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TITLE:

Microdissection of Mouse Brain into Functionally and Anatomically Different Regions

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Brain anatomy, mouse, brain, dissection, brain atlas, molecular assays

SUMMARY:

We present a hands-on, step-by-step, rapid protocol for mouse brain removal and dissection of discrete regions from fresh brain tissue. Obtaining brain regions for molecular analysis has become routine in many neuroscience labs. These brain regions are immediately frozen to obtain high quality transcriptomic data for system level analysis.

ABSTRACT:

The brain is the command center for the mammalian nervous system and an organ with enormous structural complexity. Protected within the skull, the brain consists of an outer covering of grey matter over the hemispheres known as the cerebral cortex. Underneath this layer reside many other specialized structures that are essential for multiple phenomenon important for existence. Access to these functionally important regions buried deep within the brain structure requires quick and precise dissection steps.

Mouse models are routinely used to study human brain functions and diseases. Changes in gene expression patterns may be confined to specific brain areas targeting a particular phenotype depending on the diseased state. Thus, it is of great importance to study regulation of

transcription with respect to its well-defined structural organization. A complete understanding of the brain requires studying distinct brain regions, defining connections, and identifying key differences in the activities of each of these brain regions. A more comprehensive understanding of each of these distinct regions may pave the way for new and improved treatments in the field of neuroscience. Herein, we discuss a step-by-step methodology for dissecting the mouse brain into sixteen distinct regions. In this procedure, we have focused on mouse C57Bl/6J brain removal and dissection into multiple regions using neuroanatomical landmarks to identify discrete functionally-relevant and anatomically-elusive brain regions. This work will help lay a strong foundation in the field of neuroscience, leading to more focused approaches in the deeper understanding of tissue.

INTRODUCTION:

The brain, along with the spinal cord and retina, comprises the central nervous system that executes complex behaviors, controlled by specialized, precisely positioned, and interacting cell types throughout the entire body[1]. The brain is a complex organ with billions of interconnected neurons and glia with precise circuitry performing numerous functions. It is a bilateral structure with two distinct lobes and diverse cellular components[2]. The spinal cord connects the brain to the outside world and is protected by bone, meninges, and cerebrospinal fluid and routes messages to and from the brain[2-4]. The surface of the brain, the cerebral cortex, is uneven and has distinct folds, called gyri, and grooves, called sulci, that separate the brain into functional centers[5]. The cortex is smooth in mammals with a small brain[6,7]. It is important to characterize and study the architecture of the human brain in order to understand the disorders related to the different brain regions, as well as its functional circuits. Neuroscience research has expanded in recent years and a variety of experimental methods are being used to study the structure and function of the brain. Developments in the fields of molecular and systems level biology have ushered in a new era of exploring the complex relationship between brain structures and the functioning of molecules. Additionally, molecular biology, genetics, and epigenetics are rapidly expanding, enabling us to advance our knowledge of the underlying mechanisms involved in how systems function. These analyses can be carried out on a much more localized basis, to help target the investigation and development of more effective therapies.

The mammalian brain is structurally defined into clearly identifiable discrete regions; however, the functional and molecular complexities of these discrete structures are not yet clearly understood. The multi-dimensional and multi-layered nature of the brain tissue makes this landscape difficult to study at the functional level. In addition, the fact that multiple functions are performed by the same structure and vice versa further complicates the understanding of the brain[8]. It is vital that the experimental approach executed for the structural and functional characterization of brain regions uses precise research methodologies to achieve consistency in sampling for correlating neuroanatomical architecture with function. The complexity of brain has been recently explained using single cell sequencing[9,10] such as temporal gyrus of human brain is composed of 75 distinct cell types[11]. By comparing this data to those from an analogous region of the mouse brain, the study not only reveals similarities in their architecture and cell types but also presents the differences. To unravel the complex mechanisms, it is therefore important to study diverse regions of the brain with full precision. Conserved structures and function between

a human and mouse brain enable the use of a mouse as a preliminary surrogate for elucidating human brain function and behavioral outcomes.

With the advancement of systems biology approaches, obtaining information from discrete brain regions in rodents has become a key procedure in neuroscience research. While some protocols such as lasers that captures microdissection[12] can be expensive, mechanical protocols are inexpensive and performed using commonly available tools[13,14]. We have used multiple brain regions for transcriptomic assays[15] and have developed a hands-on and rapid procedure to dissect mouse brain regions of interest in a step-by-step manner in a short time. Once dissected, these samples can be stored immediately in cold conditions to preserve the nucleic acids and proteins of these tissues. Our approach can be performed faster leading to high efficiency and permitting less chances for tissue deterioration. This ultimately, increases the chances of generating high quality, reproducible experiments using brain tissues.

PROTOCOL:

Animal handling and experimental procedures were conducted in accordance with European, national and institutional guidelines for animal care. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the US Army Center for Environmental Health Research now Walter Reed Army Institute of Research (WRAIR) and performed in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

NOTE: The procedure will be performed on six to eight week old euthanized mice of the C57BL/6j strain by cervical dislocation[16]. No perfusions are performed in our lab but this protocol may be modified where perfusions to clear blood from the vasculature can be performed. All supplies required for the dissection are listed in the **Table of Materials**. The dissection is subdivided into three components to include the removal of the brain, the removal of the pituitary gland and the brain dissection. The intent of brain tissue collection is to process them for transcriptomic assays following RNA extractions. As soon as the brain region is dissected, we immediately transfer each of the brain regions into an already labeled freezer vial and store the vial in liquid nitrogen or -80 °C.

1. Mouse Brain Removal

1.1 Clamp the maxilla of the decapitated head with a hemostat (**Figure 2i**) and use a gauze to reflect the scalp rostrally further creating a dry field on the dorsum of skull (**Figure 2ii**).

1.2 Insert the fine curved scissors into the foramen magnum to separate the adhering meninges. Here, insert the scissors at the opening on the base of skull where the spinal cord passes with the blade pointing vertically (12 o'clock position), but parallel to and pressing against the interior surface of the basal plate bone (i.e., occipital squama) and rotating the blade forty-five° to the left side and then to the right side.

1.3 Continue to rotate the wrist to pry off the basal plate bone that will snip up the middle of the bone continuing into the occipital bone and intraparietal bones prying the bones left and right until they are removed. In a similar fashion, remove the occipital bone and the intraparietal bone to expose the cerebellum.

NOTE: At this point, the hollow bony structure called the tympanic bullae, on the ventral posterior portion of the skull that encloses parts of the middle and inner ear, can be removed unless one is dissecting the posterior and anterior lobes of pituitaries that is a hollow bony structure on the ventral side (**Figure 2iii**).

1.4 Remove the muscle attachments to the temporal ridge using a curved sharp scissor blade. Place one limb of the curved sharp scissors under the lambdoid suture penetrating into the junction of the transverse and sagittal sinuses (**Figure 2iv**).

1.5 Advance the scissors rostrally along the midsagittal suture up to the bregma and cut it very gently while carefully lifting upward to avoid laceration of cerebral cortices. This is a critical step in the procedure (**Figure 2v**).

1.6 At this step, parietal bones are lifted and rotated thus scraping the inner surface of the bone to identify and cut remaining meningeal attachments. Grasp the temporal bone prying outwardly, away from brain. Remove the frontal bone from each side using curved scissors or a rongeur into the orbit and cutting coronally at a right angle to the orbital ridge, but no further than the midline (**Figure 2vii**).

1.7 Make two cuts parallel and about 4 mm apart in the sagittal plane (**Figure 2viii**).

NOTE: Do not cut both sides with one single stroke.

1.8 Remove the fragments of the frontal bone avoiding lacerating the brain surface (**Figure 2ix**). At this point, cut the dura mater using the scissors that is accessible between the olfactory bulbs.

1.9 Gently invert the skull to allow gravity to assist in removal of the brain, while continuing to identify and cut remaining meningeal attachments and cranial nerves. The brain will be released by cutting the largest trigeminal nerve attached to the brain and is visible from the base of the calvarium (**Figure 2x**).

NOTE: Transfer the brain to a cold saline solution (ice cold RNase-free sodium citrate (0.9%) or physiological (0.9%) saline) for further dissection.

2. Dissection of the Anterior and Posterior Pituitaries

NOTE: The pituitary glands are covered by a very tough tent-like membrane, with a ridge that runs laterally between the left and right trigeminal nerves. These structures are extremely soft

and delicate and, as such, it is recommended the posterior and anterior lobes of pituitary glands be dissected in stages, in situ, directly from the skull. Immediately after dissection, transfer the respective pituitary gland to a pre-labeled vial and store the vial in liquid nitrogen preferably otherwise -80 °C. The pituitary gland rests exactly over the junction of the occipital and basisphenoid bones; if they flex, the pituitary architecture is disrupted.

2.1 Keep the auditory bullae intact to make sure that the pituitary anatomy is intact and easily identifiable (**Figure 2ix**).

2.2 Dissect the posterior lobe of pituitary followed by the anterior lobe of pituitary, from the remainder of the skull.

2.3 Make an extremely small parasagittal cut on both sides in the ridge of the membranous tent and lift the posterior lobe of pituitary with ultra-fine forceps, taking care not to disrupt the anterior pituitary tissue (**Figure 2x**).

2.4 Make a sagittal cut between the lateral margins of the anterior lobe of pituitary and the nearest trigeminal nerve and lift out the anterior lobe of pituitary thereafter (**Figure 2x**).

3. Mouse Brain Dissection

NOTE: Immediately after brain and pituitary removal, further dissection is performed on a pre-chilled stainless-steel block (**Figure 3**). Post dissection, transfer the brain regions to pre-labeled vials and transfer the vials preferably to liquid nitrogen otherwise -80 °C. Structures produced by the top-down method (in chronological order) potentially include the following: cerebellum (CB), brain stem/hind brain (pons and medulla oblongata) (HB), olfactory bulbs (OB) as accessory olfactory bulbs, medial prefrontal cortex (MPFC), lateral prefrontal cortex (FCX), anterior and posterior corpus striatum (ST), ventral striatum (VS) comprised of the nucleus accumbens (NAC) and olfactory tubercle (OT), septum (SE), preoptic area, piriform cortex (PFM), hypothalamus (HY), amygdala (AY), hippocampus (HC), posterior cingulate cortex (CNG), entorhinal cortex (ERC), midbrain (MB) with thalamus and rest of the cerebral cortex (ROC) (**Table 1**). Specific regions will be discussed in order of isolation, working with a single hemisphere.

3.1 Take great care in removing the brain from the calvarium as the landmarks may be destroyed in case there are any lacerations. At this step, perform all dissections using face protection, specifically, a 7x jewelers visor, and illumination will be provided by surgical lamps positioned over each of the dissector's shoulders. Much of the dissection will be accomplished using blunt mode small curved forceps (i.e., Graefe forceps).

3.2 Place the brain on a stainless-steel block (**Figure 4i** and **Figure 4ii**). Keep the block cold by surrounding it with ice and an ice-cold saline solution. Periodically moist the tissue with ice-cold saline solution to preserve the structures.

3.3 Position the brain such that the cerebral cortices are facing upwards. Using small curved

forceps, gently reflect the CB by exposing the superior, middle and inferior cerebellar peduncles and remove the **CB** (**Figure 4iii** and **Figure 4iv**).

3.4 Make a midsagittal cut starting from the dorsum and between the **OB** and the cerebral hemispheres (**Figure 4v**) and make sure not to extend farther than the anterior commissure. At this point, the vermis can be easily separated from the lateral portions and **HB** will be obtained by a coronal cut at the anterior margin of the pons.

3.5 Separate the medulla by a coronal cut at the posterior margin of the pons.

NOTE: At this point, the diencephalon, the posterior part of the forebrain containing the epithalamus, thalamus, hypothalamus, and ventral thalamus and the third ventricle, will be turned ventral side up, and a midsagittal cut will be made from the optic chiasm rostrally. The dissection of cerebral hemispheres followed by removal of **OB** from one of the hemisphere will be at this step (**Figure 4v** and **Figure 4vi**).

3.6 Separate the cerebral hemispheres (**Figure 4v**) before dissecting the diencephalon further (**Figure 4v**). The dorsal approach will be employed by careful blunt dissection to preserve the critically salient midline landmarks. Visualize every interhemispheric connection before severing them as this will minimize the possibility of deviating from the midline.

NOTE: At this step one of the cerebral hemisphere (**Hemibrain**) can be preserved and the second hemisphere can be used for further dissection

3.7 Slip the closed blades of a small, curved forceps, beneath the corpus callosum and gently spread to retract the neocortex bilaterally. The corpus callosum is a broad band of nerve fibers that joins the two hemispheres which will be bluntly dissected by pinching with the forceps, without disturbing the midline structures lying beneath.

NOTE: Mesial faces of the hemispheres will have multiple landmarks visible such as myelinated structures like the genu of the corpus callosum, the fornices, and the anterior commissure. Also, though a few millimeters lateral to the midline, the mammillothalamic tract and the fasciculus retroflexus may also be visible.

3.8 Bisect the multiple structures that cross the midline and. This will include the corpus callosum, anterior commissure, ventral fornix commissure, posterior commissure, dorsal fornix commissure, supra mammillary decussation, superior colliculus commissure, ventral fornix commissure, and periventricular thalamic fibers.

3.9 Take special care at this step in or near the midline that might be partially compromised by the dorsal approach method. All follow-up structures are at risk if not performed carefully.

3.10 Remove the the **OB (**wedge shaped and lighter color) and accessory olfactory bulbs followed by collection of the **MPFC** (cingulate cortex area 1, prelimbic, infralimbic, medial orbital,

and secondary motor cortices [M2]) and **FCX** by a coronal cut that is made 1mm anterior to the genu of the corpus callosum (**Figure 4vii**). Divide the resulting section by a parasagittal cut 1/3 of the distance from the medial to the lateral surface, yielding **MPFC** medially and remainder of FCX laterally. The cingulate cortex is the mouse analog of MPFC.

NOTE: This tissue slice will also contain a small amount of the M2 (secondary motor cortex) [17] and is unavoidable.

3.11 Make a coronal cut at the level of the anterior commissure leading to visibility of pars anterior limb of the anterior commissure in the cross-section on the rostral face of the resulting coronal section whereas the transverse portion will be revealed in the caudal face of the section This is a confirmatory landmark for **NAC**[18]. The **VS** is composed of the **NAC** and **OT**.

NOTE: There is an anterior limb in addition to the transverse segment in the anterior commissure and it is called anterior commissure, pars anterior.

3.12 Partially cut horizontally through the transverse portion of the anterior commissure from the midline beneath the anterior horn of the lateral ventricle, to free the septal nucleus dorsally and the **VS** ventrally. Remove the small amount of the cortex from the lateral surface where **NAC** and **OT** will be removed.

3.13 Separate the rostral portion of the **ST** from the overlying cortex via curved scalpel cut just outside the external capsule taking care not to include striatal tissue in the cortical sample. At this point, the septal **nuclei /SE** will be visible and can be easily taken from this slice (**Figure 4viii**).

NOTE: The anterior and posterior limits of the **PFM** are defined by imaginary coronal planes that are in line with the anterior commissure and the mammillary bodies respectively. The medial limit is defined by the external capsule[18].

3.14 On the lateral surface of the remaining hemi-section of the diencephalon, make a partial horizontal cut along the rhinal sulcus extending caudally, but only as far as the imaginary coronal plane level with the mammillary bodies. Make a partial coronal cut, extending medially 1 mm from the lateral cortical surface in the plane of the mammillary bodies of the **HY**. Here, para-sagittal incision in the plane of the claustrum will free the **PFM** (**Figure 4ix** and **Figure 4x**).

NOTE: On the medial surface of the remaining hemi-section of the diencephalon, numerous anatomical features will be visible including the mammillary bodies, the fasciculus retroflexus, and the stria medullaris. A semicircular pattern (dorsal concave side) will be visible, which denotes the margin between the **thalamus** and the **HY.** The **HY** will be easily identified on the midsagittal section view per the optic chiasm anteroventrally and anterior commissure anterodorsally and the mammillary bodies along with the fasciculus retroflexus posteriorly. The latter landmarks have been well displayed in the atlas[17] as well as in albino mouse forebrain context[19].

3.15 Make a partial coronal cut posterior to the mammillary bodies, extending only as far laterally as the hypothalamic sulcus. At this point a parasagittal cut along the length of the hypothalamic sulcus now frees the **HY.**

3.16 Entail the eversion of the lateral ventricle to reveal additional intra-limbic connections and the remaining limbic system components. Viewing the medial face of the remaining hemi-section of the diencephalon, curved forceps will be best option as they allow the technician to manipulate around other delicate sections of the surrounding area used to sever the fornix and is inserted into the lateral ventricle and gently expanded, using blunt dissection to open the ventricle and rotate the **HC** 90° from the vertical to the horizontal plane. It may be necessary to use the #11 blade to section the choroidal artery/choroid plexus as the pointed tip can help with precise cuts.

3.17 Rotate the **CNG** 90° (but in the opposite direction from the **HC**) to be in the horizontal plane. Using forceps, gently rotate the **HC** another 180° outward and laterally, which will make the inner surface of the **ERC** visible and facing upwards.

3.18 A fan-like radiation of myelinated fibers arising from the **ERC** will be seen converging to form the angular bundle, including the perforant path and its attachment to the **HC**. Rotate the thalamus and **MB** 180° dorsally to ventrally reveal the **AY**.

3.19 If required, lift the optic tract to reveal the attachment of the stria terminalis as it will contain bands of fibers running along the lateral margin of the ventricular surface of the thalamus to the **AY** and will make the outlines of the **AY** clear.

NOTE: The **HC** and fornix will be clearly visible in their entirety, nested in the lateral ventricle and, can be easily lifted out. The lateral ventricle will be lined with pia mater and fan-like origins of the perforant path through the very transparent subpial aspect of the **ERC** will be visible[20].

3.20 The outer surface of medial **ERC** will have a visible prominent layer of large pyramidal cells. In addition, a dense convergence of Golgi-staining fibers will be a salient feature in the medial **ERC**. In this dissection procedure, after everting the lateral ventricle, these fibers will be easily visible on the medial surface through the pia mater, which lines the inner surfaces of the ventricles, forming a very prominent fan-like structure.

3.21 Notice that the fibers are gathering subpially, descending and leaving the ventral **ERC**, extending posteriorly and then ascending vertically to perforate the **HC.** This fan-like structure in the **ERC** will be used to define the margins for the purpose of dissection. Identify and remove in the order of **AY, ERC, CNG and HC** structures.

NOTE: Defining a good landmark for **AY** dissection will be a key for the next step and the junction on posterior margin where stria terminalis meets the **AY** will be a good point.

3.22 At this point, the remaining structures include the **thalamus** and the **MB.** Confirm the

identification of the thalamus by visualization of the stria medullaris on its dorsal surface extending in the midline rostrocaudal plane. Separate the **thalamus** completely from the **midbrain** by a coronal cut caudal to the habenula and rostral to the superior colliculi. The **ROC** is saved at this step.

3.23 At this point complete collection of all brain regions and, immediately (as each is obtained) store the samples flash frozen till further processing.

REPRESENTATIVE RESULTS:

Our understanding of the complex brain structure and function is rapidly evolving and improving. The brain contains multiple distinct regions and building a molecular map can help us better understand how the brain works. In this method paper, we have discussed the dissection of the mouse brain into multiple distinct regions (**Table 1**). In this protocol, the structures are identified based on the critical landmarks and is achieved by keeping the tissue moist with saline solution by retaining its sturdiness for immediate dissection. The method covers more regions than other reports[21] and is complementary to dissection methods using frozen brains[22,23]. These dissected tissues can be preserved and processed later depending on the requirements of the study. The method discussed here is immediate removal of the brain from the skull followed by dissection which provides enough tissue in a quick way for downstream assays given the size of the mouse brain. Our team has extracted RNA from multiple dissected tissues and assayed for gene expression profiling using microarrays for brain tissues; AY, HC, MPFC, septal region, corpus striatum and VS and the results have been published[15]. These harvested brains were stored at -80°C for several months before RNA extraction. This method can be adopted in combination of other methods to diversify downstream utility of brain regions. This dissection method is focused on following landmarks among anatomically distinct adjoining regions instead of being strictly coronal or sagittal dissections. The distinct brain regions along with weight data was collected under the study aggressor exposed social stress model of PTSD[16] and RNA concentrations along with spectrophotometric readings is shown as **Figure 5**.

Figure Legends

Figure 1: Representation of Mouse Brain with Distinct Tissue Types Collected. This figure is a cosmetic representation of the structures and is not scaled to any mapped database.

Figure 2: Brain Removal from the Cranial Cavity Followed by Pituitary Removal. Stepwise procedure for brain removal (i) clamping and holding after hair removal (ii) securing the clamp with a Kimwipe to keep hair away from the tissue (iii) before removing of muscles (iv) after removal of muscles (v) separation of méninges (vi) removal of globe/eye (vii) cut on orbital ridge (viii) removal of parietal and frontal bones (ix) brain display and removal (x) brain detachment (xi) pituitary view (xii) pituitary removal.

Figure 3: Dissection Setup Station. This image shows the set up for brain dissection post-brain and pituitary tissues removal.

Figure 4: Brain Dissection. Stepwise procedure for brain removal (i) top view of brain (ii) dorsal view of brain (iii) cerebellum removal (iv) cerebral separation (v) hemispheres dissection (vi) olfactory bulb removal (vii) MPFC, FCX and accessory olfactory dissection (viii) SE, VS and ST dissection (ix) PFM removal (x) Limbic system dissection

Figure 5: Data Generated after Sample Collection (A) Brain tissue weight and (B) RNA concentration and (C) OD 260/280 from each of the brain tissue is shown. Here the data is gathered from control groups (n= 3-6) from a study group as reported earlier[15,16].

Table 1: Description of Distinct Regions from Mouse Brain. This table contains list of all brain regions collected with its abbreviation.

DISCUSSION:

The mammalian brain is a complex organ composed of an array of morphologically distinct and functionally unique cells with diverse molecular signatures and multiple regions that perform specialized and discrete functions. The dissection procedure reported here can have multiple goals depending on the requirements of the lab. In our lab we assessed transcription in multiple brain regions collected from mice exposed to PTSD like stress[16] . We would like to study further the impact of strain genetic background[24] on expression levels in multiple brain regions. This protocol has multiple critical steps that needs to be considered for successful reproducibility of the experiments. Each of the localized regions of brain play a distinct role in neuropathological condition and detailed knowledge of the appropriate brain region to study is lacking. Therefore it is important to generate the dataset pertaining to brain region. Thus, the data can not only be queried by selecting brain region but also by data category (e.g., Transcriptome, protein, cell (cytoarchitectural), or other) leading to more precise information. Previously, the tissues such as olfactory bulb, frontal cortex, striatum and hippocampus in fresh rat brain tissues have been shown to be dissected using a microscope[25]. Alternately sections can be dissected while the brain is frozen[14] followed by RNA and protein extractions but this method is limited to brain regions that can be identified by clear landmarks. Total RNA extractions have been carried post microdissection[25] from major brain regions as well as using non-laser capture microscopy approach for gene expression studies[13]. Here we focus on the dissection of fresh mouse brain to separate out the specific brain structures that have dedicated control over physiological and behavioral functions. Our method explains the dissection of more regions than already published reports however it is complementary to other dissection methods available. This approach can help provide a comprehensive assessment of the tissues and its association with debilitating conditions. This dissection strategy provides a viable option to existing sample collection strategies opening possibilities for new discoveries.

With the method described herein, brain tissues are snap frozen in liquid nitrogen before transferring to -80 °C for long term storage. It is important that the tools and tissues are kept cold during the entire procedure for preservation of nucleic acids or proteins. These frozen tissues are homogenized at a later time using standard lab SOPs. Some of these brain tissues are very small and care should be taken during the homogenization and extraction process protecting target molecules from degradation at higher temperatures.

In this process, it is important to identify clear landmarks to pinpoint the specific tissue regions. This is achieved by keeping the tissue moist with saline solution to keep it from becoming quickly soft and retaining its sturdiness for a while. Our previous studies compared gene expression changes between control and aggressor exposed mouse tissues[15] and did not study any changes caused due to the saline used during dissection. In our experience, this is especially important during the incision starting from the hypothalamus and the 180° flipping as it exposes and makes the regional separation shading of the limbic regions (AY, HC, ERC) obvious and more clear. The limbic system is situated deep within the brain and gets damaged by a variety of stimuli, and hence is important diagnostically and therapeutically. There limbic system consists of brain regions however there is no universal agreement on this list[26]. Though not studied, we think that there are minimal or no effects of saline use. This is because the entire procedure lasts about 20 minutes following cold conditions during the entire process.

Regions within brain tissue are identified using landmarks mentioned in brain atlases. Using this technique, the landmarks need to be clear and this procedure should be done sequentially. The dissection has to be done while the brain is still fresh and sturdy; (has to be done with the first 15 to 20 minutes) – otherwise the landmarks will not be clear and regions will not be distinct if the brain stays for longer time and became softer.

As described above, within the brain are sub regions, containing multiple functional areas that act independently or in coordination with intrinsic connective networks. It is important to retrieve these regions with great precision in order to study its broad dimensions. This will help to integrate these concepts by combining the specialized regions where each is serving a distinct process with a therapeutic potential.

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DISCLOSURES:
The authors have nothing to disclose.

Disclaimer:
Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The opinions or assertions contained herein are the private views of the author, and are not to be construed as official, or as reflecting true views of the Department of the Army or the Department of Defense. Research was conducted under an approved animal use protocol in an AAALACi accredited facility in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory*

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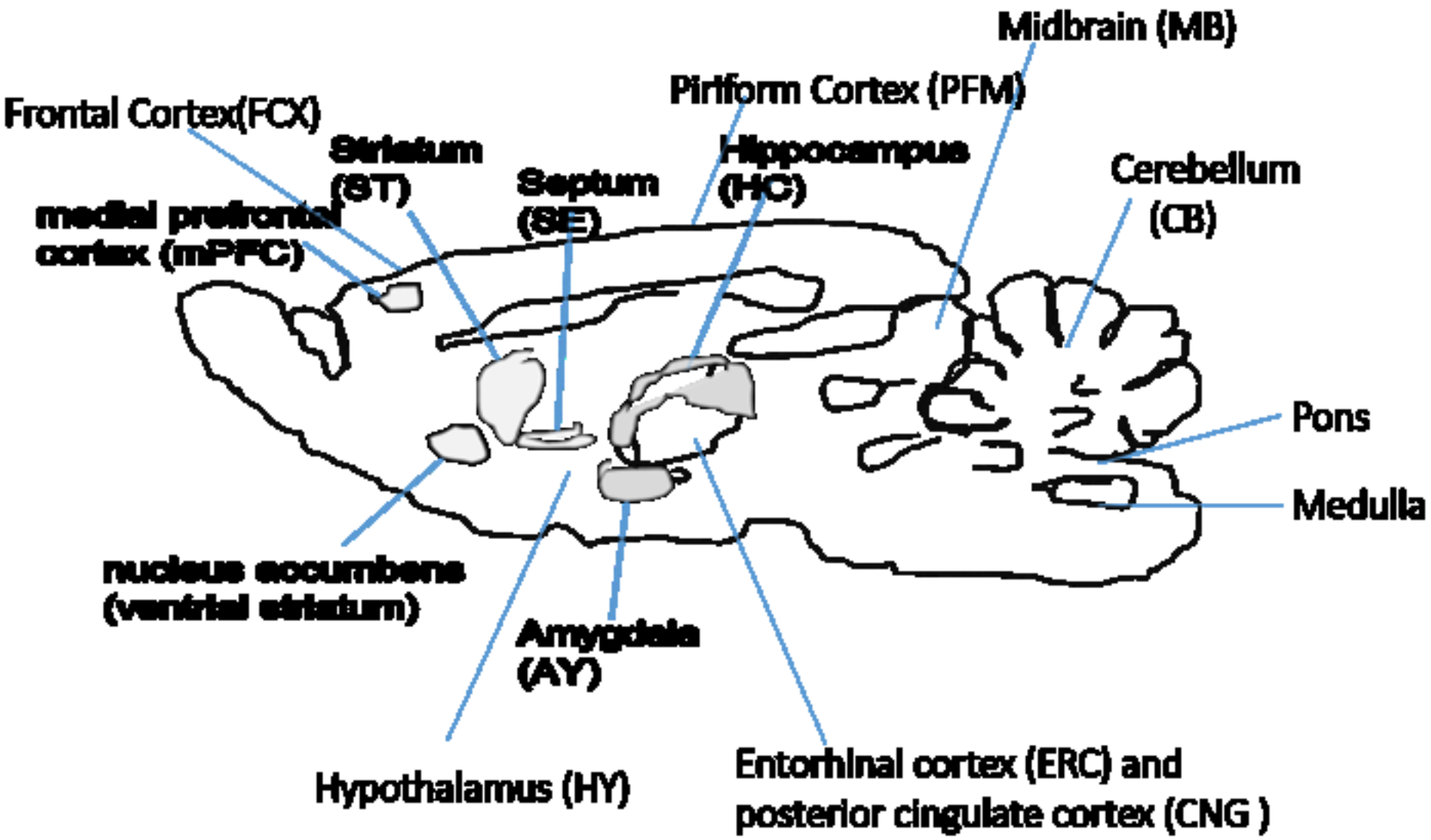

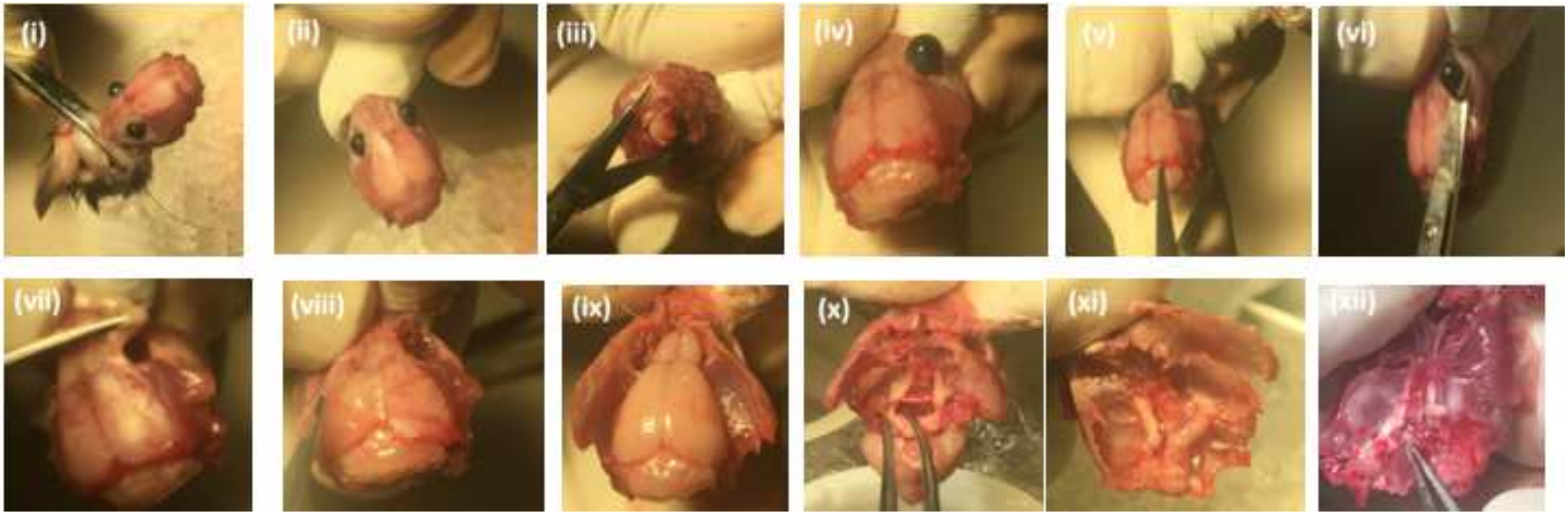

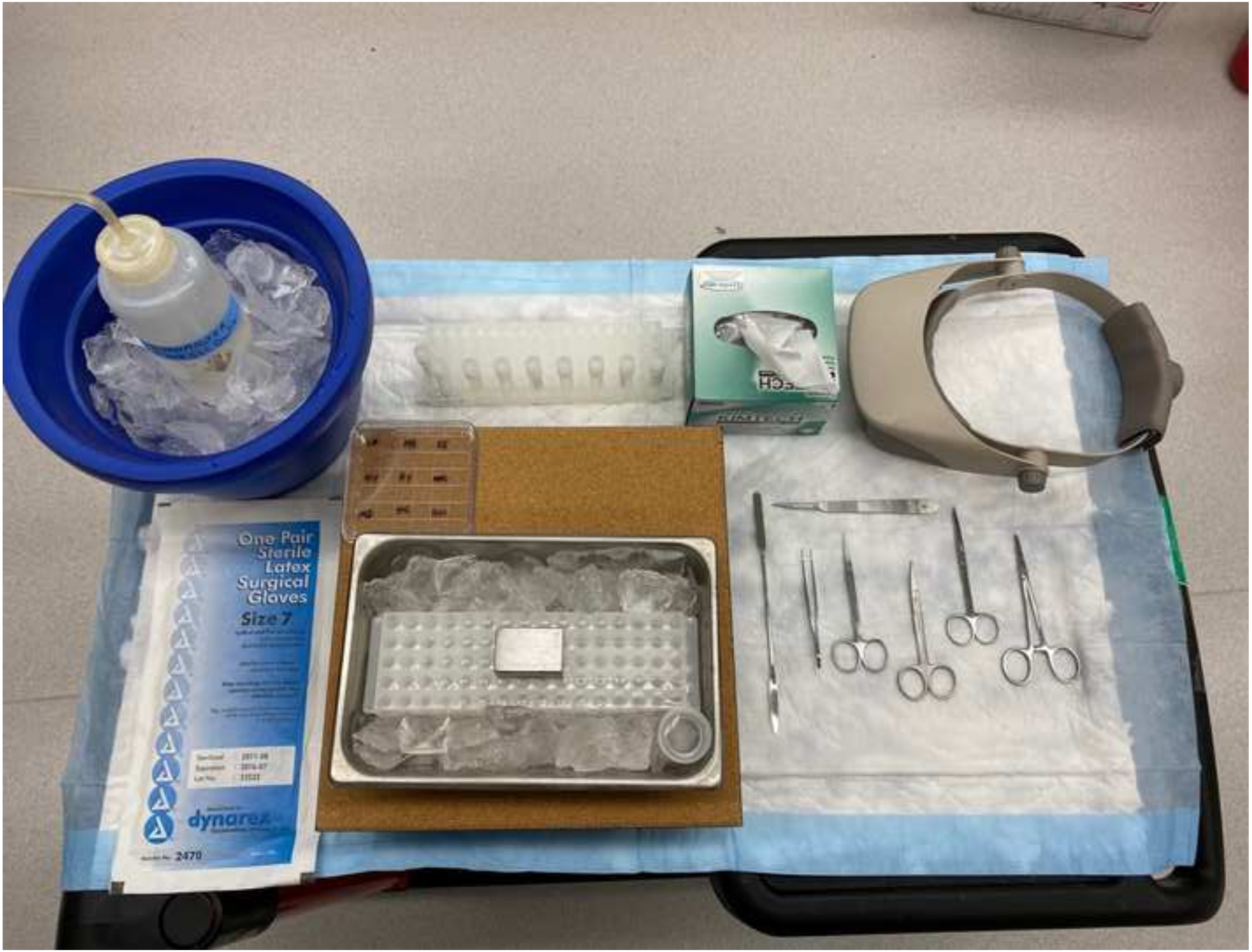

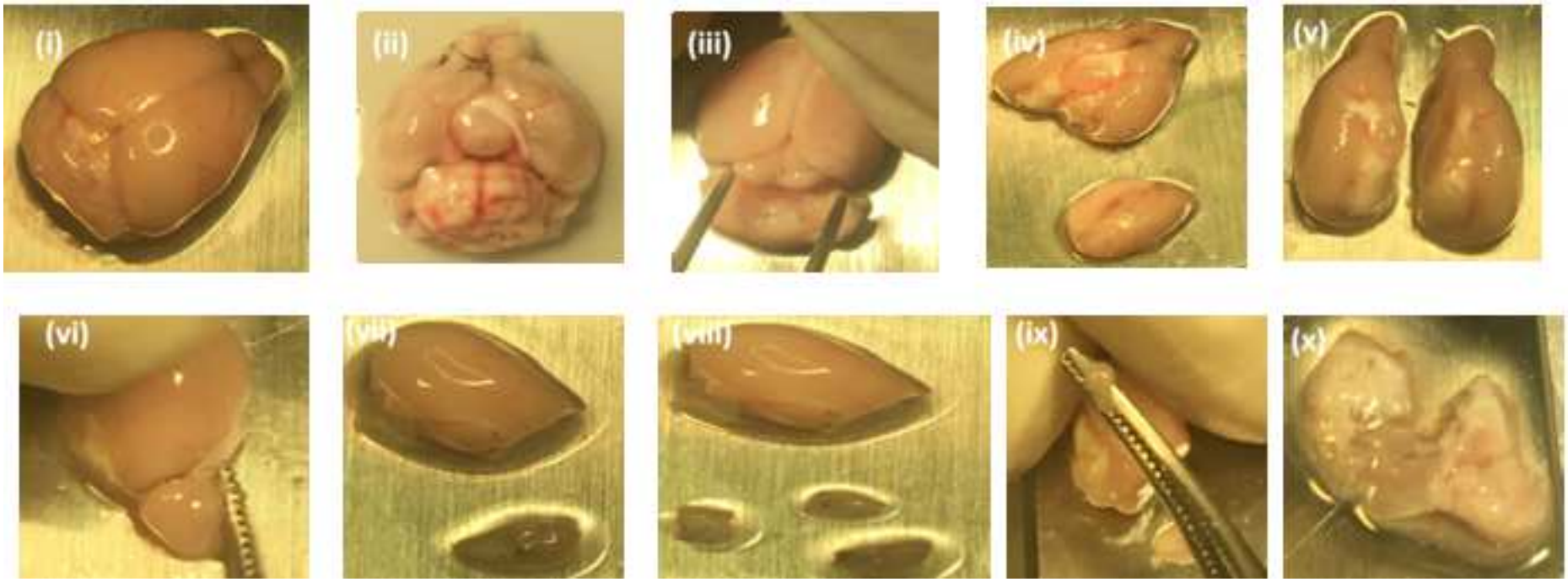

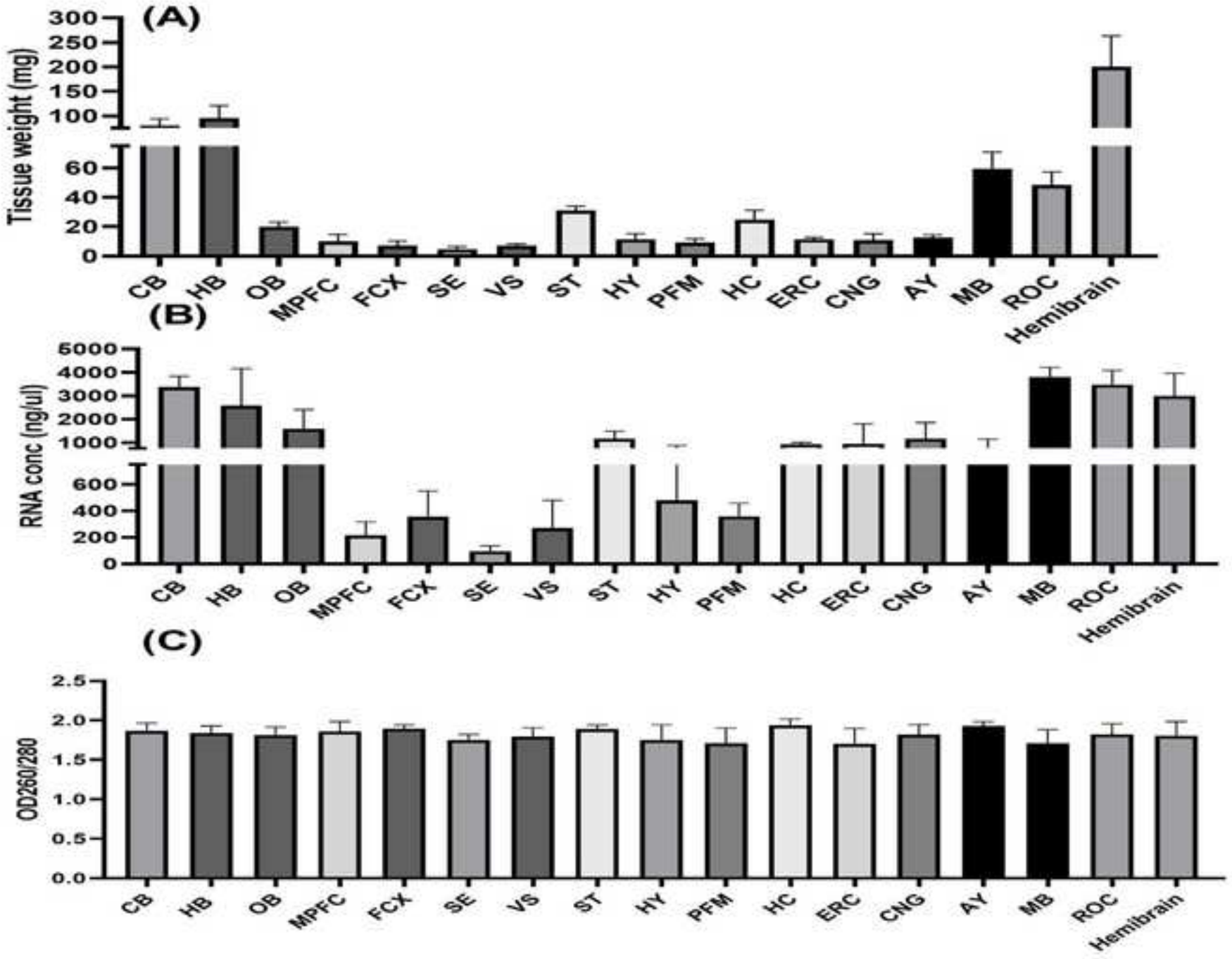

Table1: List of distinct regions from mouse Brain

#	Abbreviations	Description of brain region
1	CB	Cerebellum
2	HB	Brain stem/hind brain (pons and medulla oblongata)
Separate into the two hemispheres		
3	OB	Olfactory bulbs and accessory olfactory bulbs
4	MPFC	Medial prefrontal cortex
5	FCX	Lateral prefrontal cortex
6	SE	Septum or septal region
7	VS	Ventral striatum includes the nucleus accumbens (NAC) and olfactory tubercle (OT)
8	ST	Anterior and posterior corpus striatum
9	HY	Hypothalamus
10	PFM	Piriform cortex
11	HC	Hippocampus
12	ERC	Entorhinal cortex
13	CNG	Posterior cingulate cortex
14	AY	Amygdala
15	MB	Midbrain with thalamus
16	ROC	Remainder of the cerebral cortex

Table of Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Brain Removal			
Deaver scissors	Roboz Surgical Store	RS-6762	5.5" straight sharp/sharp
Deaver scissors	Roboz Surgical Store	RS-6763	5.5" curved sharp/sharp
Delicate operating scissors	Roboz Surgical Store	RS-6703	4.75" curved sharp/sharp
Delicate operating scissors	Roboz Surgical Store	RS-6702	4.75" straight sharp/sharp
Light operating scissors	Roboz Surgical Store	RS-6753	5" curved Sharp/Sharp
Micro spatula, radius and tapered flat ends			stainless steel mirror finish
Operating scissors 6.5"	Roboz Surgical Store	RS-6846	curved sharp/sharp
Tissue forceps	Roboz Surgical Store	RS-8160	4.5” 1X2 teeth 2mm tip width
Rongeur (optional)	Roboz Surgical Store	RS-8321 many styles to choose	Lempert Rongeur 6.5" 2X8mm
Pituitary Dissection			
Scalpel handle	Roboz Surgical Store	RS-9843	Scalpel Handle #3 Solid 4"
and blades	Roboz Surgical Store	RS-9801-11	40mm
Super fine forceps Inox	Roboz Surgical Store	RS-4955	tip size 0.025 X 0.005 mm
Brain Dissection			
A magnification visor	Penn Tool Col	40-178-6	Magnification, Rectangular
Dissection cold plate	Cellpath.com	JRI-0100-00A	Iceberg cold plate & base
Graefe forceps, full curve extra delicate	Roboz Surgical Store	RS-5138	0.5 mm Tip 4” (10 cm) long
Light operating scissors	Roboz Surgical Store	RS-6753	5" curved sharp/sharp
Scalpel handle	Roboz Surgical Store	RS-9843 (repeated above)	Scalpel Handle #3 Solid 4"
and blades (especially #11)	Roboz Surgical Store	RS-9801-11 (repeated above)	Sterile Scalpel Blades:#11 Box 100 40mm
Spatula	Amazon	MS-SQRD9-4	Round End
Tissue forceps	Roboz Surgical Store	RS-8160 (repeated above)	4.5” 1X2 teeth

Dear Editor,

Subject: Revised version of the manuscript

Number: JoVE61941 Microdissection of mouse brain into functionally and anatomically different regions

We would like to thank-you for giving us an opportunity to consider our revised paper in the journal. I am submitting the revised version of manuscript along with all the updated documents. Following is the response to the reviewer and editorial comments.

Thank-you

Aarti Gautam

Response to the Editorial comments:

Changes to be made by the Author(s):

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

Completed as suggested.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

Completed as suggested.

3. Please provide at least 6 keywords or phrases.

Completed as suggested.

4. Please reword lines 408-422 as it matches with previously published literature.

These lines have been removed from the updated version.

5. Please rephrase theShort Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Presented here is a protocol …”

Completed as suggested

6. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

Completed as suggested

7. Ethics statement should be placed before the numbered step in the protocol section.

Completed as suggested.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tensemay be added as a “Note.” However, notes should be concise and used sparingly.

Completed as suggested.

9. The Protocol should contain only action items that direct the reader to do something.

Completed as suggested.

10. Please ensure you answer the “how” question, i.e., how is the step performed?

Completed as suggested.

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.

Completed as suggested.

12. For euthanization did you use CO2 asphyxiation as well. Only cervical dislocation is not an acceptable euthanasia.

The brain dissection protocol was developed as part of our previously developed mouse model simulating features for post-traumatic stress disorder (PTSD) (Hammamieh et al 2012) where tissues were collected post cervical dislocation. Cervical dislocation was used in our study to avoid the profound effect of analgesia/ anesthesia on the cerebral functions which can mask the cerebral signatures associated with PTSD.

Rasha Hammamieh, Nabarun Chakraborty, Thereza C.M. De Lima, James Meyerhoff, Aarti Gautam, Seid Muhie, Peter D’Arpa, Lucille Lumley, Erica Carroll, Marti Jett, Murine model of repeated exposures to conspecific trained aggressors simulates features of post-traumatic stress disorder, Behavioural Brain Research, Volume 235, Issue 1, 2012, Pages 55-66,ISSN 0166-4328, https://doi.org/10.1016/j.bbr.2012.07.022.
(http://www.sciencedirect.com/science/article/pii/S0166432812004780)

13. Do you perform perfusion as before brain dissection?

No perfusion is carried out before dissection.

14. How do you identify different structures of brain during the dissection process?

The structures are identified based on the critical landmarks and is achieved by keeping the tissue moist with saline solution to keep the tissue from becoming quickly soft and retaining its sturdiness for a while.

15. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings andspacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Completed as suggested.

16. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted. Notes cannot be highlighted.

Completed as suggested.

17. Please include a figure or a table in the Representative Results showing the effectiveness of your technique backed up with data.

Figure 5 have been added in the current version.

18. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

Completed as suggested.

19. Please include at least one paragraph of textto explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, how do you figure out the functions and find these are distinct for different regions, how do you study anatomy, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

Completed as suggested.

20. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

No figure from previous publication is used. However, Figure 1 (brain cartoon) is created from the same background as used in previous manuscript (Muhie et al 2015). Supplemental file Figure S1 Additional file 1. Please let me know if any approvals are required. Thank-you

Muhie S, Gautam A, Meyerhoff J, Chakraborty N, Hammamieh R, Jett M. Brain transcriptome profiles in mouse model simulating features of post-traumatic stress disorder. Mol Brain. 2015;8:14. Published 2015 Feb 28. doi:10.1186/s13041-015-0104-3

21. As we are a methods journal, please ensure that the Discussion explicitly cover the following indetail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Discussion section includes a paragraph with suggested additions.

22. Please do not abbreviate the journal titles in the references section.

JOVE citation format is used from the Endnote

_____

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript describes a method to dissect out coarse brain regions from a fresh mouse brain.

Major Concerns:
1. The manuscript was not explicit about the applications for this procedure. Is it for bulk RNA-seq, single cell RNA-seq, epigenetics, proteomics, metabolomics? Each of these applications might require different tissue processing and handling.

The applications description from this protocol had been added in the introduction section.

2. The fresh brain was placed on acold block, with periodic wetting of cold saline solution during

dissection. There was no mention of how long the procedure took and the downstream processing of the tissue. For single cell sequencing or epigenetics profiling, it is important to keep the brain in its "native" state as much as possible. This includes submerging the tissue in ice-cold artificial cerebral spinal fluid (ACSF) during dissection. This procedure has been published elsewhere as slice preparation for electrophysiology and single cell transcriptomics sequencing. I would imagine leaving the brain "moist" and wetting with saline during the procedure would cause significant gene expression changes and cell death. The manuscript does not provide downstream results to verify the quality of the sample based on this procedure (see major concern #1).

We completely understand that use of saline solution may cause changes, however in our protocol, it is utmost important that we keep the tissue wet to preserve the landmark structures. We try to minimize the dissection method and so far, with our experience we are able to complete this protocol is about 20 minutes. The published study (Muhie et al 2015) from our team have used similar conditions of dissection and compared control to stressed mouse tissues.

Muhie S, Gautam A, Meyerhoff J, Chakraborty N, Hammamieh R, Jett M. Brain transcriptome profiles in mouse model simulating features of post-traumatic stress disorder. Mol Brain. 2015;8:14. Published 2015 Feb 28. doi:10.1186/s13041-015-0104-3

3. There is no comparison with published methods for fresh brain dissection for electrophysiology and single cell sequencing. Publishedmethods include keeping the brain in ACSF, cutting slices with a vibratome, dissection of regions under a microscope.

No direct comparisons have been made to the previous studies, however in the current version, tissue weight and RNA data from the current data have been added.

Minor Concerns:
1. There is no reference atlas drawings of the brain regions dissected. It would be helpful to have an overview of the atlas, with delineation of which brain regions are being dissected. The Allen Reference Atlas of the Mouse Brain has a 3-D rendering to aid in this.

No atlas drawing have been added but a cartoon showing all tissues have been added as Figure 1

2. Introduction and Discussion paragraphs can use some revision. They are somewhat unfocused, and there are many overstatements about the importance of studying brain structures. Subcortical brain structures should also not be deemed to have more "priority" over the cortical sheet. The entire brain is important and each region have diverse and specific functions. It is valid to study different parts of the brain to understand it as a whole, without having to claim that any particular region is more important than the others.

We agree that entire brain is important. Our team have been studying multiple brain regions and studying its molecular effect on post-traumatic stress disorder (Muhie et al 2015). We have re-worded the manuscript as a brain dissection protocol.

Muhie S, Gautam A, Meyerhoff J, Chakraborty N, Hammamieh R, Jett M. Brain transcriptome profiles in mouse model simulating features of post-traumatic stress disorder. Mol Brain. 2015;8:14. Published 2015 Feb 28. doi:10.1186/s13041-015-0104-3

3. More figures togo with the text would be useful. Text is heavy with anatomy and autopsy terms that is not easily understandable.

The manuscript has been re-worded to make it easily understandable.

Reviewer #2:
Manuscript Summary:
The manuscript by Gautam et al. describes a protocol for microdissecting mouse brains into anatomically distinct regions, which is important for advancing our understanding of brain function. The protocol is clearly described and will be of interest to the neuroscience community.

Major Concerns:
1. The figures are useful but key structures should be labelled on the images for orientation. It would also help if there was a summary diagram/illustration that depicts all of the regions that are dissected in this protocol to provide anatomical context for the reader.

Figures are labeled to show the key structures.

Minor Concerns:
1. The manuscript would benefit from careful proofreading as there are some grammatical errors throughout

Completed as suggested.
2. Line 56: the retina is also part of the central nervous system

Corrected as suggested.
3. Line61: the brain is also protected by bone, meninges and CSF and this should be incorporated into this sentence

Added as suggested.
4. Line 93: this protocol has applications that extend beyond molecular assays and imaging studies. The authors should highlight the broader applications so that the article appeals to a wider audience (e.g. neuroimmunology studies).

Rephrased as suggested
5. Line 113: it should also be mentioned that some experiments may require mice to be transcardially perfused to clear blood from the vasculature and rapidly fix tissues

Added in the updated version.

6. It should be noted that when separating the brain from the calvaria, usually the pia remains attached to the brain and the arachnoid-dura remains attached to the calvaria (in mice). It may be important for some studies to remove the pia.

Protocol rephrased