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https://dx.doi.org/10.4314/nvj.v39i4.2 **ORIGINAL ARTICLE**

Processing Rodent Brain Tissues: Review of Basic Anatomical Techniques for Scientists with Minimal Knowledge of Neuroanatomy

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SUMMARY

An understanding of brain structure, how to harvest rodent brain samples and the various histological procedures to follow is imperative in conducting researches in neuroscience especially for beginners. The general brain aversion bias and complexity of studying the brain, poses a real challenge in identifying and understanding its anatomy either grossly or microscopically. Therefore, this review attempts to provide a basic explanation for researchers with little knowledge of neuroanatomy but are interested in processing brain tissues ranging from opening the skull of the rodents, harvesting freshly perfused or unperfused brain, weighing of the brain and identifying various landmarks on the brainstem, cerebellum, and cerebrum. This review further provides a clue to the various anatomical landmarks in the brain and the functional role each structure identified in such landmarks play. Since most of the brain cytoarchitecture is viewed microscopically, knowing the appropriate staining techniques that clearly show the desired brain cell or region in question is important in determining various neuropathologies as well as in conducting various pilot studies on the brain. This review therefore provides a solid foundation for researchers who are not too familiar with the brain but need to conduct various studies on it.

Keywords: brain, histological procedures, anatomical landmarks, staining techniques, cytoarchitecture, fixation

INTRODUCTION

The nervous system and the brain in particular, is one of the most complex in the body and can pose a headache to researchers who do not have more than basic training in biology or neuroanatomy. With numerous Latin derived terminologies and unclearly delineated structures, the brain truly needs an in-depth study of gross and histological neuroanatomy, to be understood. Although across species, the basic cytoarchitecture of the nervous system is similar, morphological differences occur in the gross appearances and overall proportions, positioning and function of structures and across age groups. Therefore, a review such as this will greatly help any researcher who just simply needs basic understanding on how to harvest the rodent brain samples; of which parts to collect and which histological, histochemical or immunohistochemical procedure(s) to consider appropriate for sampling the nervous tissue, in order to perform basic experiments. This paper therefore, reviews the procedures to observe while working with brain tissues and states appropriate basic histological procedures for researchers under different experimental conditions.

Typical Rodent Brain Gross structure

The rodent brain is grossly lissencephalic in neonates and juveniles but may show slight gyrencephaly in the putative caudomedial and visual cortex in adults in some species such as the Greater Cane rat and African giant rat (Dwarika *et al.,* 2008; Olude *et al.,* 2016). The dura meninx appears typically (Fig.1). transparent and as a colourless membrane but may turn slightly whitish upon post fixation in aldehydes (Olude *et al.,* 2016). Average brain weight in rodent ranges from 0.4g in mice, 2g in adult wistar rats; 4g in guinea pigs; 7.6g in squirrels (Nieuwenhuys, 1998); 10.5g in African grasscutter (Nzalak *et al.,* 2008) to 5.60g in African giant rats (AGR) (Nzalak, 2008; Olude *et al.,* 2016). Interestingly, the olfactory bulbs of the AGR make up about 25% of the brain size in the adult AGR compared to 2.98% observed in the adult Wistar rat (Bukar *et al.,* 2015; Olude *et al.,* 2016). The cerebral cortex constitutes about 31% of the total central nervous system volume (Swanson, 1995). The hind brain comprises of the cerebellum, medulla oblongata and pons, with the last two forming portions of the brain stem. The cerebellum is observed caudal to the cerebrum and dorsal to the fourth ventricles in the region of the pons and rostral portion of the medulla oblongata. It consists of the three typical lobes of rodents in the median portion (rostral, central and caudal lobes); three lobes lateral to the median portion (lunate, ansiform, and paramedian lobes), and two prominent paraflocculars lobes (Hebel and Stromberg 1986; Olude *et al.,* 2016). The dorsal view of the cerebellum reveals a typical worm-like appearance in juvenile and adults. It is relatively smooth without the typical vermis and is slightly separated from the cerebrum by an interposing and exposed mesencephalon in neonates

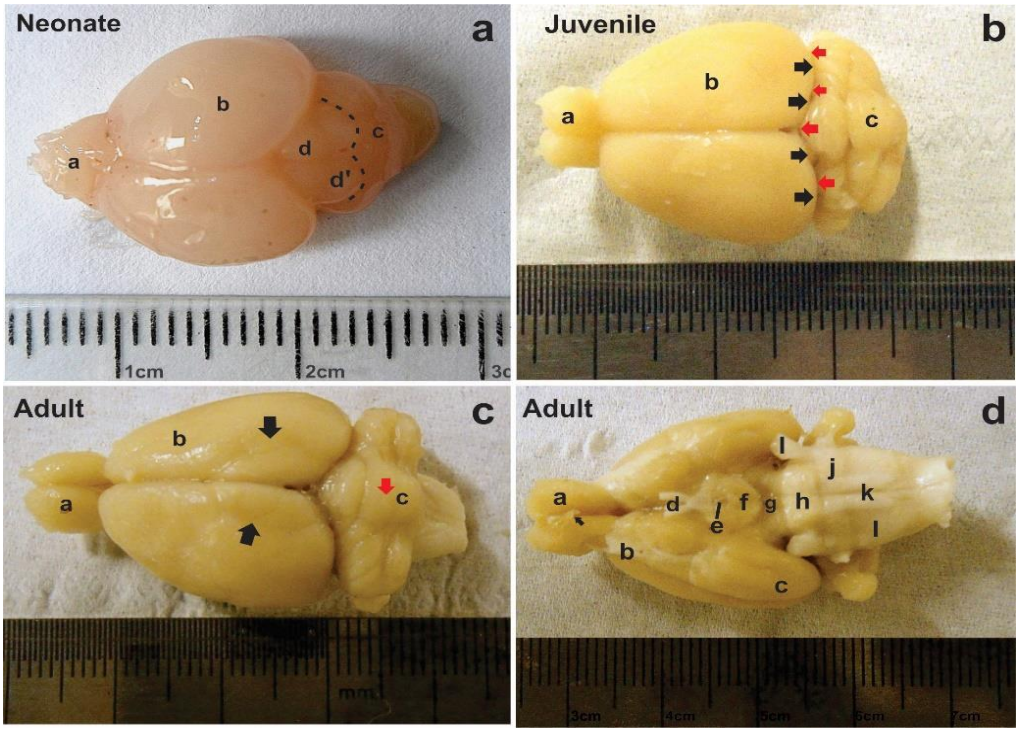

Figure 1: (a) Photograph of neonate AGR brain showing the fully extracted dorsal view. Note the separation of the cortices by the mesencephalic tectum delineated at the caudal extent by broken dotted lines. a = olfactory bulb, b = cerebral cortex, c = cerebellum, d = rostral colliculus, d' = caudal colliculus. (b) Picture showing the fully extracted dorsal view of a juvenile AGR brain with lissencephalic conformity and apposed cerebral and cerebellar cortices (black and red arrows). a = olfactory bulb, b = cerebral cortex, c = cerebellum. (c) Picture showing the fully extracted dorsal view of an adult AGR brain. The putative caudomedial visual cortex shows slight gyrencephally (black arrows). a = olfactory bulb, b = cerebral cortex, c = cerebellum. Note the cerebellar vermis (red arrow). (d) Picture showing the fully extracted ventral view of an adult AGR brain. a = olfactory bulb, b = olfactory tract, c = piriform lobe, d = optic nerve, e = stalk of hypophysis cerebri, f = mammillary body, g = interpenduncular fossa h = pons, i = trigeminal nerve (cranial nerve V), j = trapezoid body k = pyramid, l = medulla oblongata. Note the insertion of falx cerebri par bulbous olfactorius (black arrow). **Picture was obtained from Olude *et al.*, 2016**

Working with brain samples

a. **Humane and ethical treatments:** Animals should be anaesthetized with either ether, lethal dose of pentobarbitone or xylazine-ketamine combination used in appropriate doses (See PSU, (2016) for recommended anaesthetic doses in rodents). Quick cervical displacement is also widely practiced and known to be acceptable (Rijn *et al.*, 2011). It is important to ensure that death is induced as painlessly and quickly as possible.

b. **Opening the skull:** Basic implements may be required depending on the age of the animal being sampled. Neonates and smaller rodents have thin fibro-membranous

skulls that may be lifted skillfully with a pair of scissors or forceps, being careful not to pierce the underlying brain tissue (Gage *et al.*, 2012). For larger rodents and more mature animals, bone cutters may be required. Improvised bone cutters may also serve the purpose (Gage *et al.*, 2012). The procedure is achieved by first decapitating the head using a pair of scissors (optional). A midline incision along the skin is then drawn from the neck to the tip of the nose to expose the skull as the skin is parted laterally by reflection. Excess muscles are trimmed using scissors or ronguer. By accessing the foramen magnum (where the spinal cord exits from the skull), the occipital bone may be carefully nipped off on the inner parts of the bone without touching the brain tissue. This may be done with a pair of blunt-sharp scissors or bone cutter or ronguer. Next, a cut extending to the distal edge of the caudal (posterior) skull surface should be made with an identical cut also made on the contralateral side. Use the rongeurs to clear away the skull around the cerebellum. The scissors should then be carefully slid along the inner surface of the skull as the tip travels from the dorsal distal caudal (posterior) corner to the distal frontal edge of the skull, lifting up on the blade, to prevent damage to the brain. This should be repeated on the opposite side. The dorsal surface of the skull should be peeled away from the brain and sides trimmed away. A cut should be made rostral to the frontal bone suture then using a spatula, lift the olfactory bulbs and nervous connections along the ventral surface of the brain. In this process, gently tease the brain away from the head, trimming any dura that still connects the brain to the skull using iris scissors. (Fig. 2)

c. **Handling fresh perfused brain:** Certain procedures such as Horseradish peroxidase staining, biocytin staining and immunohistochemistry are best carried out on perfused brains (Gage *et al.*, 2012; Poorani and Brundha, 2015). It is true that brain harvested on ice may be fixed directly in fixative fluid. This technique may not allow deep penetration of the fluid and may take longer to fix especially for mature brain tissues. On the other hand, it is more advisable to perform transcardia perfusion (Gage *et al.*, 2012; Poorani and Brundha, 2015). Transcardia perfusion is briefly explained below.

d. **Perfusion:** In the absence of perfusion pressure pump, a modified perfusion technique which utilizes gravity may be utilized (Olopade *et al.*, 2011; Mustapha *et al.*, 2014) (Fig. 3). Fixatives generally function to harden tissue, arrest autolysis, prevent bacterial degradation and fix proteins. They either coagulate (alcohols, acids, mercuric chloride) or cross link (aldehydes) proteins (Eltoum, 2001a; Eltoum, 2001b). These fixatives are mostly corrosive to living tissue and protective wears for the body and eyes are essential during their handling. Fixatives have been known to cause weight loss (5-10%) occurring even after a day in fixatives in humans but this has been found to be negligible in African giant rats (Ibe *et al.*, 2015, Olude *et al.*, 2016). When the brain is removed

post perfusion, it may be placed in a vial of perfusate at least 10 times the volume of the brain itself and swirled occasionally for about 2-24 hours, depending on the protocol requirements (Fredenburgh *et al.*, 2001). For immunohistochemistry, it is however advisable, to post-fix the brain minimally (not more than 4-6 hours) to avoid over-fixation. It is important to note however, that although "over-fixation" can be detrimental, under-fixation has recently been appreciated as a significant problem and may be responsible for inappropriate results for some assays (Webster *et al.*, 2009). After this, the brain should be washed with phosphate buffered saline (PBS) by exchanging the media 3 times and swirling each time for 10 minutes each. The brain tissue can also be stored in 0.1% sodium azide in 0.1M PBS at 4°C. This prevents microbial growth and leakage of proteins, arrest over fixation and allows the tissue to be stored for over 1 year if need be (Gage *et al.*, 2012).

Figure 2: Skull opening steps for exposing the mouse brain **(a)** decapitated head from the rest of the body at the atlanto-occipital joint (red arrows). This joint is located behind the external ears. **(b)** Exposed skull after reflecting the skin over the head. This is usually seen as thin semi-transparent membranes of flat bones encasing the brain. **(c)** Dotted arrow indicating direction of bone cutter to lift skull from the underlying brain **(d)** Opened skull showing the cerebral cortex *(x)* and cerebellum *(y)* of the exposed brain.

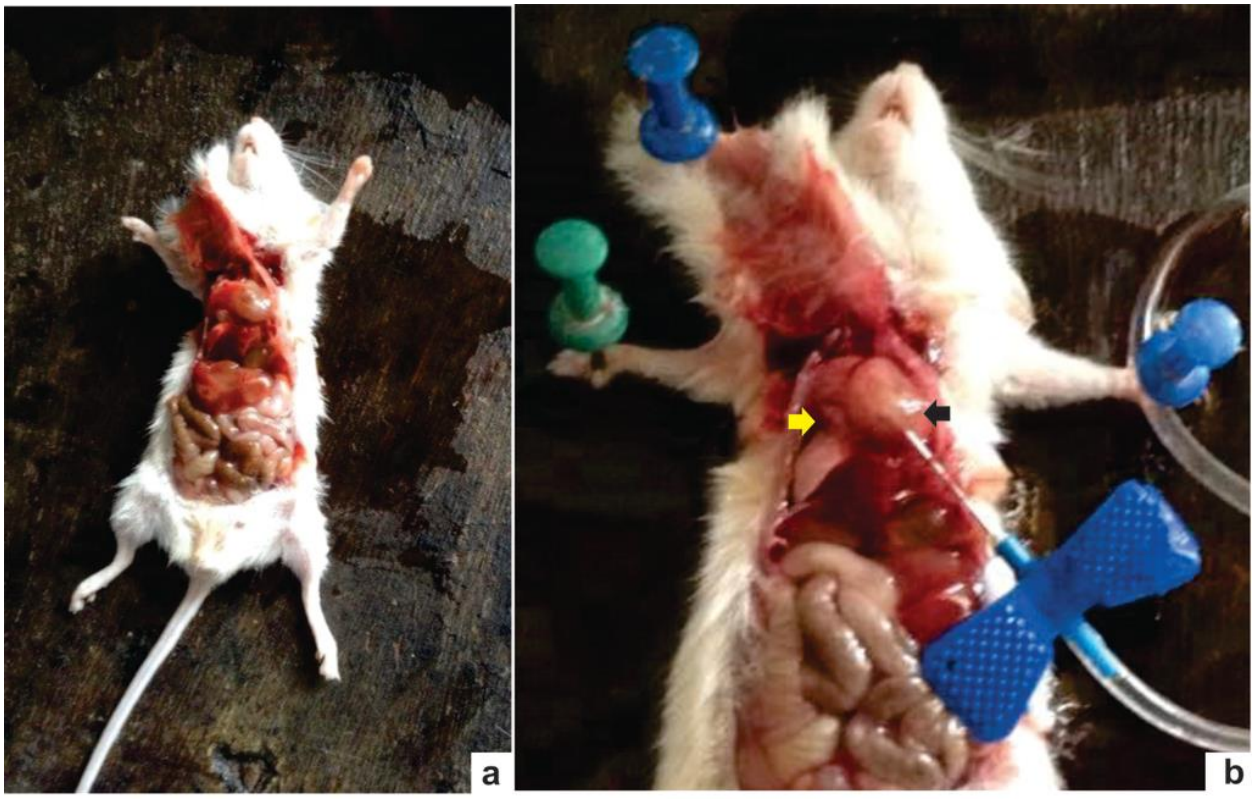

Figure 3: Diagram showing an opened abdominal and thoracic cavities **(a)** prior to the placement of the perfusion needle. **(b)** The perfusion process intubates the left ventricle (black arrow) and nips open the right atrium (yellow arrow).

e. Weighing the brain: Brain weight offers great insight into changes that may have occurred in the brain. These changes may vary from sexual dimorphisms (Ibe *et al.,* 2010), to pathological changes (Skullerud, 1985; Mustapha *et al.,* 2014). These weight parameters may be correlated against body parameters such as body (head, trunk and tail) lengths or body weights. Also, relative brain ratios such as cerebrum to cerebellum weights ratio, may offer great insights into allometric changes or even diseases that occur in age groups of animals. It is to be noted that brain weights change with age from neonatal life, increasing in the juvenile stages and decreasing in senescent stage samples (Olude *et al*, 2016).

f. Sectioning the brain: For the purpose of anatomical studies, tissue sectioning is an important step to studying the brain. Tissues should first be trimmed to fit the section of interest if the whole brain is not to be used or the brain is bigger than sectioning machine. Various methods utilized in sectioning can be made on: paraffin embedded sections (3-10µm), frozen sections (5-30µm on cryostat; 30-100µm on sliding microtome), vibratomes (40-100µm) and ultrathin sections (60nm for electron microscopy).

It is worthy of note that unlike most organs/systems of the body, including the spinal cord, the brain represents a

3-dmensional variation (Yaksh and Harty, 1981), with different structures or regions best appreciated along three principal sectioning planes, thereby resulting in three different views. These planes include sagittal, coronal and horizontal planes (Fig. 4).

Sagittal section: describes a plane that runs down the longitudinal length of the brain parallel to the median plane, dividing it into uneven halves of left and right portions (Fig. 4). However, a mid-sagittal section (also called median section) is an imaginary plane that divides the brain into two equal halves. Inasmuch as actual sectioning in the sagittal plane results in right and left portions, an anatomical sagittal section may be a two-dimensional view of the cut surface on the medial aspect of either portion (Purves *et al.*, 2004).

Coronal (Transverse) section: depicts an imaginary plane that is drawn at right angle to the median plane and divides the brain into rostral and caudal portions (Fig. 4). They are the easiest to visualize and show a lot of the nuclei of the cerebral cortex (basal ganglia and limbic systems). When done serially, it provides with opportunity to tracking the extent of these nuclei. One of the first structures that are noticed in a coronal section is the corpus callosum which appears in all coronal sections of the cerebrum thereby serving as a useful landmark (Purves *et al.*, 2004).

Horizontal (Frontal) section: It is an imaginary plane at right angle to the transverse plane and divides the brain into superior (dorsal) and inferior (ventral) parts (Fig. 4). (Purves *et al.*, 2004).

Following sectioning, the brain is typically processed for staining or assay. A summary of appropriate techniques and sample types for different procedures is shown in Table I.

g. Homogenization: Certain brain processing methods require homogenates and thus the brain tissue may be subjected to this procedure. It is briefly described below:

The section of the brain of interest such as cerebral cortex, cerebellum, pons or whole brain, is dissected and weighed then processed using a homogenizer (e.g. Dounce homogenizer®) or a handheld motor grinder. Homogenization of such brain section can either be done in ice-cold or non-ice-cold assay-specific buffer solution depending on the assay that is to be carried out on the resulting homogenate. The homogenate is usually subjected to centrifugation (usually done using a centrifuge) in order to separate the supernatant from the pellet. The supernatant or the pellet and sometimes both can be used for various assay procedures such as detection of brain disease biomarkers, protein extraction, RNA extraction and so on (Bhakta and Brodey, 2007).

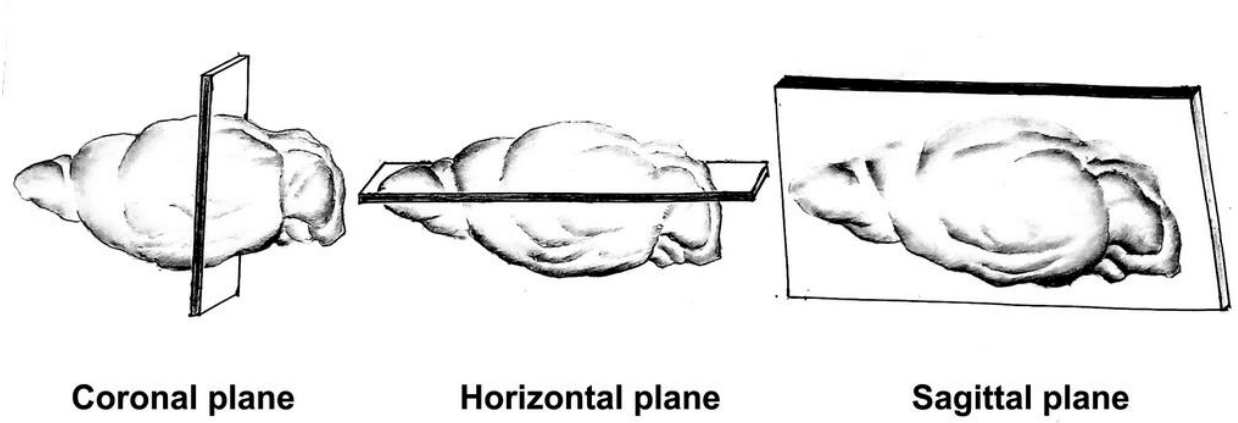

Figure 4: Schematic diagram showing the three different sectioning planes of mouse brain.

TABLE I: Summary of appropriate sample types and processing techniques in brain process

Test	Sample/Fixation Type	Stain/Technique Examples
Basic histology	Perfused brain tissues in 4% buffered formalin	Cresyl violet, Kluver-Barrera, Haematoxylin and Eosin (H&E)
Protein assay and Quantification	Whole unperfused brain	Western blotting Immunoassay (with kits)
Neuron and glia impregnation	Whole unperfused or perfused brains	Silver impregnation method (Golgi cox or Bielchowsky's)
Cellular signaling e.g. intracellular proteins targeted at neurons and glia	Perfused brain tissues in 4% buffered formalin	Immunostaining procedures against specific antibodies
Electron microscopy	Perfused brain (Karnovsky's reagent)	Transmission and Scanning Electron Microscopy

DISCUSSION

Unravelling the functions of the various parts of the brain and their influence on behavior and pathologies requires a thorough understanding of the brain's anatomy both at the organ level as well as the cell (neuron) level. To evaluate these functions, various parts of the brain require quantitative and qualitative assessment during research. However, not all scientists are familiar with the rodent brain anatomy to the extent of identifying all necessary components of neural functioning to be assessed. Hence, the need for us to provide this concise review on rodent brain processing.

There is much similarity in the structure of the brain of rodent across different species. The neocortex of most rodent brain exhibits lissencephaly (smooth brain) which is clearly

seen in *Rattus novergicus* (Kelava *et al.*, 2013). However, some rodent brains such as Armadillo (*Daspyus hybridus*), Greater cane rat (*Thryonomys swinderianus*) and adult African giant rat **(***Cricetomys gambianus***)** exhibit slight gyrencephaly in the caudomedial putative cortex (Dwarika *et al.*, 2008; Olude *et al.,* 2016). The occurrence of slight gyrencephaly is not selective in some adult rodent. It appears to be associated with large rodent brains as well as aging and this may have various neurological effects on brain functions in the adult rodents (Olude *et al.,* 2016).

Generally, anaesthetics used in rodent brain research could be injected intravenously, intramuscularly, intraperitoneally or inhaled and the choice of anesthetics used in many brain researches on rodents most times depends on the preference of the researcher (Flecknell 1989). Although we suggested the parenteral use of xylazine/ketamine combination to sedate the animal prior to perfusion in this review, many other anaesthetics such as isoflurane, pentobarbital, thiopental can also be used (Izer *et al.,* 2014; Cargle *et al.,* 2017). Several anaesthetics have been shown to interfere with the outcome of experiments involving neurotoxicity and neuroprotection (Clarkson, 2007). Having experimentally reviewed the influence of several known anaesthetics used in rodent research, Karmarkar et al. (2010) posited that many anaesthetics can cause unnoticed and unintended consequences on experimental outcomes. Therefore, it recommended that care should be taken in the choice of anaesthetics especially for researches relating to neurotoxicity and neuroprotection.

It is important to also note that the type of histological technique/protocol that the brains will be subjected to will largely determine whether to harvest perfused or unperfused brains. Moreover, the knowledge of perfusion and choice of perfusants do play critical roles in order to rightly fix the brains for procedures requiring working with perfused brain samples. Different post-fixation methods can thus be deployed since the preservation of the integrity of the brain's cytoarchitecture is important in many studies. Methods such as intravascular, transcardial or immersion perfusion fixation are well established in literature (Bolon *et al.,* 2006; Tao-Cheng *et al.,* 2007; Bolon *et al.,* 2008). However, in all cases care should be taken in optimizing the fixation protocol especially for ultrastructural and immunohistochemical studies (Jordan *et al.,* 2011). Kasukurthi et al (2009) has shown that there is apparently neither quantitative nor qualitative difference with immersion fixation in comparison with transcardial fixation methods in studies involving peripheral nerve regeneration, however, it is unclear whether the same holds true for the central nervous system.

For basic histology, perfusion with 10% formalin may be appropriate for the brain, but preparing it in buffer solution is usually more ideal (Adickes *et al.,* 1997). For Immunohistochemistry however, literature typically advises a 4% paraformaldehyde solution fixation transcardia or by direct immersion fixation for small brained species e.g. mice or neonates (Webster *et al.,* 2009). For electron microscopy, Karnovsky's fixative (Glutaraldehyde + Paraformaldehyde + Sodium hydroxide in buffer solution) will be more appropriate. On the shrinkage factor of the rodent brain, Ibe *et al.,* (2010), showed that the AGR had negligible shrinkage following 3 days post fixation in 10% formalin. This assumption is adopted by the authors also.

Sectioning of brain tissues into slices is an important part of brain processing. A large percentage of brain research involves optical imaging of brain slices for histological studies, immunohistochemical analysis, virus

tracing of synaptic connections, tracking of virus encoding various fluorescent proteins in defined brain regions and in tracing the path of a nanotube after microinjection (Zhang and Xiong, 2014). Thus, distortions and section damages which are common occurrences in brain sectioning could introduce artifacts and false-positives when slices are imaged under the microscope. Some neurobehavioural studies involve the identification of lesions in the rodent's brain after subjecting it to diverse experimental conditions. Ruining the collected brain slices due to improper sectioning can further translate to the reporting of false results. Pinsky et al. (2015) have developed a high-throughput method of whole-brain sectioning which improves the ease of section collection as well as the quality of the collected tissue. Also, Kapelsohn (2015) has outlined improved methods for cutting, mounting and staining brain tissues for neural histology.

Therefore, regardless of the type and nature of experiment to be conducted using the rodent brain, following appropriate guidelines in relation with proper anaesthetics, perfusion, fixation, sectioning, staining and imaging is undeniably important for an unbiased study and accurate reporting of experimental findings.

CONCLUSION

This paper offers a simplified approach for researchers who are not too familiar with brain anatomy but need to obtain and analyze histologically some samples from the brain in their work. More in-depth studies of neuroanatomy will definitely be required to identify all relevant nuclei or cells related to particular functions. But the authors believe that this review will go a long way in reducing the aversion or loopholes a researcher may encounter, owing to ignorance of the basics of brain research.

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