**July 5, 2016**

Dear Editor,

Thank you for your revision and suggestions. We tried to satisfy all comments received from you and reviewers.

Changes have been made and are tracked in the tracked-changes version of the manuscript.

Below in ***bold italics,*** are our answers to the editor and reviewers.

To the editor: most of the reviewers’ comments were fine. However, it looked like that one of the reviewer (#2) did not have a specific medical or neuropathology background since the very general questions. Even though, we tried to comment on his/her observations/questions as much as we could.

Thank you.

Bets regards,

***Diego Iacono***

Dear Dr. Iacono,

Your manuscript JoVE54602R2 "Symmetric Bi-Hemispheric Postmortem Brain Cutting to Study Healthy and Pathological Brain Conditions in Humans." has been peer-reviewed and the following comments need to be addressed. Please keep JoVE's formatting requirements and the editorial comments from previous revisions in mind as you revise the manuscript to address peer review comments. Please maintain these overall manuscript changes, e.g., if formatting or other changes were made, commercial language was removed, etc.

Please track the changes in your word processor (e.g., Microsoft Word) or change the text color to identify all of the manuscript edits. When you have revised your submission, please also upload a separate document listing all of changes that address each of the editorial and peer review comments individually with the revised manuscript. Please provide either (1) a description of how the comment was addressed within the manuscript or (2) a rebuttal describing why the comment was not addressed if you feel it was incorrect or out of the scope of this work for publication in JoVE.

Your revision is due by Jul 07, 2016. Please note that due to the high volume of JoVE submissions, failure to meet this deadline will result in publication delays. To submit a revision, go to the JoVE Submission Site and log in as an author. You will find your submission under the heading 'Submission Needing Revision'.

Sincerely,

Mala Mani,

Science Editor

JoVE

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Editorial comments:

• Your manuscript has been modified by your editor, please maintain the current formatting throughout the manuscript. Please use the updated manuscript located in your Editorial Manager account (under “File Inventory”) for all subsequent revisions. Updated manuscript is also attached.

• Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

• JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information. ***We have added DOIs when available.***

• **Please disregard the comment below if all of your figures are original**. If you are re-using figures from a previous publication, please obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]. ***All figures in this manuscript are original.***

• Figure 3a: Please remove “Thermo Scientific”. ***Done***

•The Protocol length exceeds 3 pg. Please highlight 2.75 pg of material or less for filming. We suggest omitting sections 1 & 4 from filming. ***We underlined sections 1&2 for filming since section 3 requires a fresh brain from the autopsy room, which availability cannot be granted for the day of filming.***

•The manuscript must be copy edited for numerous grammatical errors. Such editing is required prior to acceptance and should be performed by a native English speaker. The discussion is particularly difficult to understand. Some examples are indicated below.

-Long abstract – Please clarify “capitalize current and possibly future biomolecular/neuroimaging techniques”. **We replaced the word “capitalize” with “use”.**

-Please do not use “excellent” or similar adjectives to describe the protocol. Please use only objective phrasing. **We eliminated the word “excellent”.**

-Line 455 – “This method can be modified or adapted to the specific needs of each neuropath lab keeping though the hemispheric analysis as one of its features” – Please clarify. **We rephrased this statement with ”This method can be adapted to the specific needs of each neuropath lab (for example by reducing the number of cerebral regions to assess) but keeping the bi-hemispheric cutting procedure as one of its main features.**

-Line 458 – “only to specific type of investigation or molecular analyses”. **We clarified this statement with specific examples “…specific types of investigation (i.e. immunohistochemistry) or molecular analyses (i.e. genomic or proteomic analyses)”.**

-Line 462 – “devises”. **We replaced “devices” with “machines”.**

-Line 467 – “situation determined”. **We rephrased with ”These new types of brain lesions created an increased risk that previous brains classified as normal or control, are actually not”.**

-Line 468 – “constant amelioration”. **We rephrased with ”The constant improvement of immunohistochemistry techniques and new neuropathologic discoveries”.**

-Line 474 – “studies finalized to genomic/proteomic analyses supposed”. **We rephrased this part with “…Moreover, studies aiming to analyze genomic and proteomic aspects of human brain necessitate a brain-bank team ready to manage, in a quick manner, all brain donations procedures, legal consent arrangements, brain removals, and immediate cutting for freezing procedures.**

-Line 479 – “help to better understanding”. **We rephrased with “help the better understand the possible…”**

-Line 481 – “One of the best fits for this protocol in the context of clinico-pathologic longitudinal studies, which are often the best type of investigations to collect more exhaustively clinical, imaging, genetic and environmental data to better correlate autopsy findings and microscopic/immunohistochemistry results.” – This is not a complete sentence. **We rephrased with” One of the best uses for this protocol will be in the context of clinico-pathologic longitudinal studies, which are often the best type of investigations to collect prospective clinical, imaging, genetic, environmental, and other type of data to better correlate autopsy findings and microscopic/immunohistochemistry results with previously collected clinical information”.**

-Line 482 – “exhaustively clinical” **We rephrased this part (see changes).**

-Line 497 – “comprehensible” is not the right word. **We replaced this word with “reasonable”.**

-Line 499 – “genetic useful information”. **We eliminated the word “useful”.**

-Line 503 – “emotional feelings”. **We used just the word “emotions”.**

-Line 506 – “not-mammalian species”; “If there is a hemispheric predilection for specific pathologic processes in terms of neurodegeneration, neuroinflammation, and neuroreparative capacities in humans is not known”. **We rephrased with “…and non-mammalian species. If there is, in humans, a hemispheric predilection for specific pathologic processes in terms of neurodegeneration, neuroinflammation responses, and neuroreparative capacities is not known and it has been very rarely investigated.”**

-Line 550 – “begun”. **Corrected.**

-Line 568 – “The set of neuroanalyses currently performable on human brain tissues were”. **We rephrased with: ”The set of neuroanalyses available now to analyze human brain tissues were not imaginable a few years ago and it is highly probably that there will be further advancements in the near future”.**

-Line 572 – “In the contest of complex illnesses”. ***“,…such as the neuropsychiatric diseases..”***

-Line 607 – “comparable efforts similarly”. ***We eliminated “comparable” and use the word “similar”.***

-Please correct the grammar in the Acknowledgements section***. Done.***

•Additional detail is required:

-2.3 – How are olfactory bulbs and tracts preserved? “***Preserve olfactory bulbs and tracts with special care to avoid tissutal laceration due to their extreme frailty”.***

-2.4 – What is used to cut here? **We add: …and using a sharp knife”.**

-2.9.2 – Please clarify “de-identifying codes; neuroanatomical names”. It is not clear how things are labeled here, so please use complete sentences and correct punctuation. ***We added this: “De-identifying codes could be created by generating random or semi-random numbers for each case (i.e. AD160001, where AD stays for Alzheimer’s Disease study, 16 is the year of autopsy (2016), 0001 a progressive accession specimen number).”***

•Results:

-Please provide a figure at higher magnification showing the positive staining. Currently it is difficult to see what is referred to in the results section. ***We have added pictures obtained at different level of magnification for each type of brain lesions described in the figure.***

-Please explicitly discuss table 2 in the results section. ***Done.***

-The color code does not contribute to the clarity of the table unless it is appropriately used. It should be removed if nothing is color-coded. ***We added the color-code in Table 2 and in the legend.***

•Discussion: Please discuss what alternative methods could be performed and the significance of this protocol in relation to them. ***We added this (line 611, discussion): ”An alternative approach to the described brain cutting procedure could be the cross-sectional cutting of each entire hemispheric surface. This method though requires specialized and more expensive tools (i.e. larger microtome, larger slides, etc.) than the ones normally used. Our method, instead, proposes a more extensive collection of brain regions from both hemispheres by cutting those single cerebral regions using tools normally available (and affordable) in most of research neuropathology labs.”***

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The present manuscript describes a protocol for symmetric bi-hemispheric brain cutting. The methods are sound and the protocol can be well replicated when following the proposed steps. Standardization of this procedure is of tremendous importance given that autopsy is increasingly utilized and co-registered with e.g. neuroimaging. The discussion covers those aspects sufficiently.

Major Concerns:

N/A

Minor Concerns:

There is little to criticize, thus I have only minor recommendations:

-Please comment how the brain is fixed because the steps describe heavily depend that the brain´s hemispheres are formed identically. Normally the brain is deformed during the process of fixation just because of gravity. Are there any special preparations, which can be done to prevent this? ***The best way to prevent tissue distortions is to immediately immerse the entire brain in a sufficient amount of fixative (i.e. 1 gallon of formalin 10%) in a cylindrical container (usually, at the beginning, the brain tends to float in the container so avoiding tissue deformations).***

-Line 174: Is the brain entirely fixed after 2-3 weeks? If then please give a reference e.g.: Yong-Hing CJ, Obenaus A, Stryker R, Tong K, Sarty GE. Magnetic resonance imaging and mathematical modeling of progressive formalin fixation of the human brain. Magn Reson Med. 2005;54: 324-32. doi:10.1002/mrm.20578. ***Complete brain tissue fixation (by formalin or other fixatives) depends on different factors (type and concentration of fixative, periagonal ischemic-hypoxic conditions, presence of brain edema before and after death, tissutal pH, temperature of the corpse until autopsy, autolysis, etc.). 2-3 weeks has been the minimal time normally required to obtain a sufficient brain fixation in a non-edematous brain to perform standard neuropath evaluations, especially in clinical centers where there is the necessity of reporting autopsy findings as soon as possible (i.e. for legal reasons). A period of 2-3 weeks has been demonstrated to be sufficient enough to obtain the fixation of intact human brains and it is normally good enough for most of the histological staining and immunohistochemistry protocols. Only recently, with the advancement of imaging-pathology correlations the fixation issue (in a research setting) has become more important. We add the ref as suggested.***

-Line 250: Additionally to the photographs, I would recommend overlaying a transparent empty graph sheet with e.g. 1 mm spacing. This will enable locating structures, sizes and abnormalities more accurately. We added this: ***“We suggest using a cutting surfaces (see Figure 3) with printed millimetric grid on their sides to localize brain structures, size and possible abnormalities in a more accurate manner”.***

-Line293: Please describe what is meant by "standard procedure". ***We added a ref (ref14) that describes all proper steps needed to perform a correct tissue processing, which is a standardized process performed by automatic systems.***

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

This is a very good scientific study for definitive diagnoses for complex neuropsychiatric phenomena to know the hemispheric differences in AD, PD or ALS and other neurodegenerative diseases in comparison to brain imaging. It has confirmatory and immense value since it relies specifically upon the direct physical assessment of brain tissues. However, there are concerns that should be improved.

Major Concerns:

a) Most of the time collection of as many as brain sample, storage, autopsy timings, and analyzing brain sample may differ so how do you confirm and correlate the neuropathological changes in any particular disease? ***This is very general question that goes much behind the aims of our manuscript. We wish to emphasize the importance and necessity to study human neuroanatomy. Only though the careful and patient neuroanatomical studying it is possible to recognize abnormalities and correlate them with clinical information. It is not possible in this manuscript to describe how the loss-of-function concept helped to define anatomy and function of specific cerebral regions in humans (decades of clinico-pathologic studies…), or how using inferential processes it is possible to correlate neuropathologic changes that are typically present in certain clinical manifestations and not in others (this is part of a of the medical and neuropathology specialty training).***

b) How do you know the particular neurochemical and the morpho-functional changes after autopsy as most of the neurochemicals their have half life and may be degraded by the time of testing? ***This is the reason why it’s important to freeze the brain as soon as possible. Quick freezing represents the best method possible to preserve the “chemistry of the brain as it is” at the moment of death. Of course, increasing the number of brain donations (by age, sex, conditions, etc.) would allow a better understanding of morpho-functional changes across the life-span and conditions. Moreover, there are other disciplines such as biochemistry, molecular biology, pharmacology, genetics, radiology, etc. (and animal models) that tremendously help to understand the “biochemistry” of the brain. Here we just describe a brain cutting method that could help to better understand pathologic process present in human brain at the moment of death.***

Minor Concerns:

c) In 2.3 Section, you have mentioned marking pre and post central gyri with color pencil before sectioning but this may erase the spot at the time of sectioning so how do you confirm the spot in that case? ***We never mentioned pencils in this manuscript. We mentioned needles (as showed in Fig.1).***

d) In 2.10 Section, you have mentioned numbering of the different parts—but again there may be chances of mixing with right and left hemisphere parts. So, how do you distinguished. ***Usually you put the left hemisphere on your left and the right hemisphere of your right (see pics in Fig.1) and you need to be extremely careful during the brain cutting. Each number corresponds always to the same cerebral region for each and every brain cutting. We also suggested using histocassettes with different colors when collecting regions from different hemispheres (in Fig. 3 for example, green cassettes are for the left hemisphere, blue cassettes for the right hemisphere).***

e) How did you confirm congenital malformations, vessel abnormalities, and any other possible abnormality in the cerebral surfaces before cutting? ***There are malformations visible by naked eye: meningeal cysts, lissencephaly, cortical (external) artero-venous malformations, tumors, etc... In the manuscript we suggested some textbooks where to acquire all this knowledge (which represents a medical specialty). We cannot summarize in this manuscript the entire field of neuropathology. Our manuscript describes a brain cutting method only.***

f) How many normal and pathological brain samples did you cut bi-hemispherical in this study? What was the wrong with other brain cutting techniques? ***As described in the manuscript we analyzed 4 out of 46 control brains (from normal subjects) following our bi-hemispheric cutting so far. The study aims to analyze the pathology before its clinical manifestations (there are no pathologic brains involved in this study; these are brain from neurologically normal people). Previous brain cutting techniques have been often limited by the fact that the neuropath analyses were performed on one hemisphere only, assuming that the pathologic is symmetric, but this is not always the case.***

g) Representative results section—what is Braak staging30 systems? What is TDP43 and mention the significance of measuring it. ***Braak staging (ref33) is a method normally used in neuropathology to stage the level of severity of neurofibrillary tangles (tau pathology), a pathology present in Alzheimer’s disease. TDP43 is another type of pathology (present in frontotemporal dementia cases, for example; ref14). The general significance of measuring specific type of pathology is due the attempt to correlate the progression of that specific pathology (i.e. TDP43) across specific anatomical brain regions and to correlate them with the available clinical data.***

h) How many folds amyloid beta/ Lewy bodies were more or less in left cortex, hippocampus than right one as it was a qualitative value? ***The described values are semi-quantitative (see Braak or CERAD staging systems). Due to the limited number of cases assessed so far, it is not possible to generalize the results yet. If confirmed though, more quantitative techniques (i.e. stereology) could be used.***

i) How did you omit artifactual variations of immuno/histo-staining intensities and how did you correlate clinico -pathologic situations? ***Usually all sections for a certain immunostain are stained at the same time (to minimize the possibility of variations). All cases analyzed in this study were clinically normal so representing very important cases especially useful to individuate pre-clinical stage of amyloid and tau formation/deposition.***

Additional Comments to Authors:

No

[Editorial recommendation: Please keep JoVE’s protocol requirements in mind as you address the above comment - the protocol must contain sufficient details in order to enable users to accurately replicate your technique. We recommend NOT removing any details from the protocol text.]