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Extraction and Dissection of the Domesticated Pig Brain

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Corresponding Author:	Ryan Dilger University of Illinois at Urbana-Champaign Champaign, IL UNITED STATES
Corresponding Author's Institution:	University of Illinois at Urbana-Champaign
Corresponding Author E-Mail:	rdilger2@illinois.edu
Order of Authors:	Stephen A. Fleming
	Supida Monaikul
	Austin T. Mudd
	Reeba Jacob
	Ryan N. Dilger
Additional Information:	
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TITLE:

Extraction and Dissection of the Domesticated Pig Brain

AUTHORS:

Stephen A. Fleming, Supida Monaikul, Austin T. Mudd, Reeba M. Jacob, Ryan N. Dilger

Stephen A. Fleming
Neuroscience Program
University of Illinois at Urbana-Champaign
Urbana, United States of America
sflemin2@illinois.edu

Supida Monaikul
Department of Animal Sciences
University of Illinois at Urbana-Champaign
Urbana, United States of America
smonaik2@illinois.edu

Austin T. Mudd
Neuroscience Program
University of Illinois at Urbana-Champaign
Urbana, United States of America
mudd.austin@gmail.com

Reeba M. Jacob
Division of Nutritional Sciences
University of Illinois at Urbana-Champaign
Urbana, United States of America
rjacob25@gmail.com

Ryan N. Dilger
Department of Animal Sciences, Division of Nutritional Sciences, Neuroscience Program
University of Illinois at Urbana-Champaign
Urbana, United States of America
rdilger2@illinois.edu

CORRESPONDING AUTHOR:

Ryan N. Dilger
(217) 333-2006

KEYWORDS:

Pig; Brain; Dissection; Neuroscience; Skull; Hippocampus; Striatum; Cerebellum; Thalamus; Prefrontal Cortex; Brainstem

SUMMARY:
This protocol details the technique for removal of the pig brain in its entirety and dissection of several brain regions commonly studied in neuroscience.

ABSTRACT:
Use of the pig as a preclinical and translatable animal model has been well-documented and accepted by research fields investigating cardiovascular systems, gastrointestinal systems, and nutrition, and the pig is increasingly being used as a large animal model in neuroscience. Furthermore, the pig is an accepted model to study neurodevelopment as it displays brain growth and development patterns similar to what occurs in humans. As a less common animal model in neuroscience, surgical and dissection procedures on pigs may not be as familiar or well-practiced among researchers. Therefore, a standardized visual protocol detailing consistent extraction and dissection methods may prove valuable for researchers working with the pig. The following video showcases a technique to remove the pig brain while keeping the cortex and brainstem intact and reviews methods to dissect several commonly investigated brain regions including the brainstem, cerebellum, midbrain, hippocampus, striatum, thalamus, and medial prefrontal cortex. The purpose of this video is to provide researchers with the tools and knowledge necessary to consistently perform a brain extraction and dissection on the four-week-old pig.

INTRODUCTION:
The pig has been well documented and accepted as a translatable animal model for research in cardiovascular systems[1], gastrointestinal systems[2], nutrition[3,4], diabetes[5], toxicology[6], and surgical techniques[7]. Use of the pig in neuroscience is beginning to increase, as PubMed searches for the keywords “swine brain animal model” result in four-times more results from 1996-2005 than the preceding 10 year period[8], and even more results at present. A primary reason that the popularity of the pig model is expanding is due to its similarities in growth, structure, and function of the brain when compared with humans. In comparison to the human brain, the pig brain exhibits similar gyral patterning, vascularization and distribution of gray and white matter[9]. Moreover, the pig brain has been used in neuroimaging procedures, evoked potential recording, and in establishing neurosurgery techniques[8,9]. Unlike other animal models, however, the pig and human experience perinatal brain growth spurts, as opposed to pre- or post-natal growth spurts. At birth, the human and pig brain weigh approximately 27 and 25 percent of their adult brain weight, respectively, compared to the rat brain that weighs 12 percent of its adult brain weight and the rhesus monkey brain at 76 percent of adult weight[10].

One reason the pig has been only slowly adopted as an animal model for neuroscience is because many researchers are unfamiliar with the animal in this context. Researchers may not be aware of its potential uses in the field or may not know the proper techniques required to use such a model. As use of the pig as a biomedical and preclinical model gains attention and use in neuroscience, it is necessary to establish standardized procedures of tissue removal to ensure accurate comparison of data across studies. Although dissection and surgical techniques involving the pig brain have been published elsewhere[11-13], there is a need for simple and standardized protocols to collect pig brain tissue, especially for use in biochemical assays. As such, the aim of this video is to provide the knowledge necessary to allow researchers to perform

a standardized brain extraction and dissection. This video illustrates one proper technique to remove the pig brain while keeping the cortex and brainstem intact, and subsequently review methods to dissect several key brain regions.

PROTOCOL:

Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign

NOTE: Prior to euthanasia, the pig was anesthetized via intramuscular injection with a combination of telazol:ketamine:xylazine (50.0 mg of tiletamine HCl plus 50.0 mg of zolazepam HCl reconstituted with 2.50 mL of ketamine HCl (100 g/L) and 2.50 mL of xylazine (100 g/L) and administered at 0.06 mg/kg BW). Once anesthetized, the pig was euthanized via intracardiac administration of sodium pentobarbital (390 mg/mL administered at 1 mL/5 kg BW). For brain dissection, it is recommended that the method of euthanasia be chosen based on the desired analytical procedure of the tissue. The method of euthanasia should cause as little damage to the brain as possible.

1. Extraction of the pig brain

1.1) Following humane euthanasia, decapitate the pig by cutting above the nape of the neck, between the first and second vertebra (atlas and axis, respectively).

1.2) Secure the head in an immobilized bench vise modified to include spikes. Ensure that the head is completely immobilized before proceeding.

1.2.1) Using a scalpel, make a sagittal cut along the midline of the skull that continues to the posterior of the head.

1.2.2) Make a second (transverse) cut on the posterior of the head.

1.2.3) Make a third (transverse) cut on the posterior end of the snout and in-line with the eyes. Cut the skin as far away from the skull as possible to ensure easy access to the skull.

1.3) Using a bone saw, make two anterior-to-posterior cuts lateral to the midline, extending from the eyes to the apex of the skull curvature. Bevel the saw towards the midline and cut just deep enough to penetrate the skull.

1.3.1) Repeat the above process on the perpendicular sides, creating a rectangular “window” in the skull.

1.4) Using a meat hook, pry the rectangular section of the skull off. Begin by placing the hook into one of the corners of the section and apply upward pressure to loosen the skull piece. Take care to place the meat hook only at the level of the skull to prevent inadvertently penetrating the

brain tissue.

1.5) If any meningeal layers remain on the brain, use forceps and blunt scissors (or alternatively, a scalpel) to gently remove the layers without cutting into the brain.

1.6) Use a scalpel to cut away muscle and fat at the posterior portion of the head, exposing the posterior portion of the skull.

1.6.1) Make two transverse cuts along the posterior of the skull. Be sure not to cut into brain tissue.

1.6.2) Secure the head by placing a firm hand on the snout and apply a backwards pressure to pull the posterior portion of the skull off, exposing the cerebellum.

1.7) Remove the head from the bench vice and place on a surgical mat.

1.8) Use a long slender tool, such as the blunt end of a scalpel, to remove the brain. Invert the head and use a gentle scooping motion to coax the brain out of the skull cavity without damaging the surface of the brain.

NOTE: It will be necessary to sever cranial nerves to remove the brain. Do so gently and do not attempt to forcefully pull the brain out. The brain will fall out on its own when it is properly and completely detached from the skull and spine.

2. Dissection of the pig brain

NOTE: It may be helpful to use a brain atlas or fiber dissection guide[14] as a visual representation during dissection procedures. Make sure dissected tissue samples are stored properly according to project-specific needs upon removal of each sample (described in more detail below). Additionally, please note that for the purposes of this video, all brain regions shown were dissected from the right hemisphere, but this may differ per laboratory based on experimental objectives.

2.1) To remove the brainstem (predominantly the medulla), make a coronal cut caudal to the cerebellum.

2.2) To remove the cerebellum, make a coronal cut posterior to the cortex. Isolate desired regions (e.g., vermis, flocculus, etc.) of the cerebellum from this sample. Be sure not to include any portions of the brain stem in this sample.

2.3) Separate the two hemispheres of the brain by making a mid-sagittal cut along the longitudinal fissure. Make this cut as a continuous motion to prevent causing damage to the cortex.

2.4) To remove the midbrain, dissect a desired amount of tissue just ventral to the superior and inferior colliculi.

2.5) To remove the hippocampus, place the blunt end of a scalpel in the posterior portion of the corpus callosum and gently roll the hippocampus out, using a "J" motion starting from the posterior portion of the corpus callosum; an entire hippocampal horn is 'bean-shaped'.

2.6) The striatum (caudate nucleus is primarily shown) is a grey and white matter-striped region just below the corpus callosum and anterior to the hippocampus. Bevel the scalpel and remove this region, revealing striated tissue upon removal.

2.7) To remove the medial prefrontal cortex, dissect tissue from the frontal gyrus to the corpus callosum, removing the most medial portion of that section. The right cortex should remain after removal of the medial prefrontal cortex.

2.8) To remove the thalamus, remove the bulb-like structure at the center of the midsagittal surface of the brain and rostral to the midbrain. The thalamus has a spherical shape and will look slightly darker than the surrounding tissue.

3) Post-dissection

3.1. Upon completion of the dissection, preserve tissue samples properly for subsequent analyses. Omission of this step will result in significant autolysis and degradation within as little as 20 minutes.

3.2. Leave each brain region intact at the time of dissection if structural analyses will be performed, or mince the tissues to create a homogenous tissue sample prior to preservation. Common sample preservation methods for brain tissues include cryopreservation and chemical fixation using crosslinking agents[15].

3.3. For standard lab assays (e.g., gene or protein expression), immerse in liquid nitrogen or solutions that stabilize genetic material prior to long-term storage at -80 °C to provide convenient and cost-effective preservation methods.

3.4. If maintaining tissue structure is a priority, preserve brain tissues through traditional fixation methods (e.g., using aldehyde-based fixatives to crosslink proteins), either without or with perfusion of the animal or organ of interest prior to tissue dissection.

REPRESENTATIVE RESULTS:

This section describes examples of results obtained after correct extraction and dissection of a 4-week-old pig brain. **Figure 1** outlines the shape of each brain region for use as a guide during dissection. Part of the brainstem may remain in the skull after removal of the cerebellum (Fi**gure 1B**). This can be removed while isolating the desired region of the cerebellum. **Table 1** displays the average weight (mean ± standard error of the mean) for each of the dissected brain regions

(n=5).

Figure 1: Extracted Pig Brain. Outlines of brain regions for use as a guide during dissection. Regions shown are from the right hemisphere.

Table 1: Brain Regions Weights. Average weight of the 4-week-old pig brain and each dissected brain region (n=5).

DISCUSSION:

The techniques described herein were designed for pigs approximately 4 weeks of age. It is critical to perform these steps immediately after the pig has been humanely euthanized to ensure the integrity of brain tissue structure is maintained, especially when considering subsequent biochemical assays. It is helpful to use an atlas or fiber[16] dissection guides when first learning the techniques. It is recommended that the experimenter practice several brain extractions and dissections prior to obtaining samples for data collection. The most difficult step is removal of the skull. This step will become easier with experience as it largely requires firsthand practice to know where on the skull to saw and when the skull has been cut through. This procedure is similar to that described by Bassi et al.[12], though it does not require the need to create a hexagonal cranial window and provides a visual tutorial of how to perform the technique.

A limitation of this technique it that when working with older pigs, it may take more time to remove the skull as it becomes significantly thicker with age. If using a saw is too laborious or ineffective for thicker skulls, it may be necessary to either use a hammer and chisel, like that shown by Bjarkam et al.[13], or powered surgical equipment (e.g., bone saw). Furthermore, this technique does not always ensure the capture of the olfactory bulbs.

ACKNOWLEDGMENTS:

The authors would like to acknowledge Jim Knoblauch and Martin-Booth Hodges of the College of Agricultural, Consumer and Environmental Sciences Information Technology and Communication Services for their expertise in shooting, recording, and editing audio and video.

DISCLOSURES:

The authors declare that they have no competing financial interests.

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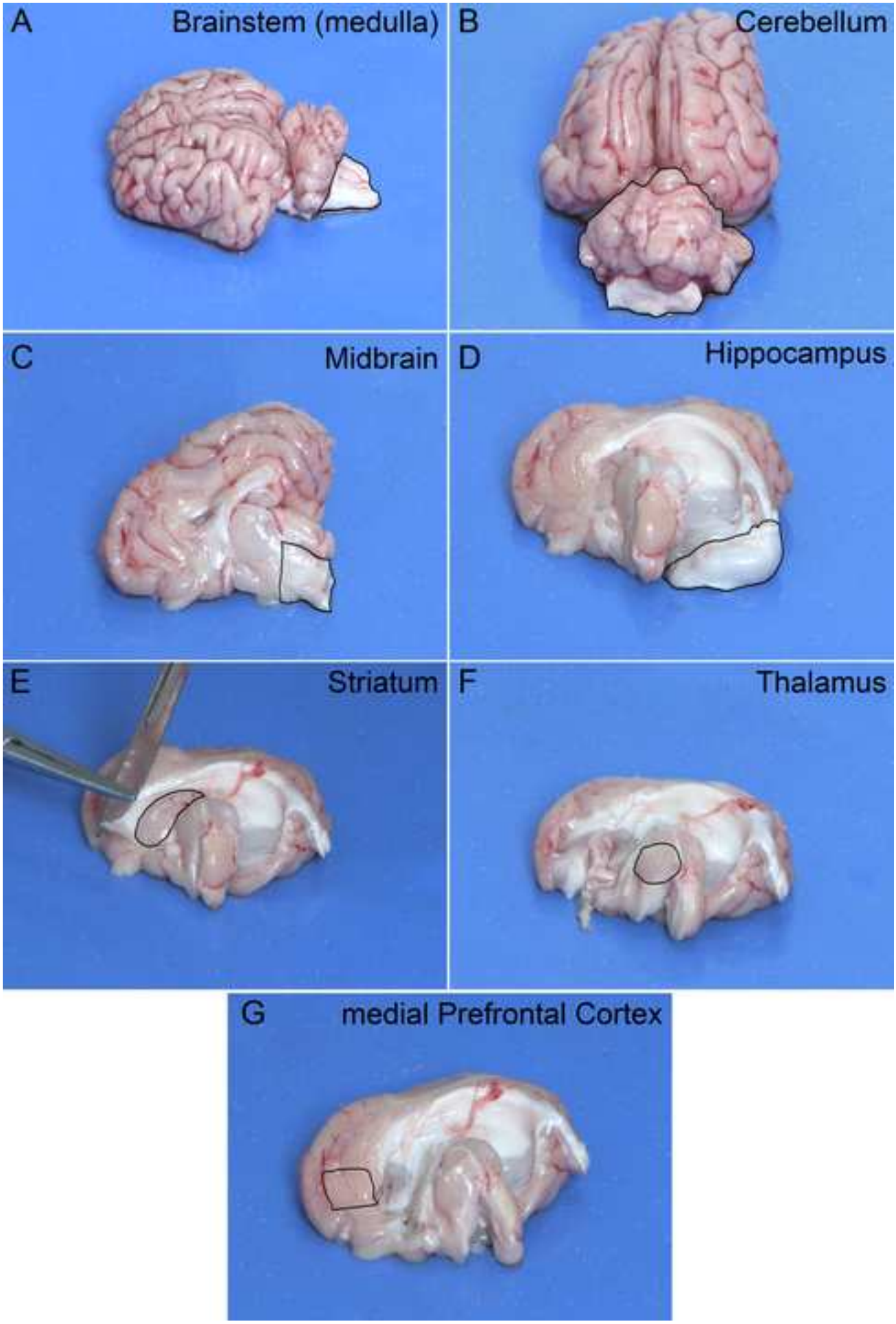

Table 1

Region	Weight (g)	SEM
Whole Pig*	8.006	0.545
Brainstem	0.829	0.132
Cerebellum	5.929	0.137
Midbrain	0.376	0.047
Hippocampus	0.500	0.051
Striatum	0.410	0.115
Thalamus	0.476	0.120
medial Prefrontal Cortex	0.459	0.122

*Weight presented as kg

Table of Materials

Click here to access/download;Table of Materials;Materials and Equipment.xlsx

Name of Material/Equipment	Company	Catalog #
#22 Scalpel Blades for #4 Handles	Ted Pella, inc.	549-4S-22
11 1/2" Satterlee Bone Saw	Leica Biosystems	38DI13425
5 1/2" Skull Breaker with Chisel End (Meat Hook)	Leica Biosystems	38DI37636
5-inch Heavy Duty Workshop Bench Vise	Pony	29050
Butcher Knife 25cm	Victorinox	5.7403.25
CM40 Light Duty Drop Forged C Clamps	Bessey	00655BC3120
Diamond Hone Knife Shaper	Chef's Choice	436-3
Shandon Stainless-Steel Scalpel Blade Handle #4	ThermoScientific	5334
Tissue Forceps	Henry Schein	101-5132
Vinyl Dissecting pad	Carolina	629006

Comments
Sharpen before use

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The complete manuscript has been proofread and amendments made throughout.
2. Please discuss any limitations of the protocol in the discussion. The discussion has been amended accordingly.

Changes to be made by the Author(s) regarding the video:

1. Export the video in a higher resolution, it is too low. The minimum acceptable resolution is 1280x720 and the current resolution is 852x480 The revised video has a resolution of 1280x720, per journal requirements.
2. Please have a narration for the concluding section of the video. Amended as requested (all audio has been re-recorded for consistency).
3. Please cut the last 4 seconds of the video. It is just a white screen. Changed as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Fleming et al. report the results of an interesting study showing a well-made and easy to follow video-manuscript. I congratulate the authors because the work adds important information for future work that uses pigs as their animal model. In order to improve the replicability and dissemination of this method into the scientific community I would suggest the following:

Major Concerns:

Dissection of the Pig Brain:

Suggest adding the measurements when dissecting the brain stem, midbrain, thalamus, and medial prefrontal cortex. For example, 3X3X3mm, etc. Whereas we appreciate these comments, we have not added measurements because these vary based on age of the animal and the dissected pieces are not necessarily uniform in their dimensions.

Discussion:

Suggest talking about limitations and other techniques to extract the pig's brain. For example please see: Bassi, Thiago & Rohrs, E & Fernandez, K & Ornowska, M & Reynolds, C.S.. (2018). Direct brain excision: An easier method to harvest the pig's brain. Interdisciplinary Neurosurgery. 14. DOI: 10.1016/j.inat.2018.05.010 Thank you, additional references have been added, along with the incorporation of limitations in the discussion.

Reviewer #2:

Manuscript Summary:

Relevant topic, but pig brain removal has previously been described in Jove see:
"Exposure of the Pig CNS for Histological Analysis: A Manual for Decapitation, Skull Opening, and Brain Removal. Bjarkam CR, Orlowski D, Tvilling L, Bech J, Glud AN, Sørensen JH.
J Vis Exp. 2017 Apr 13;(122):55511. doi: 10.3791/55511."
The authors should at least refer to this paper, and describe wherein their method is superior to the one described in 2017. Thank you, this paper has now been cited.

Major Concerns:

See above

Furthermore, the brain stem piece depicted in Figure 1 is only the medulla, in neuroanatomy brain stem = medulla + pons + mesencephalon Thank you, the figure and text have been amended to clarify that the depicted tissue is predominantly medulla.

Reviewer #3:
Fleming et al. propose a methods article describing a standardized protocol for the extraction of the pig brain and the collection of a number of key cerebral structures in unfixed specimens. The method has numerous potential applications in neuroscience. The authors should explain why they chose to collect those particular anatomical structures and enuntiate the potential research uses of each particular structure that can be harvested by use of their protocol. The title and abstract are concise and adequate for a methods article. However, referring to the procedures described as "surgical" may lead to some confusion. The authors should replace this term with "dissection". Thank you, we have amended the text to emphasize the term 'dissection' as requested.

The materials and equipment are well listed and easily accessible. In general, the written steps of the protocol and the video are clear and reproducible so that the described results can be achieved by following them. The critical steps are highlighted and advice is given on how to perform them correctly. Nevertheless, the description of the procedures may be improved as follows:

* (1.2.1): the authors should rephrase "that runs anterior to the most posterior portion of the head." It sounds confusing. Corrected
* (1.2.2) and (1.2.3): the authors should replace "laterally" with "transverse". Corrected
* (1.3): by saying "lateral to the midline" some readers might be made to think the cuts should be close to the midline. The authors should specify approximately how far lateral to the midline are the cuts to be executed. Thank you, we have appended the following verbiage to clarify: "extending from the eyes to the apex of the skull curvature"
* (1.3.1): the authors should replace "adjacent" with "perpendicular" for clarity Corrected
* (1.5): the use of the scalpel can be risky at this moment (02:48). Removing meninges with blunt scissors and forceps should be advised. The text has been amended to include this safety note.
* (2): the authors should recommend some atlases that are the most useful for this purpose. Maybe an anatomical dissection paper (for example: DOI: 10.1111/ahe.12280) could help. Thank you, this reference has now been included in the text.
* (2.1): the complete description is: "a coronal cut caudal to the cerebellum" Corrected
* (2.2): the different parts of the cerebellum should be indicated with arrows in the video around 05:40 No change was made as we were unable to edit this part of the video.
* (2.3): the authors should encourage performing the mid-sagittal cut as a continuous motion, without repeatedly lifting up scalpel from the brain, as this may cause damage of the cortex. After separating the hemispheres, the major anatomical landmarks should be indicated with arrows on the medial surface of the hemisphere (06:07) This note has been added to the text.
* (2.4): the colliculi should be shown from a lateral view (06:14). While we appreciate the input, we have no ability to provide additional video perspectives.
* (2.5): the transition at 06:32 is too abrupt. The video should show and the procedure should describe how the lateral ventricle is opened. Before that, it would be a good idea to indicate the position of the hippocampus by tracing its contour with dashed lines on an image of the brain before opening the lateral ventricle (such as the one at 06:29). We appreciate the input, but no changes have been made to the video at this timing.

* (2.6): the authors should replace "striatum" with "caudate nucleus". The term "striatum" also includes putamen and globus pallidus, which are not dissected in this video. Thank you, the text has been amended accordingly.
* (2.8): the authors should indicate with an arrow where the "frontal gyrus" and the "right cortex" are at around 07:53 and 07:59 respectively. Callouts have been added as requested.
* The authors should also describe freezing or fixing of the structures. The optimal way of handling those delicate tissues should be indicated. Thank you, the text was amended to denote common preservation methods.
* Figure 1 should include more anatomical landmarks. No changes were made here.
* The authors should include emphasize more on problems and troubleshooting. The discussion has been amended to discuss problems and limitations.

The results of the protocol are very good and, as illustrated in Table 1, reasonably reproducible.
All in all, the paper offers a robust method for the collection of pig brains and anatomical samples, which may require some practice for a beginner, but is detailed enough to provide a good support during the learning period and to assure reproducibility ones the necessary level of experience is achieved. It also has the potential to enhance the use of the pig as an animal model in neuroscience.

Reviewer #4:
Manuscript Summary:
This manuscript and accompanying video describes the Method for removing the brain from the skull of a Young pig, and the dissection of different brain regions of interest.

Major Concerns:
My comments to the video and to the manuscript both relates to the level of detail: I would have liked to have a more detailed description of which main structures are included in each sample, and by which landmarks one distinguishes one structure from others. I appreciate the difficulty in making a video, but I would suggest re-filming as the focus is sometimes a bit off, and one can tell that it is adjusting while the video is running. I would also suggest pausing a bit on each structure. We very much appreciate this input, but it is no longer possible for us to amend the video as requested. To answer the specific questions regarding general neuroanatomy, we felt these types of questions best answered by an atlas, we now cited two potential atlases in the introduction and discussion.

Manuscript-specific comments:
2. Dissection of the Pig Brain
My general comment is that this is not of enough detail. Being more specific about the landmarks used to dissect out different areas of the brain would be both helpful and important.
Brainstem: The brainstem consists of a great many important nuclei for the diffuse modulatory systems, and I would like the authors to be more precise in where they cut the brainstem from the rest of the brain (ie which landmarks ventrally), and to specify which strucutres are then included in the brainstem: eg: both the raphe nuclei and locus coerulus?
2.4)Midbrain: which strucures are included in the midbrain section when it is cut the way you describe here?
2.5) When you remove the hippocampus, how much do you include of the thin white-looking 'sheet' of tissue that connects the bean-shaped hippocampus to the brain?
Figure 1:
Here it would be of great help to the reader to also identify other landmarks than the targeted region in

each picture frame. We appreciate input from the reviewer and we have made changes where possible.
Table 1: please adjust so that the decimal point is in the same place in each row (so that the difference between body weight and brain area weight is immediately clear). Amended.

Minor Concerns:
Video: A minor criticism is the lack of lab-coats on the personnel. If the authors are to re-do the filming, I suggest that the human subject demonstrating the sawing technique wears a white lab-coat. Thank you, but it is not possible for us to re-shoot the video footage, so this change was not made.

Manuscript: Protocol (line 104, and also 115 and other places where this expression occur): please replace 'euthanasia' with 'humane killing' as euthanasia means the painless killing of an incurable patient in order to end suffering, while 'humane killing' refers to ending the life of an animal for research purposes. We understand the issue raised by the reviewer, but we respectfully decline to change our phrasing here. There appear to be differences in syntax within the scientific community, and in the animal realm, ‘euthanasia’ is generally used to mean ‘good death’. Regardless, we have amended the text to use the phrase ‘humane euthanasia’ to find middle-ground with the reviewer.

Line 152: Regarding the placement of the brain after it has been removed from the skull cavity: We find it helpful to keep it in a singular use plastic dish with a little bit of saline so that the tissue will not stick to the dish and thereby be damaged. Does this need to be taken into account with the protocol submitted here? We appreciate this input, which highlights personal preferences that exist between laboratories, but no amendments have been made to the text or video in response.