronal death following ischemic stroke remains to be established.

Several studies have provided evidence for the activation of NFκB in neurons, glial and

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endothelial cells during ischemic stroke (Gabriel et al., 1999; Stephenson et al., 2000; Nurmi et al., 2004; Tang et al., 2007). γ-secretase-mediated Notch-1 signaling is required to sustain NFκB activity and cytokine production in peripheral T cells as pharmacological inhibition of Notch attenuates the nuclear distribution of NFκB resulting in reduced transcriptional activity (Shin et al., 2006). γ-secretase-mediated Notch signaling can also enhance NFκB activation by increasing the expression of components of the active NFκB signaling complex (Oswald et al., 1998). Whether Notch induced by cerebral ischemia results in a protracted activation of NFκB and attendant transcriptional activity is not known. In the present study we provide evidence that Notch activation induced by ischemia promotes neuronal cell death and brain injury by a Ca^{2+}-dependent mechanism that can be blocked by inhibition of the Janus kinase (JAK) and NFkB-Bim pathway.

MATERIALS AND METHODS:

Cell culture and transfection: SH-SY5Y (human neuroblastoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal bovine serum (FBS) supplemented with 1% penicillin and streptomycin. These cells were maintained at 37 °C with 5% CO_2. For transfections, cells were plated at a density of $2x10^5$ per well in six-well plates one day before transfection. Cells were transfected using Lipofectamine 2000 (Invitrogen, USA) according to the manufacture's protocol, and then incubated with complete medium for 24 h. Notch1 siRNA was purchased from Santa Cruz (sc-36095). NICD, NICD-NES and NICD-NLS constructs were kindly provided by Dr. B.A. Osborne (University of Massachusetts, USA) (Shin et al., 2006).

Cortical neuron primary cultures: Dissociated cell cultures of neocortical fragments were established from 18-day Sprague-Dawley rat or 16-day C57B/6 mouse embryos as described previously (Okun et al., 2007). Cells were plated in 35- or 60-mm diameter plastic dishes and maintained at 37 °C in Neurobasal medium containing 25 mM glucose and B-27 supplements (Invitrogen, USA), 2 mM L-glutamine, 0.001% gentamycin sulfate and 1 mM HEPES (pH 7.2). Approximately 95% of the cells in such cultures were neurons and the remaining cells were astrocytes. Sodium cyanide was purchased from Sigma (USA) and was prepared as a 100X stock in culture medium.

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Cytosol and nuclear extract preparation: Mouse brain tissue pellets were suspended in 1.5 ml of Buffer A (10 mM Hepes (pH 7.9; 1.5 mM $MgCl_2$, 10 mM KCl and 1 mM EDTA) supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 0.5 μg/ml leupeptin, 1 μM pepstatin and 0.05% Nonidet P-40, and left on ice for 10 min. The nuclei were separated from the cytosol fraction by centrifugation at 4 °C at 3,000 rpm for 10 min. Supernatants were collected as cytosolic protein extracts. To prepare nuclear extracts, the pellets were resuspended in 1.2 ml of Buffer B (20 mM Hepes, pH 7.9; 1.5 mM $MgCl_2$, 420 mM KCl, 25 % Glycerol, and 0.2 mM EDTA) supplemented with 1 mM dithiothreitol, 1 mM phenylmethylsulfonylfluoride, 0.5 μg/ml leupeptin, 1 μM pepstatin, homogenized and left on ice for 30 min. Samples were centrifuged at 14,000 rpm for 20 min at 4 °C. The supernatant, containing nuclear proteins, was transferred to a microcentrifuge tube. 10 μl of the supernatant was used for a Bradford assay and the remainder was stored at -80 °C.

Luciferase assay: SH-SY5Y cells were plated at a density of 2×10^5/well in 12-well dishes and transiently transfected with 50ng of pRL-TK, 200ng of NF-κB-Luc, 400ng of Myc NICD and control pcDNA3.0 using linear PEI (polyscience). pRL-TK trasnsfection was used for normalization of transfection efficiency. Transfected cells were treated with BAPTA/AM (Calbiochem) or AG490 (A.G. Scientific, Inc.) for 12hr or kept as control. Twenty-four hours after transfection, cells were harvested in passive lysis buffer. NFκB luciferase assay performed using Dual-luciferase reporter assay system (Promega). Luciferase activity was measured in a FB12 luminometer (Berthold detection systems).

Glucose deprivation: For glucose deprivation (GD), glucose-free Locke's buffer containing (in mmol/L) 154 NaCl, 5.6 KCl, 2.3 $CaCl_2$, 1 $MgCl_2$, 3.6 $NaHCO_3$, 5 HEPES, pH 7.2, supplemented with gentamycin (5 mg/L) was used. For glucose-deprived experiments, the cultured neurons were incubated with glucose-free Locke's buffer for 3, 12, 18, or 24 h. We have selected the GD model rather than OGD in order to induce slow cell death through apoptotic mechanisms.

Western blot analysis: Protein samples were subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a Tris-glycine running buffer. Gels were then

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electroblotted using a semi-dry transfer apparatus (Bio-Rad) in transfer buffer containing 0.025 mol/L Tris base, 0.15 mol/L glycine, and 10% (v/v) methanol for 1.5 h at 15 V onto a nitrocellulose membrane (Bio-Rad). The membranes were then incubated in blocking buffer (5% non-fat milk in 20 mmol/L Tris-HCL, pH 7.5, 137 mmol/L NaCl, 0.2% Tween-20) for 1 h at 23 °C. The membrane was then incubated overnight at 4 °C with a primary antibody. After washing three times (5 min per wash) with Tris-buffered saline-T (20 mmol/L Tris-HCL, pH 7.5, 137 mmol/L NaCl, 0.2% Tween-20), the membrane was incubated with a horse radish peroxidase-conjugated secondary antibody. After washing five times (5 min per wash) with Tris-buffered saline, the membrane was incubated with chemiluminescent substrate for enhanced chemiluminescence (Pierce Endogen, Rockford, IL, USA) for 5 min, and chemiluminescent signals were visualized by exposing the membrane to x-ray film (Kodak x-ray film; InterScience, Mississauga, ON, USA).

Cell viability assay: Cell viability was determined by the trypan blue exclusion assay. For the transfected cells, cell viability was determined based on the morphology of GFP-positive cells under a fluorescence microscope.

Middle cerebral artery occlusion and reperfusion: Three-month-old C57BL/6 male mice were used for *in vivo* experiments. The focal cerebral ischemia/reperfusion model was similar to that described previously (Arumugam et al. 2004). Briefly, the mice were anesthetized with isofluorane, a midline incision was made in the neck, and the left external carotid and pterygopalatine arteries were isolated and ligated with 5-0 silk thread. The internal carotid artery (ICA) was occluded at the peripheral site of the bifurcation of the ICA and the pterygopalatine artery with a small clip, and the common carotid artery (CCA) was ligated with 5-0 silk thread. The external carotid artery (ECA) was cut, and a 6-0 nylon monofilament with a tip that was blunted (0.2–0.22 mm) using a coagulator was inserted into the ECA. After the clip at the ICA was removed, the nylon thread was advanced into the middle cerebral artery (MCA) until light resistance was felt. The nylon thread and the CCA ligature were removed after 1 h of occlusion to initiate reperfusion. In the sham group, these arteries were visualized but not disturbed. Mice were administered either 1 mg/kg compound E or vehicle (DMSO) by infusion into the femoral vein (20 μL) 15 min prior to start the surgery, which was approximately 30 min before the onset of ischemia or immediately after the ischemic period. Cerebral blood flow was measured by placing the animal's head in a fixed frame after it had been anesthetized and prepared for surgery. A craniotomy was performed to access the

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left middle cerebral artery and was extended to allow positioning of a 0.5-mm Doppler probe (Moor LAB, Moor Instruments, Devon, UK) over the underlying parietal cortex approximately 1 mm posterior to bregma and 1 mm lateral to the midline. Laser-Doppler recordings are expressed as percentages of the pre-ischemic baseline and were averaged over 30- min periods during 1 h of ischemia, and 0, 30, 90, 120 and 180 and min of post-ischemic reperfusion. The University of Queensland Animal Care and Use Committee approved these procedures.

Neurological assessment: The functional consequences of focal cerebral ischemia and reperfusion (I/R) injury were evaluated using a 5-point neurological deficit score (0, no deficit; 1, failure to extend right paw; 2, circling to the right; 3, falling to the right; and 4, unable to walk spontaneously) and were assessed in a blinded fashion, as described previously (Bederson et al. 1986).

Quantification of cerebral infarction: At 72 h of reperfusion, the mice were euthanized with a lethal dose of isofluorane. The brains were immediately removed and placed into PBS (4 °C) for 15 min, and 2-mm coronal sections were cut with a tissue cutter. The brain sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) in phosphate buffer at 37°C for 15 min. The stained sections were photographed, and the digitized images were used for analysis. The borders of the infarct in each brain slice were outlined and the area quantified using NIH image 6.1 software (National Institutes of Health, Bethesda, MD, USA). To correct for brain swelling, the infarct area was determined by subtracting the area of undamaged tissue in the left hemisphere from that of the intact contralateral hemisphere. Infarct volume was calculated by integration of infarct areas for all slices of each brain in a blinded manner, and was expressed as a % of the ipsilateral hemisphere.

Data analysis: All the results are reported as the means ± S.D. The overall significance of the data was examined by one-way analysis of variance (ANOVA). The differences between the groups were considered significant at $P<0.05$ with the appropriate Bonferroni correction made for multiple comparisons. Neurological behavior scores were analyzed by using a nonparametric Kruskal–Wallis test and Dunn's Multiple Comparison Test.

RESULTS:

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Modulation of calcium overload-triggered cell death by Notch: Calcium overload in response to ischemic injury is known to trigger cell death by activating various proteases including caspases and calpains, and by triggering other catabolic processes mediated by lipases and nucleases. To evaluate the role of Notch in calcium overload-triggered cell death, we used RNA interference to specifically knockdown endogenous Notch-1 (**Fig. 1A**) and gene transfection approaches to overexpress the notch intracellular domain (NICD) (**Fig. 1B**) in human neuroblastoma cells (SH-SY5Y). To induce calcium overload, the calcium ionophore, A23187, was added after 12 h to cells transfected with either Notch-1 siRNA or control siRNA. A23187-induced SH-SY5Y cell death was significantly reduced in cells transfected with Notch-1 siRNA compared to control siRNA (**Fig. 1C**). Next, we examined whether the Notch pathway contributes to glucose deprivation (GD) or sodium cyanide (NaCN) -induced cell death, which is known to involve calcium overload as the result of cellular energy depletion. Both GD and NaCN-induced cell death were significantly reduced in cultures transfected with Notch-1 siRNA compared to the control siRNA group (**Fig. 1D**). Conversely, ectopic NICD expression which was sufficient to induce cell death (**Fig. 1E**) further enhanced A23187-triggered cell death (**Fig. 1F**).

Modulation of cell death by NICD over-expression and its localization: NICD migrates into the nucleus and associates with a transcription factor, RBP-Jk, which in turn upregulates the expression of various Notch effector target genes. To test whether Notch-mediated cell death is correlated with nuclear translocation of NICD, we employed two modified GFP-NICD constructs (**Fig. 2A**). Specifically, GFP-NICD was modified by inclusion of either an additional nuclear export signal to increase cytosol levels (NICD-NES) or an additional nuclear localization signal to increase its nuclear retention (NICD-NLS) (Shin et al., 2006). SH-SY5Y cells were transfected with either NICD-NLS or NICD-NES, and fluorescence imaging was used to confirm their subcellular localization (**Fig. 2B**). We observed that transfection with either NICD, NICD-NLS or NICD-NES resulted in significantly increased cell death compared to vector transfected group (**Fig. 2C)**. Cell death was substantially potentiated in the NICD-NES transfected group when compared to the NICD and NICD-NLS groups (**Fig. 2C)**. These results demonstrate the that cytosol-localized NICD can trigger cell death cascades that may be independent of the expression of Notch effector target genes.

Effects of a calcium chelator, pan caspase inhibitor and Janus kinase (JAK) inhibitor on NICD-mediated cell death: Because Notch modulation can influence calcium overload-

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triggered cell death, we next investigated the relationship between calcium signaling and caspase activation in NICD over-expression-induced cell death. SH-SY5Y cells were transfected with NICD, NICD-NLS or NICD-NES, and then treated with 10 μM BAPTA/AM (a cell-permeable calcium chelator) or 10 μM Z-VAD (a pan caspase inhibitor) 2 h post-transfection (**Fig. 3A, B**). As demonstrated previously (**Fig. 2C**), cell death was enhanced by over-expression of NICD, NICD-NLS, or NICD-NES 24 h following transfection. Treatments with BAPTA/AM (**Fig. 3C**) or Z-VAD significantly ameliorated cell death induced by NICD. Key players in the intracellular response to ischemic stroke are kinase pathways that induce alterations in the pattern of gene transcription (Planas et al., 2006). To examine the contribution of kinases to Notch-induced cell death, we tested the effects of several kinase inhibitors (data not shown). Among them, the Janus kinase (JAK) inhibitor (AG490) was effective against NICD-induced cell death. Two hours after transfection of NICD, NICD-NLS, or NICD-NES, SH-SY5Y cells were treated with 10 μM AG490 and cell death was assessed 24 h later. NICD-induced cell death was significantly reduced by AG490 treatment in NICD and NICD-NLS transfected groups, but interestingly it had no effect in the NICD-NES transfected group in which cytosolic NICD retention was enhanced (**Fig. 3D**). This may suggest that JAK signaling is essential for cell death potentiation by NICD in the nucleus but not in the cytosol. Furthermore, we have a performed NFκB luciferase reporter assay in order to test if NICD-induced NFκB activity was blocked by the calcium chelator, BAPTA/AM (5μM) and the JAK2 inhibitor, AG490 (5μM). Our data indicate that both BAPTA/AM and AG490 significantly reduced the NICD-induced NFκB activity (**Fig. 3E**).

Involvement of γ-secretase in cell death induced by ischemia-like conditions: We next investigated the effect of a γ-secretase inhibitor against ischemic stroke-like cell death *in vitro* using both SH-SY5Y and primary neuronal cells. We subjected SH-SH5Y cells to GD in the presence of a γ-secretase inhibitor, either DAPT or compound E. GD-induced cell death was significantly reduced by either DAPT or compound E (**Fig. 4A**). Since we previously showed that DAPT reduced GD-induced primary neuronal cell death, here we only show the effect of compound E (Arumugam et al., 2006). Similar to in SH-SY5Y cells, compound E also significantly reduced GD-induced cell death in primary cortical neurons (**Fig. 4B**). Next we have performed immunocytochemistry analysis of NICD in primary neuronal cells following GD. NICD translocation into cytoplasm and nucleus was observed following GD in primary neurons (**Fig. 4C**). Furthermore, we investigated how the γ-secretase inhibitor protects against

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neuronal cell death under GD conditions. GD resulted in increased levels of an apoptotic protease cleaved caspase-3, nuclear factor kappa B (NFκB: Phospho-p65 and Phospho-p50) and a pro-death BH3-only protein, Bim (**Fig. 4D**). Treatment with the γ-secretase inhibitor reduced levels of cleaved caspase-3, P-p65, P-p50 and Bim (**Fig. 4D, E, F**). Furthermore, in order to test whether NFκB activation is directly linked to GD-mediated apoptosis via Bim, we treated neurons with an NFκB inhibitor (7-Methoxy-5,11,12-trihydroxycoumestan, 1 μM; Sigma, USA). The NFκB inhibitor reduced expression levels of both cleaved caspase-3 and Bim after 12 and 24 h of GD. In addition, we observed that at 24 h the level of apoptosis inducing factor (AIF) induced by GD was attenuated by the NFκB inhibitor (**Fig. 4G)**.

γ-secretase inhibitor treatment *in vivo* protects against neurological deficit and infarct development after ischemic stroke: We have previously shown that the γ-secretase inhibitors, DAPT and DBZ, reduce brain damage and neurological deficits measured 3 d after cerebral ischemia-reperfusion. The cerebral blood flow measurements obtained immediately before and after MCAO showed >90% reduction in blood flow to the MCAO infarct region, which did not differ between groups. In order to test whether a γ-secretase inhibitor might protect against stroke-induced brain injury by similar mechanisms as those observed *in vitro* (see Fig. 4), here we used the γ-secretase inhibitor, compound E, which has not previously been tested in a model of stroke. Treatment with compound E by intravenous infusion 30 min before or immediately after ischemia resulted in reduced brain damage (**Fig. 5A**) and improved functional outcome (**Fig. 5B**). Nonparametric Kruskal–Wallis test and Dunn's Multiple Comparison Test shows that both pre- and post- compound E treated group were not significantly different from the sham group. However, only the vehicle-treated group showed a significant increase in neurological deficit score compared with sham animals. Our data clearly show that the compound E-treated groups had a reduced neurological deficit score. Next, we analyzed the protein levels of NICD and Bim in the ipsilateral brain region following 1, 3 or 24 h of reperfusion (**Fig. 5C**). As expected, we found that both NICD and Bim were elevated in the brain following ischemic stroke (**Fig. 5C**). Next, we measured the levels of NICD, p-P65 and Bim to assess whether compound E might protect against ischemic stroke-induced brain injury by affecting these proteins (**Fig. 5D**). Our data confirmed that levels of NICD, p-P65 and Bim were all significantly reduced in the compound E-treated group compared to the vehicle-treated group (**Fig. 5D, E, F, G**).

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DISCUSSION:

A major finding of this study is that Notch signaling endangers neurons following ischemic stroke by modulating the NFκB, prodeath protein Bim, calcium signaling, and caspase pathways, and that this neuronal damage can be inhibited *in vitro* and *in vivo* by treatment with a γ-secretase inhibitor. We have previously shown that activity of γ-secretase and subsequent Notch activation are elevated after ischemic stroke (Arumugam et al., 2006). Furthermore, γ-secretase activity was elevated in cultured cortical neurons after ischemic conditions, and γ-secretase-mediated Notch signaling endangers neurons in ischemic stroke by increasing their vulnerability to apoptosis, and by activating microglial cells and stimulating the infiltration of proinflammatory leukocytes (Arumugam et al., 2006). Thus, intravenous treatment with γ-secretase inhibitors DAPT or DBZ improved functional outcome and reduced brain damage (Arumugam et al., 2006). Thus, here we have further investigated the mechanisms by which Notch mediates cell death following ischemic stroke.

Ca^{2+} is a major intracellular messenger that mediates many physiological responses in neurons (Foster, 2007). Studies of animal and cell culture models have clearly demonstrated that neuronal Ca^{2+} overload is pivotal to the death of neurons following ischemic stroke (Broughton et al., 2009). Ca^{2+} induces oxidative stress through several different mechanisms. These include activation of oxygenases, perturbation of mitochondrial Ca^{2+} and energy metabolism, and induction of membrane-associated oxidative stress. Ca^{2+} overload also triggers apoptosis, a form of programmed cell death (Mattson et al., 2001; Berna-Erro et al., 2009; Broughton et al., 2009). This might occur by Ca^{2+}-mediated activation of pro-apoptotic proteins such as Bax, Par-4 and p53, leading to mitochondrial membrane permeability changes, release of cytochrome c, and caspase activation (Mattson et al., 2000). There is much evidence to suggest that γ-secretase is also involved in the regulation of cellular Ca^{2+} homeostasis. Mutations in the γ-secretase subunit, presenilin, perturb endoplasmic reticulum (ER) Ca^{2+} homeostasis such that greater amounts of Ca^{2+} are released upon stimulation, possibly as a result of alterations in inositol 1,4,5-trisphosphate (IP3), ryanodine receptor channels, calcium-ATPases and the ER stress protein Herp (LaFerla, 2002; Smith et al., 2005; Tu et al., 2006).

These observations implicate the involvement of γ-secretase-mediated Notch signaling in neuronal cell death under the conditions of overloaded intracellular Ca^{2+} following ischemia. To directly determine if γ-secretase -mediated Notch signaling is involved in Ca^{2+}-induced

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cell death, we recently examined the effect of γ-secretase inhibitors on Ca^{2+}-triggered cell death in B103 rat neuroblastoma cells (Choi et al., 2010). However, the role of Notch signaling in Ca^{2+}-mediated neuronal cell death following ischemic stroke is still poorly understood. Using siRNA to target Notch-1, and NICD transfection to increase Notch signaling, here we found evidence for an effect of Notch in Ca^{2+}-triggered cell death of cultured human neuroblastoma cells. Furthermore, we showed that cell death induced by ischemic stroke-like conditions (i.e. chemical hypoxia and GD) is significantly reduced in Notch-1 -depleted cells. These conclusions regarding the role of Notch in Ca^{2+}-triggered cell death were strengthened by the observation of reduced death of NICD-transfected cells in the presence of BAPTA-AM.

In order to investigate the mechanisms by which NICD induces cell death, we next studied if NICD localization in the cytosol or its translocation to the nucleus was related to NICD-induced cell death. Both cytosol and nucleus localization of NICD caused significant cell death, but NICD localized in cytosol further potentiated the cell death. Cell death induced by NICD transfection was significantly reduced by either the pan caspase inhibitor Z-VAD or the selective chelator of intracellular Ca^{2+} stores, BAPTA AM. These results suggest that NICD over-expression leads to calcium-induced cell caspase-mediated apoptosis. However, treatment with a JAK inhibitor only blocked NICD-induced cell death in nuclear NICD over-expressed cells, whereas it had no effect in the cytosol-transfected NICD group. This result suggests that cytosolic NICD-induced cell death occurs independently of JAK transcription.

Several studies have provided evidence for the activation of NFκB in neurons, glial and endothelial cells during ischemic stroke (Gabriel et al., 1999; Stephenson et al., 2000; Nurmi et al., 2004; Tang et al., 2007). The activation of NFκB is mainly controlled at the post-transcriptional level by complex formation with the inhibitory subunit IκB in the cytoplasm. Activating stimuli (e.g. TNF) activate the IκB kinase leading to phosphorylation of the inhibitory subunit, which is then ubiquitinylated and degraded by the proteasome (Wallach et al., 2002). This triggers liberation and translocation of the NFκB complex into the nucleus, where it initiates transcription by binding to regulatory DNA sequences. γ-secretase-mediated Notch signaling is required to sustain NFκB activity and cytokine production in peripheral T cells since inhibition of Notch by γ-secretase inhibitors attenuates the translocation of NFκB to the nucleus and downregulates its activity (Shin et al., 2006). γ-secretase-mediated Notch

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signaling can also enhance NFκB activation by increasing the expression of components of the active NFκB signaling complex (Oswald et al., 1998). Notch signaling may also increase NFκB activity by binding to and enhancing the nuclear distribution of the active NFκB signaling complex (Shin et al., 2006). We have found that Notch is activated in cultured neurons subjected to ischemia-like conditions, as demonstrated by an increase in levels of Notch intracellular domain (NICD) - the active component of Notch signaling, during glucose deprivation (GD).

Furthermore, our studies have found that γ-secretase inhibitor treatment reduces GD-induced NFκB-p65 and NFκB-p50 levels in neurons. To date, only a few NFκB-regulated genes have been identified to be involved in neuronal cell death. NFκB has been shown to have a significant association with the pro-death BH3-only protein, Bim, in mediating apoptosis following ischemic stroke (Okuno et al., 2004). Recent evidence implies that Bim is essential for pro-apoptotic Bax and Bak activation (Okuno et al., 2004). Bim targets the BH3 domain of Bax, causing its conformational change, which leads to its oligomerization and integration into the outer mitochondrial membrane, and the formation of channels for release of cytochrome *c* and other mitochondrial apoptogenic factors (Donovan et al., 2002), which in turn lead to apoptosis. Our data shows that a γ-secretase inhibitor not only reduces p65 and p50, but also Bim, *in vitro* and *in vivo* following conditions of ischemic stroke. We have further confirmed that inhibition of NFκB activity in ischemic conditions in neurons reduces the level of Bim, which further supports the conclusion that γ-secretase-mediated Notch signaling induces neuronal cell death via NFκB-Bim pathway.

We have previously shown that after 3 days, the degree of brain damage and neurological deficit resulting from cerebral ischemia-reperfusion is reduced in mice transgenic for antisense Notch compared to nontransgenic mice (Arumugam et al., 2006). Here we report similar results obtained using another potent γ-secretase inhibitor, Compound E. Notably, Compound E was effective in reducing infarct volume and improving functional outcome when administered 30 min before the onset of ischemia or immediately after the ischemic period. It was shown previously that compound E cross the blood-brain-barrier and inhibits cortical γ-secretase activity, determined ex vivo using the recombinant substrate (Grimwood et al., 2005). Our *in vivo* data also confirm the neuroprotective mechanisms of Compound E observed *in vitro* in that levels of NICD, p65 and Bim were reduced in Compound E-treated

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animals compared to vehicle-treated control animals. Our findings suggest that agents that target γ-secretase and Notch signaling pathway may prove effective in reducing neuronal cell death and brain injury following ischemic stroke.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Thiruma V. Arumugam, Vardan T. Karamyan, Sung-Chun Tang, Sic L. Chan, Tim Magnus, Christopher G. Sobey and Dong-Gyu Jo.

Conducted experiments: Thiruma V. Arumugam, Yi-Lin Cheng, Yun-Hyung Choi, Yuri Choi, Sunghee Yang, Young-Kwang Yun, Jong-Sung Park, Dong Kwon Yang, John Thundyil, Mathias Gelderblom.

Performed data analysis: Thiruma V. Arumugam, Yi-Lin Cheng, Yun-Hyung Choi, Mathias Gelderblom and Dong-Gyu Jo.

Wrote or contributed to the writing of the manuscript: Thiruma V. Arumugam, John Thundyil, Vardan T. Karamyan, Sic L. Chan, Tim Magnus, Christopher G. Sobey and Dong-Gyu Jo.

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Footnotes

[#] These authors contributed equally to this work.

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FIGURE LEGENDS:

Figure 1: Modulation of cell death by Notch-1: (**A**) knockdown of endogenous Notch-1 using Notch-1 siRNA and (**B**) overexpression of notch intracellular domain (NICD) in human neuroblastoma cells (SH-SY5Y). (**C**) A23187, a calcium ionophore, -induced SH-SY5Y cell death was significantly reduced in cells transfected with Notch-1 siRNA compared to control siRNA. ** P<0.01 compared to the control siRNA group; # P<0.05 compared to the control siRNA group. (**D**) Glucose deprivation (GD) or sodium cyanide (NaCN)-induced cell death was significantly reduced in cultures transfected with Notch-1 siRNA compared to the control siRNA group. ** P<0.001 compared to the control siRNA group; ### P<0.05 compared to the control siRNA group. (**E**) NICD transfection itself significantly increased cell death after 24 h compared with vector-transfected cells. ** P<0.01 compared to the vector group. (**F**) A23187-induced calcium-mediated cell death was significantly increased when NICD was over-expressed in human neuroblastoma cells. *** P<0.001 compared to the vector group.

Figure 2: Modulation of cell death by NICD localization: (**A**) Schematic diagram shows that GFP-NICD was modified by inclusion of either an additional nuclear export signal to increase cytosol levels (NICD-NES) or an additional nuclear localization signal to increase its nuclear retention (NICD-NLS). (**B**) Representative immunofluorescence shows that the GFP-NICD, NICD-NLS or NICD-NES expression vectors were transfected into SH-SY5Y cells. (**C**) Transfection with NICD, NICD-NLS, or NICD-NES resulted in significantly increased cell death compared to vector-transfected group. *** P<0.001 compared to the vector group.

Figure 3: Effect of a calcium chelator, caspase inhibitor and kinase inhibitor against NICD-mediated cell death: (**A and B**) NICD, NICD-NLS, or NICD-NES -transfected SH-SY5Y cells treated with BAPTA/AM or Z-VAD. (**C**) NICD, NICD-NLS, or NICD-NES –induced cell death was significantly reduced by treatment of BAPTA/AM or Z-VAD. ** P<0.001 compared to the vector group; ++ P<0.05 compared to the NICD vehicle group; ## P<0.05 compared to the NICD-NLS vehicle group; $$ P<0.01 compared to the NICD-NES vehicle group. (**D**) Janus kinase (JAK) inhibitor (AG490) was significantly reduced NICD- or NICD-NLS-induced cell death. Figure. Increased activity of NF-κB by NICD was decreased by BAPTA/AM and AG490. * P<0.01 compared to the vector group; ** P<0.001 compared to the vector group; ++ P<0.01 compared to the NICD vehicle group; ## P<0.01 compared to the NICD-NLS vehicle group. (**E**) NF-κB luciferase reporter (200ng) plasmid and pRL-TK (50ng) plasmid of an internal control were transiently transfected with NICD (400ng) or

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empty control (400ng) into SH-SY5Y cells. Transfected cells were treated with the calcium chelator, BAPTA/AM (5μM) and JAK2 inhibitor, AG490 (5μM) for 12 h. Cells were harvested for dual luciferase assay after 12 h exposure to BAPTA/AM, AG490 or the vehicle (DMSO). ***P < 0.0001 compared to the control value.

Figure 4: γ-secretase inhibitor protects against ischemic neuronal cell death: (**A**) GD-induced SH-SY5Y cell death was significantly reduced by either DAPT or compound E. ** P<0.01 compared to the GD group. (**B**) Compound E significantly reduced GD-induced cell death in primary cortical neurons. ** P<0.01 compared to the GD group. (**C**) NICD translocation into cytoplasm and nucleus following GD in primary neurons. (**D, E and F**) Treatment with the γ-secretase inhibitor significantly reduced levels of cleaved caspase-3, P-p65, P-p50 and Bim in primary neurons following 12 h GD condition. *** P<0.05 compared to the GD group; n=6 in each group. (**G**) The NFκB inhibitor, 7-methoxy-5,11,12-trihydroxycoumestan, reduced expression levels of both cleaved caspase-3 and Bim after 12 and 24 h of GD. The level of apoptosis inducing factor (AIF) was also attenuated by the NFκB inhibitor at 24 h following GD.

Figure 5: γ-secretase inhibitor treatment *in vivo*: (**A**) Treatment with compound E by intravenous infusion 30 min before the onset of ischemia or immediately after ischemia resulted in reduced brain damage and (**B**) reduced neurological deficit. *** P<0.05 compared to the vehicle treated group; n=6-14 in each group. Nonparametric Kruskal–Wallis test and Dunn's Multiple Comparison Test shows that both pre- and post- compound E treated groups were not significantly different from sham group. Neurological data are shown as grouped with mean only values. (**C**) The protein levels of NICD and Bim in the ipsilateral brain region were increased following 3 or 24 h of reperfusion. (**D, E, F and G**) The protein levels of NICD and Bim were significantly reduced in the compound E-treated group compared to the vehicle-treated group. ** P<0.05 compared to the vehicle treated group; *** P<0.01 compared to the vehicle treated group; n= 6-8 in each group.

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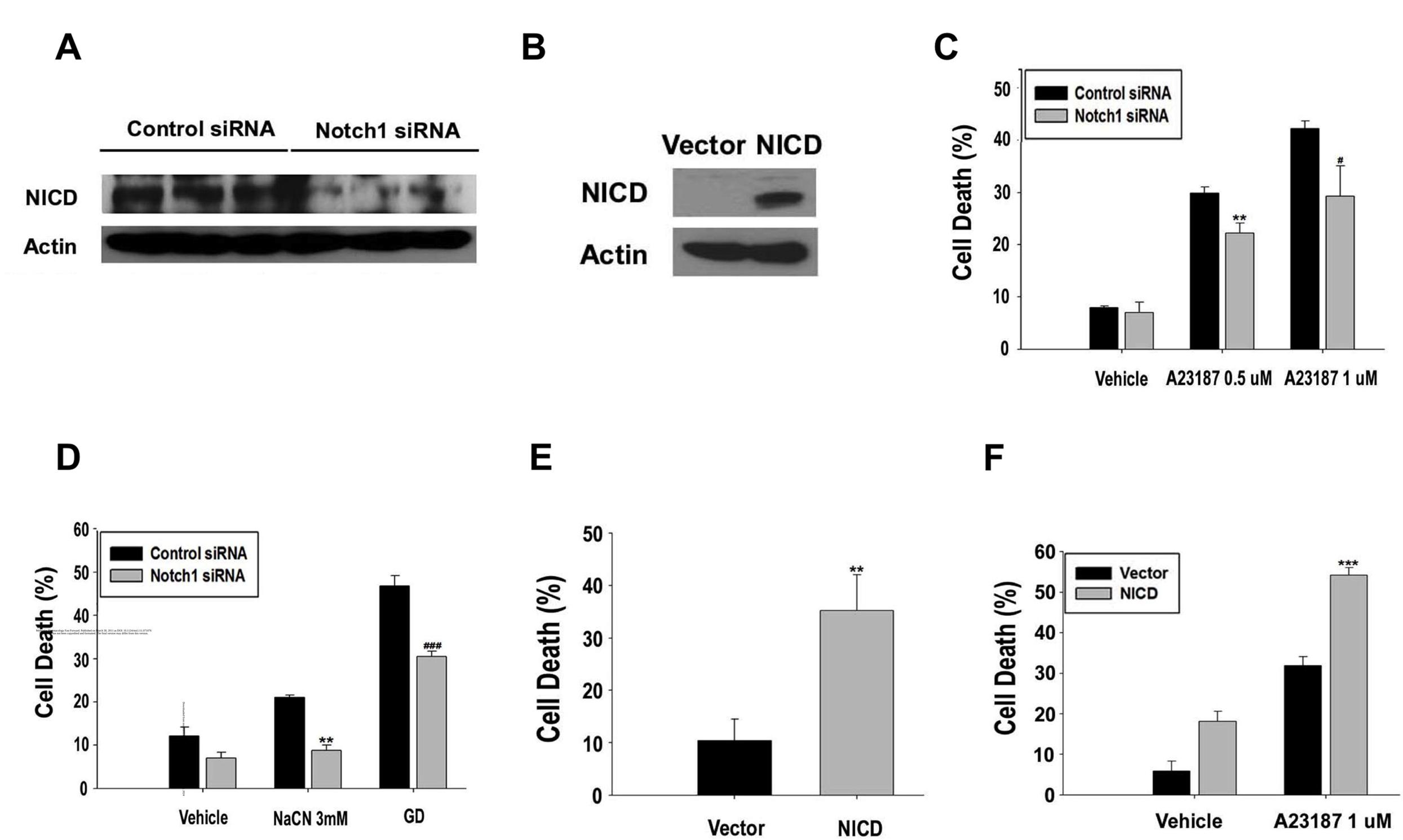
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NICD
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Cell Death (%)
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A23187 1 uM
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Notch1 siRNA
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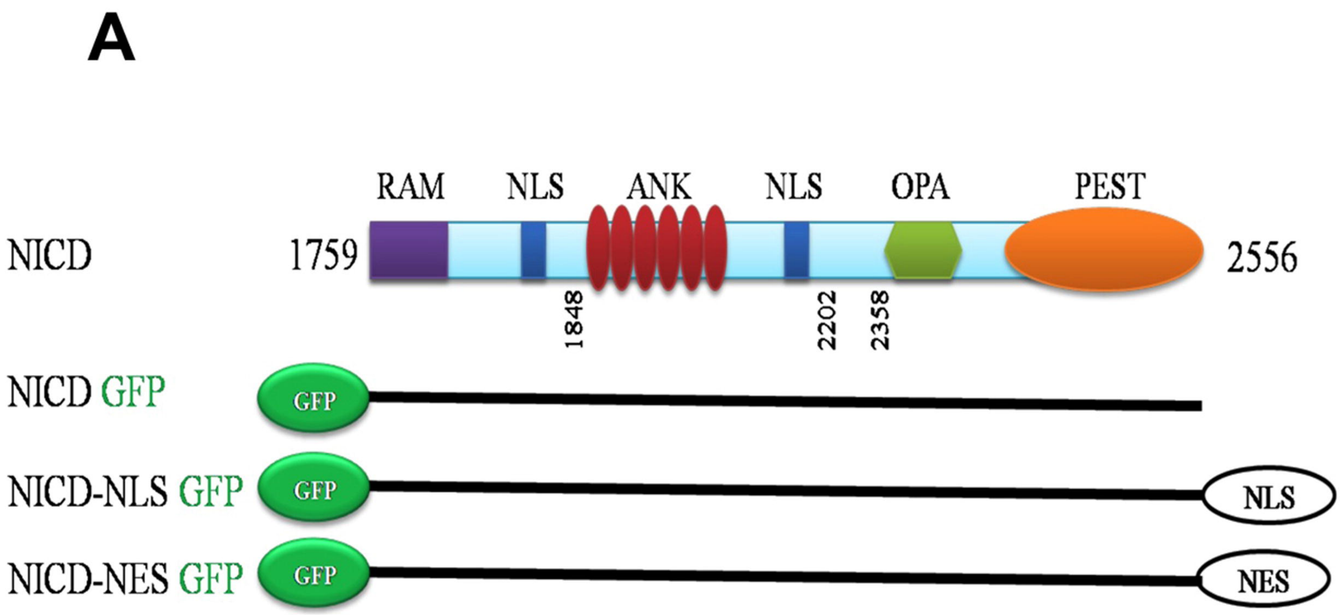

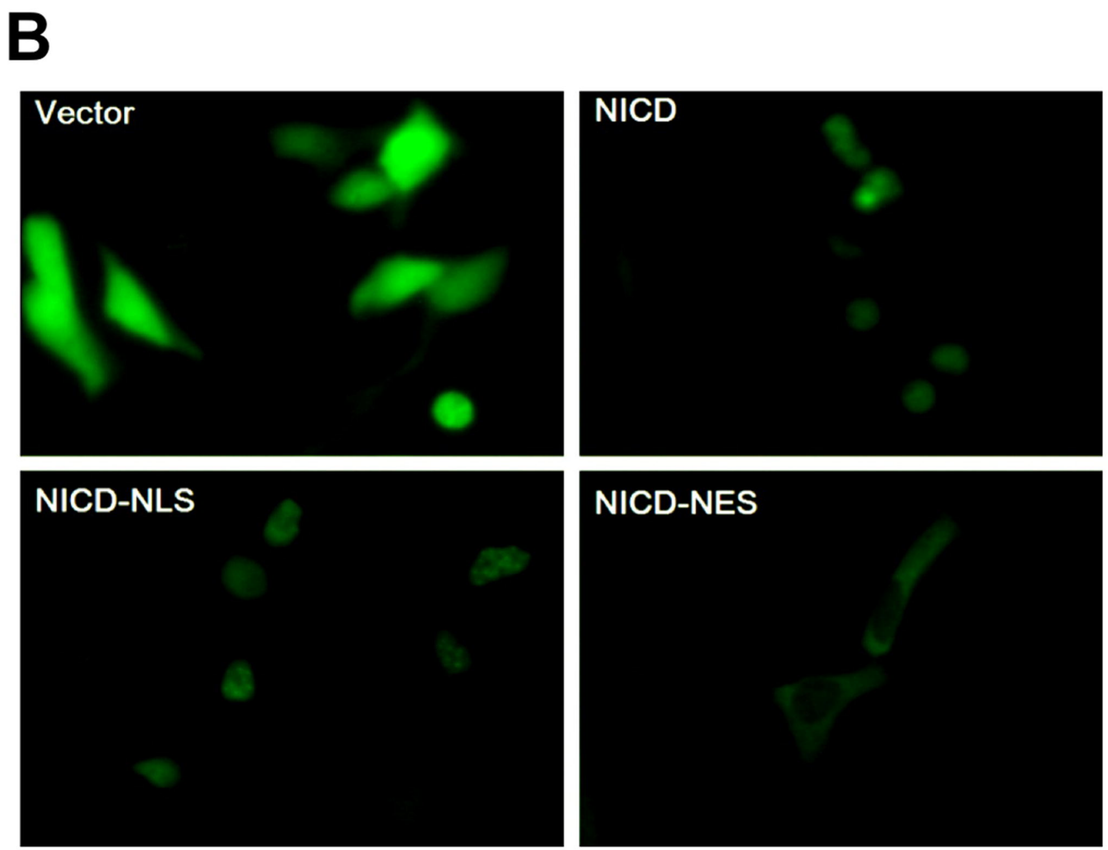

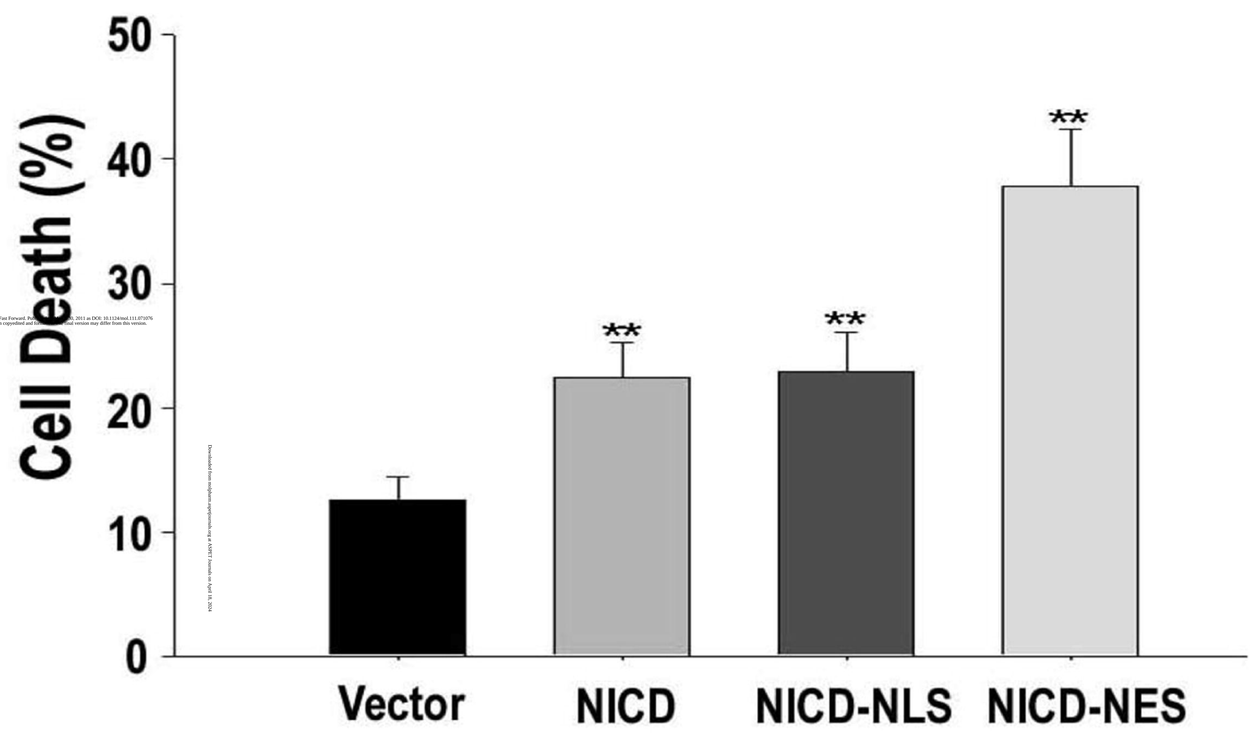

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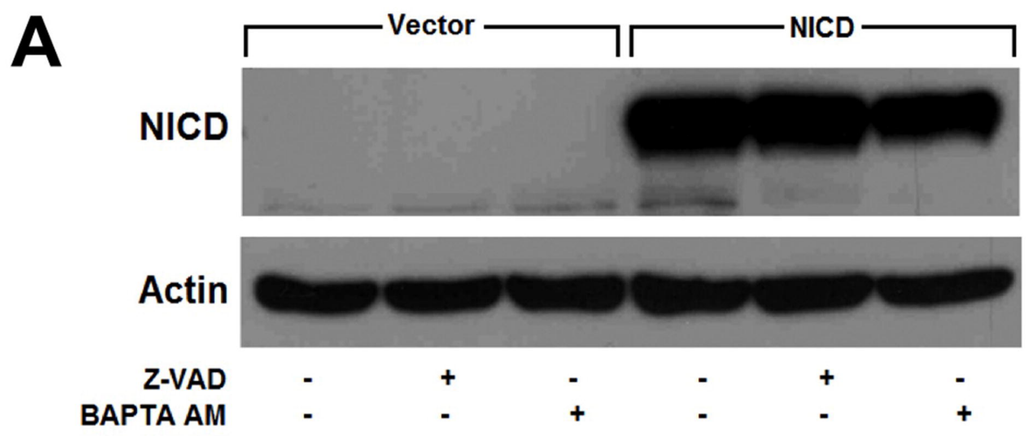

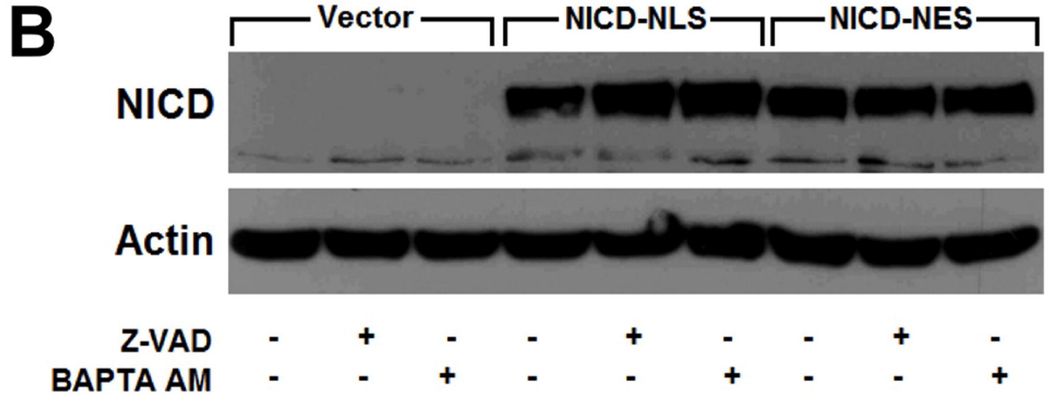

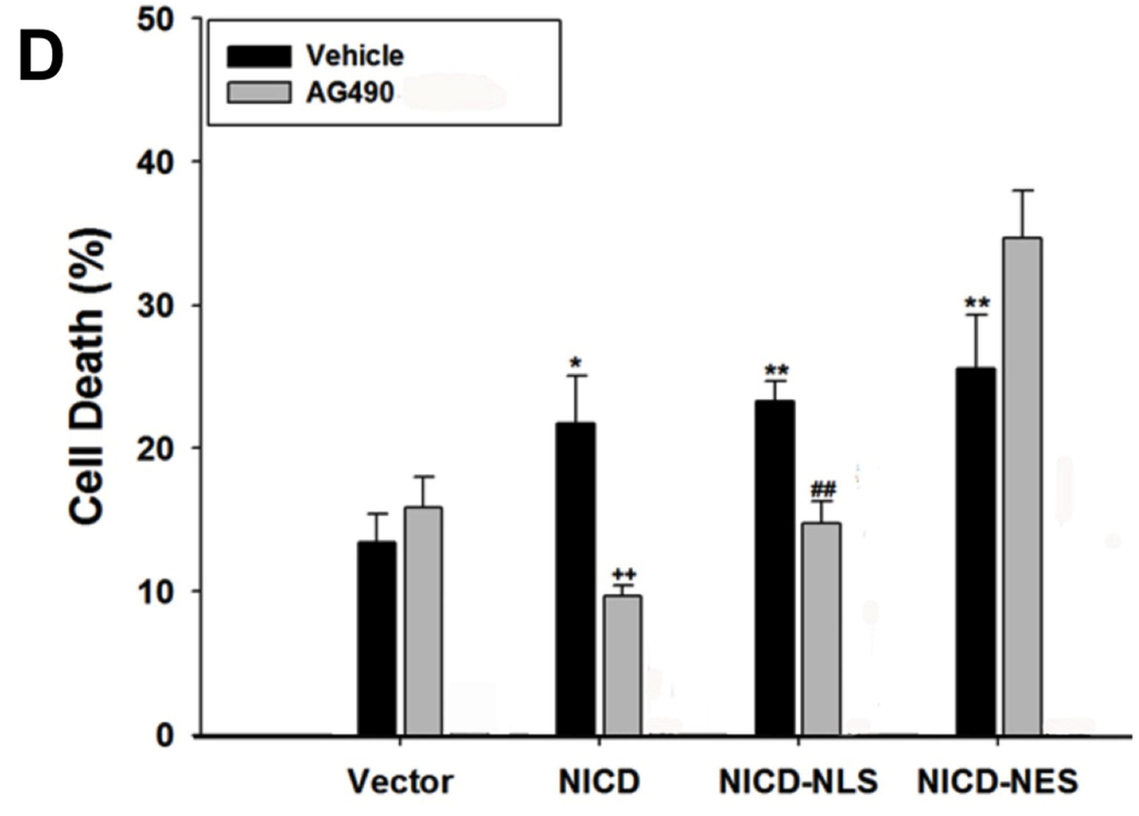

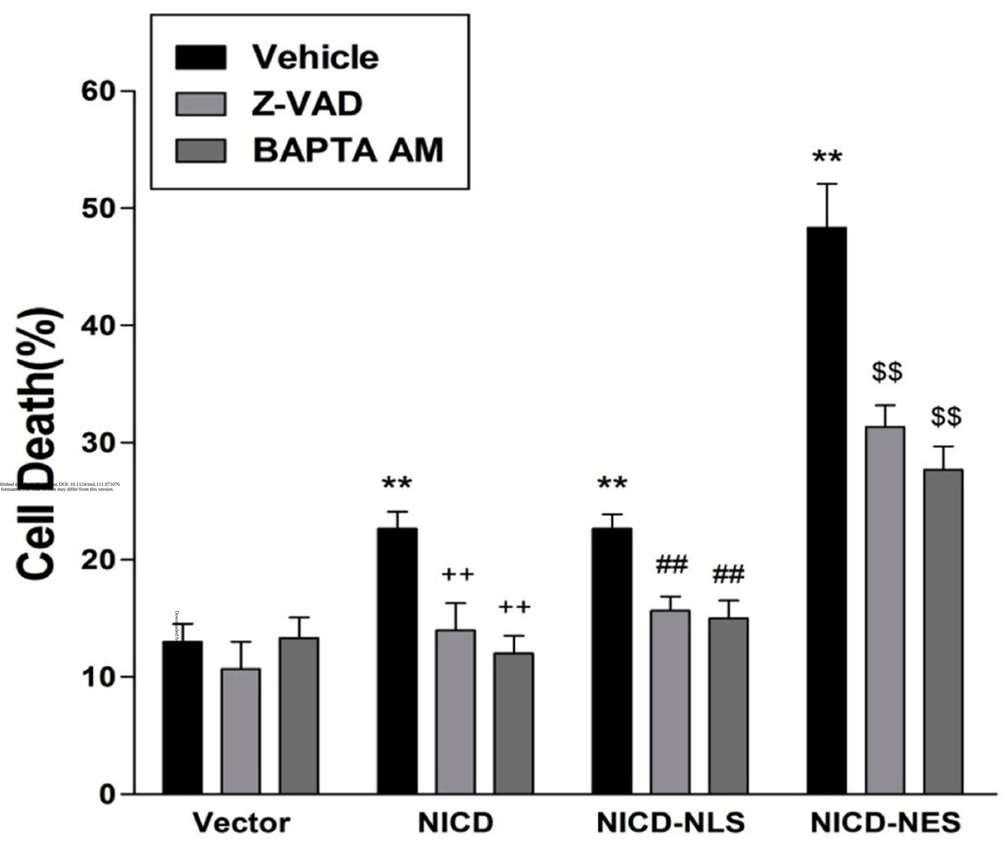

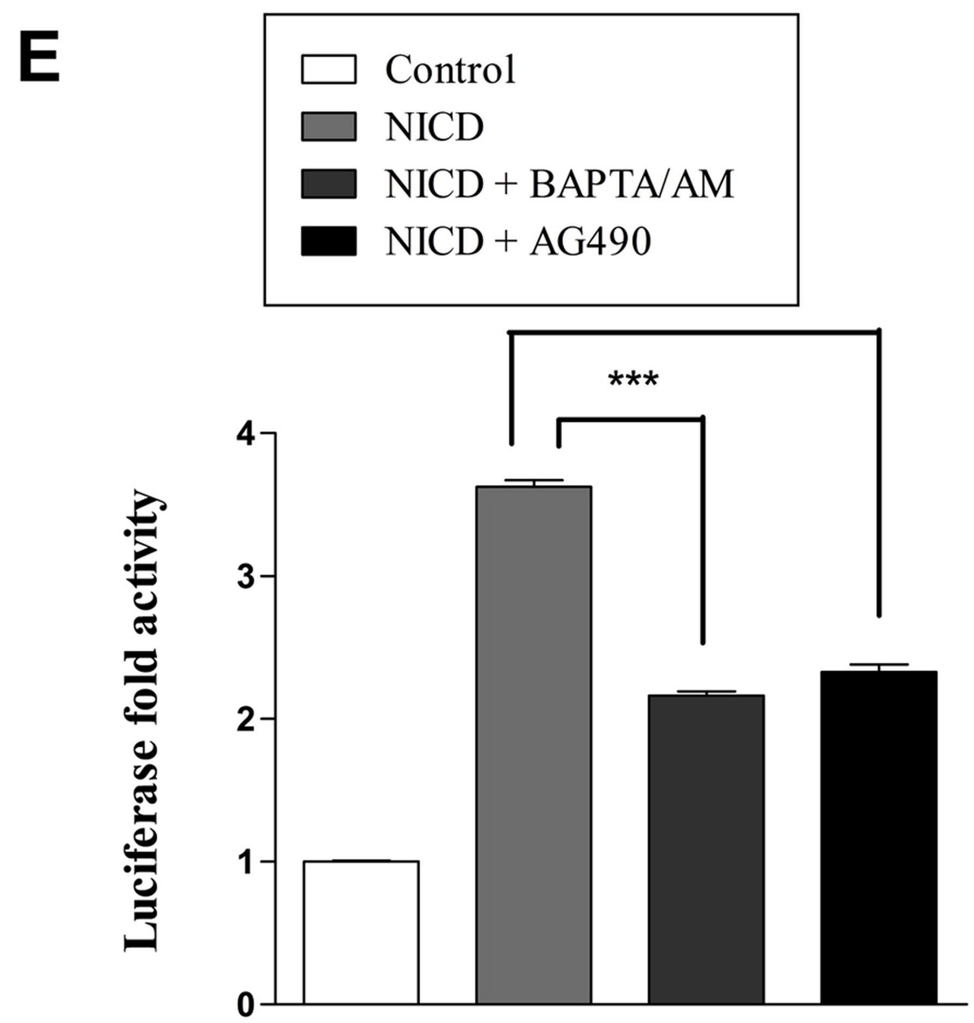

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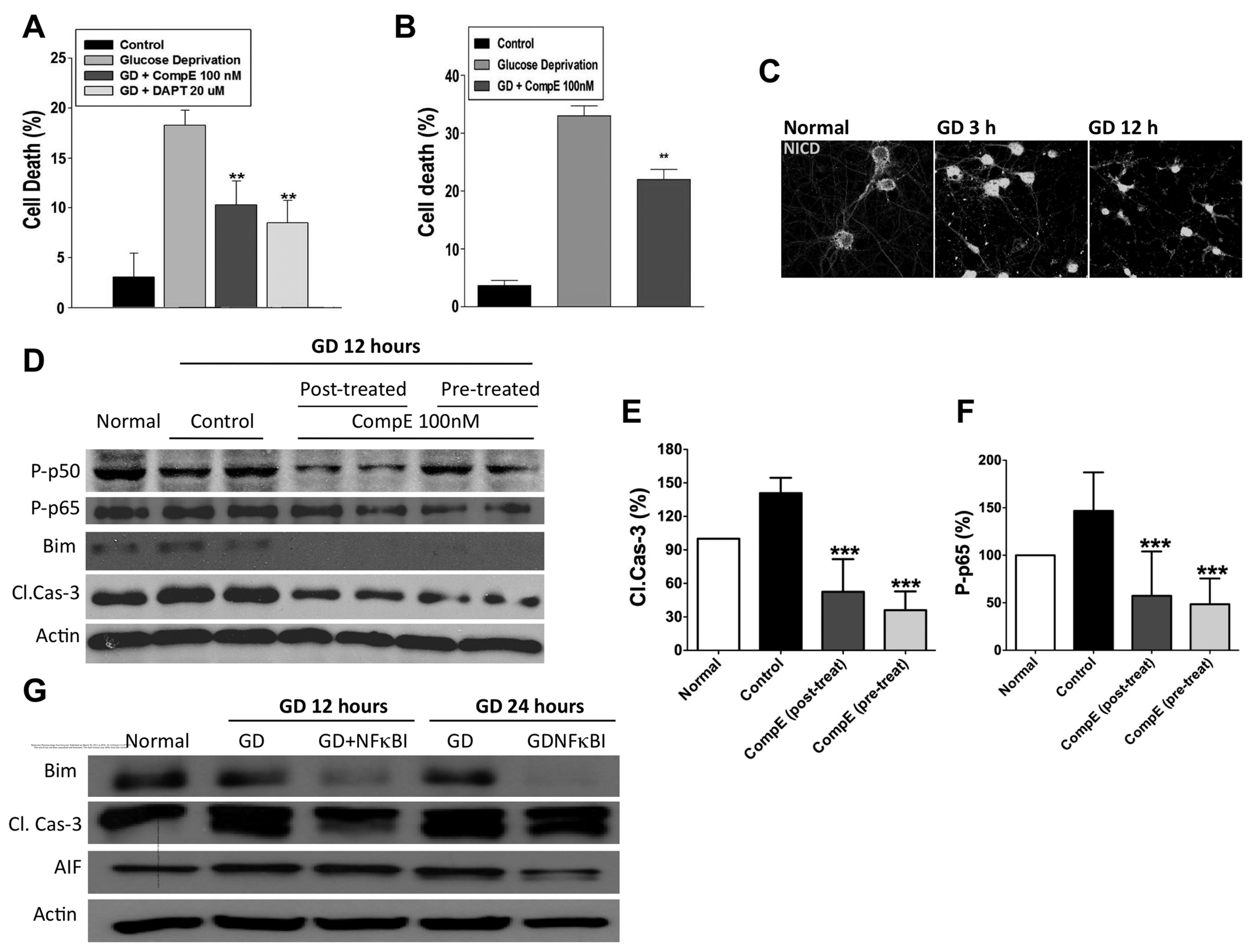
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GD + DAPT 20 uM
Cell Death (%)
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B
Control
Glucose Deprivation
GD + CompE 100nM
Cell death (%)
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10
20
30
40
**
C
Normal
GD 3 h
GD 12 h
NICD
D
GD 12 hours
Post-treated
Pre-treated
Normal
Control
CompE 100nM
P-p50
P-p65
Bim
Cl.Cas-3
Actin
E
Cl.Cas-3 (%)
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120
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180
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Normal
Control
CompE (post-treat)
CompE (pre-treat)
F
P-p65 (%)
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CompE (post-treat)
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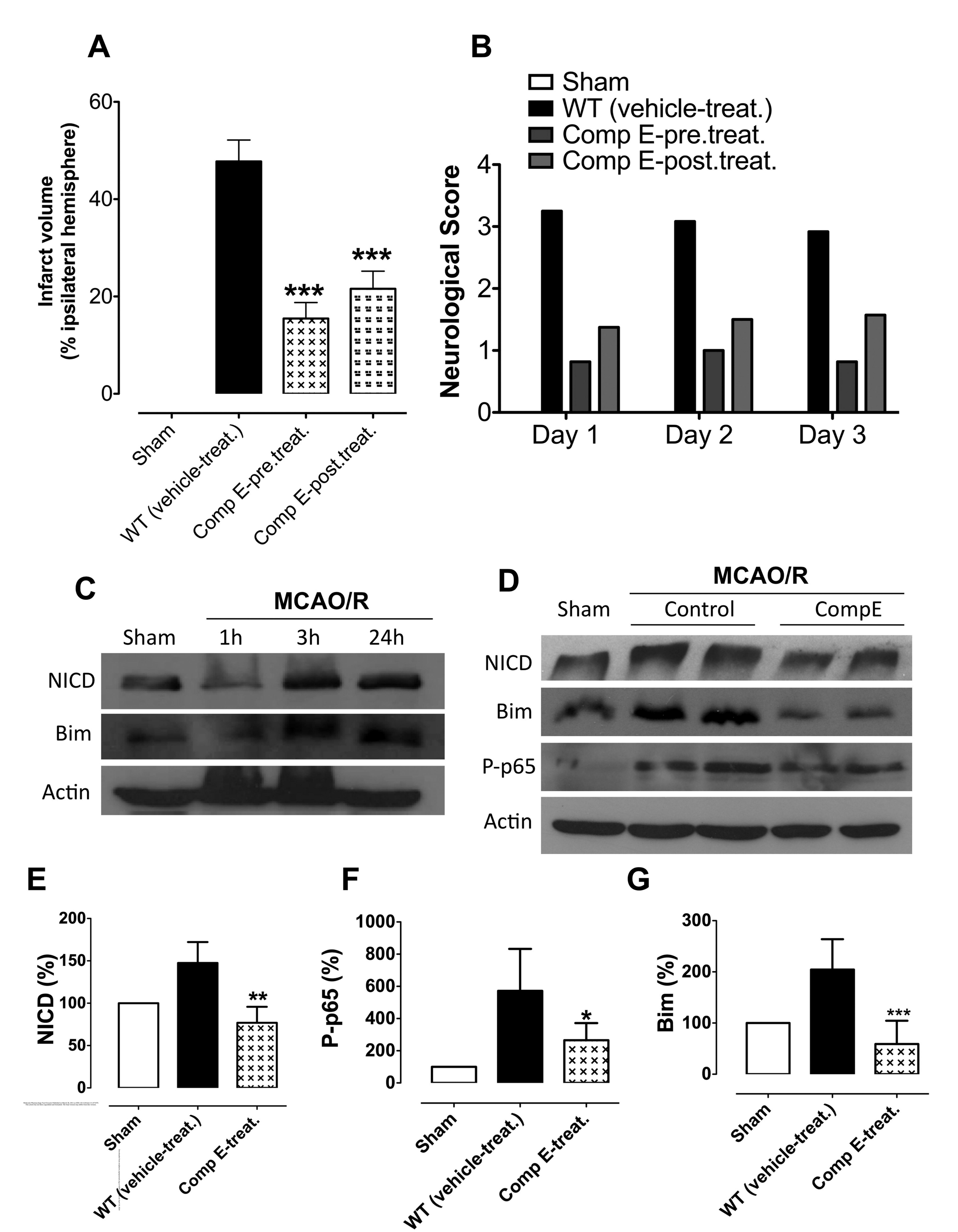

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