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Neuroimaging of the Amygdala: Quantitative Mechanistic Approach

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Additional information is available at the end of the chapter

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1. Introduction

Radiology has evolved fantastically since the discovery of X-rays on 8th November 1895, when William Roentgen amazed the world with a new source of energy, the X rays, capable of penetrating opaque matter making it transparent to non-invasive observation.

Radiology Imaging has counted with constantly improving technology that boosted this great evolution, not only because of science but also due to the influence of strong economic components operating in health and biology sciences, probably considering the massiveness of potential applicability and its vast and always growing field of action.

Neuroimaging technology has been part of these changes increasing its potential more dramatically in the last decades; everything indicates these continuous improvements will not decrease. Little did we know two decades ago that we would be able to assess in the same exam, in a totally non-invasive manner, morphology, biochemistry and function of the Central Nervous System with the degree of accuracy, detail and image quality we can in fact obtain nowadays. Constantly evolving technology will help us to do it even better, faster, and with a much more exquisite level of detail, allowing us to reach our challenge of generalization and reproducibility of findings that patients deserve, science pursues and we expect.

Since the very beginning radiology imaging has mostly been a qualitative visual based discipline capable of studying opaque matter, internal structures, otherwise not observable in a non-invasive manner. It has helped scientists, clinicians and technicians to know more about the human body in general and the central nervous system in particular, interfering less with the studied object, by means of a diversity of devices, producing images of remarkable quality.

Radiology has demonstrated to be an excellent qualitative tool to assess the central nervous system's normal features. However and since not long ago, it has started a firm

transformation from being a mere qualitative observation technique to become a precise quantitative discipline, at present able to study volume changes throughout time, with an excellent level of precision, capable of registering physiology-induced image variations, which lead to become a mechanistic specialty.

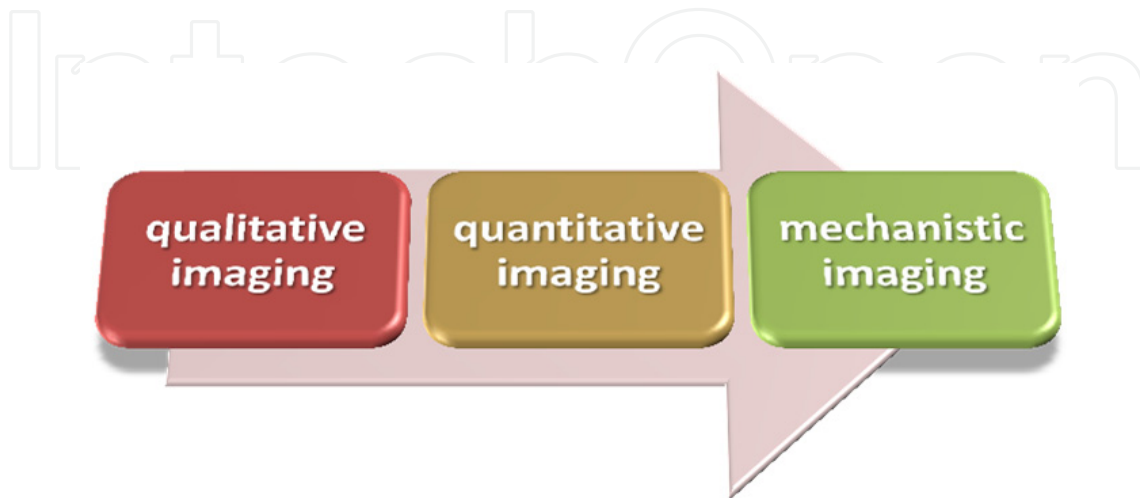


Figure 1. Paradigm shift in Brain Imaging

Imaging improvements have been dramatic in the last few years during which a paradigm shift has taken place, evolving from not only obtaining good images of the central nervous system but also accurately identifying precise boundaries of brain tissue, quantifying its volume, assessing neural connections, feeding vessels, hemodynamics, perfusion parameters, molecular and biochemical properties, and CNS functions, becoming mechanistic radiology [Fig 1] (Buckler, et al. 2011).

The changes leading to mechanistic imaging are also referred to as functional or molecular imaging. The new paradigm of quantification is reshaping the qualitative picture taking model of imaging in use until not long ago, by adopting concrete measurement-making processes not only in research but also in usual clinical practice.

In recent years, reputed authors in the field of radiology formally commenced a robust discipline of quantification in diagnostic imaging, formerly used to assess quality more or less vaguely –or with less reproducibility and precision- now named Quantitative Radiology, in all possible radiology imaging modalities, although more emphatically in computed tomography (qCT) and magnetic resonance imaging (qMRI). The latter, quantitative Magnetic Resonance Imaging (qMRI), is the modality of choice to image the amygdala. The amygdala, or *corpus amygdaloideum* in Latin, from Greek “almond”, is a large nuclear complex situated in the dorsomedial portion of the temporal lobe adjacent to and richly connected with the hippocampus. Both form an essential part of the Limbic System (Nieuwenhuys et al 1988). The functions of the amygdala appear related to emotions and modulation of stimuli interpretation, also intervening in mood modulation. The limbic

system regulates a number of essential behaviors controlling appropriate responses to stimuli with social, emotional or motivational relevance (Sokolowski and Corbin 2012). qMRI techniques permit to demonstrate cross sections and volumes, boundaries, signal intensity characteristics, topography, in vivo biochemical properties of neural tissue, connections, biochemistry and functionality of this important grey structure in the limbic system (Nieuwenhuys, Voogd and van Huijzen 1988) [Fig 2].

Magnetic resonance is the procedure of choice to image the amygdala and the remainder of the limbic system (Hirai, et al. 2000). Computed Tomography (CT) can be used to study the limbic system although to a lesser extent; besides, its grey-white matter dissociation is not as high as in MRI while it delivers ionizing radiation, to be avoided if possible, due to its biological effects. Extra consideration should be given to the administration of iodinated contrast material to enhance grey matter on CT since it has deleterious effects over the kidneys (Klatzberg and Barrett 2007).

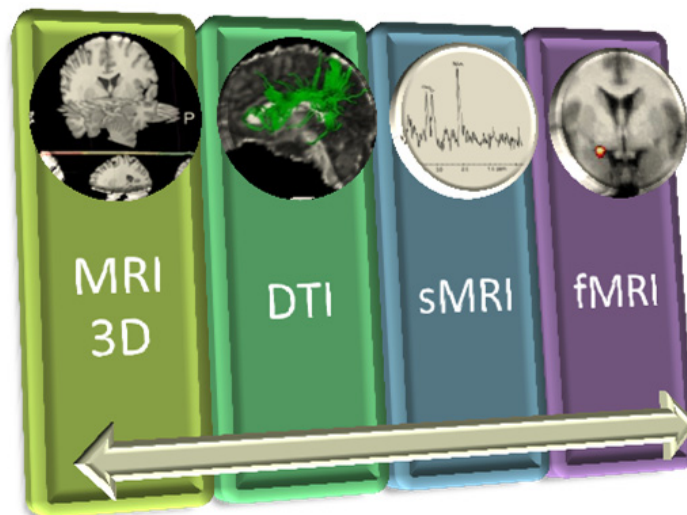


Figure 2. Usual possibilities of interrogating the subject with MR in neuroimaging the amygdala

Neuro imaging techniques to demonstrate the amygdala morphologically are therefore mainly and almost exclusively based on magnetic resonance imaging (MRI), capable of rendering a detailed neurostructural analysis, and also to assess neural connectivity, in vivo biochemical properties and functionality at a synchronous moment without moving the studied subject from the same imaging modality unit in which the exam is being performed. MRI also provides the essential capability of an excellent spatial (Jacobs, Ibrahim and Ouwerkerk 2007) and temporal resolution (Li, et al. 2002), remarkably important for mechanistic imaging. Other procedures based on nuclear medicine principles are single proton emission computed tomography or SPECT, positron emission tomography or PET (Karow, et al. 2010), the combination of PET with computed tomography, PET-CT, and magnetic resonance imaging or PET-MRI. The last ones are obtained in units capable of carrying out both modalities simultaneously, fusing images with a high degree of accuracy,

reliability and reproducibility (Judenhofer, et al. 2007). However, temporal resolution remains as an essential element to be considered, being the capability to register stimuli in rapid succession, which is not doable with nuclear medicine technologies (Ranger, et al. 2007). The latter registers metabolic activity from a known amount of radiotracer administered to the subject before the exam. The nervous tissue is avid of glucose, its main feeding metabolite. If it is marked with a radiotracer, neural tissue becomes visible in direct proportion to its metabolic activity. Although signal fades with time, its rate is not as fast as needed in an adequate stimulus-response model. Given the temporal resolution drawback of nuclear medicine studies, functional exams are performed in a magnetoencephalography (MEG) or functional MRI (fMRI) unit.

Magnetoencephalography (MEG) has an exquisite temporal resolution and a very good signal localization into space although needs to be fused to MRI obtained structural study to increase spatial resolution to the required level. Therefore an MRI must be obtained immediately before or after MEG, with appropriate skin markings to be untouched throughout both procedures. These skin markers will be essential to successfully fuse structural images from MRI to signal from MEG permitting a unified visual impression (Qingmao, et al. 2008).

MRI of the amygdala can be satisfactorily done at a variety of magnetic field strengths obtaining very good quality images from units with a magnetic field strength from 0.5, 1 to 1.5 Tesla (Maubon, et al. 1999). Although an important number of clinicians and researchers continue working at 1.5 Tesla (Korvenoja, et al. 2006), there is an increasing tendency to study subjects under 3 Tesla units (Spampinato, et al. 2011), 7 Tesla and 9.3 Tesla in clinical settings. Preliminary results seem to suggest that ultra high field strength MR units will be able not only to improve signal to noise even more dramatically but also to obtain MR spectra from other elements as sodium, an apparent excellent marker of conditions as Alzheimer's type cognitive impairment years before the clinical onset, abnormality already proven to be detectable in brain tumors (Ouwerkerk, et al. 2003).

The amount of available signal in conventional magnetic resonance imaging (MRI) is inextricably tied to the static magnetic field strength (B_0) of the imaging system. Until recently, most clinical MRI scanners operated at field strengths at or below 1.5 Tesla, however, due in part to improvements in magnet design and shielding which ease siting requirements, 3 Tesla clinical scanners are now widely available and there is a push for even higher field whole body scanners (7- 11 Tesla) throughout the industry.

Single proton computed tomography (SPECT) and Positron emission tomography (PET) are nuclear medicine studies capable of rendering excellent functional images. However, the spatial and temporal resolution of both methods are not the best to assess the amygdala although in selected cases, as in mesial temporal sclerosis and other refractory seizure disorders, PET fused with CT may be of help. PET-MRI units are ready to enter the clinical field having passed several processes of assessment by selected centers and clinical researchers however not yet available to the vast majority of researchers, neuroscientists or clinical specialists.

2. MRI acquisition protocol

MRI acquisition protocol must be suitable to the purposes of the undertaken research. It varies whether it intends to be mainly structural, functional, combined or including aspects such as tissue biochemistry and connectivity.

Series	Description	Purpose
1	Ax T2	Neurostructural, pathology
2	Ax SPGR T1	Neurostructural
3	Ax GE	Susceptibility effect
4	Ax FLAIR	Water linked to macromolecules
5	Ax DWI >16 directions	Tractography
6	sMRI	Biochemistry
7	fMRI	Steady state and post stimuli

Table 1. General purpose MRI protocol. It may take 45-60 min depending on the MR unit, magnetic field strength, gradients, coils, software, sequences, operator and used paradigms for functional series (MRI: magnetic resonance imaging; Ax T1 3D: axial series T1 weighted 3 Dimensional; Ax T2. GE and FLAIR: axial series T2 weighed, Gradient Echo T2 weighted, Fluid Attenuation Inversion Recovery; DWI>16 dir: Diffusion Weighted Images acquiring in sixteen directions or more; sMRI: spectroscopy MRI; fMRI: functional MRI)

The acquisition protocol concerning the amygdala and rest of the limbic system may be intended for pure anatomical evaluations including the assessment of intra and intercellular diffusion of water molecules, tissue biochemistry or tissue functionality. A standard protocol covering all aspects [Table 1] usually takes between 45 and 60 minutes, depending on factors such as magnetic field strength, gradients, receiver coils, software, pulse sequences, operator and used paradigms to assess functionality. Considering an initial adequate positioning and if the subject is beforehand explained as satisfactorily as possible about the study, the importance of being still throughout and clearly what he/she is expected to do during the delivery of stimuli in fMRI sequence, an acquisition study following the protocol mentioned in Table 1 would take approximately 75 minutes in a 1.5T and 60 minutes in a 3T unit.

It is crucial to discuss important aspects of the acquisition since results may vary significantly if they are neither strictly observed nor kept in mind at all times leading to potential error.

There are several important image parameters to assess quality being the most relevant magnetic field strength and Signal to Noise ratio, Contrast to Noise ratio, Modulation Transfer Factor, slice thickness and acquisition matrix.

The magnetic field strength is very important, influencing all of the others. Signal to noise ratio reflects signal intensity capable of forming image. It is considered a crucial parameter regarding image quality, although not the only one (Jacobs, Ibrahim and Ouwerkerk 2007). Contrast to noise ratio, type of coils, patient immobility, adequate positioning to minimize

motion artifacts and pre saturation pulses to decrease artifacts from blood flowing from the heart with high kinetic energy are also relevant factors contributing to image quality (Brown and Semelka 1999). Other crucial factor to be kept in mind is the intrinsic noise of the system in use, quantitatively expressed by the Modulation Transfer Function or MTF (Ranger, et al. 2007). It is a ratio between existing and registered information, which by definition ranges between 0.99 and 0.01 being 1 the ideal and 0.01 the worst.

All of these factors affect the correct MRI assessment of amygdala's structure, connectivity, biochemistry and function. Neuroimaging of the limbic system as a whole or its components has usually a well-defined purpose, which helps to define the suitable study protocol.

Volume acquisitions are typically gradient echo sequences (GE) T1, Proton Density or T2 weighted images, prescribed in just one anatomical plane of axial, sagittal or coronal contiguous slices forming a volume composed by identical isovolumetric voxels usually ranging from 0.5 to 1 mm³. If appropriate settings and algorithms are selected, high quality T1, Proton Density or T2 weighted images are obtained in 3 to 7 minutes, time that varies according to the MR unit, make, model and radiographer in charge, among other factors. The subject positioning and cooperation throughout the acquisition provide an additional quality factor. During the acquisition a *k*-space filling takes place, consistent in the initial data collection of each series to be used for reconstructing of the images. Its center represents image contrast while its periphery represents image anatomy. The *k*-space is an MRI concept naming where data forming the MR images are collected, in which morphology and contrast do not necessarily coincide in the same or adjacent point. As a practical consequence, if the subject moves near the end of the series, contrast may not be that affected as form. The subject must be still throughout the acquisition; the less motion affects scanned volume, the higher its imaging properties will be. These concepts are to be kept in mind by the research team especially to validate results testing consistency. Isovoxel based morphometry (IVBM), the technique used for manual, semi automated or fully automated segmentation, is based on signal intensity differences from voxels forming the volume. Therefore image quality factors influencing signal need to be thoroughly checked, mainly although not exclusively Modulation transfer factor (MTF), signal to noise ratio (SNR) and contrast to noise ratio (CNR).

The most common and useful series concerning neurostructural analysis are Gradient Echo T1 weighted series (with a narrow flip angle typically less than 20°). As other gradient echo sequences, they show high signal intensity (bright) within rapid flow vessels as carotid siphons, elements of the Circle of Willis and –usually- the initial segments of both mid cerebral arteries (MCAs). Grey-white matter differentiation is usually of remarkable high quality, suitable for post processing (Bertoni 1998; Bertoni 2010).

3. Assessing connectivity: MR tractography

Diffusion tensor images (DTI) obtained from diffusion-weighted images in at least 16 directions are usually a minimum requirement for a satisfactory tractography (N. Ratnarajah, A. Simmons, et al. 2012) capable of rendering 3D images of intra cerebral tracts

with the quality of an atlas, a very useful initial approach to assess the whole neurostructure following techniques already described [Fig 4] (Catani 2008). For the limbic system, particularly the amygdala, the circuit of Papez is an important element to work on (Chan 1997, Kwon 2010), for which high resolution source diffusion weighted images (DWI) series are required as the source to post process, preferably in at least 64 directions (Ratnarajah 2011).

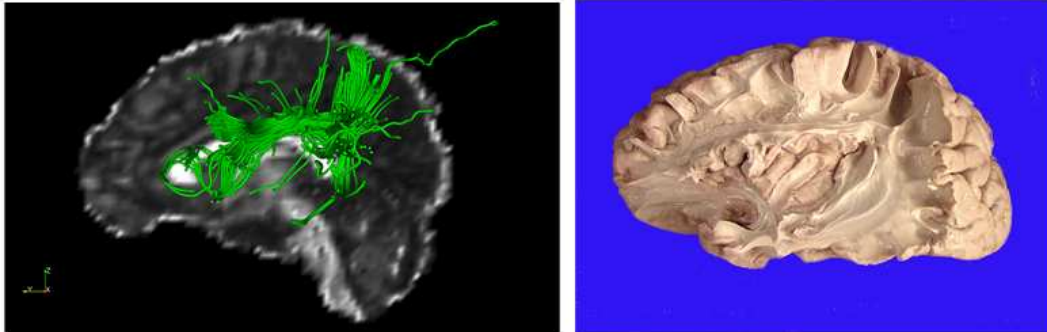


Figure 3. Demonstration of the arcuate fasciculus with DTI following Cattani's atlas technique for virtual dissection and Hojjatoleslami-Nagulan dual tensor technique to depict the tracts

Techniques vary according to the software in use and source images. Double tensor methods tend to provide useful tractography when compared with *in vitro* dissections (N. Ratnarajah, A. Simmons, et al., Residual bootstrapping on classified tensor morphologies 2011). Again, careful comparison of source images is crucial. It must be remembered that good source images should have enough number of directions and enough resolution, besides all the other essential factors mentioned before concerning image quality. These sets of will be several and composed by hundreds of images whose format source is DICOM, standing for Digital Imaging and Communications in Medicine, the accepted convention protocol to store and interact with medical images, containing not only the image but all the relevant subject and institutional identity, acquisition parameters and other relevant information concerning the procedure. Conversion from DICOM must be carried out carefully, especially when renaming the source images. Some software programs require standardization before running a tractography depiction. Newer MR units offer separate workstations including tractography-rendering software. However, some researchers prefer to carry them out under the same software program for all steps a mechanistic approach implies, to be able to fuse different post processing sets of images in a more flexible manner. Better results are usually obtained with dual tensor algorithms of reconstruction to carry out *in vivo* dissections like those already performed as *in vivo* dissections to form atlases of brain tractography (Catani and Thiebau de Schotten 2008).

4. Assessing biochemistry: MR spectroscopy (sMR)

Magnetic Resonance Spectroscopy, MRS or sMR, provides a measure of neural tissue chemistry. Resonance takes place by setting precession frequencies with hydrogen (^1H proton), sodium (^{23}Na) or phosphorus (^{31}P) for *in vivo* studies in human medicine. Hydrogen

spectroscopy is the widest in use, easiest to perform with a much higher signal-to-noise ratio than the other two elements.

Metabolite	Indicates	ppm
Lipids	Brain catabolism	0.8 – 1.3
Lactate	Anaerobic glycolysis	1.3
NAA	Neuronal marker	2.0
Glutamine/GABA	Neurotransmitters	2.2 – 2.4
Creatine	Energy metabolism	3.0
Choline	Cell membrane	3.2
<i>myo</i>-Inositol	Glial-cell marker	3.5
glucose	Intermediate metabolism	3.4 / 3.8

Table 2. Elements usually detected on spectroscopy MR of the brain, what they indicate and their concentration in parts per million

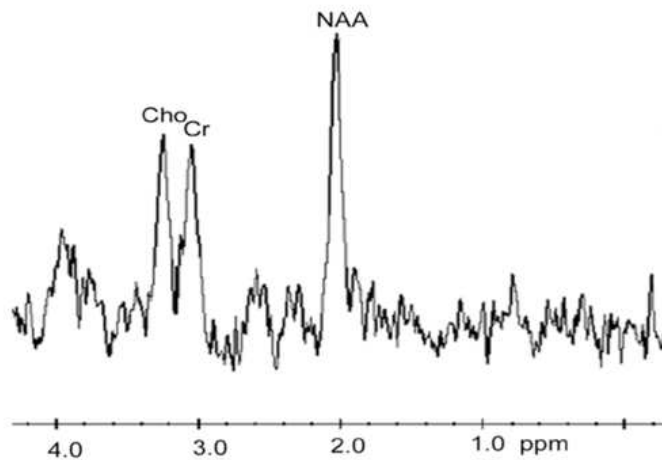


Figure 4. MR normal brain spectrum (Andre, et al. 2006)

In vivo spectroscopy can be performed in the range of 10 to 15 minutes being part as a usual MR protocol to study the brain. It can be used to determine and to monitor biochemical changes in normal brain and pathological conditions whether they are congenital, inflammatory, degenerative, neoplastic or even miscellaneous. MR spectra do not label diagnoses but must be interpreted within the appropriate clinical context, in the light of other neurostructural and functional findings.

sMRI implies measuring spectra in multiple voxels throughout the brain (multivoxel automated technique) or performing in selected areas always measuring the contralateral mirror area, as a manual technique. In the case of the amygdala, as for other components of the limbic system and considering their anatomy, the latter seems to be more convenient and reliable sMRI (Agarwal, et al. 2010), of increasing use in psychiatry.

Usually found peaks mark metabolites as lipids, lactate, N-acetyl aspartate, glutamine-gamma amino butyric acid, creatine, choline and *myo*-inositol. They tend to correlate or

indicate brain destruction or catabolism, anaerobic glycolysis, neuronal tissue marker, neurotransmission, energy metabolism, cell membrane marker and glial cell marker, respectively (Smith, Smirniotopoulos and Rushing 2008).

The medial temporal lobe structures tends to show high levels of choline, presumably reflecting different cellular compositions between allocortex and neocortex (Arslanoglu, et al. 2004)

5. Assessing functionality: fMRI, MEG, SPECT, PET

Main neuroimaging MR techniques to assess in vivo functionality of neural structures are Magnetoencephalography or MEG, and functional MRI (fMRI), the latter within the acquisition protocol already described intended to study both, morphology and function [Fig 5].

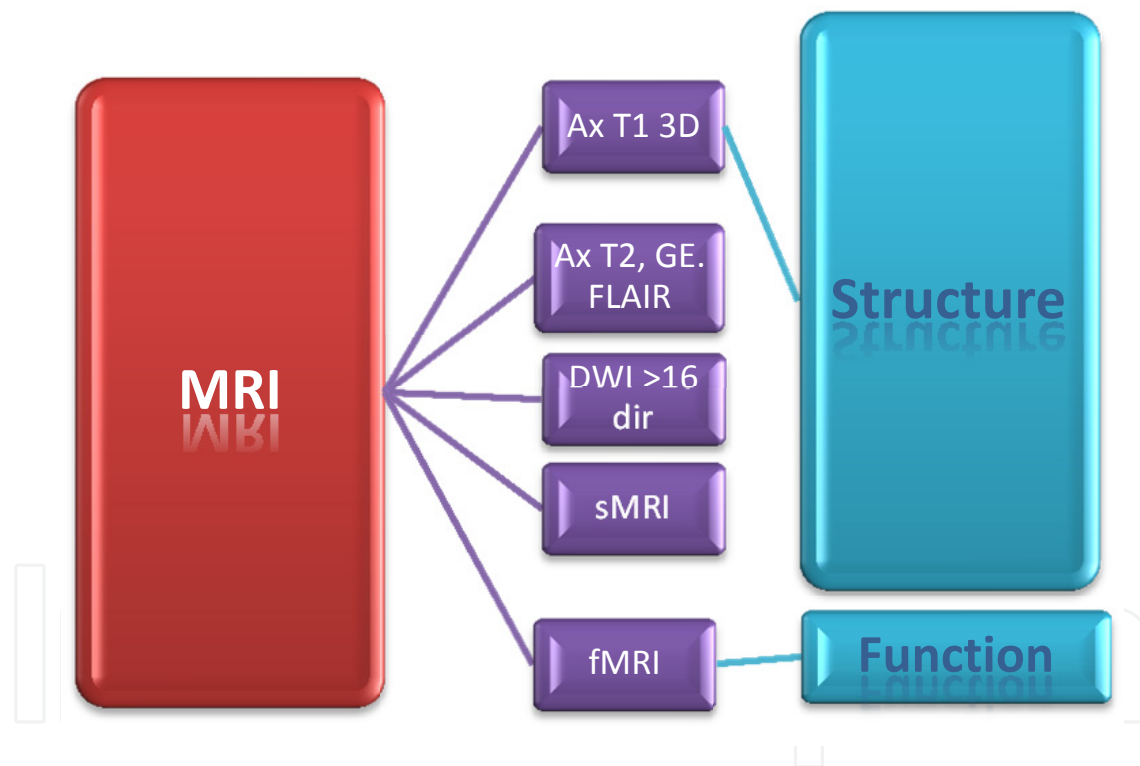


Figure 5. Standard acquisition protocol. MRI: magnetic resonance imaging; Ax T1 3D: axial series T1 weighted 3 Dimensional; Ax T2, GE and FLAIR: axial series T2 weighed, Gradient Echo T2 weighted, Fluid Attenuation Inversion Recovery; DWI>16 dir: Diffusion Weighted Images acquiring in sixteen directions or more; sMRI: spectroscopy MRI; fMRI: functional MRI)

5.1. Magneto encephalography (MEG)

Magnetoencephalography or MEG measures electrical activity of the brain with similar principle to MRI , the Faraday's Law of induction (Bitar, et al. 2006), although registering

and recording data as an electroencephalogram, frequently used to study epilepsy (Moore, et al. 2002) especially those cases refractory to treatment in which hippocampal sclerosis is suspected in view of a potential surgical approach to improve quality of life otherwise seriously compromised (Capizzano, et al. 2001).

It must be carried out in a separate and devoted MR unit, basically formed by a smaller but powerful magnet placed as a helmet in a Faraday's cage in which the subject is exposed to visual or auditory stimuli. Brain activity is registered and recorded with 120 channels according to Faraday's principle stating that every electrical current generates a magnetic field and vice versa. Stimuli are applied repeatedly throughout time with predetermined intervals.

Immediately before or after MEG the brain of the subject is studied with MRI to assess neurostructures. On both exams the subject wears the same set of external markings in predetermined areas, usually nasion, occiput, temporal areas and vertex, which permit adequate post processing for image fusion, locating with precision where signal originates. Therefore, the exquisite spatial resolution of MRI and the reliable electrical signal from neural structures are depicted showing both, structure and function, usually color coded in a background of grey scale neuroimages, 2D or 3D.

5.2. Functional MRI (fMRI)

fMRI is carried out with an additional pulse sequence based in magnetic susceptibility effect that measures the rate of consumption of deoxy-hemoglobin (Chavand, et al. 2009) in a given amount of tissue. Magnetic susceptibility effect is obtained on T2* series, gradient echo pulse sequences with no rephasing pulse (Bitar, et al. 2006), what makes it particularly useful to detect elements distorting the magnetic field such as iron particles, being also sensible to other elements as calcium; it marks hydrogen distribution alteration in the vicinity of atoms from elements such as those mentioned before. Since changes can be detected rapidly varying throughout time, this is particularly useful for functional studies. Iron in the molecule of hemoglobin makes it prone to be detected. Its changes from deoxy-hemoglobin to oxy-hemoglobin indicate oxygen consumption by the brain tissue, a direct marker of activity. Nuclear medicine studies based in administering glucose marked with radiotracers, although exquisitely sensitive to mark metabolism, do not offer an identical temporal resolution to detect rapidly changing effects in the studied tissue, which makes it not suitable for functional studies (Jäger, et al. 2002).

Image fusion with neurostructural series does not require additional markings and the subject completes all required steps of the exam, morphology, biochemical, connectivity and functionality assessment, at the same time and in the same unit (Korvenoja, et al. 2006) without changing position, what enhances its advantages. Fusion can be thoroughly carried out without as many precautions required in other techniques. If a break is required in between series localizers must be obtained again and -preferably- an additional set of 3D for a precise image fusion.

There is an increasing tendency to study subjects with fMRI also in the steady state, not applying external stimuli, not only as a baseline series but also as a non-stimuli mediated investigation in normal and abnormal clinical scenarios. Steady state is a condition of equilibrium in between longitudinal and transverse magnetization when the subject is placed in the isocentre of a strong magnetic field (longitudinal magnetization), while receiving an external radiofrequency (transverse magnetization). Although applicable to other MRI concepts and sequences, for fMRI represents a relaxed, basal state hence suitable to be registered as a baseline and to compare with post stimuli registrations.

6. Stimuli

Paradigms can be predesigned in clinical settings by vendors of fMRI packages and workstations (Smiths, et al. 2006). Usually visual and auditory stimuli are applied in bursts with no stimuli gaps in between while the sequence is running. The patient has to respond generally by pressing buttons or by doing the required action. Pre exam discussion and subject cooperation play an essential role in these series. They do not significantly vary in either implementation or in interpretation if compared with other functional techniques (Faro and Mohamed 2006).

7. Post processing

Post processing encompasses all the processes taking place once data collection has been successfully carried out, usually on independent consoles or computers. It means transforming the available data to obtain further information. When applied to neuroimaging, post-processing purposes comprehend 3 dimensional image rendering, segmentation, evaluation of the cortex, evaluation of neural tracts and fusing images.

Segmentation, the process discussed in the following paragraphs, intends to thoroughly assess morphology of Central Nervous System in the endocranium, to measure volumes of grey matter (GM), white matter (WM) and Cerebro-spinal Fluid (CSF) with accuracy, precision and convenience in the totality or in the segments forming the CNS; and to assess the brain cortex. These objectives may vary among protocols, clinicians and researchers, depending on the clinical setting or type of research; their importance could be similar sometimes; or some could predominate over the others.

Neurostructural analyses are based in post processing of images carried out in independent consoles, usually by members of the research team. Workstations may be basically vendor provided or set by the clinical-research team. The former are usually designed by the vendor and less flexible, although straightforward and stable, i.e.: not prone to system crashes. The latter needs careful considerations regarding hardware, software and an adequate learning curve to maximize results. However, they have the remarkable advantage of flexibility. Hardware requirements are important for intended calculations of complex matrixes need to be considered. Most of the selected software programs explicitly mention minimum needs. PC, Linux and Mac computers are successfully used to run these calculations offering

stable conditions to work through, satisfactory image rendering, good masking, segmentation and fusion options.

Quantitative magnetic resonance imaging of the brain to measure intracranial structures has become an essential clinical tool to evaluate and accurately assess both normal and abnormal individuals and a myriad of neuropsychiatric conditions in common practice permitting measurements with standardized techniques and procedures (Tofts 2004)

Volume loss of the brain versus age, involution changes and gender versus age, perinatal and childhood neurological anomalies, neurostructural analyses in patients with dementia (Krüger, Bertoni and Curran 2011), mainly those with Alzheimer's disease, frontotemporal dementia and Pick's disease, schizophrenia, subjects with refractory seizures and suspected mesial temporal sclerosis and those undergoing functional examinations of the brain to fuse images, tend to constitute the some of the groups of individuals more commonly examined with this technique.

Source images will almost invariably be in DICOM format (Digital Imaging and Communications in Medicine), which is computer language universally adopted and accepted in radiology imaging departments, standard for handling, storing, printing and transmitting information in medical imaging. The importance of DICOM lies not only in its universal applicability but also on the implied processes. DICOM archives are not only original processed signal or attenuation from the studied object as it was acquired but also much more information which is not part of the image but labels it adequately.

Once the study has been done, the images are transferred onto the DICOM archive and post-processing consoles can open them as part of the DICOM protocol. At this point, if an independent system is being used rather than a vendor provided one, images are to be downloaded to a separate archive. Studies have to remain completely anonymous, coded and transformed to formats suitable for post processing. It is essential to leave images with no traces of identifiable information but only a heading code to distinguish cases. Masking the identity of the subject data in DICOM images requires at least a reasonable degree of knowledge and expertise to be carried out effectively and to a bioethical acceptable extent. It must be remembered that DICOM archives are not just images but more complex collections of information, which may translate in images hence, extra care should be paid to these processes.

For segmentation, in general, the useful set of source images is provided by a high-resolution fast three-dimensional gradient echo T1 or T2-weighted pulse sequence (Jaume 2009). These series tend to produce a range of 160 to 180 slices which can be post processed on DICOM or other data formats, according to the software in use, to perform segmentation and sub segmentation of structures allowing appropriate volume measurements that can be carried out in manual, semi-automated or automated manner. Images should be formed by voxels of 1 or 0.5 mm with no gaps in between slices, which provide a satisfactory data collection for further three-dimensional image. A second likewise essential element to consider is to achieve the best possible signal intensity grey-white matter differences leading to a correct segmentation (Abidi 2012)

There is a range of post-processing software with several excellent programs, academically available at no cost, developed by reputed research centers, universities and institutes, including FLS, Surfer, Brain Suite 2, MIPAV (McAuliffe 2012) and Slicer, among others. Most of them are explicitly downloadable not for clinical but for research purposes. Their relevance, usefulness and reliability may vary. It is crucial that the team members become familiar with them perhaps using one as the main post processing tool and a second one to validate. Platforms they operate on are PC, Linux and Mac.

7.1. Source images

Several centers and authors prefer to change DICOM to other formats assigning them a coded number to identify and to differentiate between studies without breaching confidentiality of both, subject and institution, a legal and ethical bond which must be always carefully observed. This conversion usually facilitates building up and maintaining an appropriate database. Usual image formats could be ANALYZE™, hdr, img, nii, NIfTI and several others as [seriesname].mmr in the case of files created with Slicer.

Most databases can usually swap and convert from one format to another although extra care should be paid not only to avoid losing information in the transformation but also to check anonymity details and potential left-right flipping which is not infrequent and may well go undetected for a while as it happens with organs following mirror corresponding parts on both sides with only a subtle *petalia* principle to help differentiate dominant side (Chang Chui and Damasio 1980) not usually picked up by the untrained observer. At this point, it is useful to consider an internal validation process, where the research team performs segmentations in several known control subjects under the formats to be used, comparing consistency and results. Calculations could be carried out in validated cases such as the ones available on LONI. Once consistency is proven and taken for granted, the method should be continued without significant changes until new validations take place.

7.2. Alignment, homogenization and skull erosion

Homogeneity and orientation have to be checked initially. The former can be corrected in some software programs as MIPAV. For alignment purposes, the axis from the anterior to the posterior grey commissure is essentially anteroposterior. The one extending throughout the inter-hemispheric cisterns determines the sagittal orientation plane. When possible, the plane extending from one petro-mastoid ridge to the other marks the third plane to align volume properly. If marked asymmetries of the brain and skull are present, automated segmentation techniques will not be reliable and should be carried out manually or in a semi-automated manner with constant visual check throughout the whole procedure.

Then, skull erosion has to be carried out [Fig 8] deleting structures from the skin to the inner aspect of the *duramater*. Some software programs require these steps explicitly giving some choices when it comes up to erode the skull. Some others carry out the process just naming it in the background although in all of them results tend to be equivalent. An important factor to be kept in mind here is a thorough visual inspection of the resulting surface; not

infrequently islets –on occasions more than islets- of high signal intensity soft the process of erosion run by the program cannot adequately delete tissues and, if small volume changes are determined, results may be altered significantly leading to error. Visual analyses, expectably by someone trained in neuroanatomy, are essential to take for granted that segmented and measured volumes do correspond to the anatomical structures they are meant to correspond.

7.3. Standardization of images

The obtained volume must be aligned properly in all three axes of space, which could be done automatically or manually. The latter requires marking structures as anterior and posterior commissures and inter-hemispheric cisterns. Marking the most anterior, most posterior, superior, inferior and each one of both laterals as Talairach coordinates, essential to confine the atlas voxels to the intended area, carries out further standardization. Images must be taken from source to AC-PC (anterior commissure to posterior commissure) alignment and then to Talairach space to apply the voxels. Once done, images are taken back to AC-PC alignment and then back to normal to calculate volumes of each structure. This process is explained in detail by MIPAV (Medical Image Processing, Analysis and Visualization) (McAuliffe 2012)

7.4. Masking

Further steps vary significantly depending on the type of program. MIPAV gives choices to segment with automated masking providing both images and figures with the results. Others run similar calculations with no visual representation in which case, visual control of each step is required [Fig 6].

Masking is reducing tissue signal intensity differences to three or four main ones following mathematical algorithms, which narrows ranges between different magnitudes as having a rather quantic scale rather than continuum-like one. Grey-white matter and CSF segmentation can be however carried out without masking, by selecting the range of signals the measured tissue presents statistically an expression of mean signal plus minus 1 standard deviation. Cases with normal volunteers tend not to represent particular difficulties. However, some cases with atrophy and/or conspicuous changes in signal intensity due to pathology require manual calculations and careful slice-by-slice evaluation of these processes in order not to miss register results. Masking on CNS images follows mathematical algorithms on images to obtain two, or three signal intensity differences corresponding to grey, white matter and cerebrospinal fluid, which try to mimic gross specimen stains in pathology to help with visual discrimination of tissues in CNS. If the studied subject has no obvious signal intensity alterations in grey matter forming hippocampus or amygdala, this could help. Usually, masking could lead to consider some of the temporal cortices adjacent to the amygdala in the resulting final image, to be carefully checked case by case. Validation between manual and automated measurements is a useful step to carry out to gain certainty especially before considering studying extensive cohorts.

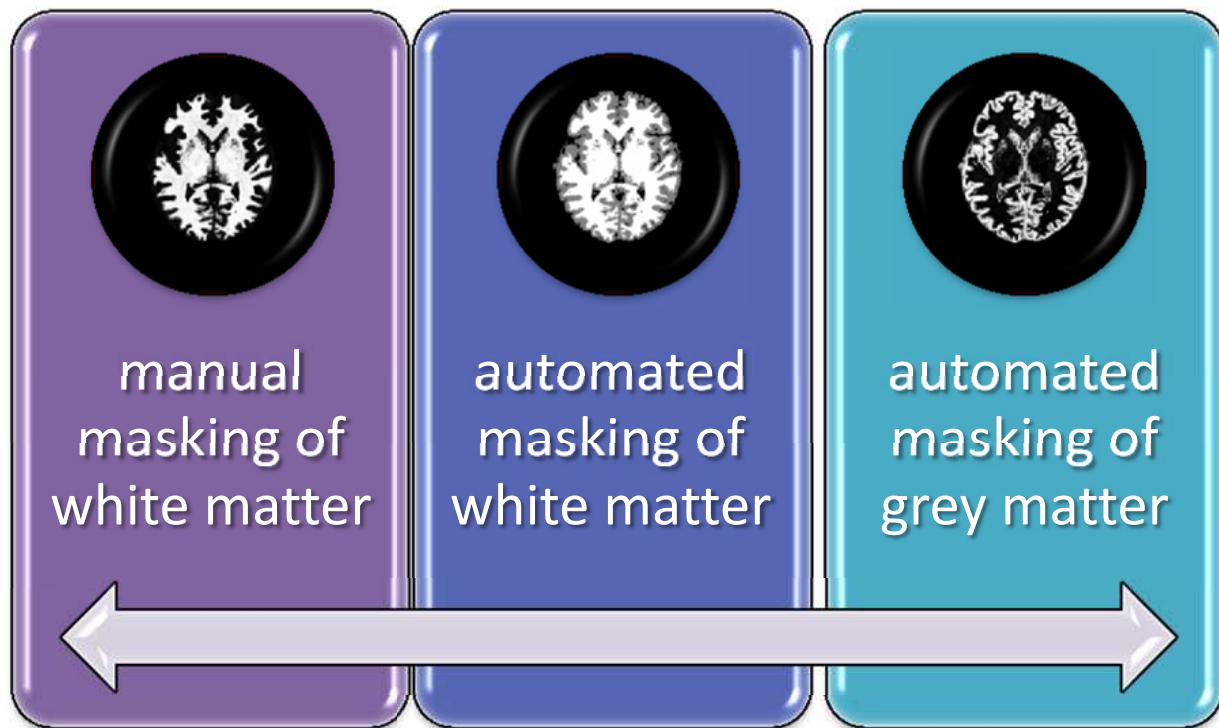


Figure 6. Masking for initial gross segmentation in grey matter, white matter and CSF

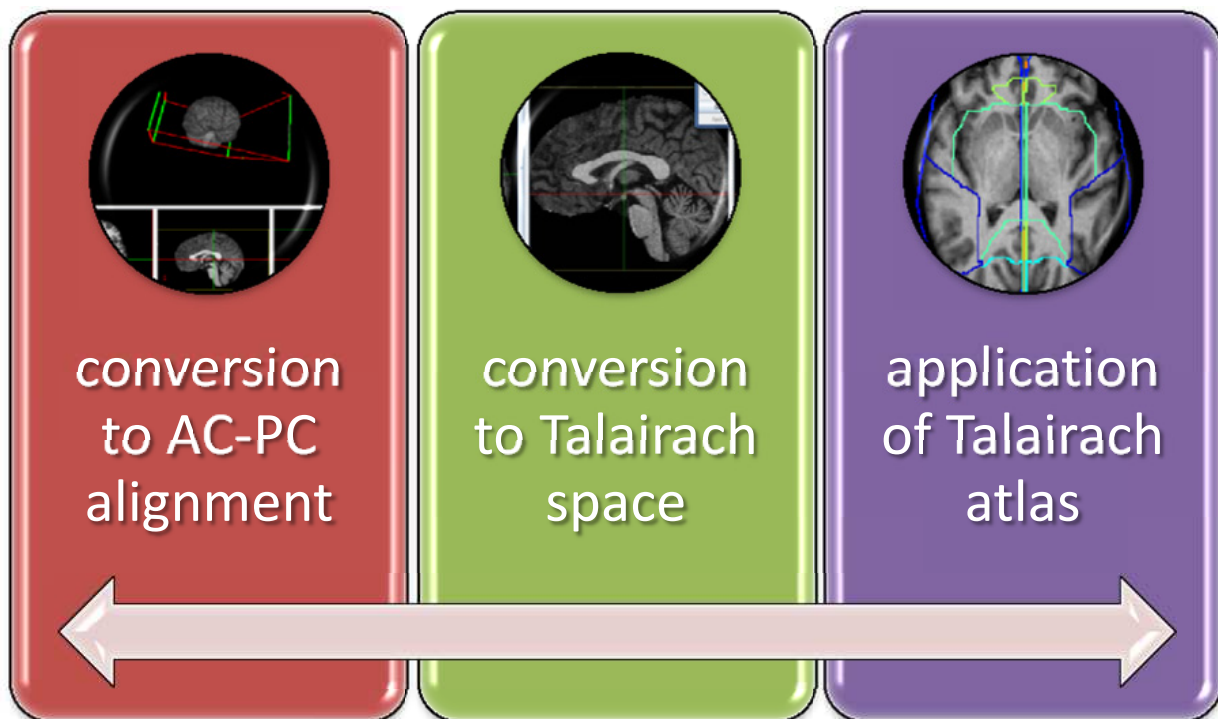


Figure 7. Alignment to anterior commissure-posterior commissure plane and further transformation into Talairach space before applying the atlas

3.2.5. Sub segmentation can carry on following an atlas, which usually is a validated one such as ICBM [Fig 9], Talairach, Talairach-Tournoux, Montreal, or even institution designed, the latter for as long as a rigorous validation process has taken place. Atlases will provide predetermined boundaries applied with precision from the central planes starting in sagittal, axial and coronal landmarks. Boundaries permit to measure volumes determined by signal intensity within the selected VOI (volume of interest), for automated processes [Fig 7]. Semi automated processes are based on moving the cursor throughout the slice to obtain graphs of isosignal intensity like isobars on a map. The observer accepts what he thinks is reasonable. Manual segmentation implies marking contours slice-by-slice measuring, as the other methods, signal intensity voxels within an accepted range. The latter is very useful in cases of frank signal intensity anomalies such as mesial temporal sclerosis affecting hippocampus and amygdala in which following Jack method (Jack, et al. 1992) is very helpful to determine a cutoff plane to divide parahippocampal gyrus from the hippocampal-amygdala complex. However, sub segmentation using Talairach atlas at an appropriate level carries it out automatically.

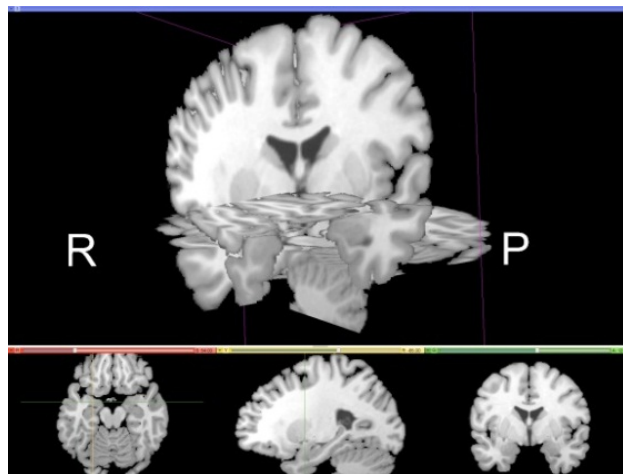


Figure 8. Segmentation with Slicer: skull erosion has already taken place

The study of the temporal lobe has been typically carried out in clinical settings for the study of refractory epilepsy, in most institutions, or all epilepsy cases in some others although tertiary centers do perform it as part of many other protocols with routine 3D acquisitions. High resolution T2 weighted images in coronal plane perpendicular to the Sylvian fissure are reliable to study signal intensity changes of the amygdalae and hippocampi, an essential assessment before attempting to carry out automated segmentation of the limbic system. It must be kept in mind that automated segmentation procedures are based on signal intensity differences. If the latter are not statistically significant any masking or not masking segmentation process may well be not reliable in which case it is better to carry out manual segmentation.

7.5. Manual segmentation

Carried out slice by slice by tracing contours of the amygdala, is the safest procedure when the researcher is trained enough in anatomy and imaging. (Bertoni 2010).

Any plane can be selected to depict the amygdala which, given its rounded-ovoid shape, permits a good delineation of boundaries in almost all possible planes, axial, coronal, sagittal or oblique, to be used on manual segmentation method. However, segmentation must be carried out following the same plane throughout the manual procedure making sure that progressively increasing number of contiguous slices, contours are traced from the start to the end on each side. If further certainty is needed, the same researcher can calculate again following a different plane to check if results do match. Some structures are easier seen for segmentation purposes on one or two of all possible planes, which is influenced not only by researcher decisions but also his training and experience manipulating anatomical images. The hippocampal –amygdala complex volume calculations are usually carried out on oblique coronal images in most of the non-3D acquisitions to minimize boundaries determination errors on 2D slices, although at the same time, it implies that more slices are required to cover the whole of the anatomical area to be fully depicted. However, slices in the axial oblique reformatted plane from 3D source images tend to be much more useful in these regards being able to depict the entire hippocampal structure in fewer images (Bertoni 1998). Inter-observer and intra-observer variability are useful parameters to assess consistency of measurements as an initial evaluation of robustness of the method selected by the team.

7.6. Semi automated segmentation

Almost all the available post processing software programs permit to detect lines by highlighting sequences of points of identical signal intensity when the cursor is dragged on each image. Once the appropriate contour has been selected a click is usually enough to freeze it. Again, as it must be carried out in non-automated segmentation procedures, following the same plane throughout the calculation is essential.

7.7. Automated segmentation

Automated segmentation is based on the application of atlases on sets of images sequentially arranged, usually named “stack images” which the operator can scroll at will. Before applying any atlas, the operator must make sure all the required conditions are met to assure an adequate, precise, reproducible and thorough process in order to validate the results with certainty.

The basic principle to apply an atlas is to run a process of signal intensity measurement for which the source images must be of the highest possible quality, 3 dimensional, isovolumetric of not more than 1mm³, properly aligned and oriented, among other important conditions mentioned before.

In applying the atlas, the observer measures a volume of densities within a range (not masked) or a precise mathematical value (masked). Independently of choosing to calculate volumes from masked or unmasked pictures, images should be checked one by one before running the calculation, preferably by more than one observer to grant consistency before running any other subsequent validation method.

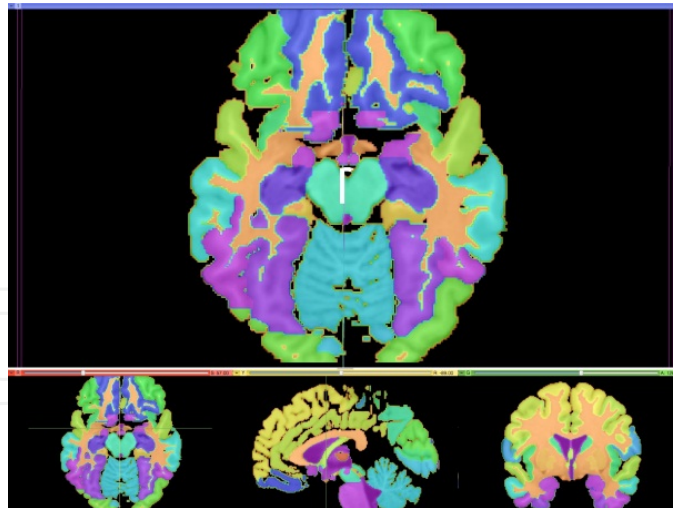


Figure 9. Segmentation and subsegmentation applying ICBM atlas on Slicer

A strong validation process usually results in publishing an academically available atlas. They vary according to the institution, research or clinical team, and author. Useful ones are Talairach, Talairach-Bazin, ICBM and Montreal, among others. There are normal and disease-specific ones. Their applicability may vary with the software program in use; the researcher should consider becoming very familiar with at least one, validating a reasonable number of cases by calculating structures with manual, semi-automated and automated procedures to gain certainty, skills and accuracy before trying to segment cohorts of larger numbers.

Some of them are intended to volumes and some are intended to neural tracts. All academically available segmentation software programs permit to carry out calculations based in atlases developed by the researcher which must fulfill the adequate internal and external validation requirements to be confidently used. Although in the past only real dissections from post mortem specimens were doable and acceptable, technology available today permits to carry out in vivo dissections from source radiological images, which have also become the accepted source to demonstrate the whole of the neural structures including connectivity, mapping and functionality (The Human Connectome Project) (LONI, UCLA 2012). Their essential principle is to apply volume of interest limits, slice by slice throughout the 3D series, to then measure signal intensity differences inside the selected volume. A proper alignment usually according to anterior-posterior grey commissures, inter-hemispheric cisterns and skull base-petrous planes are of crucial importance.

It is important to consider assessing selected images not only mathematically but also visually; signal intensity differences may lead to potential error if contours are not checked slice by slice throughout the entire selected volume. It is essential to consider that software programs can run mathematical calculations with precision; however, the appropriate determination of boundaries to avoid including unwished pieces of tissue which would lead to incorrect results, remains as a fundamental responsibility of the operator (Chen 2006).

The Talairach atlas (Nowinski, et al. 2005) offers a robust and validated process of segmenting and subsegmenting CNS structures, bilaterally or unilaterally. Once initial steps have been

carried out including homogeneity check and skull erosion, an appropriate alignment according to anterior commissure (AC) to posterior commissure (PC) plane completed with selection of two points in the interhemispheric space to ensure a plane, after which Talairach coordinates are set to mark the most prominent points anteriorly, posteriorly, superiorly, inferiorly and on both sides. Images are taken from the source set to AC-PC plane and from it to Talairach space, mathematically created, normalized, to permit placement of different volumes of interest (VOIs) as each atlas level permits. As the VOIs are placed, the researcher must ensure no segment is left outside the area of interest, going through the whole set of images to check image boundaries. Then images with applied VOIs must be taken back to AC-PC and from it to the source set of pictures with all selected VOIs appropriately copied. Volume calculations for each VOI are then matter of selecting the appropriate signal intensity for grey or white matter in each volume. If the region selected, for instance, includes the amygdala, by selecting a signal intensity corresponding to grey matter, all those 1 mm³ voxels having grey matter signal will be included in the result leaving aside all those with white matter signal. Signal intensity ranges for unmasked images (ie: 0-100 for cerebrospinal fluid, 150-250 for grey matter and 400-600 for white matter), or values for masked ones (1 for CSF, 2 for grey and 3 for white matter), need to be entered manually to determine each result in each voxel. This is what provides volume values for neural structures, voxel by voxel. If selected voxels include the whole of the boundaries of the brain, then segmentation values will show the totality of CSF, grey and white matter for the whole of the brain. If each hemisphere is selected and the voxel boundaries have been correctly set, each hemisphere, then results will be for each one of them. The same applies when subsegmentation takes place to determine volume for each lobe, region, subcortical structure and even Brodmann areas, which can be calculated if level 5 segmentation is selected when using the Talairach atlas.

Unlike other imaging procedures based on attenuation of energy measured in standardized units with calibrated devices such as X Rays, computed tomography or ultrasound, MRI signal originates in resonance of hydrogen atoms placed in an external magnetic field and therefore intensity values for each pixel or voxel can vary from MR unit to MR unit, from one subject to the other or even within the same subject if his temperature of hydration change (Ramani, Hensen and Helpert 2006).

If multiple regions (several VOIs) are selected to calculate volumes from all of them simultaneously, the researcher should keep in mind the computing resources in use and the computer memory allocated to the calculation. More modern software program versions tend to be much more stable than earlier ones; likewise, more powerful computers and improved platforms result in a smoother and successful calculation. In any case, some extra care should be paid to these details since this is sometimes the trickiest step in automated calculations. Validations against valid calculations or databases are similar demanding computer calculations.

ICBM atlas is other powerful resource to be considered by the research team. It runs smoothly on Slicer where fusion with uploaded volumes, subject-by-subject, result in an extremely useful step to facilitate correct VOIs placements. Calculations are then straightforward processes.

Independently from the chosen software program and for as long as the researchers are familiar and skilled operating it, if computing resources are adequate and internal-external validation processes take place, results will tend to be reliable, reproducible and consistent.

A careful analysis of all these factors will be of help to assess inconsistencies in results reported in the literature perhaps due to differences in cohorts numbers, field strength of the MR units, type of equipment, acquisition and calculation techniques.

Factors which may favorably influence robustness and confidence in segmentation results are the increasing availability of super high field MR units, larger cohorts of studied individuals and better software programs.

Several neuroscientists, authors and clinical researchers have carried out segmentation and functional studies of the amygdala with a diversity of purposes producing a very useful framework to consider. The study of the amygdala is of increasing interest to try to understand its role in cognition, impulses modulation, its participation in mood regulation and many other important aspects in the neurosciences field.

Neuroimaging the amygdala in the clinical domain has been more extensively dedicated to study its role in epilepsy, especially in refractory epilepsy causing dramatic alterations in lifestyle, therapeutic approach and outcome. Refractory epilepsy of medial temporal origin usually implies a thorough assessment of the amygdala-hippocampus complex because if a surgical therapeutic approach is accepted, resection of both structures take place aiming to improve quality of life.

Other important source of interest in the amygdala in the clinical domain originates in psychiatry. Cognitive impairment, memory problems and dementia constitute an increasing public health problem given today's extended life expectancy with its deep impact in individual, familiar and social life.

Clinical research concerning segmentation of the amygdala was initially based only on manual segmentation processes. They are very useful and accurate especially if there are signal intensity anomalies in the limbic system. They are also useful and accurate to study the hippocampus due to its intricate anatomy, and the amygdala given its proximity to the hippocampal head. The location of these two structures, not only adjacent to one another but also to the rest of the temporal cortex of the uncus and parahippocampal gyrus tend to be difficult to solve when volume calculations exclusively rely on automated segmentation processes. These calculations need to be corroborated manually to confirm consistency of results.

Volume changes have been found on a myriad of normal and abnormal conditions including mood disorders, schizophrenia, attention deficit disorder, depression, attention deficit disorder, anxiety and obsessive-compulsive as the main although not the exclusive entities linked to neurostructural changes, biochemical and functional alterations (Goodwin 2000).

Familiarity with the anatomy, connections, neural biochemistry, functionality, MRI, image archiving and manipulation, software use, atlases application, image fusion to study morphology, biochemistry, connectivity and function are essential aspects to increase certainty, accuracy and testing results when studied cohort increase in number and quality.

A useful way of starting a robust process of segmentation is by carefully building and checking the database for image quality, noise and type of archives. DICOM format will always tend to be the most useful and universal, although perhaps not the most convenient for further post processing.

When the set of stack images has been selected, it should be copied from the archive to an ad-hoc folder in the computer in order to be used for calculations. Images must be checked one by one, usually in the range of 140 to 200. Axial plane is the most convenient to apply atlases on MIPAV such as Talairach, due to the original work carried out on Fleischig-type axial brain slices. ICBM as other atlases however, can be used in all three directions on the same screen simultaneously as it happens using Slicer.

Image noise, its homogeneity, positioning corrections and slice selection to include only slices from the base of the skull to the convexity are preliminary steps to be followed to grant a good quality control. Image preparation must be carried out independently to the software in use. The voxel dimensions and eventual resizing must be considered. In Mac environment, Osirix™ offers capability of resizing and rendering in a selected plane if source images have been obtained in a different plane or with a different voxel dimension. MIPAV offers that capability and can be conveniently implemented on PC.

Skull and soft tissue erosion usually represent the following step. Extra care should be paid to check results image by image in all three planes. Sometimes islets of fatty tissue are not satisfactorily removed due to their high signal intensity and positioning (as base of the skull, occiput or convexity) altering results, especially if little volume changes need to be detected. These cases do require manual removal because further erosion will probably delete noble tissue, necessary for a correct calculation.

3D sets of images can subsequently be manipulated to study the surface of the brain identifying gyri and sulci with remarkable detail. Further reorientation is necessary for an adequate segmentation aligning the whole volume in the anterior commissure to posterior commissure plane to then mark Talairach coordinates, formed by the most superior, inferior, anterior, posterior and lateral planes.

It is safer to combine a sufficient number of studies doing two segmentation methods, for instance manual and automated, to look for consistency of results before validating them with external and more extensive databases.

Independently of these steps, a careful direct observation and measurement of signal intensities throughout the limbic system should be carried out before applying atlases in automated calculations to make sure measurements and boundaries delineation are consistent since they rely on signal intensity differences between adjacent areas.

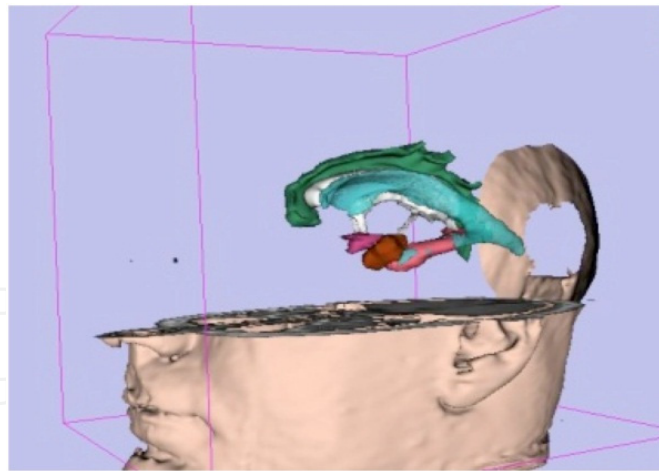


Figure 10. Subsegmentation of the amygdala with Slicer and ICBM atlas. The amygdala is shown in purple; hippocampus pink; pericallosal gyrus in green; corpus callosum in white; ipsilateral ventricle in light-blue

Manual measurements appear to be the safest method in present conditions to confirm that what we delineate and measure correspond to what the research team has intended to. Axial-oblique slices parallel to the largest head-tail hippocampal axis are a reliable method to manually delineate the amygdala, tracing its contours and studying its signal intensity (Bertoni, Sclavi and Sauer, Volumetry of hippocampus and amygdala with magnetic resonance imaging 1998). It is also a useful check up for consistency of results, randomly or systematically, in automated methods of segmentation (Bertoni and Sclavi, Isovoxel based morphometry of hippocampi and amygdalae: a comparison of manual and automated volume measurements 2010).

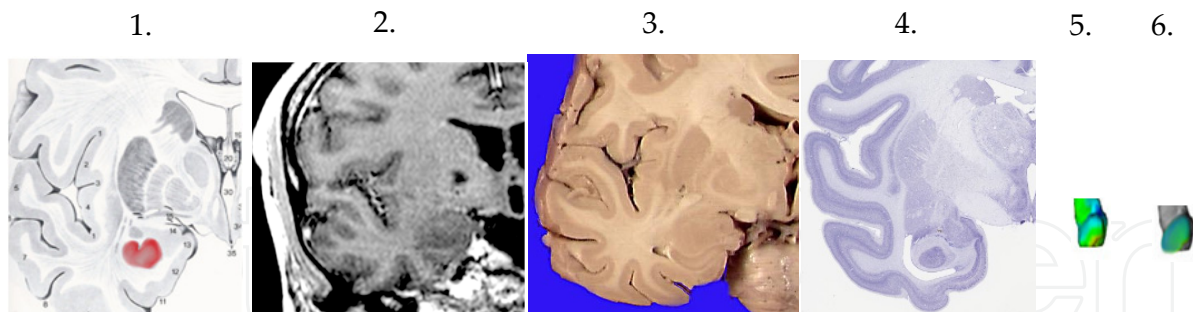


Figure 11. Right amygdala; coronal slices correlating atlas section, MRI, gross specimen, stained gross specimen, 3D reformat with part of the hippocampus and just the amygdala, with contours smoothed by the software

(1) Modified from Nieuwenhuys (Nieuwenhuys, Voogd and van Huijzen 1988); (2) coronal reformat MRI from source axial images; (3) gross specimen; (4) stained gross specimen, brainmaps.org, US; (5) Modified from Timoner (Timoner, et al. 2002)

Thorough assessment of signal intensities in the region of interest becomes essential in subjects with underlying neurostructural abnormalities. It should be remembered that in some cases of medial temporal sclerosis, for instance, as other neuropsychiatric conditions, a frankly abnormal range of signal measurements are obtained in areas of intricate anatomy.

Manual measurements provide an adequate method of reassurance in these difficult cases to avoid miss registration of volumes.

Mean normal volumes of the amygdala vary according to different authors, probably due to studied cohorts and technique differences. They vary in a range between 0.5 (Bertoni 1998) and 1.63cc (Bickart 2011). These volume variations may be due to differences in calculation procedures, techniques and MR units. Some of the technical details mentioned before concerning image quality parameters as noise, resolution, voxel dimensions, procedure of determining boundaries and validation are not thoroughly mentioned in all of the available research that provide volume measurements. Some series, however, include more extensive cohorts and therefore seem to give firmer bases to consider results. In any case, to study more extensive cohorts with strict quantitative mechanistic protocols and thorough validation processes are still due.

Disorder	Structural MR	DTI	fMR	sMR
Schizophrenia	1.Decreased frontotemporal and cerebral volumes, increased ventricular volume 2.Cortex thinning	Decreased prefrontal bundles, uncinate fas and corpus callosum	Decreased prefrontal, cerebellum and striate activity	1.Decreased NAA in prefrontal and cingulate 2.Increased Glu in prefrontal areas and hippocampus in risk subjects
Bipolar disorder	Increased T2 hyperintensity in white matter	Decreased prefrontal and corpus callosum bundles	Decreased prefrontal and cingulate activity	Decreased NAA in prefrontal
Major depressive disorder	Decreased prefrontal cortex, anterior cingulate, medial temporal and basal ganglia volumes	Decreased prefrontal bundles	Decreased cingulate, prefrontal and amygdala activity	Decreased NAA in prefrontal and hippocampus
Anxiety disorders	Decreased hippocampus	Decreased cortico striato cortical circuit	Decreased prefrontal and hippocampal activity	Decreased NAA in striatum
Attention deficit disorder	Decreased anterior cingulate, cortex thinning	Decreased frontostriatal bundles	Decreased prefrontal activity	Increased Glu in prefrontal and striatum

Figure 12. Reported findings in major psychiatric conditions (Agarwal, et al. 2010)

The increasing knowledge in the field of psychiatry has promoted more adequate, thorough and precise neuroimaging assessment of subjects with different conditions. Moreover, continuous updates on information permit to conceive systematic neurostructural studies to assess normal and abnormal subjects. These include detailed neurostructural analyses, whole brain and limbic system segmentation including the amygdala, extensive tractography for connectivity purposes, in vivo spectroscopy and functional studies in both, the steady state and post stimuli.

Multiple morbid and abnormal conditions are linked to amygdala volume and functional changes, including bipolar, depressive and anxiety disorders, schizophrenia and several others, among some of the most cited entities in which this limbic nucleus shows volume alterations, biochemical anomalies, altered neural connectivity and abnormal functionality in the steady state or after specific stimuli (Agarwal, et al. 2010).

8. Conclusion

Segmentation and volume determinations of the amygdala are image post processing techniques of increasing importance to assess healthy individuals and subjects with morbid conditions in neurosciences. A large number of studies have reported volume variations of the amygdala in usual physiological adjustment, neurobiology of mood, premorbid and pathological conditions reporting changes in a myriad of conditions. The latter include post traumatic stress, anxiety, depression and obsessive compulsive disorders, among others, probably the amygdala role in the modulation of emotions and sensory pathways. Although some studies have found sexual dysmorphism and different regression volume values of the amygdala depending of age (Bertoni 1998), others seem to suggest just the opposite (Hirai, et al. 2000).

Decreased volumes of the amygdala have been usually linked to almost all those cases with underlying pathological entities, from alcoholism (Thomas et al, 2011) and other addictions to schizophrenia (Wang, et al. 2008, Goodwin 2000).

Whether it be manual, semi-automated or fully-automated, segmentation results should be carried out not only thorough and carefully but also internally and externally validated. The former using at least two methods (manual and semi-automated or fully automated) and two different software programs until to grant consistency. External validation comparing results against more extensive databases is a further step to grant consistency, safer and more reliable results concerning neuroimaging the amygdala to assess its volume, boundaries, connectivity, metabolism and functionality.

Wider availability of high and very high magnetic field MR units, more powerful computing systems, systematic and more friendly quantitative mechanistic determinations and standardized paradigms of stimuli will probably facilitate and encourage further research in the near future to improve our present knowledge of the amygdala increasing the understanding of its complex modulatory functions influencing human behaviour

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