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Article type : Original Article

Bile-duct ligation renders the brain susceptible to hypotension induced neuronal degeneration: implications of ammonia

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Running title: Hypotension in hepatic encephalopathy

Keywords: minimal hepatic encephalopathy, hypotension, liver transplant, neuronal death, bile duct-ligation

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/JNC.15290

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Conflict of interest: The authors declare no conflicts of interest related to this study.

Financial support: This work was supported by a grant from the Canadian Institutes of Health Research. MAC is a recipient of a graduate research award from the Canadian Liver Foundation. CRB and MMO were supported by Fonds de recherche du Québec – Santé (FRQS) doctoral research bursaries.

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Acknowledgment: Part of the data presented in this manuscript have been published as the Master's thesis of the first author, MAC, at Université de Montréal: https://papyrus.bib.umontreal.ca/xmlui/bitstream/handle/1866/13482/Clement_Marc-Andre_2015_memoire.pdf.] The authors thank Mallinckrodt Pharmaceuticals for providing OP. The authors thank the behavior phenotyping core facilities of CRCHUM for behavior instruments.

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List of abbreviations: BDL, bile-duct ligation; BP, blood pressure; CBF, cerebral blood flow; CLD, Chronic Liver Disease; GFAP, glial fibrillary acidic protein; HE, Hepatic Encephalopathy; HRQOL, health-related quality of life; HT, hypotension; LT, Liver Transplantation; MHE, Minimal Hepatic Encephalopathy; NeuN, neuronal nuclei antigen; OHE, Overt Hepatic Encephalopathy; OP, Ornithine phenylacetate

Abstract

Hepatic encephalopathy (HE) is a debilitating neurological complication of cirrhosis. By definition, HE is considered a reversible disorder, and therefore HE should resolve following liver transplantation (LT). However, persisting neurological complications are observed in as many as 47% of LT recipients. LT is an invasive surgical procedure accompanied with various perioperative factors such as blood loss and hypotension which could influence outcomes post-LT. We hypothesize that minimal HE (MHE) renders the brain frail and susceptible to hypotension-induced neuronal cell death. Six-week bile duct-ligated (BDL) rats with MHE and respective SHAM-controls were used. Several degrees of hypotension (mean arterial pressure of 30, 60 and 90mmHg) were induced via blood withdrawal from the femoral artery and maintained for 120 minutes. Brains were collected for neuronal cell count and apoptotic analysis. In a separate group, BDL rats were treated for MHE with the ammonia-lowering strategy ornithine phenylacetate (OP; MNK-6105), administered orally (1g/kg) for 3 weeks before induction of hypotension. Hypotension 30 and 60mmHg (not 90mmHg) significantly decreased neuronal marker expression (NeuN) and cresyl violet staining in the frontal cortex compared to respective hypotensive SHAM-operated controls as well as non-hypotensive BDL rats. Neuronal degeneration was associated with an increase in cleaved caspase-3, suggesting the mechanism of cell death was apoptotic. OP treatment attenuated hyperammonemia, improved anxiety and activity, and protected the brain against hypotension-induced neuronal cell death. Our findings demonstrate that rats with chronic liver disease and MHE are more susceptible to hypotension-induced neuronal cell degeneration. This highlights MHE at the time of LT is a risk factor for poor neurological outcome post-transplant and that treating for MHE pre-LT might reduce this risk.

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Introduction

Characterized by cognitive, motor and psychiatric disturbances, hepatic encephalopathy (HE) is a neuropsychiatric syndrome affecting close to 30-84% of patients with chronic liver disease (CLD, cirrhosis) (Shawcross *et al.* 2016). HE is classified into two forms: overt HE (OHE) and covert/minimal HE (MHE). OHE encompasses several clinical signs ranging from asterixis, gross disorientation, stupor and coma, which are associated to increased morbidity and mortality (Shawcross *et al.* 2016). MHE is described when no overt or obvious symptoms of HE are observed, and diagnosed using sensitive neuropsychological and neurophysiological tests (Waghray *et al.* 2015).

Highly underdiagnosed, MHE is characterized by decreased concentration, poor memory, reduced speed of information processing, impaired motor abilities and is associated with an increased risk of accidents (Lauridsen *et al.* 2016). These subclinical abnormalities have a significant impact on patients' health-related quality of life (HRQOL), and on their ability to function daily (Ridola *et al.* 2018). As much as 80% of patients with end-stage liver disease suffer from MHE, and this underestimated phenomenon leads to a 4-fold increased risk of developing severe or OHE (Hartmann *et al.* 2000b).

The ultimate solution for treating HE is to replace the diseased liver with a healthy one. Indeed, liver transplantation (LT) is the only curative treatment to date that demonstrated to significantly prolong the lives of patients with CLD. The number of liver transplantations is on the rise, partially due to an increase in the number of organs available from donors (cadaveric and living), but also due to an upsurge in the number of patients on the LT list. With a 90% 1-year and 75% 5-year survival rate (Kim *et al.* 2018), LT is no longer considered an experimental high-risk procedure. Thus quality of outcome, including neurological status and quality of life are important considerations in post-LT evaluation.

Historically, HE has always been considered to be a reversible metabolic disorder and therefore it was expected to completely resolve following LT. However, following the implantation of a new liver, persisting neurological complications remain a common problem affecting as many as 47% (8 - 47%) of LT recipients (Amodio *et al.* 2007; Kornerup *et al.* 2019; Piñero *et al.* 2018; Sotil *et al.* 2009; Cheng *et al.* 2018). These enduring neurological

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complications following LT (no longer HE since the diseased liver has been replaced with a healthy one) continue to weigh severely on the patients' quality of life, leading to longer stays in the hospital and thus causing further financial burden on health care systems (Campagna *et al.* 2010). Retrospective studies have reported that multiple episodes of overt HE pre-LT are associated with patients enduring neurological complications following LT (Cheng *et al.* 2018; Sotil *et al.* 2009). However, the impact of HE and MHE *at the time* of LT on post-LT outcome remains undefined.

Both perioperative and postoperative factors could negatively impact the brain (Lening *et al.* 2018; Weiss and Thabut 2019). Indeed, LT is a major invasive surgical procedure accompanied by intraoperative stress and confounding factors, including blood loss (hypovolemia) and hypotension (de Boer *et al.* 2005; Dhar *et al.* 2008). Due to its high-energy demand (consuming close to 20% of the body's energy even though it accounts for <2% of body weight), the brain is particularly vulnerable to hypotensive conditions (Magistretti and Allaman 2015). However, in the setting of MHE, we hypothesize the compromised brain becomes predisposed to what would normally be an innocuous hypotensive insult, resulting in cell injury and death. This could explain the anticipated susceptibility of patients with MHE to cerebral damage following intraoperative stress (i.e a hypotensive insult) resulting in enduring neurocognitive dysfunction following LT (Rose and Jalan 2004). The aim of this study is to evaluate the impact of MHE on neurological outcome following a hypotensive insult in a cirrhotic rat model. Moreover, to fully assess the impact of MHE, ornithine-phenylacetate (OP), an ammonia-lowering strategy will be used to treat MHE as previously demonstrated (Davies *et al.* 2009; Jover-Cobos *et al.* 2014).

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Materials and methods

Experimental design

The study was not pre-registered. Throughout the experimental protocol, 124 male Sprague-Dawley rats (8 weeks old, 175-200g; Charles River, Montreal, QC, Canada; RRID:RGD_70508) were housed 2-3/cage in standard conditions with *ad libitum* access to food and water. After 2 days of acclimation, rats were anaesthetized with isoflurane (induction 4% in oxygen 1 L/min followed by maintenance under 2% in oxygen 0.5 L/min for the duration of surgery) to perform bile duct-ligation (BDL) or control-operations (SHAM) as previously described (Bosoi *et al.* 2011). Rats were assigned to the specific hypotension/treatment group arbitrarily at this timepoint. Since rats are very similar at this age, they were group-assigned before any intervention, the bias of their complication development (by surgery and cirrhosis) was thus avoided. Carprophen (0,05 mg/kg subcutaneous) and Bupivacaine (0.25%, 0.2 ml/kg, surgical site infiltration) were administered for analgesia. Vitamin K (50 μg/kg) was administered weekly for bleeding prevention. Animals presenting with weight loss > 20% following surgery (n=5) or bleeding complications (pallor, dark feces and prostration) towards the end of the model (n=7) were prematurely euthanized in CO_2 chambers under isoflurane anaesthesia, as allowed in our facility. All animals that survived underwent the hypotension procedure, with no other exclusion criteria. No death occurred during the hypotension procedure.

After 6 weeks, hypotension was induced for two hours in BDL and SHAM operated rats, total 112 rats, n = 3 - 6 for each experimental group as noted for each experimental procedure (Fig. 1A). In another set of animals, 3-week BDL rats were treated with OP (MNK-6105, Mallinckrodt Pharmaceuticals; 1g/kg, gavage) for the remaining 3 weeks. Hypotension was induced in OP-treated BDL rats and respective controls (Fig. 1B). Animals were sacrificed at the end of the hypotension period by decapitation, while still under anaesthesia. No blinding was performed. All experiments were conducted following the Guidelines of Canadian Council on Animal Care and were approved by the Animal Protection Committee of CRCHUM (S11033CRr).

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Induction of hypotension

Hypotension (HT) was induced by blood withdrawal from the femoral vein under isoflurane anaesthesia (as described above in the first paragraph of the Experimental design section), in order to simulate hypotensive conditions during transplant surgery. Isoflurane flow was adjusted at minimum for a better anaesthesia control over the 2h of hypotension. The femoral artery was surgically exposed to insert a 24G catheter, which was then connected to a sphygmomanometer. Paired SHAM and BDL rats were connected at the sphygmomanometer and subjected to same degree of hypotension at the same time. Two pairs of animals were performed daily, one in the morning, one in the afternoon.

Blood was removed gradually in small aliquots until desired degree of hypotension was achieved. Control non-hypotensive rats (mentioned as SHAM and BDL throughout the paper) were identically manipulated, except that blood was not withdrawn. In controls, BP was maintained at normal values. During the 2h of hypotension, BP remained stable in SHAM rats at all HT degrees. In BDL rats, during hypotension, saline was administered to maintain BP when it dropped. Body temperature was monitored and maintained at 37 °C with the use of a heat pad.

Measurement of the cerebral blood flow

Cerebral blood flow (CBF) and renal blood flow (RBF) were assessed through the fluorescent microsphere technique according to De Vissher & al (De Visscher *et al.* 2006). Briefly, fluorescent microspheres were injected (1/10^6 microspheres/ml, Invitrogen F8844, USA) through the brachial artery 5 minutes before sacrifice at the end of the hypotension period. The fluorescent microspheres embolize capillaries proportional to the blood flow. Blood flow was calculated based on spectrofluorometric measurement after tissue digestion, microsphere extraction and solubilization. 6 SHAM and 4 BDL rats were used in each group.

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Tissue preparation

Frontal cortex was dissected and homogenized mechanically using Potter- Elvehjem homogenizers and completed by ultrasonication in cold lysis buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 1/500 Protease Inhibitor Cocktail; Sigma, Canada). Homogenates were centrifuged at 12,000g for 40 min at 4°C. The supernatant was used as the brain cytosolic fraction. Protein content was determined according to the method of Lowry (Lowry *et al.* 1951).

Cresyl violet neuronal count

For histological analysis, neurons were stained with cresyl violet (0.01%) as described by Lange et al (Lange *et al.* 1999). Rats were perfused with saline followed by 10% formalin before brain collection. Brain slices (50µm) were cut in coronal sections with a vibratome and mounted on microscopy slides. Sections were stained with a 0.01% solution of cresyl violet (Sigma, C5042, Canada) in sodium acetate buffer (pH 3.7) for 15 min followed by a brief rinse in tap water to remove unbound dye. Slides were dehydrated by immersions in 70, 95% and 100% ethanol for 1 min each. The tissue was covered in mounting media and covered with coverslips. Images were taken within 2 days and neuronal count was done was automatically performed with the Image J software. An n = 5 animals was used for each group.

NeuN and Cleaved Caspase-3 Western blot expression

All antibodies used are listed in Table 1. Protein (20 µg) was loaded on 9% sodium dodecyl sulphate-polyacrylamide gel, electrophoresed and transferred on polyvinylidene difluoride membranes. After being blocked in 5% fat-free milk powder in TBS-T buffer (1mM Tris pH 7.5, 10mM NaCl and 0.5% Tween-20) for 1h at room temperature, membranes were incubated in a dilution of 1:1,000 of NeuN (Millipore, MAB377), caspase-3 (Cell Signaling, 9662) antibody in 5% milk-TBS-T buffer for 1h. NeuN, neuron nuclei, is a neuronal specific protein (Mullen *et al.* 1992) and cleaved caspase 3 is a well-known apoptosis marker (Hartmann *et al.* 2000a; Namura *et al.* 1998). Membranes were washed 6 times in TBS-T buffer for 5 minutes and incubated 1h at room temperature with their corresponding

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secondary antibody coupled to horseradish peroxidase (1:10,000). After 6 washes of 5 minutes in TBS-T, membranes were exposed to chemiluminescence reagent and probed on X-ray film. Densitometry of images was automatically performed with the Image Lab software. For control of protein loading, GAPDH (Sigma, G9545) was used at a dilution of 1:100,000. An n = 6 SHAM; 6 BDL; 3 SHAM+HT90; 3 BDL+HT90; 5 SHAM+HT60; 4 BDL+HT60; 6 SHAM+HT30; 6 BDL+HT30; 6 BDL+OP+HT60 animals were used.

Immunofluorescence

In a separate set of experiments, rats were perfused with saline followed by 10% formalin before brain collection. Brain slices (50µm) were cut in coronal sections with a vibratome and were transferred in 24 well-plates containing PBS. Assessment of neuronal cells and cleaved caspase-3 in prefrontal cortex was made by immunofluorescence using NeuN and cleaved caspase-3 antibody. Astrocytes were evidenced using GFAP, glial fibrillary acidic protein, a specific marker. GFAP contributes to the astrocyte's cytoskeleton, with important roles in cell communication and the functioning of the blood brain barrier. GFAP mainly stains astrocitic soma and processes, but not the nucleus (Zhang *et al.* 2019). Brain slides were blocked with PBS-0.5% Triton X-100-10% donkey serum for 30min. After incubation, they were washed 3 times for 5 minutes in PBS. The slices were exposed to the primary antibody (cleaved caspase-3: 1:200, Cell Signalling 9579, NeuN: 1:200, GFAP: 1:400, Sigma, G3893) overnight at 4°C. After 3 washes, slices were then exposed to the secondary antibody (donkey antimouse IgG coupled to Alexa488 fluorophore 1:200, Jackson Immuno Research, 715-545-150 or donkey antirabbit IgG coupled to Alexa594 fluorophore, 1:200, 711-585-152) in PBS-0.5% Triton X-100 and incubated for 30 minutes, in the dark, at room temperature. Following washes, DAPI (4',6-diamidino-2-phenylindole) was added (1µg/ml) and rinsed with PBS. Slices were then mounted on a microscope slide for fluorescence microscopy analysis (Carl Zeiss AG). Images were acquired with Zeiss AxioImager.M2 ApoTome.2. Fluorescence was analyzed using the software Image J (National Institutes of Health, Bethesda, USA). For NeuN staining, individual neurons were counted using the "analyze particles" function of ImageJ. 3 different fields at a magnification of 20x were counted for each animal. An n = 3 SHAM; 4 BDL; 5 SHAM+HT90; 5 BDL+HT90; 6

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SHAM+HT60; 6 BDL+HT60; 5 SHAM+HT30; 5 BDL+HT30; 6 BDL+OP+HT60 animals were used.

Behavioral testing

Open Field

Rodents exert both innate fear for open areas and curiosity to a new environment. This test is used to assess both motor function (by measuring the total distance the animal travels) and anxiety (the amount of time the animal avoids the exposed center area of the field and remains in close proximity to the walls) (Bémeur *et al.* 2016). On the day of sacrifice, before induction of hypotension, rats were placed in an open field arena: 90 x 90 x 40cm with black plastic walls. Rodent behaviour during 10min in the arena was tracked by the Panlab system and the distance travelled (cm) and time spent (sec) in peripheral wall areas compared to the center area were assessed. A n = 4 SHAM; 6 BDL and 6 BDL+OP were used.

Elevated-plus maze

This test assesses anxiety since rats are conflicted by their explorative nature and their fear of open spaces, therefore the closed arms of the maze provide a safer environment compared to the open arms (Bémeur *et al.* 2016). Four days before sacrifice, rats were placed in the elevated plus maze (EPM) apparatus for 10min. The maze consists of 2 closed and 2 open arms (10 x 45cm). Panlab tracking system was used to quantify the time spent in open vs closed arms and evaluate anxiety and explorative behaviours. A n = 4 SHAM; 6 BDL and 6 BDL+OP were used.

Blood ammonia

Plasmatic ammonia levels were assessed in SHAM, BDL and BDL treated with OP groups using a commercial kit (Sigma, St. Louis, MO). Blood was immediately centrifuged and plasma aliquots were snap frozen, kept at -80°C and assessed within one week. The method is based on the reaction of ammonia with α-ketoglutarate and reduced nicotinamide adenine dinucleotide phosphate in the presence of L-glutamate dehydrogenase. Oxidation

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rate of reduced nicotinamide adenine dinucleotide phosphate was recorded by the absorbance decrease at 340 nm. Ammonia concentration was calculated according to the manufacturer's protocol. A n = 4 SHAM; 6 BDL and 6 BDL+OP were used.

Statistical analysis

No sample size calculation was performed. Our n was based on our previous similar experiments (Bosoi *et al.* 2011; Bosoi *et al.* 2012). Data are expressed as mean ± standard error of the mean (SEM). Data was tested for outliers with the ROUT test and for normality with the Shapiro-Wilk normality test. Significance of difference was tested by t-test or ANOVA followed by Tukey's post-test for normal distributed data; by the Mann Whitney test or the Kruskal-Wallis test for non-parametric data. Correlation was calculated with the Spearman test. Statistical analysis was made using GraphPad Prism4. Probability values of $p<0.05$ were considered statistically significant.

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Results

Neuronal count

Hypotension was induced following blood withdrawal from the femoral artery. Reduction in blood pressure to 90, 60, or 30 mmHg did not lead to mortality in either group. The quantity of blood withdrawn to achieve respective hypotension degree was similar in SHAM and BDL rats. While small quantities (maximum 1 ml, withdrawn over 5 minutes) lead to achieve 90 mmHg, withdrawal of higher volumes was needed to achieve 60 mmHg (≈ 5 ml, over 15 minutes) and 30 mmHg (≈ 10 ml, over 30 minutes).

At hypotension of 30 and 60 mmHg, cresyl violet neuronal staining revealed a significant reduction in the number of neurons in the frontal cortex in hypotensive BDL vs SHAM rats (60 mmHg, $p<0.01$; 30 mmHg, $p<0.001$). Similar differences were also found between the BDL groups; BDL vs BDL+HT60 ($p<0.01$) and BDL vs BDL+HT30 ($p<0.001$). There was no significant change in neuronal counts between BDL and SHAM following hypotension at 90 mmHg. Similarly, no significant difference was found in the number of neurons in the frontal cortex between non-hypotensive BDL and SHAM, nor between the SHAM groups following different degrees of hypotension (Fig. 2A). Fig. 2B illustrates representative images for SHAM+HT60 and BDL+HT60. A significant correlation was calculated between neuronal counts following cresyl violet staining and the degree of hypotension ($r = 0.8710$, $p<0.001$, Fig. 2C).

The results found with cresyl violet staining were supported by immunofluorescent staining using the specific neuronal marker, NeuN. Hypotension at 60 and 30 mmHg in BDL rats resulted in a decrease in NeuN fluorescence compared to their respective SHAM-operated controls ($p<0.01$). NeuN staining in BDL+HT30 and BDL+HT60 was significantly reduced compared to BDL without hypotension ($p<0.05$ and $p<0.01$, respectively). No change was found between BDL and SHAM; non-hypotensive or 90 mmHg. Comparing all SHAM groups; non-hypotensive, HT90, HT60 and HT30, no significant difference in NeuN fluorescence was found (Fig. 2D). Fig. 2E shows representative images for SHAM+HT60 and BDL+HT60. Furthermore, Western blot confirmed these results (Fig. 2F,G) where NeuN

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protein expression was reduced in BDL+HT60 and BDL+HT30 compared to their respective BDL and SHAM groups ($p<0.01$, $p<0.05$ respectively).

Since 60 mmHg was the smallest drop in blood pressure to induce significant reduction in neuronal markers in the frontal cortex, HT60 was used for the rest of the experiments.

Apoptosis

To further support the findings displayed in Fig. 2, we measured cleaved caspase-3, a marker of apoptosis, in frontal cortex and found it increased in BDL+HT60 compared to SHAM+HT60 as well as compared to BDL ($p<0.001$, Fig. 3A,B). There was no difference between control SHAM and BDL rats without hypotension (Fig. 3A,C) as well as at HT90 (data not shown). However, similar results were observed at HT30 ($p<0.001$, Fig. 3C,D). Cleaved caspase-3 co-localized with the neuronal marker NeuN, not with the astrocytic marker GFAP (Fig. 3E).

Blood Flow

Following HT60 for 2 h, CBF was reduced in BDL rats by 75% compared to non-hypotensive BDL rats, while SHAM rats presented a slight 38% increase compared to non-hypotensive SHAM rats, with a significant difference of the delta change between the groups ($p<0.01$ SHAM vs BDL) (Fig. 4A). RBF presented similar decreases in both groups (72% in SHAM vs non-hypotensive SHAM and 97% in BDL vs non-hypotensive BDL (Fig. 4B). RBF was used as positive control since the kidney is extremely responsive to hypotension, contrary to brain (Shirley *et al.* 1991).

Effect of OP on plasma ammonia and cognition

OP was administered orally to BDL rats as an ammonia-lowering strategy (19). The significant 3-fold increase in plasma ammonia levels observed in BDL rats compared to SHAM-operated controls ($p<0.01$) was significantly reduced in BDL treated with OP ($p<0.05$, Fig. 5A).

BDL rats placed in the OF spent more time in the peripheral area (associated with anxiety) and less time in the middle area (associated with curiosity) compared to SHAM (Fig. 5B,C). OP treatment improved anxiety-like behaviour: treated BDL rats spent less time in the

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peripheral area and more time in the middle area, similar to SHAM (Fig. 5B,C). When calculating total distance, BDL rats travelled less (total distance in cm) than SHAM-operated rats ($p<0.01$, Fig. 5D). Again, OP treatment improved locomotion in BDL rats ($p<0.05$ in OP-BDL vs BDL, Fig. 5D). In the EPM, both BDL groups spent less time in the open arms compared to SHAM-operated controls, however it did not reach statistical significance (Fig. 5E).

Effect of OP on neuronal degeneration and apoptosis following hypotension

Neuronal cell degeneration in BDL rats following HT60 was protected following OP treatment. In BDL+HT60, OP prevented the significant reduction in NeuN immunofluorescence (Fig. 6A,B) as well as protein expression (Fig. 6C,D) found in BDL+HT60 vs SHAM+HT60 ($p<0.01$ and $p<0.001$ respectively). However, apoptotic marker, cleaved caspase-3, remained unchanged between BDL+HT60+OP and BDL+HT60 but both groups expressed significantly higher protein compared to SHAM+HT60 (Fig. 6E,F).

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Discussion

Results of the present study demonstrate that MHE renders the brain frail thus susceptible to hypotensive insults resulting in neuronal cell degeneration. This irreversibility becomes very difficult to treat and could be a contributing factor to neurological impairment observed following LT in spite of resolution of liver disease. Treatment of MHE with the ammonia-lowering strategy OP protects against hypotension-induced loss of NeuN protein. These results postulate the impact of MHE upon delivering the patient into a major surgery such as LT; therefore treatment of MHE would reduce the risk of developing neurological complications post-LT.

Neurological complications after LT are classified into minor and major, on the basis of clinical findings and severity (Amodio *et al.* 2007). Minor complications include tremor, headache, sleep and mood alterations and are usually reversible, while seizures, cerebellar syndromes and, consciousness alterations represent major complications with serious consequences (Amodio *et al.* 2007; Piñero *et al.* 2018). The burden of these persisting neurological complications post-LT is extensive, especially since the patient has been "treated" for liver disease. In addition to increased hospitalizations and longer stays in the hospital, these enduring neurological complications severely weigh on the patients HRQOL (Amodio *et al.* 2007; Weiss and Thabut 2019).

Recent studies have demonstrated that a history of HE leading up to LT has a negative impact on neurological outcome following LT (Cheng *et al.* 2018; Sotil *et al.* 2009). Indeed, these retrospective studies documented that cirrhotic patients with a history of existing bouts of OHE display impaired neurological resolution following LT which leads to an increased risk of mortality and morbidity (Sotil *et al.* 2009; Lin *et al.* 2014; Garcia-Martinez *et al.* 2011). In addition, patients with cirrhosis and with no history of HE pre-LT, rarely develop neurological complications following LT (Sotil *et al.* 2009), supporting the negative impact of HE pre-LT. However, it is important to note that in these studies, the history of HE was recorded at various times pre-LT (between 3 - 7 months). The neurological status of the patients *at the time* of LT was never documented. Our results strongly suggest that MHE

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present *at the time* of LT increases the risk of developing persisting neurological complications post-LT.

LT is a major invasive surgery where intraoperative stress is endured involving blood loss (hypovolemia) and hypotension (de Boer *et al.* 2005; Dhar *et al.* 2008). We simulated these conditions in 6-week BDL rats, previously characterized to have MHE (Bosoi *et al.* 2011; Bosoi *et al.* 2012), by inducing hypotension (withdrawing blood) and investigating neuronal cell death.

Historically, HE has been defined to affect glial cells. Since astrocytes play an important physiological role and are a vital neighboring cell for proper neuronal function, during HE, astrocyte swelling, and senescence have been described (Görg *et al.* 2018). Interestingly, premature astrocyte senescence is believed to contribute to persistence of cognitive disturbances after resolution of episodes of overt HE (Görg *et al.* 2015).

However, there is anecdotal evidence supporting neuronal cell degeneration in HE. A decrease of neuronal count was evidenced in an animal model with a history of repeated or long episodes of HE and was suggested to be more prevalent in alcoholic cirrhosis (Butterworth 2007; García-Lezana *et al.* 2017). Described mechanisms of neural death in HE include excessive stimulation of the N-methyl-D-aspartate (NMDA) receptor by glutamate, mitochondrial dysfunction, lactic acidosis, oxidative stress and inflammation (García-Lezana *et al.* 2017; Jeong *et al.* 2018). In BDL rats with different degrees of hypotension, the degeneration of neurons was proportional to the degree of hypotension. Similar results were not found in SHAM-operated controls or non-hypotensive BDL rats. Whether neuronal cell degeneration only occurs during certain cases or conditions of HE remains to be recognized.

Caspases (cysteinyl aspartate-specific proteases) are an important family of signalling molecules, mediators of programmed cell death. Cleaved caspase-3, reflecting activation, is a marker in many diseases such as stroke and neurodegenerative diseases (Glushakova *et al.* 2017). Cleaved caspase-3 has been associated with the initiation of the "death cascade" and is therefore an important marker of the cell's entry point into the apoptotic-signalling pathway (Brentnall *et al.* 2013). Our data demonstrate apoptosis was activated in the frontal

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cortex of BDL rats following hypotension of 60 and 30 mmHg as cleaved caspase-3 was found increased in comparison to corresponding SHAM rats. Apoptosis was previously described in the brain in different rat model of HE (Bustamante et al., 2011) as well as dysregulation of apoptotic genes was described in patients with HE (Gorg et al., 2013) However, in both studies apoptosis was not explicitly assessed in neurons. Our immunofluorescence results, demonstrate that activation of apoptosis was precisely found in neurons, not in astrocytes. However, GFAP as an astrocytic marker is of limited use as it does not stain the whole astrocyte (Zhang *et al.* 2019).

The brain is a highly metabolic organ requiring a high amount of energy to properly function and therefore hypotensive conditions may lead to detrimental effects (Magistretti and Allaman 2015). The delivery of energy substrates is tightly coupled with the energy demand in the brain, and neural cells (including astrocytes and neurons) are sensitive to shifts in oxygen or glucose, which depend on an adequate CBF and arterial blood pressure. Following hypovolemia, CBF reflex regulatory mechanisms such as arterial blood pressure, intracranial pressure, neural activity and metabolic demand are activated to maintain cerebral perfusion. However, with increased duration and severity of hypovolemia, these mechanisms become inefficient and cerebral perfusion decrease (Rickards 2015). Moreover, cirrhotic patients are often hemodynamically instable and strict precautions during LT are taken to prevent a significant drop in mean arterial pressure (MAP) (<40 mmHg), which otherwise would result in severe adverse effects during the perioperative period of LT (Perilli *et al.* 2016; Reich *et al.* 2003). CBF was affected in BDL rats, consistent with central hypovolemia, hyperdynamic circulation and decreased CBF described in cirrhotic patients (Møller and Bendtsen 2018; O'Carroll, R.E. *et al.* 1991). Kidney responsiveness to hypotension was evidenced in both SHAM and BDL groups since RBF presented a similar decrease. This suggests in BDL rats, CBF regulatory mechanisms are impaired and most likely accompanied by a great energy cost that endangers neurons and leads to neuronal degeneration. BDL rats present with low-grade brain edema, which is due to both vasogenic and cytotoxic mechanisms (Bosoi *et al.* 2011; Bosoi and Rose 2013; Bosoi *et al.* 2012). Accumulation of water in the brain can lead to an increase in intracranial pressure, which

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consequently can negatively impact CBF (Kashif *et al.* 2012). Brain edema and increased intracranial pressure in traumatic brain injury lead to apoptosis as early as 2h following injury (Yang *et al.* 2005). This suggests the presence of brain edema might impact both the hypotension-induced CBF decline and apoptosis and therefore may increase the risk of and contribute to neuronal degeneration following hypotensive insults.

Our results indicate that in the setting of MHE, the already compromised brain becomes extremely sensitive to what would normally be an innocuous hypotensive insult, resulting in cell injury and death. This could explain the anticipated susceptibility of patients with MHE to cerebral damage following intraoperative stress (i.e., a hypotensive insult), as well as the increased risk of developing enduring neurocognitive dysfunction following LT (Rose and Jalan 2004).

Ammonia is the major factor involved in the neuropathophysiology of HE (Bosoi and Rose 2009). L-Ornithine phenylacetate (MNK-6105, Mallinckrodt Pharmaceuticals) is an ammonia-lowering strategy (Jalan *et al.* 2007) that has demonstrated to be effective in reducing blood ammonia levels and protecting against the onset of HE in various models of liver failure/disease (Davies *et al.* 2009; Kristiansen *et al.* 2014; Oria *et al.* 2012; Ytrebø *et al.* 2009). Indeed, intravenous injection of OP prevented the development of intracranial hypertension in pigs with acute liver failure (Kristiansen *et al.* 2014; Ytrebø *et al.* 2009) and intraperitoneal administration of OP prevented occurrence of HE following blood ingestion in rats with portacaval anastomosis (Oria *et al.* 2012) as well as significantly reduced ammonia and cerebral edema in BDL rats (Davies *et al.* 2009).

The treatment strategy reduces ammonia levels in 2 steps: (1) L-ornithine transaminates to glutamate which acts as a substrate for glutamine synthesis from ammonia in skeletal muscle and (2) phenylacetate conjugates with the formed glutamine to produce phenylacetylglutamine, which cannot be re-metabolized to glutamate to reform ammonia and instead is excreted by the kidneys (Jalan *et al.* 2007). Intravenous OP was safe and well-tolerated in decompensated cirrhotic patients (Ventura-Cots *et al.* 2013); the drug is currently entering a Phase 3 clinical trial (ClinicalTrials.gov Identifier: NCT04128462) aiming

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to evaluate its efficacy in patients with cirrhosis and hyperammonemia associated with an episode of HE.

Our results demonstrate for the first time that the oral formulation of OP has a beneficial ammonia-lowering effect. BDL rats who were administered OP daily by gavage for 3 weeks attenuated a significant rise in hyperammonemia which lead to an improvement in behavior. In addition, OP-treated BDL rats were protected against hypotension-induced neuronal cell death. Interestingly, in BDL+HT60+OP group, cerebral cleaved caspase-3 remained elevated; suggesting the protective effect of OP may be in delaying cell death, since apoptotic pathways appear to be activated. The protection observed following OP administration sustains our hypothesis that MHE present *at the time* of LT increases the risk of the development of persisting neurological complications post-LT. Moreover, this advocates that MHE is not negligible and merits to be evaluated and treated, providing patients the best chance of benefiting from receiving a new liver.

It is difficult to depict to what extent cognitive impairment following LT is a persisting complication of pre-LT HE or newly developed after the surgical procedure since current neuropsychological tests for HE performed after LT can not distinguish the cause (Kornerup *et al.* 2019; Tryc *et al.* 2014). Aside pre-existent HE and perioperative hypotension, other factors that could explain the development of neurological complications following LT are poorly functioning grafts, infections and immunosuppressive medication which account for early onset neurological dysfunction post-LT, while diabetes and hypertension account for late onset complications (Amodio *et al.* 2007; Kornerup *et al.* 2019; Campagna *et al.* 2010; Weiss and Thabut 2019). Interestingly, patients with LT present a >30% rate of neurological complications, much higher than 4% following cardiac or 0.5% after renal transplant (Weiss and Thabut 2019). This emphasizes the important role of HE in liver disease which sensitizes the brain to peri-transplant complications and persistent neurological dysfunction.

In order to understand the impact of hypotension-induced neuronal cell degeneration on cognitive behaviour, reperfusion would be required in order for the animals to recover and become freely moving. It is known that reperfusion independently can lead to cell injury and

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death (Wu *et al.* 2018) and therefore it becomes challenging to identify the effects of hypotension vs reperfusion on cognitive function and neurobehavior.

The present study reveals the impact of HE (particularly MHE) on hypotension-induced neurological complications in a cirrhotic rat model of MHE. Therefore, evaluating and identifying patients with MHE days or hours leading up to liver transplant using sensitive neuropsychiatric tests will allow for proper treatment of MHE before LT, which in turn will reduce the risk of developing neurological complications after LT.

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Table 1: Antibodies list

Name	Citation	Supplier	Cat no.	Clone no.	RRID
NeuN	PMID: 26303010	Millipore, USA	MAB377	A60	RRID:AB_2298772
Cleaved caspase-3		Cell signaling	9579		RRID:AB_10897512
GFAP	PMID: 16680766	Sigma, Canada	G3893		AB_477010
Donkey antimouse IgG coupled to Alexa488 fluorophore	PMID: 31801075	Jackson Immuno Research, USA	715-545-150		RRID:AB_2340846
Donkey antirabbit IgG coupled to Alexa594 fluorophore	PMID: 31612853	Jackson Immuno Research, USA	711-585-152		RRID:AB_2340621
Caspase-3		Cell Signaling,	9662		RRID:AB_331439

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		USA			
GFAP	PMID: 19091986	Sigma, Canada	G3893	G-A-5, monoclonal	RRID:AB_477010
GAPDH	PMID: 22215680	Sigma, Canada	G9545		RRID:AB_796208

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Figure legends

Figure 1: Graphical time-line of animal experimental procedure. (A) 6-weeks bile duct-ligated (BDL) or control-operated (SHAM) rats underwent a two hour hypotension (90, 60 and 30 mmHg) protocol and were sacrificed right after; (B) 3-weeks BDL rats were treated with ornithine phenylacetate (OP) for the remaining 3 weeks and underwent behaviour testing as explained in the text followed by two hour hypotension at 60 mmHg and sacrifice.

Figure 2: Effect of hypotension on neuronal counts in the frontal cortex in rats with bile duct-ligation (BDL), BDL with hypotension (HT) and SHAM-operated controls after 2 h of hypotension maintained at 90, 60 and 30 mmHg (HT90, 60, 30 respectively). (A) Neuronal counts following Cresyl violet staining (% of SHAM). (B) Representative images of Cresyl violet staining in SHAM+HT60 and BDL+HT60 groups. (C) Correlation between neuronal counts following Cresyl violet staining and degree of hypotension. (D) Neuronal counts following immunofluorescent staining for NeuN (neuronal nuclei antigen) (% of SHAM). (E) Representative images of immunofluorescent staining (NeuN antibody, green) in SHAM+HT60 and BDL+HT60 groups. (F) Western blot quantification relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase, % of SHAM) and (G) Cropped Western blot images of NeuN. n = 3 -6 rats/group. Data are expressed as mean ± SEM. ANOVA followed by Tukey's post-test for (A), (D), (F); Spearman test for (C) . *p<0.05, **p<0.01, ***p<0.001 vs BDL; #p<0.05, ##p<0.01, ###p<0.001 vs respective SHAM.

Figure 3: Effect of hypotension on the apoptotic marker cleaved caspase-3 in the frontal cortex in rats with bile duct-ligation (BDL), BDL with hypotension (HT) and SHAM-operated controls after 2 h of hypotension maintained at 60 and 30 mmHg (HT60, 30 respectively). (A,C) Western blot quantifications relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase, % of SHAM)) and (B,D) images of cleaved caspase-3 in all groups. (E) Representative immunofluorescence co-staining of cleaved caspase-3 (red) with neuronal nuclei antigen (NeuN, green, represented in the first two columns) and with glial fibrillary acidic protein (GFAP, green, last column), showing its localization in neurons, not astrocytes. Nuclei (DAPI) are shown in blue in SHAM+HT60 and BDL+HT60 groups n = 3 -6

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rats/group. Data are expressed as mean ± SEM. ANOVA followed by Tukey's post-test for all. ***p<0.001 vs BDL; ###p<0.001 vs respective SHAM.

Figure 4: Blood flow in the (A) brain and (B) kidney of rats with bile duct-ligation (BDL) and SHAM-operated controls after 2 h of hypotension maintained at 60 mmHg (HT60) (delta change vs non-hypotensive controls). n = 4 rats/group. Data are expressed as mean ± SEM. Mann Whitney test for (A) and t-test for (B). #p<0.05, ##p<0.01 vs respective SHAM.

Figure 5: Effect of ornithine phenylacetate (OP) on blood ammonia and cognition in rats with bile duct-ligation (BDL) and SHAM-operated controls. (A) Arterial plasma ammonia (% of SHAM). (B) Time spent in wall area, (C) middle area (s) and (D) total distance (cm) in the open field. (E) Time spent in open arms of elevated plus maze. n = 4 -6 rats/group. Data are expressed as mean ± SEM. ANOVA followed by Tukey's post-test for all. *p<0.05, **p<0.01 vs SHAM. #p<0.05 vs BDL.

Figure 6: Effect of ornithine phenylacetate (OP) on neuronal count in the frontal cortex in rats with bile duct-ligation (BDL), BDL with hypotension (HT) and SHAM-operated controls after 2 h of hypotension maintained at 60 mmHg (HT60). (A) Neuronal counts following immunofluorescent staining for NeuN (neuronal nuclei antigen, % of SHAM+HT60). (B) Representative images of immunofluorescent staining (NeuN antibody, green). (C) NeuN Western blot quantification relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase, % of SHAM+HT60)) and (D) Representative Western blot images. (E) Cleaved caspase-3 Western blot quantification relative to GAPDH (glyceraldehyde-3-phosphate dehydrogenase, % of SHAM+HT60) and (F) Representative Western blot images. n = 4 -6 rats/group. Data are expressed as mean ± SEM. ANOVA followed by Tukey's post-test for all. *p<0.05, **p<0.01, ***p<0.001 vs BDL; ##p<0.01, ###p<0.001 vs respective SHAM.

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Fig.1

A

BDL/SHAM surgery

Day 0
n = 17 SHAM; 22 BDL; 8 SHAM+HT90; 12 BDL+HT90; 16 SHAM+HT60; 22 BDL+HT60; 11 SHAM+HT30; 14 BDL+HT30
n = 10 dead following surgery or bleeding complications

6 weeks:
2h hypotension at 90, 60 and 30 mmHg

Brains collected for:
- **Histology/ Immunofluorescence (n=48)**
- **Western blot (n=44)**

Fluorescent microspheres Injection (n=20)

B

BDL surgery

OP treatment
Gavage (1g/kg daily)

Behavioural testing

Day 0
14 BDL+OP+HT60
n = 2 dead following surgery

3 weeks

6 weeks:
2h hypotension at 60 mmHg

Brains collected for:
- **Western blot (n=6)**
- **Immunofluorescence (n=6)**

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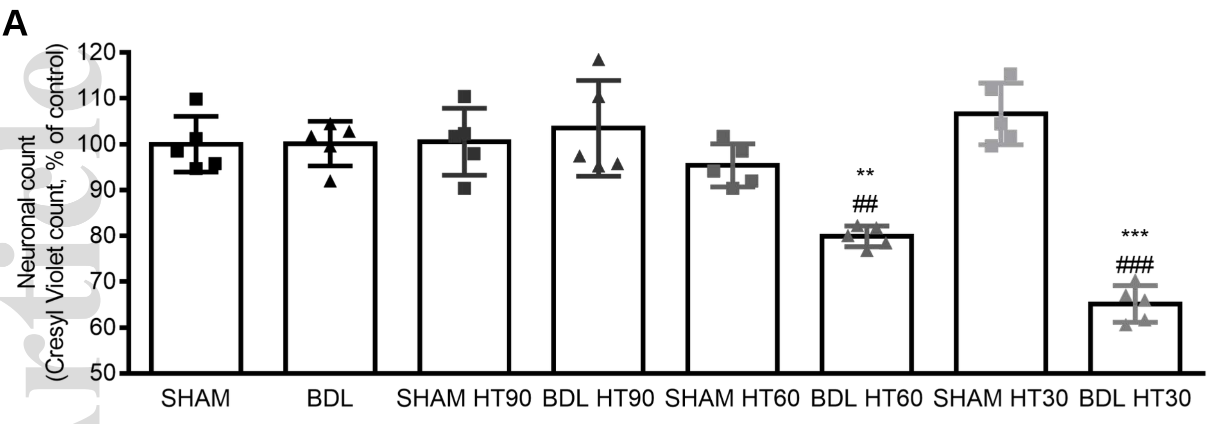
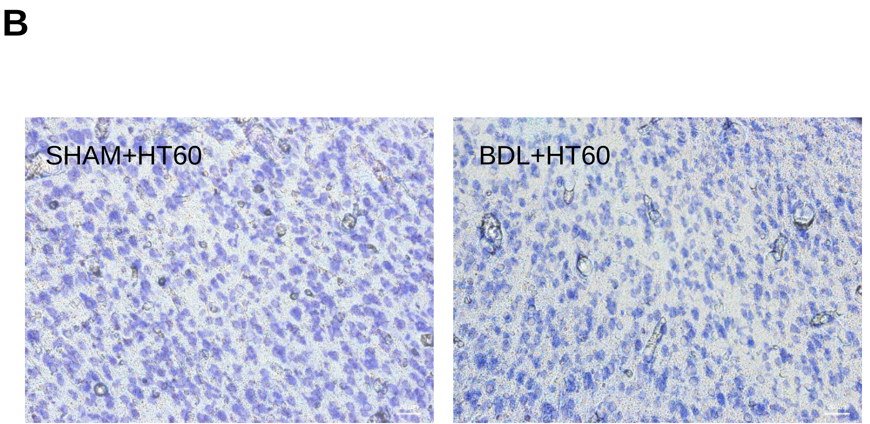
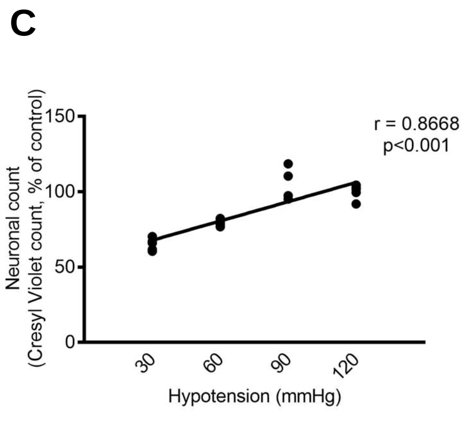
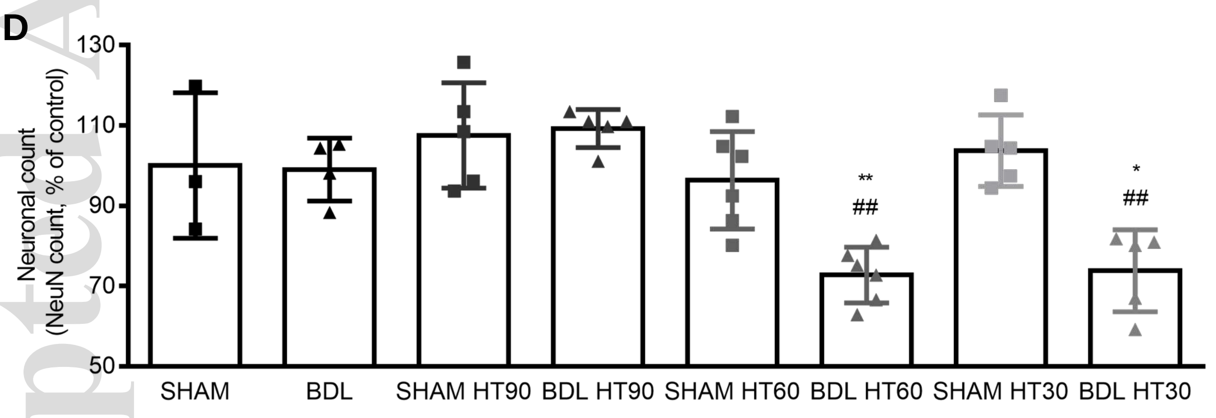
Fig.2

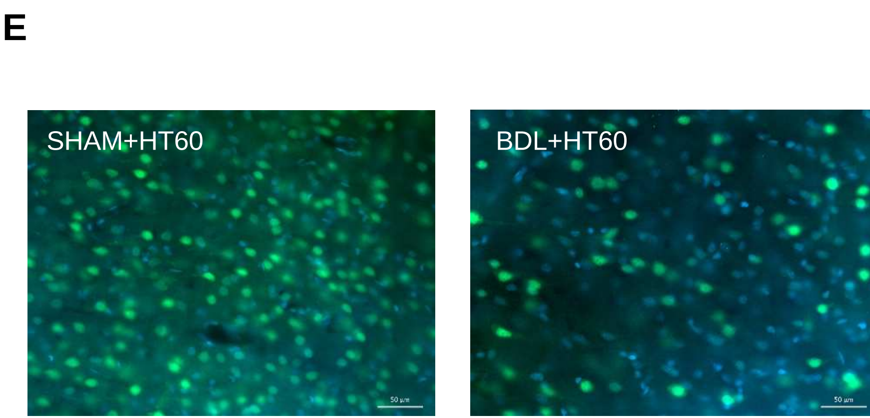
jnc_15290_f2.pdf











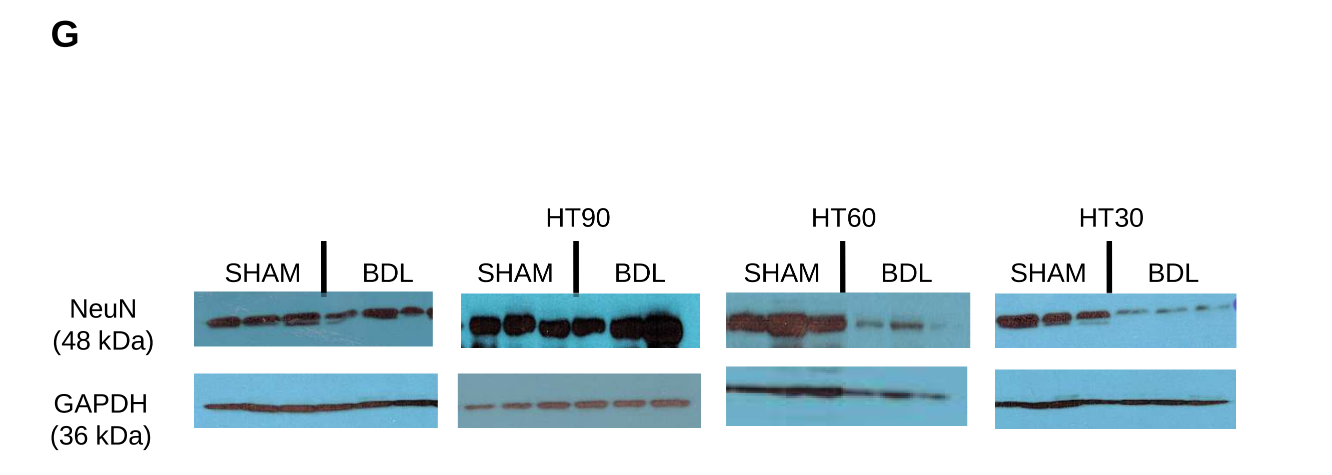


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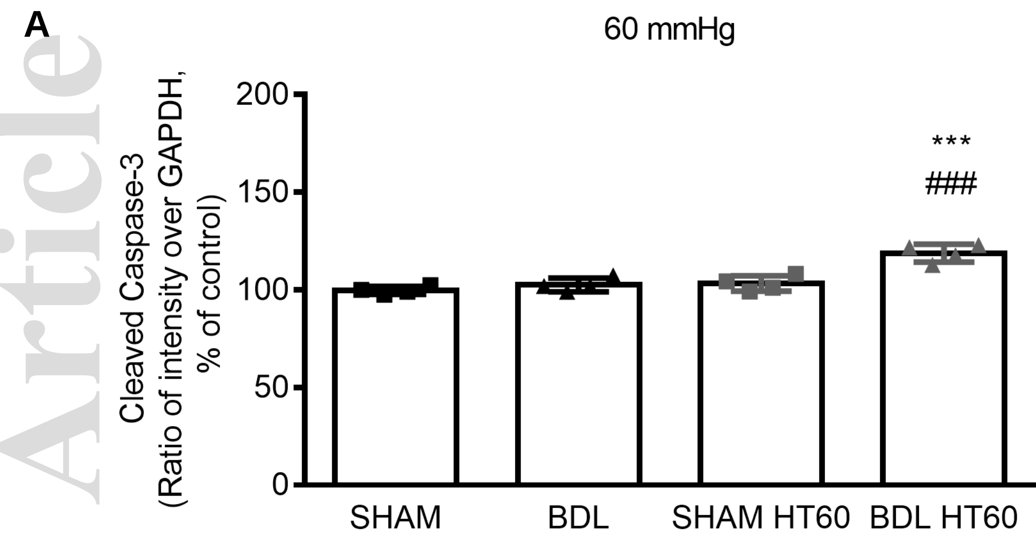
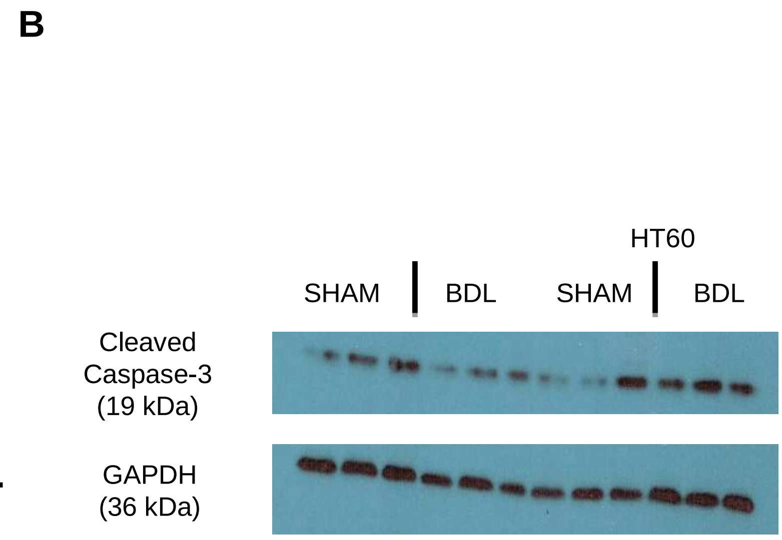
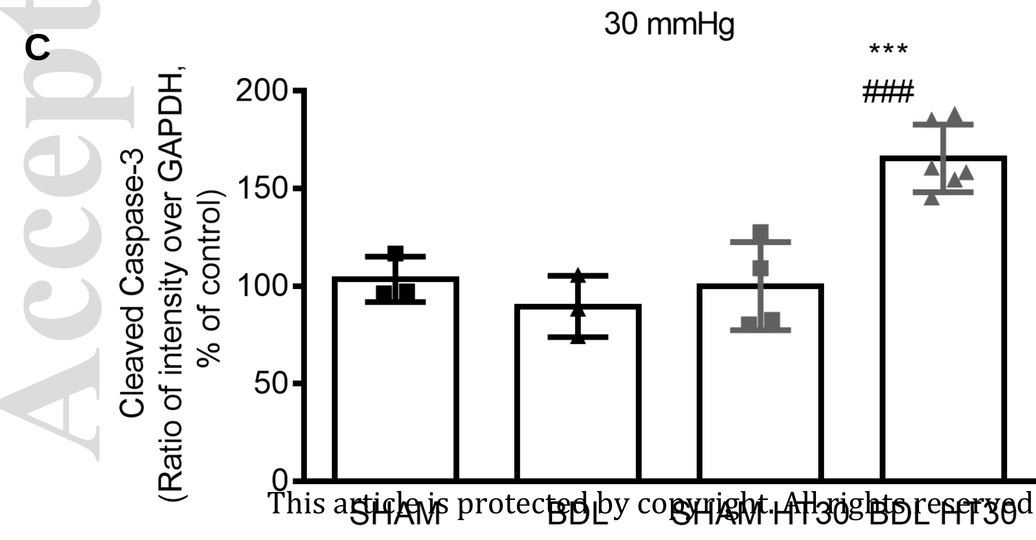
Fig.3







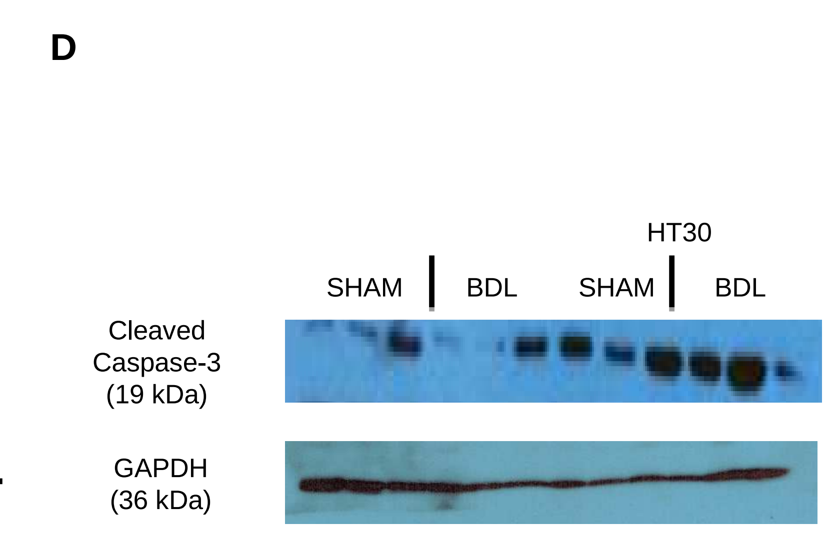


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Fig.3

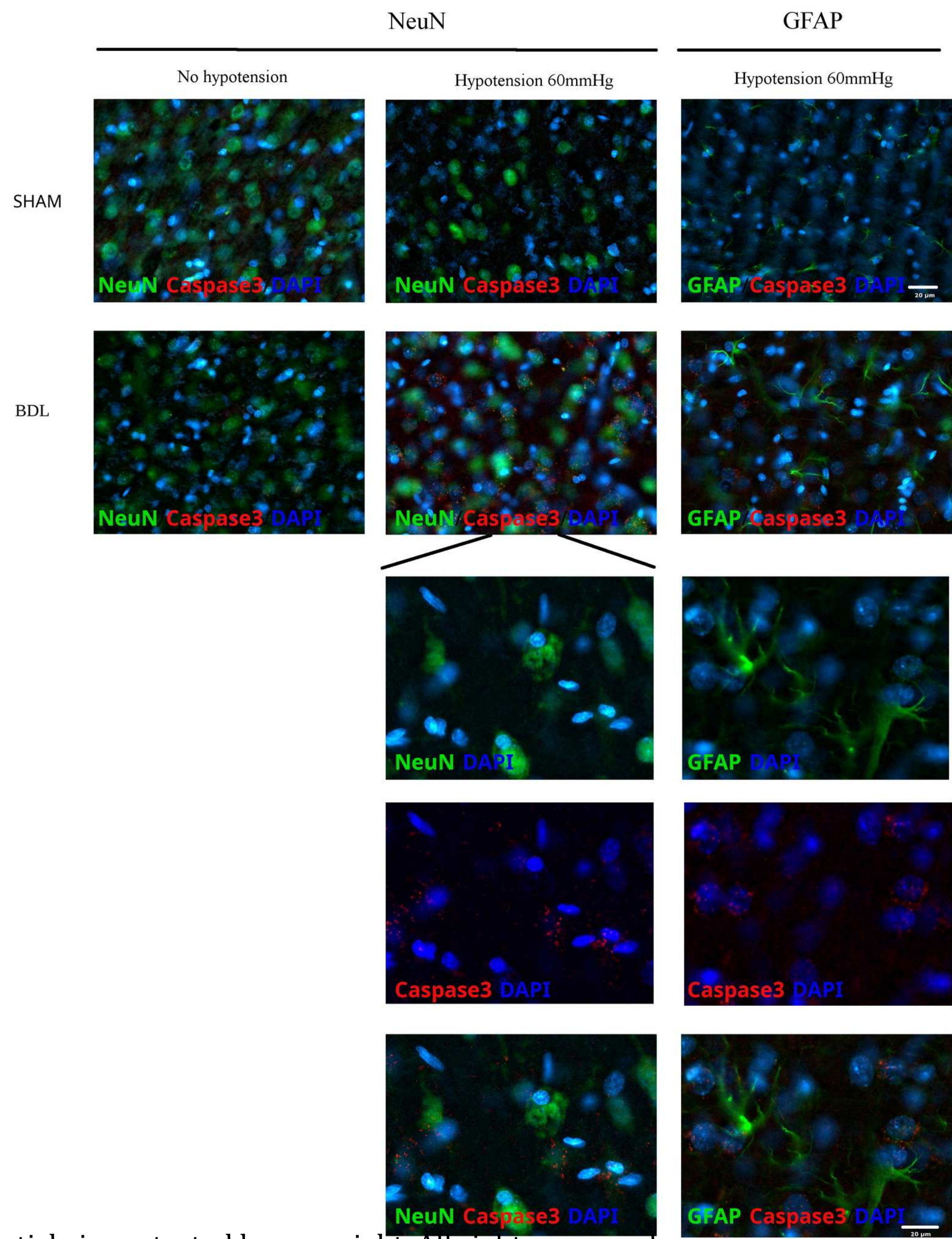

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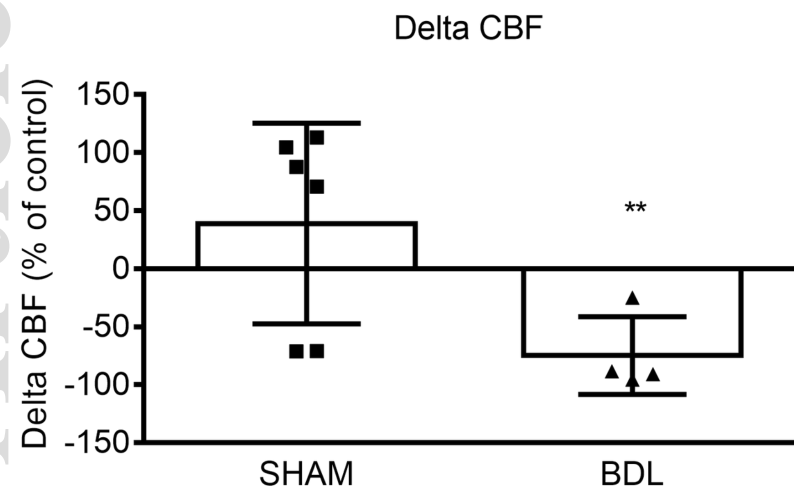
Fig.4

A



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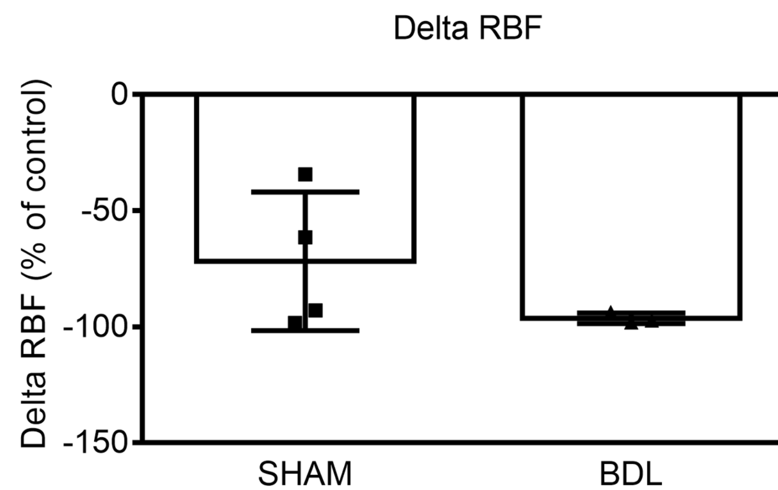

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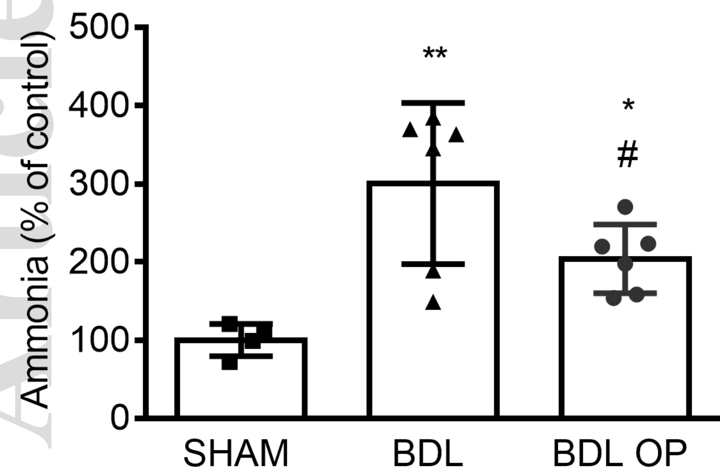
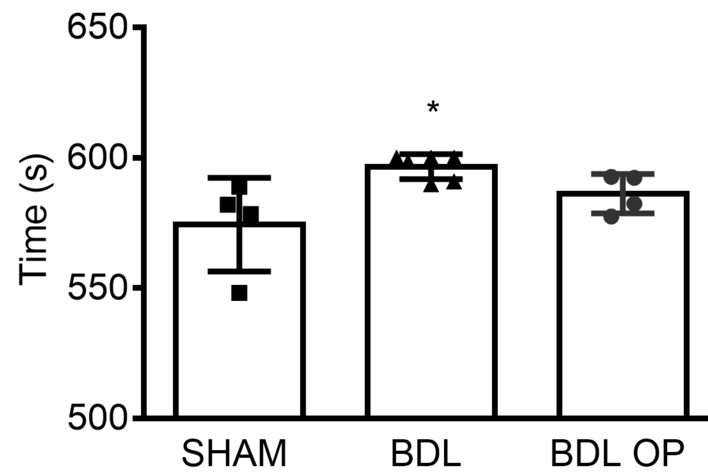
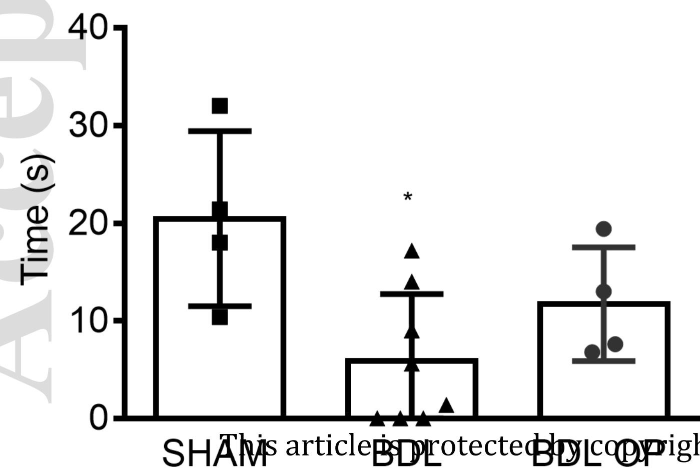
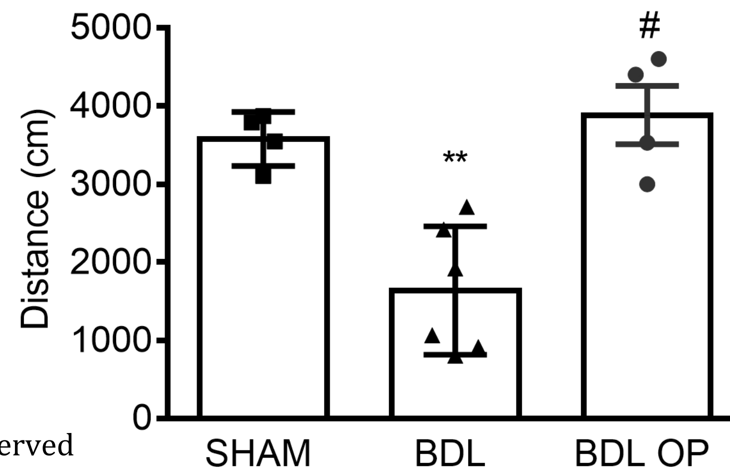
Fig.5









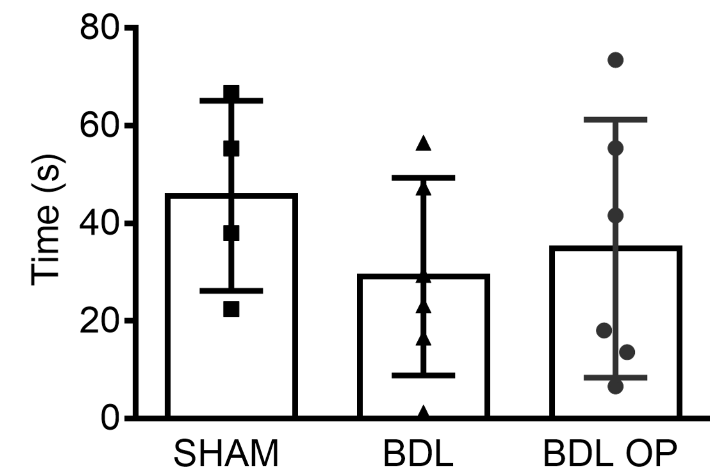


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Fig.6

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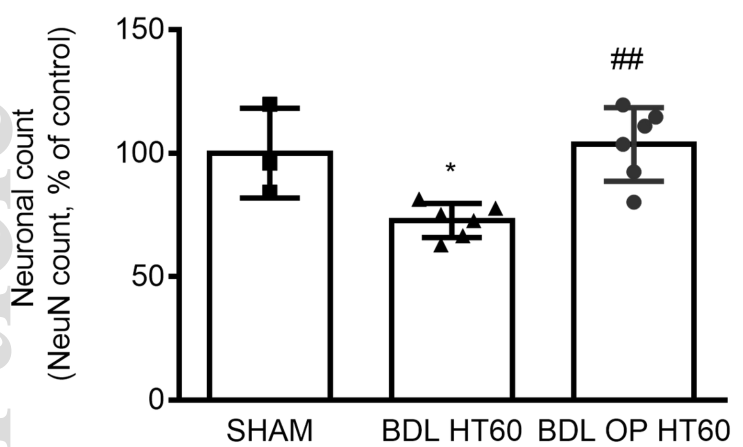

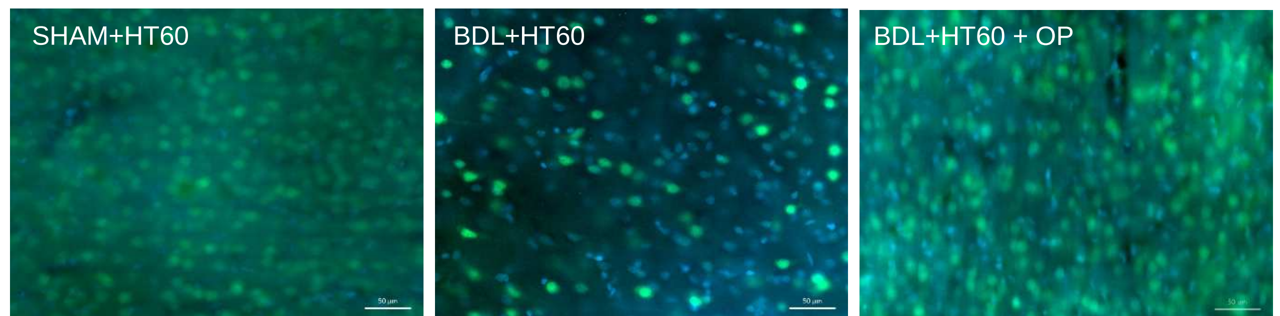

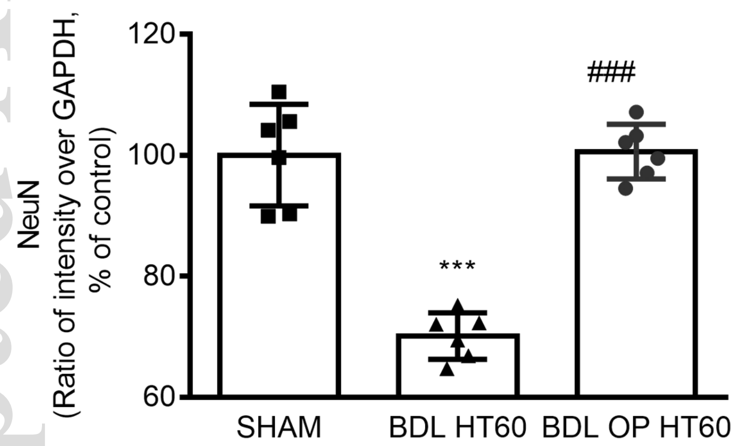

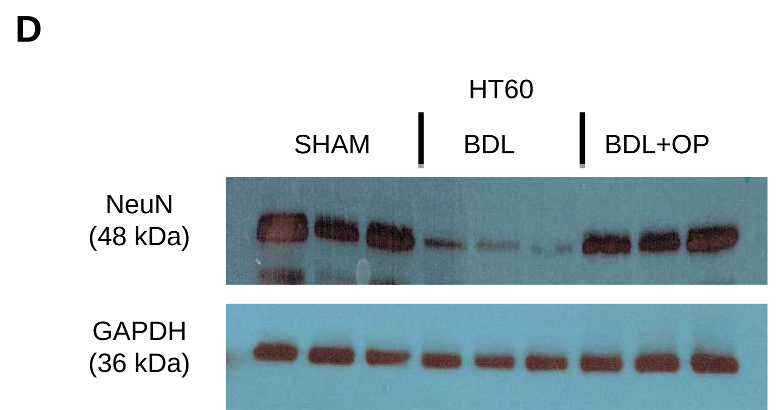

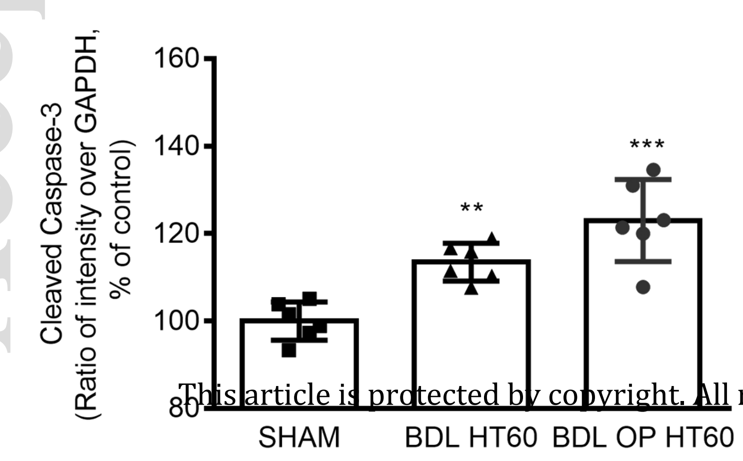

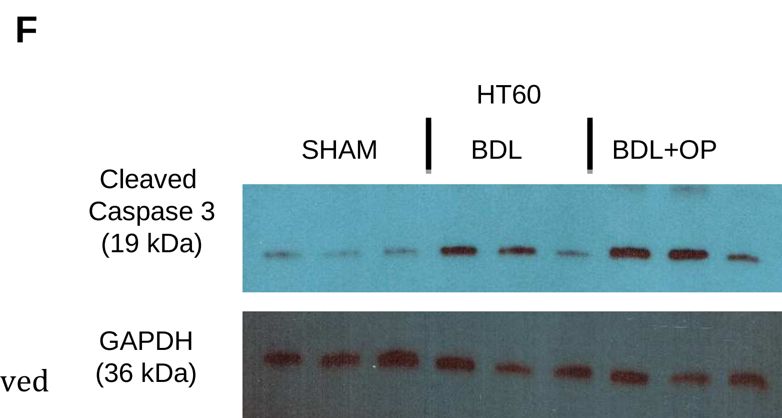

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