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14. ABSTRACT- The purpose of this study was to determine if xenon has a neuroprotectant effect in an vivo animal model of TBI. The Specific Aims were to determine the effect of inhaled xenon on brain histopathology, behavior, in short- and long-term fluid percussion (FP) and controlled cortical impact (CCI) rat models of TBI compared to controls. A unique xenon-recirculation delivery device in which the concentration of xenon and oxygen are reproducibly and accurately controlled and conserved was developed and manufactured. Outcome measures planned included histology and neurobehavioral measures. Data from initial experiments reported in 2010 using the CCI model and 50% xenon administration could not be utilized, as we discovered that although IACUC and VA Research Approvals were in place, ACURO approval was not in place. ACURO application documents were submitted again directly. Furthermore, the rat line that was utilized for the TBI model was discontinued by the supplier, necessitating a change to a different rat line. Extensive administrative and financial revisions to the SOW and budget subsequently ensued to try to feasibly achieve the revised scientific ends of the project. Administrative and financial issues between DoD and the primary recipient organization were not resolved before the final expiry of the project and before any studies could proceed with appropriate approvals in place.

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INTRODUCTION

Xenon has numerous neuroprotective effects, potentially through pleiotropic effects. Xenon could potentially reduce TBI-related secondary neuronal damage resulting from excitotoxicity, from release of glutamate, calcium, reactive oxygen species and inflammatory cytokines, from ischemic changes, and from edema (1-4). Xenon can block multiple neuronal receptors is anti-apoptogenic, may regulate cerebral blood flow, blocks excitotoxic dopamine release, and has anti-inflammatory effects, as well as other mechanisms. The progressive secondary neuronal damage from TBI is dependent upon these mechanisms. Xenon is a noble anesthetic and sedative gas and is rapidly absorbed by inhalation, distributed well to tissues, including the brain, and has rapid induction and emergence. It has minimal cardiovascular side effects, is inert, is not metabolized, and is rapidly eliminated (5,6). Xenon does not affect ventilation, pulmonary function, respiratory mechanics or airway resistance. It is used in concentrations with oxygen that have a low risk of hypoxia (7-9). Animal studies indicate xenon has other tissue protective effects (6) that occur at sub-anaesthetic doses (10). Xenon is non-flammable and non-combustible, making it potentially safe to deploy in the field. Xenon is obtained from the atmosphere during the production of oxygen, but is very expensive, due to its rarity.

Xenon mechanisms of action. Xenon has pleiotropic effects at cellular levels. It inhibits the calcium ATPase pump, controlling calcium efflux in synaptic cell membranes (11). In myocardial cells xenon activates protein kinase C-epsilon and its downstream target, p38 mitogen-activated protein, reducing infarct size (5). Xenon also upregulates expression of activity-dependent neuroprotective protein (ADNP) in neonatal rat brain (12). Xenon may affect neutrophil adhesion to endothelium during ischaemia/reperfusion injury through removal of leukocyte adhesion molecules PSGL-1 and L-selectin from neutrophil surface in vitro (13). In the brain, xenon acts as an N-methyl-D-aspartate (NMDA) receptor antagonist, conferring neuroprotection (9, 14, 15). However, it appears to block, in cortical neurons, other receptors, including -amino-3-hydroxy-5-methyl-4-isoxazolole propionate (AMPA) and kainate receptors, which have been implicated in neuronal excitotoxicty (16). Xenon has also been found to have anti-apoptogenic effects in neurons (17). Xenon can also block hypoxia-induced excitotoxic dopamine release in dopaminergic cells, in a calcium-dependent mechanism (18). Xenon likely has additional neuroprotective cellular effects that have yet to be determined.

In vitro studies of xenon in TBI. Coburn et al (19) found xenon, even 3 hrs post-injury, was effective in reducing neuronal injury measured by propidium iodide fluorescence, in the only study of xenon in TBI.

In vivo studies of xenon in ischemic and excitatory tissue injury. There are no published studies of inhaled xenon being used for neuroprotection following TBI, but there have been for cerebral or cardiac ischemia. In several animal models of transient hypoxia or of transient middle cerebral artery occlusion. xenon inhalation for one to three hours in concentrations ranging from 20-70% improved both functional neurologic and histologic outcomes, including in the striatum, often resistant to neuroprotective interventions (20-25). In rat models, xenon achieved maximal neuroprotection at 50%, even when administered up to 4 hours after excitotoxic NMDA injection and up to at least 2 h after induction of transient brain ischemia (24, 25). Xenon administered for 60 minutes to rats undergoing cardiopulmonary bypass operation attenuated neurologic injury and neurocognitive defects compared to controls (26). Although 80 % xenon anesthesia increases cerebral blood flow in normal pigs (27), it does not raise intracranial pressure (ICP) or cerebral blood flow further in pigs with elevated ICP (28).

Clinical use of xenon. Xenon has been used as an anesthetic agent intermittently since 1946. A study in 16 patients undergoing cardiopulmonary bypass surgery showed that inhaled xenon from 20-79% was safe and tolerable (29). Among 142 surgical patients xenon anesthesia was well tolerated except for more nausea and vomiting compared to those randomized to propofol (30). Several other studies in surgical patients reported xenon was well tolerated as an anesthetic (31-34). In a pilot study of critical care sedation for 8 hours, 21 patients randomized to either propofol or inhaled xenon (9-62 %) had comparable sedation. Xenon-treated patients had more stable blood pressures and recovered significantly faster than those treated with propofol (35).

The purpose of this study was to determine, at the level of proof of principle, if xenon has a neuroprotectant effect in *in vivo* animal models of TBI. The Statement of Work was revised for a focus on closed cortical impact as the TBI model, rather than including fluid percussion.

BODY

OBJECTIVES/SPECIFIC AIMS. We will test the hypothesis that inhaled xenon administered after TBI reduces neurologic and behavioral deficits in an in vivo rat model.

Task 1. Determine the effect of inhaled xenon on brain histopathology in a short-term controlled cortical impact (CCI) rat model of TBI compared to controls.

1a. Approvals will be obtained from and Research and Development Committee (VAMC) and ACURO. Approvals from VA Research and Development Committee and Brigham and Women's IACUC were obtained for this study. The ACURO application had been submitted to BVARI for forwarding to DoD. We believed we had received all necessary approvals and started animal work in good faith. We discovered late that ACURO had not received the application after all, and all animal work was halted on the study. Data generated from the initial experiments cannot be used, as ACURO approval was not in place at the time. Furthermore, we discovered that the company that provided the rats we were originally working with discontinued the line. Therefore, we planned to change the rat line to Sprague-Dawley. An amended approval from Brigham and Women's Hospital IACUC for using this rat line and for using CCI to cover moderate injury models was obtained. VA Research and Development Committee approval for this study was obtained. ACURO application documents were submitted by Dr. Bruce Kristal directly to ACURO. The investigators have not received an indication of an ACURO approval. Animal work has not proceeded and the studies did not proceed beyond the initial studies reported previously in the 2010 interim report. Our understanding was that no data could be used from the initial experiments prior to ACURO approval. This resulted in an extensive rebudgeting of the entire project. It is our understanding that the project could also not proceed until all budget issues were resolved and DoD approval was granted to proceed. Budget negotiations between BVARI and Brigham and Womens' Hospital and then between BVARI and DoD were extended. The final budget negotiations between DoD and BVARI were not resolved when the grant, in no-cost extension, expired. The PI had contacted DoD project officers to try to extend the project further in order to try to achieve the scientific objectives of the project. Due to the complexity of the budget and compliance issues, the categorization, and negotiation of funds have been handled directly by BVARI and DoD, rather than by the PI. Funding dispositions and resolutions will be handled directly by BVARI with the funding agency. The PI, although budgeted to, has not received salary support for this project.

1b. Equipment and xenon procurement. Manufacture and procurement of the devices and xenon for this project required a longer time than anticipated, due to the specialized nature of all the equipment and gas required. Industrial grade xenon (200 liters) with regulator and flow meter were obtained from Air Liquide. Administration of the xenon required design and manufacture of a specialized xenon recirculation device. This unique design was developed by collaboration with Dr. Jose Venegas at Massachusetts General Hospital (MGH) and constructed at the MGH Bioengineering Workshop. The custom designed xenon recirculation chamber is sufficiently sized for a single rat. This was delivered to Dr. Bruce Kristal's laboratory at the Department of Neurosurgery, Brigham and Women's Hospital. A commercial Insovt GKM-03 xenon/oxygen analyzer and CGS-06 side-stream and main-stream xenon/oxygen sensors (Alfa Impex, Helsinki, Finland) was acquired. This xenon/oxygen analyzer is currently recognized as the most accurate on the market, in my discussions with colleagues familiar with xenon administration and analyzers. We also acquired an AC power inverter (12 Volt DC to 220 Volt, 50Hz , EPS60012V, 600 watts continuous, 1000 watts peak, EDX Inc., Wilmington, NC) , 12 volt battery (34 Ah Rechargeable Battery, Toyo, amazon.com) , and battery recharger (Schumacher SSC-1500A, amazon.com) and transferred to Dr. Kristal's laboratory to allow operation of the xenon/oxygen analyzer . A Beam Walk Device and Dragonfly (model HPD-1700) Variable Pressure Waveform Generator with transducer, charge amplifier, and remote triggering device were manufactured by Dragonfly and were delivered to Dr. Kristal's laboratory.

1c. Methods development. *Xenon/air delivery.* Methods for gas delivery were developed. We have developed a unique method of xenon gas administration to a single rat at a time. We consulted with researchers at the Imperial College of London to review their method of administration of xenon, but developed our own method. The device is a box, with a small fan, trays of silica for water vapor removal, and soda lime for CO2 absorption, input for oxygen and xenon. A balloon is inside the device which contains air and removal of a volume of gas (either oxygen or xenon) will allow input of an equal volume of gas into the recirculation device. The box is flushed with 100% oxygen and the concentration of xenon can be controlled by removing a known volume from the balloon inside the box. Since the volume of the box is also known, the concentration of the xenon added to the box will be proportional to the volume removed from the balloon. This is verified by direct measurement of oxygen and xenon using the Alpha-Impex xenon/oxygen analyzer. Decrease in oxygen concentration can be adjusted by adding oxygen to the system. The oxygen analyzer underestimates by

about 3% and the xenon analyzer underestimates by about 4-5%, despite calibration. The rat exposed to 50% xenon/50%air becomes lethargic within several minutes, but continues to spontaneously breathe during xenon

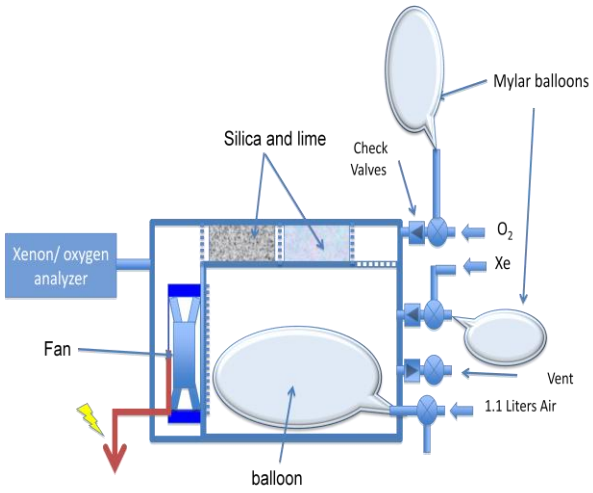

Fig. 1: Schematic of xenon recirculation box.

Figure 2: Xenon analyzer, xenon gas supply and recirculation box with connecting tubing and a Mylar reservoir.

administration. In the experimental plan, half of the rats were to be treated with 50% xenon/21% oxygen and half will inhale room air, both for 3 hours, in the recirculation box for 3 hours post-TBI

TBI methodology development. Methodology for TBI experiments was initiated and established at Dr. Bruce Kristal's laboratory, Department of Neurosurgery, Brigham and Women's Hospital. Please see section 1d below for detailed methodology for performance of CCI trials. Initial experiments were performed in male, specific pathogen free (SPF), Fischer 344 x Brown Norway F1 (FBNF1) rats. It was subsequently discovered that this rat strain was discontinued by the sole supplier. This necessitated a plan to switch to Sprague-Dawley rats. Please see the above section *1a*, regarding the issues with approvals that impacted this modification to the TBI models. With each rat strain there is also a need to conduct initial "impact dose" studies. For CCI this consists of an impact velocity and/or depth titration to assess impact intensity with 12 rats for CCI.

1d. Conduct of CCI trials. *Please see the discussion in Section 1a regarding approvals and impact on the study, as well as section 1c.* Study methodology (revised) involves 7 rats in each arm (moderate CCI with xenon, or air, sham injury with air) for a total of 21 animals. Each surgery day has one animal from each group. Animals are sacrificed in 1 week using brain perfusion /fixation method. The general method follows:

C*ontrolled Cortical Impact:* Rats are subjected to TBI utilizing the controlled cortical impact model (CCI). This model utilizes a non-penetrating, localized deformation of the cortex induced by a pneumatic impactor. Animals survive 1 week following impact. Animals subjected to injury with the CCI model receive a unilateral cortical impact to the left parietal cortex utilizing a beveled impacting tip (5 mm diameter) with a contact velocity as determined above and a final depth to be set. With each rat strain there is also a need to conduct initial "impact dose" studies. For CCI this consists of an impact velocity and/or depth titration to assess impact intensity with 12 rats for CCI. The diameter tip and velocity is set to produce a consistent and pronounced morphological alteration in the cortex, stopping just short of the hippocampus (ie, a moderate injury). Sham animals would receive a craniotomy exposing the cortical surface identical to the impact group. The impactor tip is lowered manually to the cortical surface, but no impact ensues.

Blinding and allocation strategy: Experimental conditions (e.g., impact depth, gas etc) for any given day are chosen at random (random number chart). We cannot blind our surgeon to impactor depth and gas delivery as only one person is in the room. A blinded examiner does all slide analysis. We follow an "intent-to-treat" style analysis, although we can consider retaining a condition longer if the initial poor results appear largely due to deaths prior to treatment (unlikely in this paradigm).

1) *Adaptive design.* We run all analysis with a broad adaptive design, meaning that we stop if it becomes statistically apparent that xenon is either protective or not. This is standard in Dr. Kristal's IACUC approved TBI protocol. Specifically (quoted directly from Dr. Kristal's main TBI protocol [bracketed elements added for clarity]):

"(i) Adaptive design strategy: We will run all studies with a continual adaptive design. That is, we will seek to stop studies as soon as possible (the equivalent of futility trials in humans), so as to save critical resources, eg, money, time, effort, and to enable deeper studies of key compounds and further reduce animal use. Per Dr.

Kristal's protocol:

"From the time we run the 3rd replicate of each drug [here, condition], we will begin to actively monitor the study to determine if we can stop testing some drugs [here, condition]. For example, [if] drugs [here: xenon] that are toxic can be eliminated at this point [not expected]. By the time we reach an N of 4, we can begin to use Bayesian and frequentist adaptive designs (the former by programming, we have a Bayesian statistician in lab]; the latter using the program EASTfrom Cytel, which we have in the lab). We will continue to monitor after each set of animals has been run. This design would normally not be used in an efficacy trial because it would increase the chance of stopping a trial too early, but this shouldn't matter here where we are trying to get rid of non- protective drugs [here: or prioritize hits, eg, xenon]. This approach allows us to maximally reduce animal usage."

1e. Histopathologic assessment. Dissected brains are kept in Bouin's fixative before embedding. Blocks are sectioned and stained with hematoxylin and eosin. We examine and score injured and non-injured cortex/white matter and hippocampus. Damaged area is assessed both qualitatively (hippocampus is/is not damaged) and quantitatively (area of region of injury). Histopathology is currently done using only H&E staining, but a secondary series of stains may be tested (e.g., iba1, GFAP). We use at least two stains in the current work. In general, samples are fixed immediately and go to the pathology lab approximately 1 week after sacrifice. They are ready for microscopic study about 2 weeks later. Initial experiments reported in the interim annual report of 2010 cannot be used (Please see the discussion in section 1a above for this).

1f. *Data analysis and statistics.* The quantitative data are presented as means ± St Dev. Analysis in three stages: (i) Untreated, TBI rats compared to Sham (no impact) rats (positive control validation). (ii) xenon-treated TBI rats compared to air-exposed TBI rats via Student's t-test (if data is normal via D'Agostino's omnibus test) or by Mann-Whitney U test (if data is non-normal). If region-specific data require comparisons they will build on the above analysis using t-tests coupled with Sidak's correction for multiple comparisons.

1g-i. Conduct of FP trials: In the revised Statement of Work for this Hypothesis Development Award, these experiments were not to be undertaken, given the budgetary, time and feasibility constraints. It was hoped that work with another TBI model could be undertaken under a different funding opportunity in the future. The general method for fluid percussion follows: Rats are subjected to FP-induced TBI. Rats are subjected to TBI utilizing the lateral fluid percussion (FP) model. Briefly, the skull is opened via a craniotomy (4.8 mm) centered between the bregma and lambda and between the sagittal suture and right temporal ridge and fitted with a Leur-loc fitting and the animal is attached to the Dragonfly (model HPD-1700) Variable Pressure Waveform Generator and subjected to an injury at ~2.5 atm. (Note: final pressure to be set pending final methods development). Sham treated animals are treated identically except for the pressure wave. Blinding and allocation strategy, andadaptive design as in section 1d.

Task 2. Determine the effect of inhaled xenon on behavior in short-term fluid percussion (FP) and controlled cortical impact (CCI) rat models of TBI compared to controls.

2a. Conduct of trial as in Task 1. *See section 1g-I re FP.*

2b: Neuroscore/SNAP assessment with CCI: This is a short term assessment of neurological function. Neuroscore/SNAP is a standard assessment for all TBI studies, and is conducted on days 1 and 3 post injury. Neuroscore as described by Hoover *et al* is a composite score based on combining scores from tests of forelimb reflex, hind limb flexion, lateral pulsion, and ability to stay on an angled board. We have modified the system of Shelton et al (termed SNAP) of neurological evaluation after TBI in mice (36) for use in our studies with rats. The system consists of 8 different tests that are primarily observational categories. We construct our score by using 6 out of the eight categories: Interactions (or avoidance to being handled upon removal from the cage), Cage Grasp (the manner in which the animal releases from holding onto the cage bars on the top of the cage), Visual Placing (the manner in which the animal reaches for an approaching table top), Gait and Posture (noting abnormalities while the animal is moving freely about a space), presence or absence of Head Tilt , and Baton (level of coordination used to grasp a stick with all four feet). Each category is scored on a scale of 0 to 5 and added to produce a neuroscore. For the baton, which is the proper size and weight for the animal to grasp comfortably, we use a wooden dowel (currently ¼ inch diameter x 3 ft long). With the exception of being allowed to move about freely on a counter top briefly and grasping a baton, the above tests or categories of observation all occur in situations commonly experienced by the animals during normal husbandry practices.

Neuroscore results following CCI: Data from initial experiments were reported in the interim annual report from 2010. However, these data cannot be used, as discussed in section 1a.

2c: Rotarod assessment: Rotarod testing is implemented using the programmed, accelerating Rotarod. The duration in seconds at the point at which the animal either completes the task (maximum of 2 minutes), falls from the rods, or grips the rods and spins for two consecutive revolutions rather than actively walks, is

recorded as the Rotarod score. Post-injury assessment begins at 24 hours post-injury and is performed every 24 hours thereafter to complete 3 days total. The exact testing schedule can change based on our experience with these animals.

Results with Rotarod following CCI: Data from initial experiments were reported in the interim annual report from 2010. However, these data cannot be used, as discussed in section 1a.

2d. Beam walk test. The elevated beam is constructed so as to detect non-compensatory foot-fault deficits in brain-injured rats (Dragonfly, Inc., Ridgely, WV). The length of the beam is tapered such that the starting width of the walking surface is 5.5 cm and the ending width is 1.5 cm. Along each side is a 2 cm wide ledge positioned 2 cm below the beam surface. The ledge allows the rat to avoid compensatory changes in posturing and weight distribution. Each ledge is equipped with mechanical sensors and digital recorders to detect the number of left or right steps onto it. Each step onto the ledge is recorded as a foot-fault. There is a platform at the starting end and a darkened box at the finishing end. Once in the darkened box, the animal will be allowed to stay there for 30 seconds, for positive reinforcement. We monitor beginning at 24 hours post-injury and every 24 hours thereafter to complete 3 days total. The exact testing schedule can change based on our experience with these animals. Foot-faults are added from three consecutive runs.

Results on Beamwalk following CCI: Data from initial experiments were reported in the interim annual report from 2010. However, these data cannot be used, as discussed in section 1a.

2e: Cylinder reach test. Animals with unilateral injuries reach preferentially with the paw on the non-injured side. Rats are placed in a clear cylinder and allowed to explore for 5 minutes. Wall touches and duration of contact with each front paw are measured. Test is conducted on days 1 and 3 post injury.

Results of cylinder test following CCI: Data from initial experiments referred to in the interim annual report of 2010 cannot be used, as discussed in section 1a.

Task 3. Determine the effect of inhaled xenon on behavior and histopathology in long-term fluid percussion (FP) and controlled cortical impact (CCI) rat models of TBI compared to controls.

Methods: as in Task 1. *See section 1g-I re FP.*

Results: Data from initial experiments were reported in the interim annual report from 2010. However, these data cannot be used, as discussed in section 1a.

KEY RESEARCH ACCOMPLISHMENTS:

- Designed and manufactured a unique xenon-recirculation box in which the concentration of xenon and oxygen are reproducibly and accurately controlled and conserved.

REPORTABLE OUTCOMES:

- Designed and manufactured a unique xenon-recirculation box in which the concentration of xenon and oxygen are reproducibly and accurately controlled. This can be used for a variety of xenon-related experiments.
- There are considerable issues related to the expense, delivery and monitoring of xenon in vivo.

CONCLUSION: We have successfully established a reliable and reproducible system for xenon recirculation. Data provided in the interim annual report of 2010 cannot be further reported, as discussed in section 1a above. Although xenon has pleiotropic effects and plausible biologic mechanisms of action in TBI, we speculate that it is likely that the short half life of xenon may limit its efficacy in the progressive injury of TBI. There is considerable expense with the need to reliably deliver and monitor xenon administration.

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APPENDIX: REVISED STATEMENT OF WORK

This proposed revised draft Statement of Work is provided at the request of DoD, to work within the time frame available, and a revised budget in order to try to address the essential objectives of the research project within the context of logistical, budgetary, time line, scientific, and administrative challenges. We are actively working to address those challenges, in order to proceed with this Hypothesis Development project. The major change proposed would be a focus on closed cortical impact as the TBI model, rather than including fluid percussion. Dr. Kristal trusts the quantitative precision of this model at this point and the number of rats to be used is limited. This offers the best path forward for scientific resolution of the objective for the project.

All work would be conducted under the direction of Dr. Bruce Kristal, Department of Neurosurgery, Brigham and Women's Hospital/Department of Surgery, Harvard Medical School, subject to approval by Brigham and Women' s Hospital, Dr. Kristal, BVARI, appropriate research committees, and the funding agency. Dr. Kristal's office and main laboratory is located at 221 Longwood Ave, LM322, Boston, MA. The lab's animal facilities for TBI research are located at 65 Landsdowne St., Cambridge, MA. All experiments would be conducted by or under the supervision of Dr. Kristal's laboratory veterinarian, Caryn Porter VMD or other appropriate laboratory staff. Drs. Bruce Kristal, Caryn Porter, and Jussi Saukkonen will conduct and analyze and report the studies.

RATIONALE/PURPOSE. Xenon has neuroprotective effects, through blocking multiple neuronal receptors (NMDA, AMPA, kainate and others) to block excitotoxicity, but is also anti-apoptogenic, may regulate cerebral blood flow, blocks excitotoxic dopamine release, and has anti-inflammatory effects, as well as other mechanisms. The progressive secondary neuronal damage from TBI is dependent upon these mechanisms antagonized by xenon. *The purpose of this novel study is to determine, at the level of proof of principle, if xenon has a neuroprotectant effect in in vivo animal models of TBI.*

OBJECTIVES/SPECIFIC AIMS. We will test the hypothesis that inhaled xenon administered after TBI reduces neurologic and behavioral deficits in an *in vivo* rat model.

Task 1. Determine the effect of inhaled xenon on brain histopathology in a short-term controlled cortical impact (CCI) rat model of TBI compared to controls. *(Estimated time frame from now until March 31, 2012: months 1-6*

1a. Approvals will be obtained from and Research and Development Committee (VAMC) and ACURO. (Estimated time frame: months 1-3) Since the company that provided the rats we were originally working with has discontinued the line we would be changing the rat line to Sprague-Dawley. This will require amended approvals from BWH IACUC (which is in place), and VA Research and Development Committee for using this rat line and for using CCI to cover moderate injury models per DOD review. Approval from ACURO will be sought for this project. Dr. Kristal will continue to oversee BWH paperwork; Dr. Saukkonen will oversee VAMC paperwork. BVARI will oversee the transfer of ACURO paperwork between Dr. Kristal and DOD.

1b. Equipment and xenon procurement.. Dr. Saukkonen has already procured the chamber, xenon analyzer, power inverter, battery, and xenon gas necessary for these experiments and these have already been transferred to Dr. Porter in Dr. Kristal's laboratory.

1c. Methods development (estimated time frame (month 2-4 -- will begin as soon as approval in place) *Methods* for gas delivery are in place. We will need to conduct initial "impact dose" study for the new rat strain (i.e. Sprague-Dawley). This will consist of an impact velocity and/or depth titration to assess impact intensity with 12 rats for CCI (Dr. Caryn Porter to oversee).

1d. Conduct of CCI trials (estimated timeframe, months 3-6). Study involves 7 rats in each arm (moderate CCI with xenon, or air, sham injury with air) for a total of 21 animals. Each surgery day will have one animal from each group. Animals will be sacrificed in 1week using brain perfusion /fixation method. The method follows:

C*ontrolled Cortical Impact:* Rats will be subjected to TBI utilizing the controlled cortical impact model (CCI) which Dr. Kristal's lab is now using. This model utilizes a non-penetrating, localized deformation of the cortex induced by a pneumatic impactor. Animals will survive 1 week following impact. Animals subjected to injury with the CCI model will receive a unilateral cortical impact to the left parietal cortex utilizing a beveled impacting tip (5 mm diameter) with a contact velocity as determined above and a final depth to be set. This diameter tip and velocity will be set to produce a consistent and pronounced morphological alteration in the cortex, stopping just short of the hippocampus (ie, a moderate injury). Sham animals will receive a craniotomy exposing the cortical surface identical to the impact group. The impactor tip is lowered manually to the cortical surface but no impact will ensue.

Blinding and allocation strategy: Experimental conditions (e.g., impact depth, gas etc) for any given day will be chosen at random (random number chart). We cannot blind our surgeon to impactor depth and gas delivery as only one person is in the room. A blinded examiner will do all slide analysis. We will follow an "intent-to-treat" style analysis, although we will consider retaining a condition longer if the initial poor results appear largely due to deaths prior to treatment (unlikely in this paradigm).

1) *Adaptive design.* We will run all analysis with a broad adaptive design, meaning that we will stop if it becomes statistically apparent that xenon is either protective or not. This is standard in Dr. Kristal's IACUC approved TBI protocol. Specifically (quoted directly from Dr. Kristal's main TBI protocol [bracketed elements added for clarity]):

"(i) Adaptive design strategy: We will run all studies with a continual adaptive design. That is, we will seek to stop studies as soon as possible (the equivalent of futility trials in humans), so as to save critical resources, eg, money, time, effort, and to enable deeper studies of key compounds and further reduce animal use.

"From the time we run the 3rd replicate of each drug [here, condition], we will begin to actively monitor the study to determine if we can stop testing some drugs [here, condition]. For example, [if] drugs [here: xenon] that are toxic can be eliminated at this point [not expected]. By the time we reach an N of 4, we can begin to use Bayesian and frequentist adaptive designs (the former by programming, we have a Bayesian statistician in lab]; the latter using the program EAST
from Cytel, which we have in the lab). We will continue to monitor after each set of animals has been run. This design would normally not be used in an efficacy trial because it would increase the chance of stopping a trial too early, but this shouldn't matter here where we are trying to get rid of non- protective drugs [here: or prioritize hits, eg, xenon]. This approach allows us to maximally reduce animal usage."

1e. Histopathologic assessment (estimated time frame: months 3-7) Dissected brains will be kept in Bouin's fixative before embedding. Blocks will be sectioned and stained with hematoxylin and eosin. We will examine and score injured and non-injured cortex/white matter and hippocampus. Damaged area will be assessed both qualitatively (hippocampus is/is not damaged) and quantitatively (area of region of injury). We note that our histopathology is currently done using only H&E staining, but we will be testing a secondary series of stains (e.g., iba1, GFAP). We will use at least two stains in the current work. We welcome any comments on this. In general, samples are fixed immediately and go to the pathology lab approximately 1 week after sacrifice. They are ready for microscopic study about 2 weeks later.

1f. *Data analysis and statistics.(Estimated time frame: ongoing, but also month 6-7)* The quantitative data will be presented as means ± St Dev. Analysis in three stages: (i) Untreated, TBI rats will be compared to Sham (no impact) rats (positive control validation). (ii) xenon-treated TBI rats will be compared to air exposed TBI rats via student's t-test (if data is normal via D'Agostino's omnibus test) or by Mann-Whitney U test (if data is non-normal). If region-specific data require comparisons they will build on the above analysis using t-tests coupled with Sidak's correction for multiple comparisons.

1g-i. Conduct of FP trials: *It is proposed that for this hypothesis development award these experiments not be performed, given the budgetary, time and feasibility constraints. It is hoped that work with another model be undertaken under a different funding opportunity.*

Task 2. Determine the effect of inhaled xenon on behavior in short-term controlled cortical impact (CCI) rat models of TBI compared to controls. *(timeframe, months 3-6).*

2a. Conduct of trial as in Task 1 (same animals will be used).

2b: *Neuroscore/SNAP assessment* This is a short term assessment of neurological function. Neuroscore/SNAP will be a standard assessment for all TBI studies, and it will be conducted on days 1 and 3 post injury. Neuroscore as described by Hoover et al (37) is a composite score based on combining scores from tests of forelimb reflex, hind limb flexion, lateral pulsion, and ability to stay on an angled board. Shelton et al developed a system (termed SNAP) of neurological evaluation after TBI in mice (36). We will modify this system for our rats. The system consists of 8 different tests that are primarily observational categories. We eliminated 2 of the 8 categories for our use due to equipment constraints (lack of availability of an open field box for assessment of circling and pacing behavior) and inconsistent results (difficulty scoring visual response to a novel object). We will construct our score by using 6 out of the eight categories: Interactions (or avoidance to being handled upon removal from the cage), Cage Grasp (the manner in which the animal releases from holding onto the cage bars on the top of the cage), Visual Placing (the manner in which the animal reaches for an approaching table top), Gait and Posture (noting abnormalities while the animal is moving freely about a space), Head Tilt (is it present?), and Baton (level of coordination used to grasp a stick with all four feet). Each category is scored on a scale of 0 to 5 and summed to produce a neuroscore. A baton is needed that is the proper size and weight for the animal to grasp comfortably. We will use a wooden dowel (currently 1/4 inch diameter x 3 ft long). Other modifications to the originally described technique may be required to enhance the

accuracy of the observations and the comfort of the animal. With the exception of being allowed to move about freely on a counter top briefly and grasping a baton, the above tests or categories of observation all occur in situations commonly experienced by the animals during normal husbandry practices. Additional observations may be added to further refine the score, for example time to return of righting reflex post anesthesia. We may adapt slightly if needed for the Sprague-Dawley model.

2c: *Rotarod assessment:* Given the limitations on this study and our experience in working with this type of testing, we would plan to forego this in favor of the other neurologic and behavioral outcomes, if this remains acceptable, as it previously was. If there is sufficient time and resources to utilize this assessment we may be able to include it. The methodology for Rotarod testing is implemented using the programmed, accelerating Rotarod. The duration in seconds at the point at which the animal either completes the task (maximum of 2 minutes), falls from the rods, or grips the rods and spins for two consecutive revolutions rather than actively walks, is recorded as the rotarod score. Post-injury assessment will begin at 24 hours post-injury and will be performed every 24 hours thereafter to complete 3 days total. The exact testing schedule may change based on our experience with these animals.

2d. Beam walk test, **(estimated time frame months 3-6)** The elevated beam is constructed so as to detect non-compensatory foot-fault deficits in brain-injured rats (Dragonfly, Inc., Ridgely, WV). The length of the beam is tapered such that the starting width of the walking surface is 5.5 cm and the ending width is 1.5 cm. Along each side is a 2 cm wide ledge positioned 2 cm below the beam surface. The ledge allows the rat to avoid compensatory changes in posturing and weight distribution. Each ledge is equipped with mechanical sensors and digital recorders to detect the number of left or right steps onto it. Each step onto the ledge is recorded as a foot-fault. There is a platform at the starting end and a darkened box at the finishing end. Once in the darkened box, the animal will be allowed to stay there for 30 seconds, for positive reinforcement. We will monitor beginning at 24 hours post-injury and every 24 hours thereafter to complete 3 days total. The exact testing schedule may change based on our experience with these animals. Foot-faults will be summed from three consecutive runs.

2e: Cylinder reach test Given the limitations on this study and our experience in working with this type of testing, we had previously removed this from the SOW, a change that had been approved. This was done in favor of the other neurologic and behavioral outcomes.

2f. ***Data analysis and statistics.*** As in section 1f, with corrections via Sidak's correction for multiple comparisons adapted for correlated variables if appropriate and/or FDR for uncorrelated variables.

Task 3. Determine the effect of inhaled xenon on behavior and histopathology in long-term controlled cortical impact (CCI) rat models of TBI compared to controls. We would proceed with this work only if the short-term data suggested that there was, in fact, a neuroprotective effect for xenon studies It is certain that additional funding for this study would need to be sought in order to proceed with these studies If there is not, then it would not make sense to proceed with these studes,

3a: Injury models conducted as described in tasks 1d and 1g

3b: H&E histopathology as described in tasks 1e

3c: Neuroscore/SNAP assessment as described in task 2b.

3d: Rotorod assessment as described in task 2c.

3e: Beam walk assessment as described in task 2d.

3f: Cylinder assessment as described in task 2e.

3g. Morris water maze test Given the limitations on this study and our experience in working with this type of testing, we had previously removed this from the SOW, a change that had been approved. This was done in favor of the other neurologic and behavioral outcomes.

3i. Staircase test*.* Given the limitations on this study and our experience in working with this type of testing, we had previously removed this from the SOW, a change that had been approved. This was done in favor of the other neurologic and behavioral outcomes.

3g: Data analysis and statistics as described in section 2f.

Task 4. Final data analysis (estimated time frame Months 6-7)