

fMRI Analysis of Brain Refractory Period Activity in Nicotine-Addicted Rat Models

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AUTHORSHIP PAGE

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ABSTRACT

The objective of this study was to use functional Magnetic Resonance Imaging to investigate the brain activity of nicotine-addicted rats when the stimulus was introduced during the refractory period. The results confirmed that regions of the brain normally activated by nicotine are relatively inactive during the refractory period. These results coupled with other studies of drug reaction can be used in the development of pharmaceuticals aimed at specific regions of the brain or at determining targeted drug delivery schedules.

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CHAPTER 1 - INTRODUCTION

Cigarette smoking, hereafter referred to as “smoking,” is the largest single risk factor for premature death in developed countries. Approximately one fifth of the deaths in the United States can be attributable to smoking with 28% of the smoking-attributable deaths involve lung cancer, 37% involve vascular disease, and 26% involve other respiratory diseases. More than 400,000 deaths per year and 30% of all cancers in the United States are attributable to smoking. If reduction in smoking prevalence were to be observed, morbidity and mortality attributable to smoking would decline in the future. A substantial decline in adult male smoking in the United States was observed from the 1960s through the 1990s. However, that decline has since slowed. The prevalence of current smoking among adults in the United States, defined as smoking daily or smoking on some days, is now about 23% in women and 27% in men [Bergen et al.,1999].

Addiction to nicotine has been established as the psychopharmacologic mechanism that maintains cigarette-smoking behavior. Nicotine activates the brain’s mesolimbic dopaminergic reward system. This produces dependence resulting in physical and neurobiological withdrawal symptoms on abrupt cessation [Bergen et al., 1999].

In order to understand the underlying mechanism of addiction and provide therapeutic treatment to smokers, the neural activities of the brain in nicotine-addicted subjects need to be investigated.

The goal of this project was to analyze and quantify the brain activity level in nicotine-addicted subjects when a dose of stimulus is given during the refractory period. The refractory period of a drug is a period of time after stimulation during which the brain does not fully respond to a second stimulus.

Functional Magnetic Resonance Imaging (fMRI) was used to observe the patterns of neuronal activity across different regions of the brain in the animal subjects. The data was acquired and the images were registered, segmented and analyzed using the Medical Image Visualization and Analysis software (MIVA) and MATLAB. These results coupled with the studies of drug reactions beyond the refractory period can be used in the development of pharmaceuticals aimed at specific regions of the brain or at determining targeted drug deliveries schedules which can improve control efforts.

CHAPTER 2 - LITERATURE REVIEW

Smoking and nicotine

Interestingly, although smokers report that smoking cigarettes improve their mood, anxiety and concentration, they fail to recognize that they may be using tobacco as a means to prevent or treat the unpleasant symptoms of withdrawal such as increased anxiety, sadness, agitation, and worsening concentration. Smoking temporarily alleviates these symptoms but reinforces the cycle of repeated use. The symptoms are best addressed through pharmacological treatments for tobacco dependence. Research studies of cognitive functioning has demonstrated that nonsmokers outperform smokers in nearly all tasks and the “benefits” of smoking seem be restricted only to a modest increase in attention during simple, repetitive tasks [Williams et al. 2004].

Nicotine sustains addictive tobacco use. The essence of drug addiction is loss of control of drug use. Molecular biology suggests that the $\alpha_4\beta_2$ nicotinic acetylcholine receptor subtype is the main receptor mediating nicotine dependence. Nicotine acts on these brain nicotinic cholinergic receptors and facilitates release of dopamine and other neurotransmitters. These neurotransmitters produce pleasure, stimulation, and mood modulation and reduce anxiety and tension. When a smoker stops smoking, a nicotine withdrawal syndrome ensues, characterized by irritability, anxiety, increased eating, dysphoria, and hedonic dysregulation, among other symptoms. Smoking is also reinforced by psychological conditions that urge a smoker to smoke. These include the taste and smell of tobacco, as well as particular moods, situations, and environmental cues [Benowitz 2008].

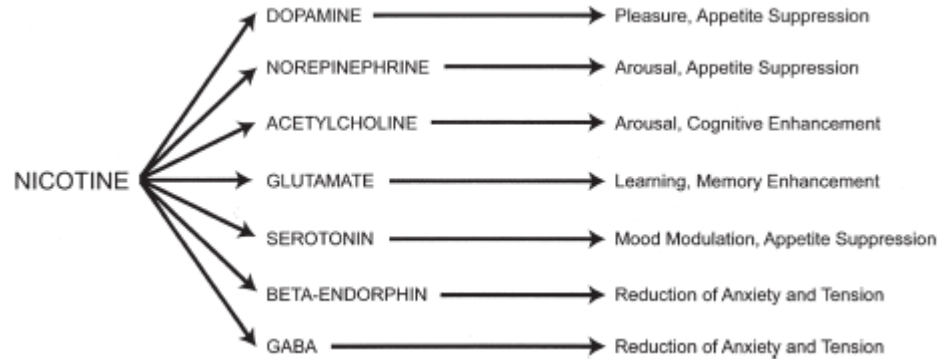


Figure 1 - Nicotine receptor activation promotes the release of neurotransmitters, which may then mediate various effects of nicotine use [Benowitz, 2008].

Nicotine influences the dopamine (DA) release within the mesolimbic system. After nicotine activates the brain's mesolimbic dopaminergic reward system, it produces dependence, which is thought to be the primary neural substrate of motivation and reinforcement associated with both natural reinforcers and drugs of abuse. Nicotine acts as an agonist for neuronal nicotinic acetylcholine receptors (nAChRs)—pentameric ionotropic (Na^+ and Ca^{2+}) receptors found presynaptically throughout the central nervous system (CNS) and postsynaptically in the autonomic nervous system that modulate the release of neurotransmitters and ganglionic potentials [Bergen et al. 1999].

The mesolimbic pathway originates with dopaminergic (DAergic) neurons in the ventral midbrain or VTA. The neurons project primarily to limbic sites including the Nucleus Accumbens (NAcc), Hippocampus (HP) and Prefrontal Cortex (PFC). The NAcc receives projections from the amygdala, HP and PFC and sends projections to the ventral pallidum (VP) and VTA. The HP exchanges reciprocal connections with the PFC. The mesolimbic system and associated brain regions are thought to mediate reward through the action of several neurotransmitters [King, NIH- R01 MH067096-01].

Sensitization

Like other addictive drugs, nicotine induces sensitization in subjects; this means that subsequent doses of this drug produces greater locomotor activity than the initial dose where locomotor activity is defined as the distance moved in time. Current data suggest that the neurological adaptations underlying sensitization begin with the first dose. Following its induction, sensitization persists as a latent state. The activity is normal when nicotine is absent, but when nicotine is re-administered, sensitization is expressed as augmented locomotion. In an animal model of addiction, the distance traveled by the animals in time increases after subjection to each dose of stimulus [Li et al. 2008].

It is important to initially determine whether nicotine sensitization is subject to a refractory period. It is crucial to our understanding of the role of sensitization in nicotine addiction to know it is subject to a refractory period. However, no study has directly evaluated this question. Assuming that sensitization is something a smoker can experience and sensitized responses are blocked within a refractory period, a novice smoker who smokes only a few cigarettes per week might experience sensitization with every cigarette. However, a typical smoker who lights up every 45 minutes might not experience sensitization unless a cigarette was smoked after a period of abstinence, such as overnight. Smokers do report more effect from the first cigarette of the day than the second, even though the second cigarette produces higher nicotine level [King, NIH- R01 MH067096-01].

In previous studies [Li et al.], sensitization to nicotine was assessed in animal models using functional magnetic resonance imaging (fMRI). However after the animals were

fully sensitized to nicotine, the neural activity of the brain within the refractory period was not assessed. The refractory period of a drug is defined as a period of time immediately after a stimulation during which the brain does not fully respond to a second stimulus. After it is determined whether nicotine sensitization is subject to a refractory period, the brain neural activity can be analyzed. To determine whether nicotine sensitization is subject to a refractory period and to analyze the brain neural activity, a behavioral study and an fMRI study need to be performed. It is hypothesized that during the refractory period of the sensitized rat, the areas of the brain will not fully respond to a challenge dose of nicotine. This hypothesis is based on the fact that the nicotine receptors are saturated with neurotransmitters and need time to recover before they can react to more nicotine.

The expanding use of fMRI in this project demands an understanding of the underlying MR principles to glean the most out of the modality. In the next section, we review briefly the basic principles of fMRI.

Magnetic Resonance Imaging (MRI)

Understanding the underlying mechanism of addiction requires an understanding of the mysteries of the brain. Functional magnetic resonance imaging (fMRI) is entirely non-invasive method with the spatial and temporal resolution to resolve patterns of neuronal activity across the entire brain in the order of 30 seconds [Kulkarni, 2005].

Functional MRI is based on increases in blood flow to the local vasculature that accompanies neural activity in the specific brain regions. It indirectly detects neural activity in different parts of the brain by comparing contrast in MR signal intensity prior to and following stimulation. When an area of the brain has an increased synaptic and

neuronal activity, it requires increased levels of oxygen to sustain its activity. Enhanced brain activity is accompanied by an increase in blood flow and blood volume to this area. The enhanced blood flow usually exceeds the metabolic demand and exposes the active brain area to high level of oxygenated hemoglobin. The Magnetic Resonance (MR) signal is sensitive to the level of oxygen within the tissue. Oxygenated hemoglobin increases the MR signal intensity that can be detected in MR scanner [Ogawa et al. 1990].

The changes in MR signal induced by changes in blood flow, volume, and oxygenation are the basis for fMRI methods. The main functional imaging method used in this study is the Blood Oxygen Level Dependent (BOLD) Technique [Ogawa et al. 1990]. The goal of BOLD fMRI studies is to map patterns of local changes in MR signal in the brain as an indicator of neural activity associated with a particular stimulus such as nicotine [Ogawa et al. 1990].

The Source of the MR Signal

A correct description of what happens when tissue is subjected to a magnetic field relies on quantum mechanics. However, all the theory necessary for MRI can be based on a simple classical model in which certain nuclei that spin around their own axes behave like small magnets [van Geuns et al., 1999].

For clinical imaging, hydrogen is the most frequently used nucleus [Van Geuns et al., 1999]. The vertebrate body is primarily fat and water. Fat and water that make the tissue have many hydrogen atoms and thus approximately 63% of the body is made of hydrogen atoms. [Hornak,1996].

Under normal circumstances, the hydrogen nuclei, the tiny magnets, are randomly distributed in space and their magnetic moments cancel each other out, and thus the net

magnetic vector is zero. However, when the patient is submitted to a strong external magnetic field (B_0) the nuclei adopt one of two possible orientations: parallel or antiparallel to the external field (Figure 2). The energy difference between the parallel and antiparallel energy states is very small; the population ratio is approximately 100,000 to 100,006. A net magnetization vector (M_z) aligned to the external magnet results from the difference between the two populations of nuclei [van Geuns et al., 1999].

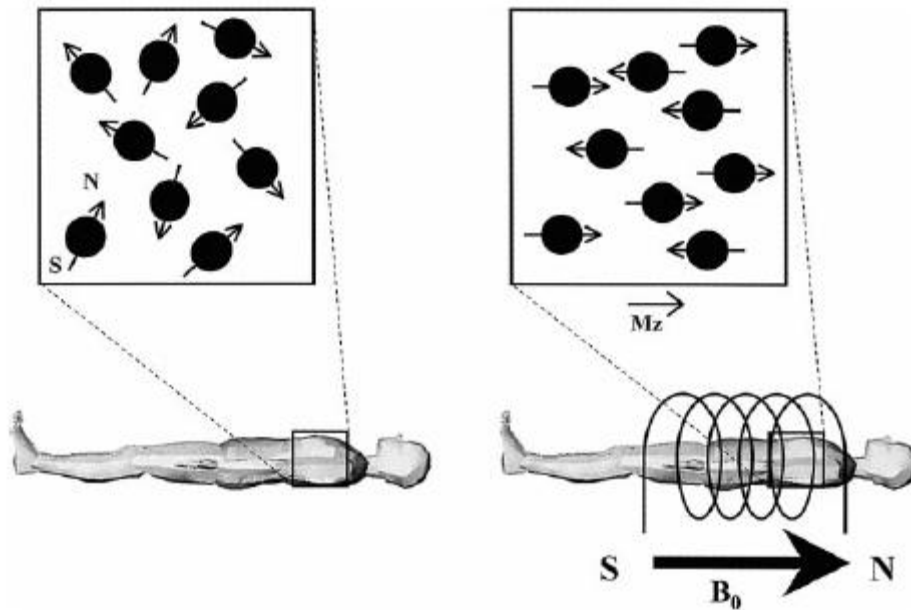


Figure 2 - Without a magnetic field the magnetic moments of the nuclei are distributed at random directions and therefore the net magnetization factor is zero. With the presence of a strong external magnetic field (B_0), the spinning nuclei align parallel or antiparallel to the external field (B_0) with a few more parallel than antiparallel. This results in a net magnetization vector (M_z) parallel to the external magnetic field [van Geuns et al., 1999].

Individual nuclei do not completely line up with the magnetic field but wobble or precess around the direction of the external field (Figure 3). The frequency of this precession is given by the Larmor equation,

$$F = \gamma B_0 / 2\pi$$

Where F is the precessional or Larmor frequency, B_0 is the strength of magnetic field,

and γ is the gyromagnetic ratio of the nucleus. It is of note that the phase of precession around the axis of the magnetic field is different for each individual nucleus [van Geuns et al., 1999].

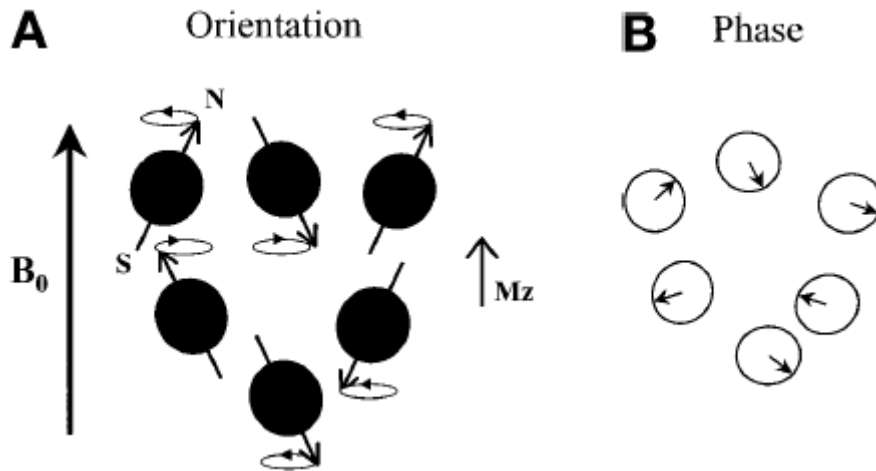


Figure 3 – (A) The individual nuclei spin around their own axes and precess around the direction of the external field (B_0) with an angle. (B) The phase of the precession around the axis of the external magnetic field is for each individual nucleus [van Geuns et al., 1999].

Excitation

The net magnetization vector from the nuclei inside the magnet in its equilibrium state, M_z is static and does not produce a measurable signal. To obtain information from the spins, the precessing spins are excited by applying energy, in the form of radiofrequency (RF) energy pulses with a frequency equal to the Larmor frequency. This frequency is called the resonance frequency. When an RF signal is given at the resonance frequency the protons absorb energy to jump from the parallel state to the higher level of the antiparallel state and the spins are “whipped” to precess in phase. The effect of all this is that the net magnetization (M_z) flips 90° from the positive, longitudinal z-axis to transverse plane (Figure 4) [van Geuns et al., 1999].

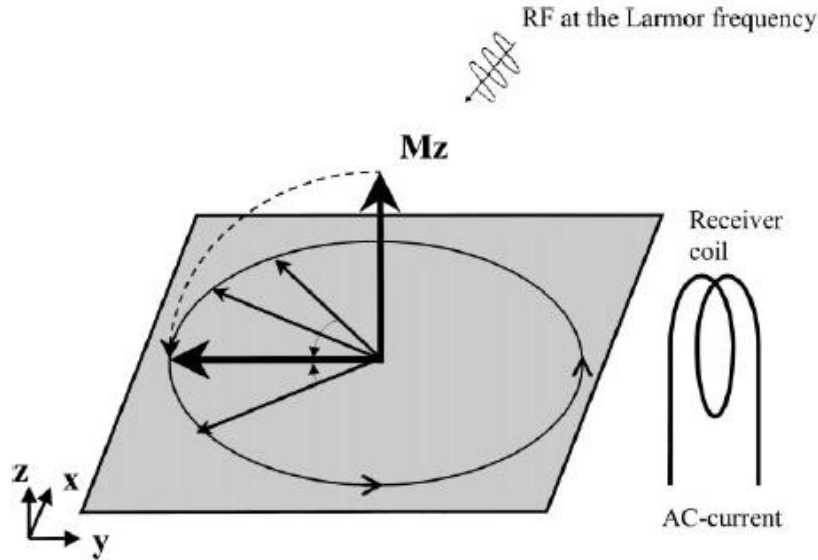


Figure 4 - The net magnetization excited with the RF pulse with the same Larmor frequency, flips 90° and the spins are “whipped” to precess in phase. The rotating net magnetization vector induces an AC in a receiver coil [van Geuns et al., 1999].

The net magnetization in the transverse plane rotates around B_0 at the Larmor frequency. This rotating transverse magnetization can be measured as it induces an alternating current (AC) in the receiver coil placed around the subject [van Geuns et al., 1999].

Return to equilibrium

After excitation, the RF frequency transmitter is switched off and the equilibrium state will be sought. The magnetization therefore decays over time, which is represented by a decreasing magnitude of M_z in the transverse plane. Consequently, the induced signal in the receiver coil will decrease in time as shown in figure 5. This decreasing signal is called the free induction decay (FID) [van Geuns et al., 1999].

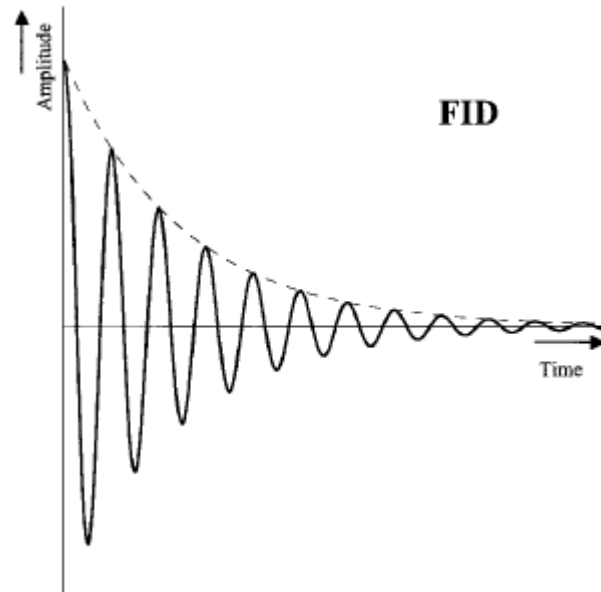


Figure 5 – The receiver coil detects the signal. The FID signal decreases over time when the net magnetization vector returns to its original orientation [van Geuns et al., 1999].

The relaxation time is the time required for the signal to return to equilibrium. Two independent relaxation processes exist: transverse relaxation and longitudinal relaxation. The process of realignment to the external magnetic field is called the longitudinal relaxation process and is characterized by the T₁, relaxation time. The T₁ relaxation time is defined as the time required for the system to recover to 63% of its equilibrium value after it has been exposed to a 90° RF pulse (Figure 6) [van Geuns et al., 1999].

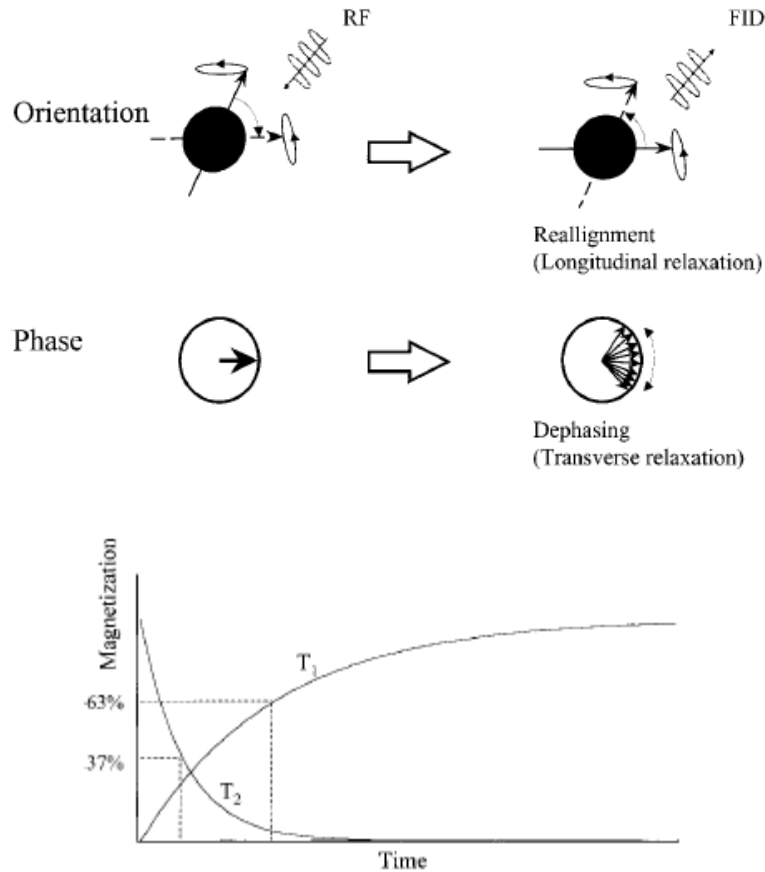


Figure 6 - Longitudinal relaxation (upper row) is the realignment of the net magnetization to the external magnetic field. Transverse relaxation (lower row) is the dephasing of the precessing spins [van Geuns et al., 1999].

The second process of relaxation, the transverse relaxation, depends on the spins precessing around the magnetization vector. Initially, after the excitation by the RF pulse, the spins precess completely in phase. Due to spin-spin interaction in the tissue and inhomogeneity of the main static magnetic field B_0 , the observed signal decreases over time since the spins begin to dephase. This process is called the transverse relaxation or spin-spin relaxation and is characterized by T_2 relaxation time (Figure 6). The T_2 relaxation time is defined as the time it takes for dephasing to decay the signal to 37% of its original value (Figure 6). Various human tissues have different T_1 and T_2 values (Figure 7). T_2 time is always shorter than the T_1 time [van Geuns et al., 1999].

Tissue	T ₁ (ms)	T ₂ (ms)
Skeletal muscle	870	47
Myocardium	600	40
Liver	490	43
Fat	260	84
Blood	1,210	35
Venous arterial blood	1,210	250

Figure 7- T₁ and T₂ values of different tissues at 1.5T [van Geuns et al., 1999]

Spatial Encoding

To create a meaningful MR image with spatial information, the MR signal from the protons has to contain information about where these protons are positioned in the subject. This is done by slice selection, frequency encoding, and phase encoding. To select an imaging slice through the body, a magnetic gradient is added along the main magnetic field in the caudal to cranial direction. Because the frequency at which the spins can be excited is dependent on the local strength of the magnetic field, a gradient with a narrow bandwidth of frequencies will only excite a thin slice of spins through the subject (Figure 8) [van Geuns et al., 1999].

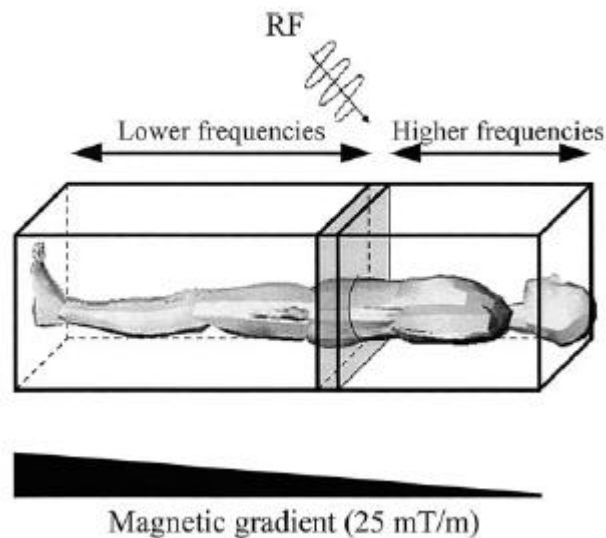


Figure 8 - By superimposing a small magnetic gradient on the main magnetic field in cranial-caudal direction, a single thin slice through the body is selected [van Geuns et al., 1999].

With a change in the excitation frequency another parallel slice can be acquired later. To obtain slices in other directions, the direction of gradients for the slice encoding is altered to an anterior-posterior gradient. A slice in any arbitrary direction through the subject can be acquired by using combinations of gradients in all three directions [van Geuns et al., 1999].

The frequency and phase encoding are also used to obtain information for the individual points within a slice and the picture elements or pixels. In the phase encoding process, a short change in the magnetic field is applied between the RF excitation pulse and the readout of the signal that will influence the frequency of precessing and results in a shift in the phase of precessing of the spins depending on the duration of this gradient switch. By repeating this process with different duration of the temporary gradients, signals with a different phase encoding are acquired [van Geuns et al., 1999].

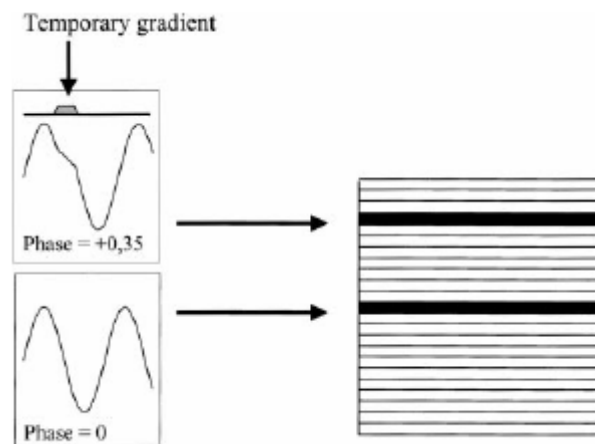


Figure 9 - During phase encoding a temporary gradient is applied. After the gradient is switched off, the spins will precess with the original frequency, but a small change in the phase of precessing will remain. The process has to be repeated to acquire multiple AC signals [van Geuns et al., 1999].

The pixels with the same phase encoding are differentiated using the frequency encoding. A magnetic gradient during readout of the signal results in a specific shift of

the resonance frequency, likewise the effect of the slice-encoding gradient, for pixels with the same phase shift (Figure 9) [van Geuns et al., 1999].

Phase and frequency encoded information are combined to allow the creation of a grid, called K-space, in which each pixel has a defined combination of phase and frequency codes (Figure 10) [van Geuns et al., 1999].

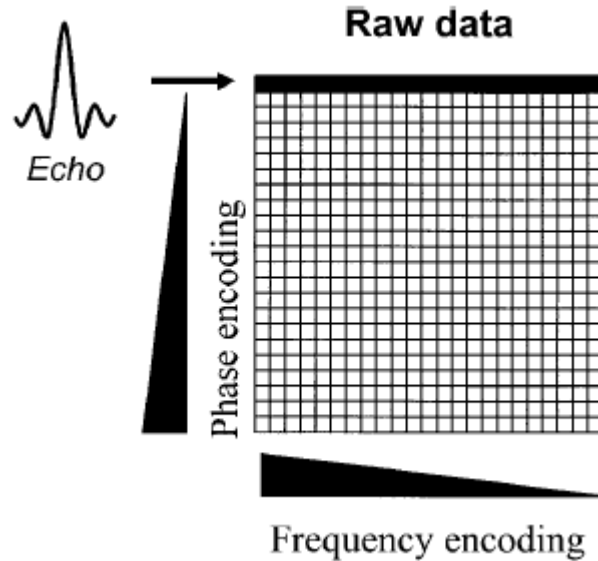


Figure 10 – To differentiate pixels with the same phase encoding, a Frequency encoding, with a gradient is used [van Geuns et al., 1999].

The data in K-Space, which represent amplitude as a function of time, are transformed into a curve that represents amplitude as function of the frequency using Fast Fourier Transform (Figure 11). The amplitude of each frequency represents the intensity of each pixel. A two-dimensional Fourier Transform is performed in both the frequency and phase encoding direction. The imaging time for a single image depends on the number of image lines desired. For example, for an image of 256 x 256 pixels, 256 signals have to be acquired [van Geuns et al., 1999].

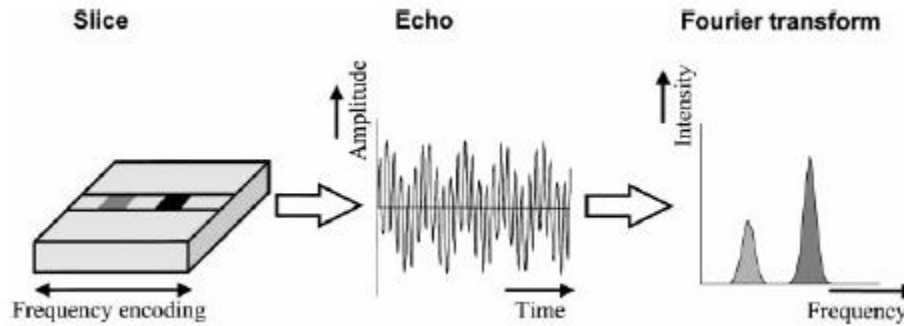


Figure 11 – An AC signal of a single image with two pixels with different proton densities will result in an AC signal echo with interference pattern of 2 sinusoidal AC currents [van Geuns et al., 1999].

The Echo Signal, Spin-Echo Imaging

There is a definite time necessary to perform the spatial encoding, and even with present fast MR scanners this cannot be performed before the FID declines. The creation of a second AC signal gives opportunities to modify the contrast in the images depending on the T1 and T2 values of the tissues. Therefore the initial FID signal is not used for clinical imaging [van Geuns et al., 1999].

To induce a second AC signal, a second RF pulse is applied. This RF pulse flips the spins by 180° , and also reverses the dephasing process as shown in figure 12. The amplitude of the signal increases as the spins rephase. The new signal, called the echo signal, is measured at its maximum (time of echo = TE) [van Geuns et al., 1999].

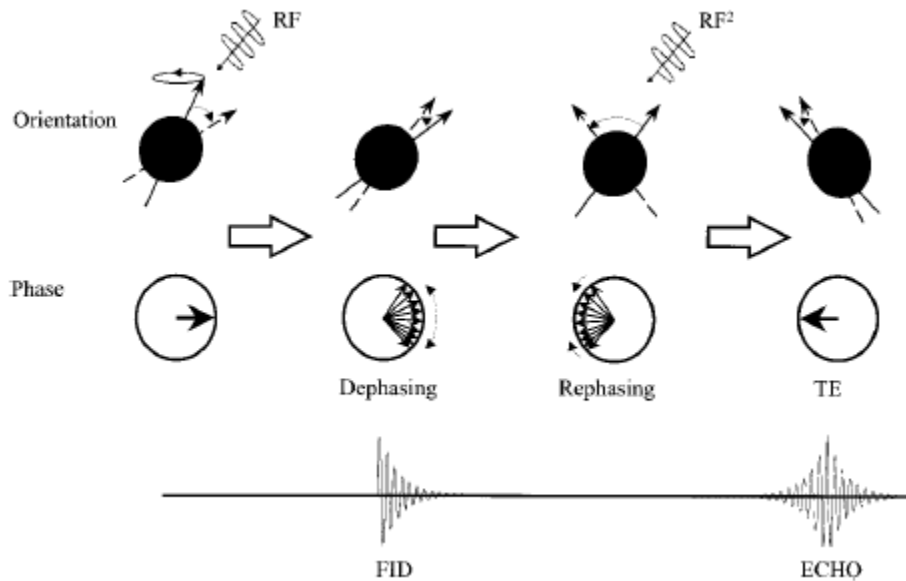


Figure 12 - The FID signal rapidly decreases before the longitudinal magnetization returns to zero. A second RF pulse flips the spins by 180° and reverses the dephasing process. When the spins are in phase again, a second AC signal is generated. Time of echo (TE) is the interval between the second RF pulse and the echo signal [van Geuns et al., 1999]

Different MR techniques use a combination of a 90° and a 180° RF pulse to generate spin-echo pulse sequences [van Geuns et al., 1999].

Contrast

Using the differences in T1 and T2 relaxation times, a contrast between different soft tissues in MRI is obtained. This contrast is superb compared with x-ray computer tomography. If the time for the next repetition of RF pulses, TR, is shorter than the time necessary for total longitudinal relaxation, the contrast in the image will be mainly influenced by T1 value of the tissues. Using a long TR and a long TE, the contrast will be dependent on T2 differences in the tissue. Finally, a combination of a long TR with a short TE will produce a contrast that is only on the proton density of the tissue [van Geuns et al., 1999].

Resolution

In MRI, pictures are composed of a matrix of elements, called picture elements or

pixels. The image represents the field of view (FOV). The image matrix defines the number of pixels used to create an image. The number of pixels is determined by the number of frequency encodings (e.g. 256 on the x-axis) and the number of phase-encodings (e.g. 256 on the y-axis) for a certain FOV. Therefore, the volume of each pixel is determined by the FOV, the matrix size used, and the slice thickness [van Geuns et al., 1999].

Resolution can be increased by changing the pixel size (the smaller, the higher the resolution), but the signal-to-noise ratio (SNR) is the limiting factor. When the pixels become too small, they do not contain enough spinning protons to produce a measurable signal [van Geuns et al., 1999].

Functional MRI (fMRI)

It was established, as long ago as 1890 that physiological functions in the brain correspond to regional brain activity. Magnetic resonance has the capability to measure the brain activity due to physiological functions. The most common current technique uses blood oxygen level dependent (BOLD) contrast, which is based on the magnetic susceptibility of hemoglobin (Hb). Deoxygenated Hb is paramagnetic, while oxygenated Hb is diamagnetic. The presence of the paramagnetic deoxygenated Hb distorts the static magnetic field. Therefore, changes in blood oxygenation can cause changes in the MR decay parameters [Noll et al. 2001].

Since the deoxygenated-Hb effect is quite small relative to the noise of the system it is typically not visible in a single experimental condition or time slice, but a composite effect can be identified after statistical tests [Kulkarni,2005].

A critical step after image acquisition in any functional MRI study is data analysis

and result compilation. In this project, a quantitative analysis strategy optimized for the rat brain was used. In this strategy, each subject is registered to a complete volume segmented atlas, a reference rat brain. All registered images are then segmented based on atlas into several regions of interest (ROIs). Statistical tests are performed on each subject and a statistical composite is created for each ROI by summing up individual analyses within ROIs. The detailed description of the strategy and algorithm is described in next chapters.

Controlling for motion

Motion artifact is a considerable problem in fMRI studies. In this study, the fMRI experiment is performed on fully conscious rats and high quality images of brain are acquired. Any head movement distorts the image and may also create a change in signal intensity that can be mistaken for stimulus-associated changes in brain activity. Motion artifact can be reduced by the use of general anesthesia; however, since we like to study the neurobiology, this is not a feasible solution as it precludes the study of brain activity involving cognition and emotion. Furthermore, anesthetics depress neuronal activity reducing MR signal [King et al., 2005].

One entirely noninvasive system developed by Insight Neuroimaging Systems (Worcester, MA, USA) was used in this study to set up an animal in just a few minutes. Just prior to the imaging session, animals are lightly anaesthetized with isoflurane gas. The head is then secured into a stereotaxic-like support system with ear bars and nose clamp. The body of the animal is placed into a body. This design isolates all of the body movements from the head restrainer and minimizes motion artifact. The restraint system is shown in figure 13 [King et al., 2005].

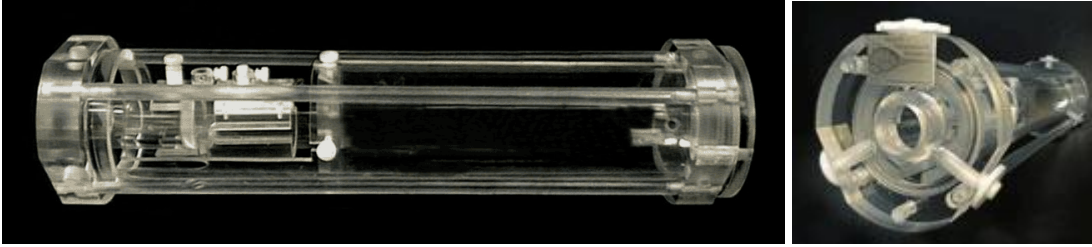


Figure 13 – The constraint system used in the fMRI study is shown above [Insight Neuroimaging Systems (Worcester, MA, USA)]

After the animal is set up, the isoflurane gas is removed and the restraining system is positioned in the magnet. Animals are fully conscious within 10–15 min and functional imaging can proceed. Residual effects of anesthesia required for setting up animals influence central nervous system activity in restrainers but are minimized by using anesthetics such as isoflurane with rapid elimination from the body [King et al. 2005]. All animal care and handling procedures were approved by UMass Medical School IRB committee.

Controlling for stress

During a functional imaging trial on fully conscious animals, the stress caused by immobilization and noise from the MR scanner is a major concern. To address this problem, animals are routinely acclimated to the imaging procedure prior to their first scanning session. The acclimation procedure is essentially a simulated scanning session so the animals can get used to the surrounding environment. Animals are anaesthetized with isoflurane and secured into the restrainer. When fully conscious, the animal is exposed to simulated experiment by placing the restraining unit into a black opaque tube ‘mock scanner’ with a tape-recording of an MRI pulse sequence. This procedure is repeated every day for 4 days for duration of 60 minutes. Following acclimation, rats show a significant decline in body temperature, motor movements, heart rate compared to

their first day of restraint as shown in figure 14 [King et al. 2005].

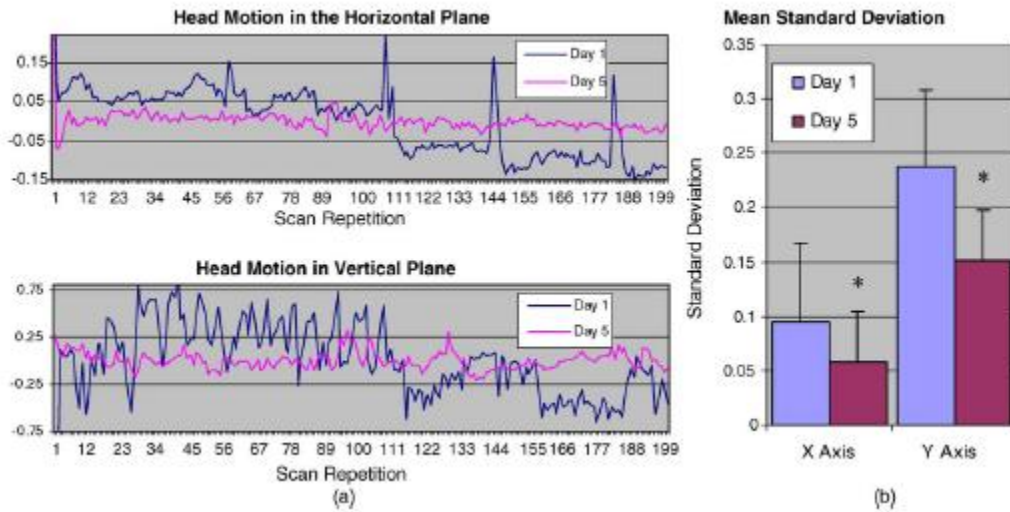


Figure 14 – Compared with day 1, the head movement in horizontal and vertical planes is significantly reduced by day 5 [King et al. 2005].

Acclimating in animals increases the Signal to Noise Ratio (SNR). The reduction in motor movement in head decreases the baseline level of noise and results in better signal resolution [King et al. 2005].

Registration, Segmentation and Data Analysis

To Analyze and quantify the brain activity due to a stimulus, the functional MRI data need to be registered, segmented and statistically analyzed. These processes were done using Medical Image Visualization and Analysis (MIVA) software and MATLAB. MIVA was developed at the Center for Comparative Neuroimaging (CCNI), WPI [Kulkarni, 2005]. As a part of this work an fMRI data analysis segment was developed as one of the application modules in MIVA. MIVA provides a user-friendly graphical interface for analyzing fMRI images. The following sections describe each step of fMRI data analysis.

Registration

Registration is a necessary step in the three dimensional reconstruction of the data. The

objective of this step is to map MRI anatomy images of all subjects to a fully segmented rat atlas using minimal user intervention. When the images acquired from all subject are registered or aligned to each other, a composite image is obtained which can then be segmented and statistically analyzed. In MIVA, the ‘Swanson’ rat brain atlas is used as a reference brain anatomical map in MIVA [Kulkarni, 2005].

Consider the following matrices,

$[S]$ = Global co-ordinates of a Subject

$[A]$ = Global co-ordinates of the Atlas or Reference

In the registration process, we seek the co-ordinate transformation matrix $[T]$ that aligns the subject with the Atlas space. In other words,

$$[A] = [T][S]$$

In this equation, $[T]$ is a 4x4 *matrix* that provides complete linear transformation (rotation, translation, scaling) [Kulkarni, 2005].

In the registration process, the subject images are first aligned to each other, *intra registration*, and then registered to the rat atlas, *inter registration*. In intra registration, the objective is to align all subjects to one standard subject. One of the N subjects is arbitrarily chosen as the standard subject and all other (N-1) subjects are aligned to this standard subject. In other words,

$$\{\text{Standard}\} = [T_{\text{Subject-to-Standard}}]_j \{\text{Subject}\}_j$$

where $j=1$ to $N-1$.

In inter registration, the standard subject is aligned to the segmented reference atlas; in other words,

$$\{\text{Atlas}\} = [T_{\text{Standard-to-Atlas}}] \{\text{Standard}\}$$

When the *subject-to-standard* transformation matrix is multiplied by *standard-to-atlas* transformation matrix, all subject are registered to the atlas.

$$\{\text{Atlas}\} = [T_{\text{Standard-to-Atlas}}][T_{\text{Subject-to-Standard}}] \{\text{Subject}\}_j$$

$$\{\text{Atlas}\} = [T_{\text{Subject-to-Atlas}}]_j \{\text{Subject}\}_j$$

There are several registration methods used in this study [Kulkarni, 2005].

Manual registration

Manual registration is based on the visual feedback from the user's computer visualization system. In this method the user is required to do the registration process and manipulate images through 3 orthogonal views of the brain, axial, coronal and sagittal based on a real-time visual feedback. The registration is performed by translation, rotation and scaling factors in x,y and z direction until good alignment is achieved [Kulkarni, 2005].

4-point Fiducial Registration

The 4-point fiducial registration is a faster method of registration compared to the manual registration. In this method, the ideal 4 points from an equilateral tetrahedron on one subject (the Standard subject) is mapped to another subject equilateral tetrahedron. First, a triangle (the base of the tetrahedron) is created on one slice and a single point on a distant slice (the tip of the tetrahedron) in the standard subject. The same 4 points is chosen in the other subject. MIVA has the ability to align these 4-points onto each other and therefore register the subject to the standard subject [Kulkarni, 2005].

The same procedure can be applied to align all subjects to the standard subject (intra registration) and finally applied to align the standard subject to the atlas (inter

registration) [Kulkarni, 2005].

Genetic Algorithm

The sequential Genetic Algorithm (GA) mimics the pathway of an expert manually doing registration [Huang, 2007]. Huang demonstrated that genetic algorithm registration provides high alignment accuracy reliability for brain tissues. The genetic algorithm avoids local minima and maxima traps of conventional optimization techniques. GA does not require any preprocessing of conventional registration methods such as threshold, smoothing, segmentation, or definition of base points or edges.

The Genetic Algorithm uses a finite element method to get the displacement of each element node by applying a boundary mapping. This method provides an accurate image registration with excellent boundary alignment of each pair of slices with minimal user involvement and finally aligns the entire volume automatically [Huang, 2007]. We used the proposed registration strategies to align multiple subjects to the standard rat atlas, which is aligned to a segmented reference atlas. All subjects were aligned to the segmented atlas and a full fMRI analysis was possible.

Genetic algorithm is a search algorithm based on the mechanics of natural selection and natural genetics that can be used as a registration method. A possible registration solution is presented as a chromosome in a string structure with each element representing one parameter in the solution. Several chromosomes are then form a population, which then produces another generation through a search process. The search process uses a ‘fittest survivors’ rule after a structured yet randomized information exchange within the existing generation to produce a new generation. After the new set of

chromosomes is produced with the mutation operation, we then select some of them, together with the original chromosomes, to be the new generation with the aid of the fitness function. After these steps are applied multiple times, the chromosomes will approach to the ideal [Chow et al. 2001].

Figure 15 shows the steps applied in the GA approach of registration.

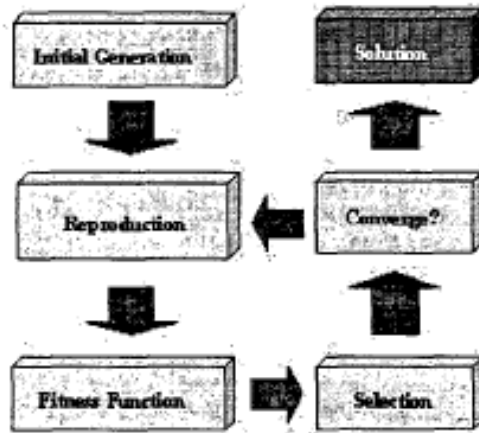


Figure 15 – The above steps are applied in a Genetic Algorithm (GA) approach [Chow et al. 2001].

Figure 16 and 17 show the image alignment before and after registration was performed using manual registration.

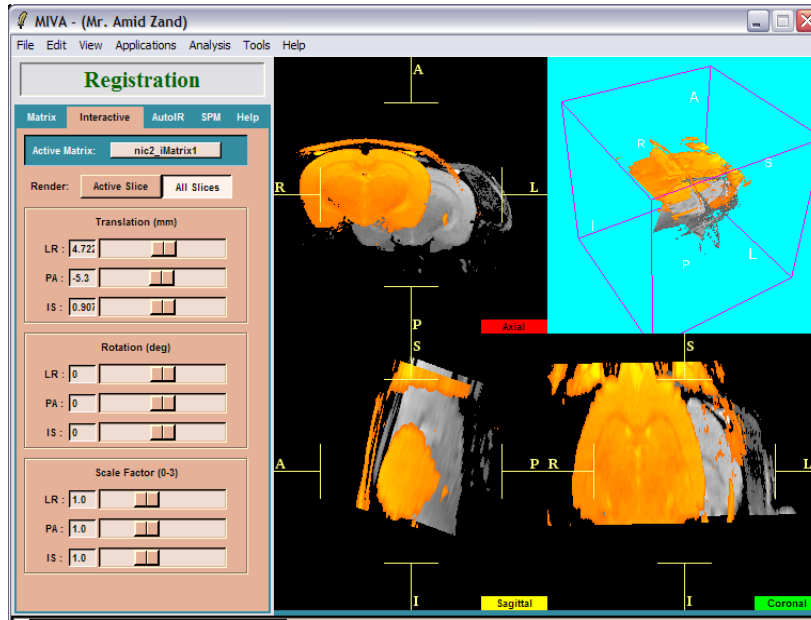


Figure 16 - image alignment before registration was performed using manual registration is shown above.

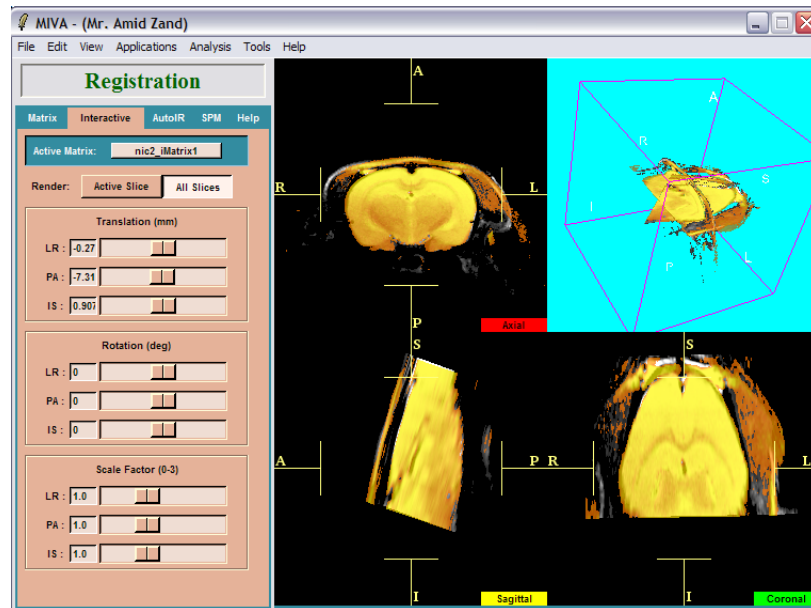


Figure 17 - image alignment after registration was performed using manual registration is shown above.

Segmentation

The purpose of segmentation is to divide an image into meaningful sub-regions and label those regions for further analysis. This step is critical in fMRI studies. The

brain can be segmented into different regions of interest (ROI) and activation level can be reported. In this study, we define different anatomical regions within the brain by superimposing the image onto Atlas and then extracting the segmented boundaries. For this purpose, a completely segmented 3D Rat Brain Atlas was developed in MIVA [Kulkarni 2005].

Mapping MRI image

In functional Magnetic Resonance Imaging (fMRI), an anatomy volume image is collected from each subject with a 256 x 256 with 14 slices through the brain. After the anatomy volume image is obtained, a temporal sequence of functional images are collected at a lower resolution of 64 x 64 while retaining the same 14 slices through the brain.. Although this method reduces the spatial resolution, it allows a more rapid cycle frequency and enhances transient data associated with the control and stimulation time dynamics of the MRI study [Kulkarni 2005].

Since the anatomy volume image has a higher resolution, it is used for registration and segmentation. Also since atlas resolution [512x512x300] is much higher than subject functional image resolution [64x64x14], results in multiple atlas voxels taking up a single subject voxel. In this study, the subject voxel was classified based on the centroid atlas voxel information where the type of material in the center of the subject voxel was chosen for the whole voxel [Kulkarni 2005].

Statistical Analysis

Statistical t-tests need to be performed on each subject with a 95% confidence level, one-tailed distributions, and heteroscedastic variance assumptions. Since multiple t-test analyses are performed, a false-positive detection controlling mechanism need to be

introduced. This subsequent filter guarantees a false-positive detection rate of 0.05. Those pixels deemed statistically significant retain their percentage change values (stimulation mean minus control mean) relative to control mean and all other pixel values are set to zero. The t-test tab in MIVA displays the various test parameters. The user needs to specify these parameters based on the design of the experiment. The same test can also be performed using MATLAB [Kulkarni 2005].

Results Summary

The result of the analysis MIVA is presented as an image for visualization as well as numerical summary used for statistical validation.

CHAPTER 3 - PROJECT APPROACH

After initial stimulation from a drug such as nicotine, the brain responds primordially to the influx of neurotransmitters. This stimulation causes an array of responses. The brain creates memory of its physical reaction and chemical reaction. This memory is involved in sensitization of the rat brain to nicotine [Li et al. 2008].

Previous studies have explored the rat brain during full response after sensitization and prior to sensitization [Li et al. 2008]. During these regular daily injections the rats have elevated locomotor activity and overstimulation within the areas of interest within the brain. However the refractory period of nicotine has not been explored. In order to explore this refractory period experimentation will be conducted in the period of time between regular doses of nicotine. The length of the refractory period of nicotine in the Sprague-Dawley rat model is not known. The refractory period will thus be explored with a number of experiments followed by extensive analysis.

Hypothesis

It is hypothesized that during the refractory period of the sensitized rat, the areas of the brain will not fully respond to a challenge dose of nicotine. This hypothesis is based on the fact that the nicotine receptors are saturated with neurotransmitters and need time to recover before they can react to more nicotine.

Assumptions

The refractory process was assessed behaviorally through a black box observation and neurologically through fMRI imaging. Both of these techniques are trying to gauge the

rats' reaction to the stimulus. The rat should not be exposed to any other kind of sensory stimulation or it may influence the results. All steps were taken to minimize any outside influences however, they cannot be completely eliminated.

In the black box observation, the rats are given an injection subcutaneously and then placed in the box. Red lights were placed around the box and the rat was placed in. The tracking system was placed in the room and the operators (students and lab technician) were present throughout the procedure. It is assumed that the presence of people did not incite stress within the rats and since these rats have been handled by people throughout their life.

During image acquisition the animals are placed in the restraints and then in the MRI magnet. The bore of the magnet is open on both sides. After the rat is placed in the machine a baseline imaging of 5 minutes is done, at which point an operator goes into the magnet room and injects the stimulation through the syringe that has been already placed on the tail vein. This is a 30 second process after which the operator leaves the room once more. It is assumed that the rat does not respond to the visual and auditory stimulation of seeing and hearing the operator. Furthermore, while analysis is conducted, the data taken during and 2 minutes after the injection are discarded in order to minimize stimulation that may have been caused by the operator.

The sensitization of the behavior study and fMRI study occur at different times for practical purposes. The assumption is that this will not change the way the rats go through the sensitization process and the rat activity is compared with its own baseline, so the assumption is that the change that the stimulation will cause will be the same. However rats are much more active during the night than during the day period.

The ROIs were chosen based on the hypothesis that the mesolimbic system is involved in the response of drug abuse.

CHAPTER 4 - DESIGN

Problem Definition

The goal of this project was to identify the brain activity during the refractory period of nicotine. In order to accomplish this goal, an experimental procedure was created to test the hypothesis. This experiment must verify the hypothesis within established constraints. The experiment must identify the presence and time frame of the refractory period, it must provide functional MRI images during the refractory period, and it must produce data that compares the brain activity of nicotine to a control group. These goals created consecutive steps which determined the design of experimentation for each successive step. As such, an overall approach was first developed and then was revised after the results of each step.

Brainstorming of Experimental Components

It is known that nicotine stimulation causes increased locomotor activity. To find the refractory period of nicotine an animal model is required. The animals should be first sensitized to nicotine, and then given challenge doses of nicotine. Their movements should be quantified in reaction to nicotine throughout the sensitization process as well as during the challenge dose. These should then be compared to the control group. A number of considerations must be taken in the design of the experimental study.

- The animal model to be used. Previous studies of nicotine addiction have been conducted on animal models such as pig [Zhu et al., 2005], squirrel monkey [Stolerman 1994], rat [King et al., 2008], and mouse [Picciotto et al. 2007].

- Once the refractory period is identified, the fMRI study can use the middle of the refractory period, the beginning of the refractory period, or the end of the refractory period for stimulation and imaging.
- The fMRI study can be conducted on the same group of rats as the behavior study or another group of rats.
- Imaging should be taken for half an hour [King et al. 2008]
- Injection can be given subcutaneously, intravenously, same for both studies, or different for both studies based on practicality.
- Images should be taken using the BOLD sequence since we want to see functionality.
- The threshold of activation should be placed at 0.01 to determine activation significance.
- Analysis software for fMRI include AFNI, Stimulate, MIVA, MATLAB and other commercial software.

After the data was acquired the images were registered to each other and then segmented to the brain map. There are various methods of registration. After registration testing with each one, the manual technique was adopted as the gold standard and used for further manipulation of the data.

Constraints

The MRI magnet at the CCNI center has a 40cm bore diameter. This physical limitation allows only small animals to be imaged. This constrains our choice of animal model for experimentation of nicotine addiction. Furthermore as of now the facility only

has approval for work with rats and mice. Rats have been used in past studies to analyze nicotine addiction and were the animal model used for this study.

The initial experiment performed to identify and locate the refractory period was a locomotor activity experiment. This experiment is relatively cheaper and easier to conduct compared to an MRI study. For this experiment, 8 rats were provided. This confines the number of subjects for the experimental and control group.

After the behavior study was done, it was found that the refractory period was within 12 hours after the regular drug administration. This determines the stimulus time period for the imaging study. This means that after addiction is reached the challenge dose will be given 12 hours after regular doses, and this is the time at which imaging would take place. Due to magnet usage times and drug administration times, the 8 animals would have to be sensitized at a time beginning at 4 different days. This is because an hour needs to be allocated for the imaging of each subject and the evening are relatively free for magnet usage.

The operators of the experimental procedure (MQP students/ authors of this paper) needed to become familiar with animal handling procedures, good clinical practices, MRI functionality, usage and understanding, as well as data acquisition and analysis software usage . This places a time constraint of 7 weeks for experiment design and execution and 7 weeks of analysis.

The Software available for analysis were excel, MIVA and MATLAB.

Final Design

The hypothesis to be tested first requires the identification of the refractory period. Past studies have shown that reaction to nicotine stimulation causes an increase

in locomotor activity in rats for at least 30 minutes after stimulation is administered [Li, 2008]. The rat was chosen as the model because it is easily available, cheap, easier to work with than the mouse, which is smaller, and the facility has AICUC permission for mouse and rat work. It is also practical for the available restraint and MRI magnet.

After the consideration of the project constraints and comparison of choices from the brainstorming ideas a final procedural study was established. It was determined that the rats would be given the injection subcutaneously in the behavioral study since this is a proven method of stimulus administration. For the fMRI study the rats would be given the injection intravenously through the tail. This was because the tail was easily accessible on the animal and giving the stimulus into the blood would ensure immediate delivery to the brain which would create a more immediate response, ideal for imaging.

To test this hypothesis and find whether nicotine does have a refractory period and when it exists in this model, a behavior experiment was established. Eight rats were divided randomly into an experiment group and a control group. The experimental group was sensitized to nicotine and the control group was given saline. In this manner both groups would be used to the injections. The rats would then be observed in a relatively dark and quiet environment such that their stress level remained at a minimum.

From this point the fMRI study would be conducted. The rats would be sensitized as in the behavioral study, and then acclimated to eliminate stress as described in the literature review. The data would be gathered from the MRI and analyzed. Figure 18 shows the flow of the thought process of the experimental design.

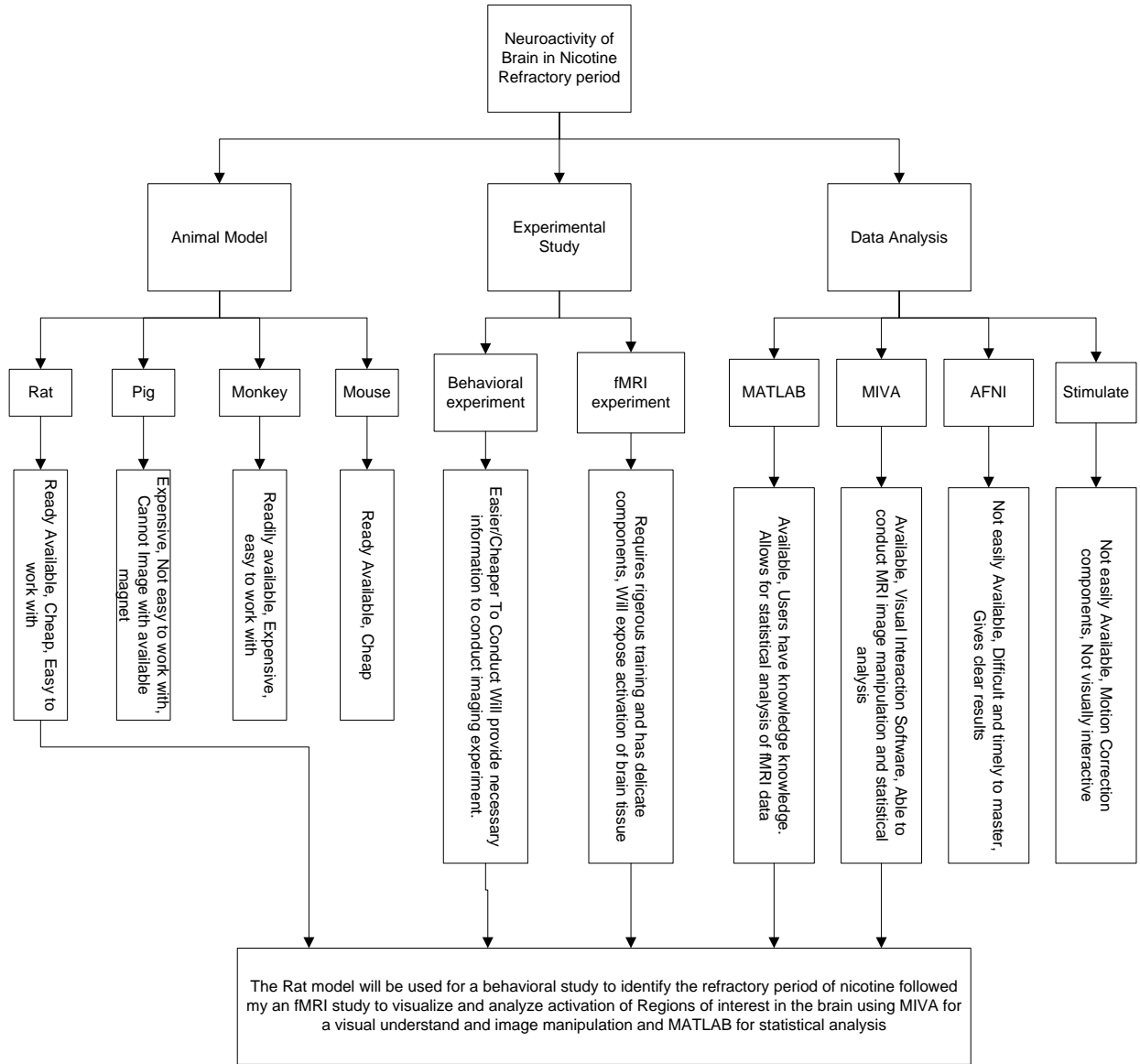


Figure 18 - The above objective tree was used in the design process

CHAPTER 5 - METHODS

Animal Care

Subjects were male Sprague–Dawley rats (275–300 g) obtained from Charles River Laboratories (Wilmington, MA) and were housed in Plexiglas cages (two in a cage). Animals were maintained in ambient temperature (22–24 °C) on a 12-hour light, 12-hour dark schedule with lights on at 09:00h. Food and water were provided *ad libitum*. The procedures were approved and monitored by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC).

Drug Administration

The experimental and the control group were injected with nicotine and saline respectively. For the experimental group, Nicotine tartrate was dissolved in distilled water at a concentration of 0.4 mg/kg, measured as the base, and the solution was adjusted with Na₂PO₄ to a physiologic pH. For the control group, sodium chloride was dissolved in de-ionized water to a final concentration of 0.9%. All injections were in volumes of 1 ml/kg body weight. Both drugs were purchased from Sigma Chemical, St. Louis, MO.

Experiment #1 - Behavioral Study

To sensitize animals to nicotine a dose of 0.4mg/kg was injected subcutaneously (SC) on a daily basis. Adult male Sprague–Dawley rats were matched for body weight and randomly assigned to nicotine or saline treatment groups (n=4 per group). On each day, each animal was placed in a non-reflective black open field of 121 cm by 121 cm for 5 minutes to habituate to the environment and. The animals were the injected with a dose of saline or nicotine according to their group. Immediately following each injection, the distance traveled was tracked using Ethovision® XT video tracking system (Noldus

Information Technology, Wageningen, Netherlands) for a duration of 10 minutes.

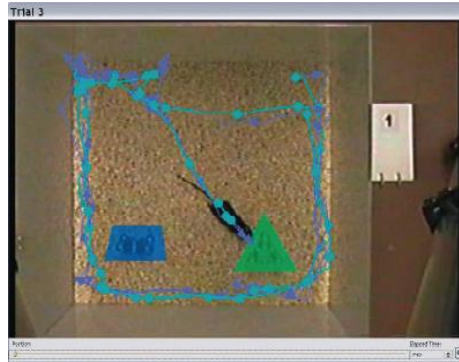


Figure 19 - each animal was placed in a black open field. Immediately following each injection, the distance traveled was tracked.

Animals received daily SC injections for 6 consecutive days. Sensitization was achieved circa day 3 [See results]. On day 6 the locomotor activities were also measured after 3 hours, 6 hours, 9 hours or 12 hours immediately after the last injection. The locomotor activity was documented and employed to confirm that the nicotine is subject to a refractory period and thus fMRI analysis can be performed.

Experiment #2 – fMRI study

fMRI study

All animals were first acclimated and then randomly separated into two groups (n=4). The experimental group received a dose of nicotine for 6 days followed by a final dose of nicotine after 12 hours of last injection. The second group received saline in the same manner. Each animal was imaged once during this last injection. The doses of nicotine and saline were administered via the lateral tail vein while the animal was in the magnet.

fMRI Imaging procedure

The acclimated rats were restrained in a multi-concentric, dual-coil, animal restrainer (Insight Neuroimaging Systems, LLC, Worcester, MA). They were lightly

anesthetized with 2.5% isoflurane. A plastic semicircular headpiece with blunt supports was fitted into the ear canals. The animal's canines were secured over the bite bar of a head holder and the earpieces were secured with adjustable screws and fitted into lateral sleeves. The adjustable surface coil built into the head holder was pressed firmly on the head and locked in place. The body was then placed in a sleeve that was suspended down the center of the cylindrical chassis and fitted with rubber gaskets to dampen motion. The headpiece locked into an immobile mounting post on the front of the chassis. The flow of isoflurane gas was terminated once the animal was secured in the head holder. A volume coil was then placed over the head restrainer and locked in position. The setup procedure took about 10 minutes, by which time the animals were fully conscious. Figure 20 shows the final outcome of the procedure.



Figure 20 - A dual coil rat restrainer system used for fMRI study

Animals were then placed in a Bruker Biospec 4.7T scanner (Oxford Instrument, Oxford, UK) equipped with a Biospec Bruker console (Bruker, Billerica, MA). Once the animal was secured in the magnet, high-resolution anatomical images were obtained using fast spin echo pulse sequences (echo time, 48 ms; repetition time, 2000 ms; field of view, 30 mm; 1.2 mm slice thickness; 256°—256 data matrix; RARE (rapid acquisition relaxation enhanced) factor, 8). After the anatomical images were obtained, BOLD fMRI images were continuously acquired over a 30 min period to include a 5 min baseline and

a 25 min period after administration of the challenge dose.

fMRI Data Analysis

The percent change in BOLD activation was calculated on a pixel-by-pixel basis by comparing the average values obtained during the 5min before and 25min after the injection of stimulate. Regions of interest (ROIs) used for analysis included the prefrontal cortex, anterior cingulated cortex, nucleus accumbens, ventral pallidum, hippocampus, ventral tegmental area, visual cortex and septum. To generate an activation map for each subject, voxels whose BOLD percent change was significant at $p < 0.05$ were overlaid on individual specimen atlases created using the anatomical images co-registered with fMRI data using MIVA software and MATLAB. The t-test statistics use a 95% confidence level, one-tailed distributions and heteroscedastic variance assumptions. The pixels considered statistically significant, were identified and statistically averaged within their respective anatomical regions.

CHAPTER 6 - RESULTS

Experiment #1 – Behavioral Study

The result of the behavioral study is shown below.

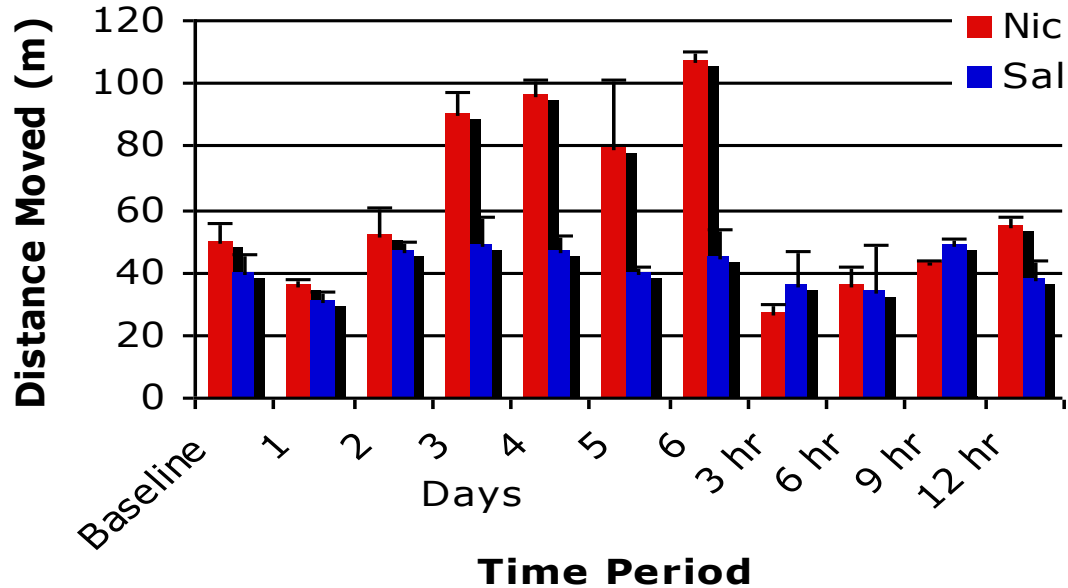


Figure 21 - After 6 daily injection of nicotine, the rats were sensitized. After the injection on day 6, each rat was given an extra dose of drug and the refractory period was observed.

Experiment #2 – fMRI Study

The fMRI composite results of the experimental (nicotine) and control (saline) groups through all 14 slices of the brain are shown in Figures 22 and 23. The activated regions of the brain are shown in color.

The fMRI composite results of the experimental (nicotine) and control (saline) group in 3-Dimensions are also shown in figures 24 and 25.

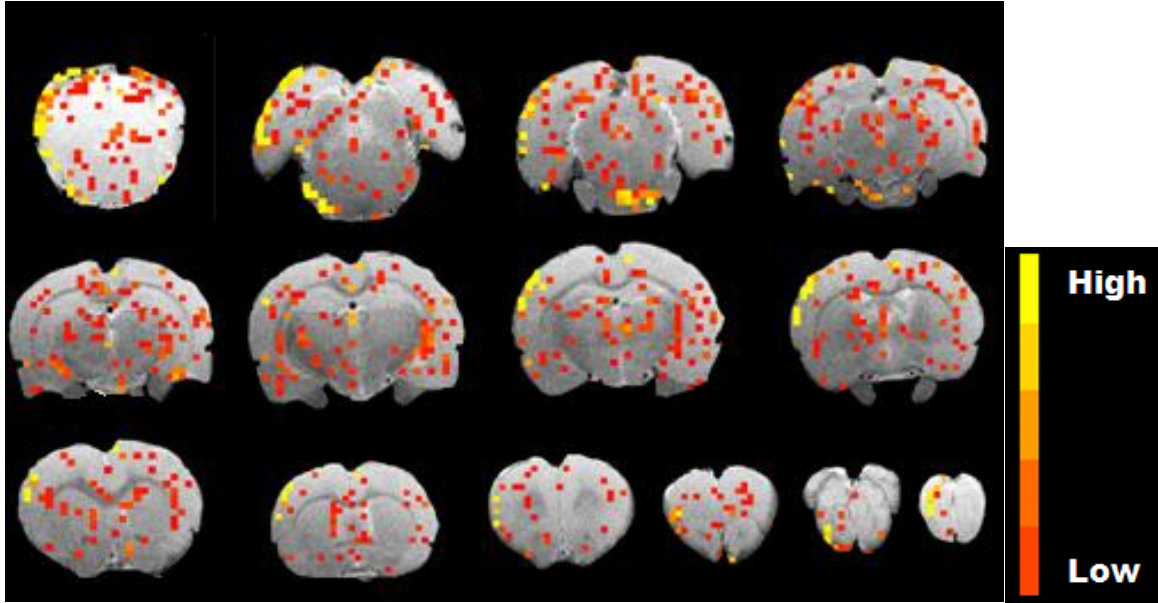


Figure 22 - Composite brain activation in the nicotine (experimental) group

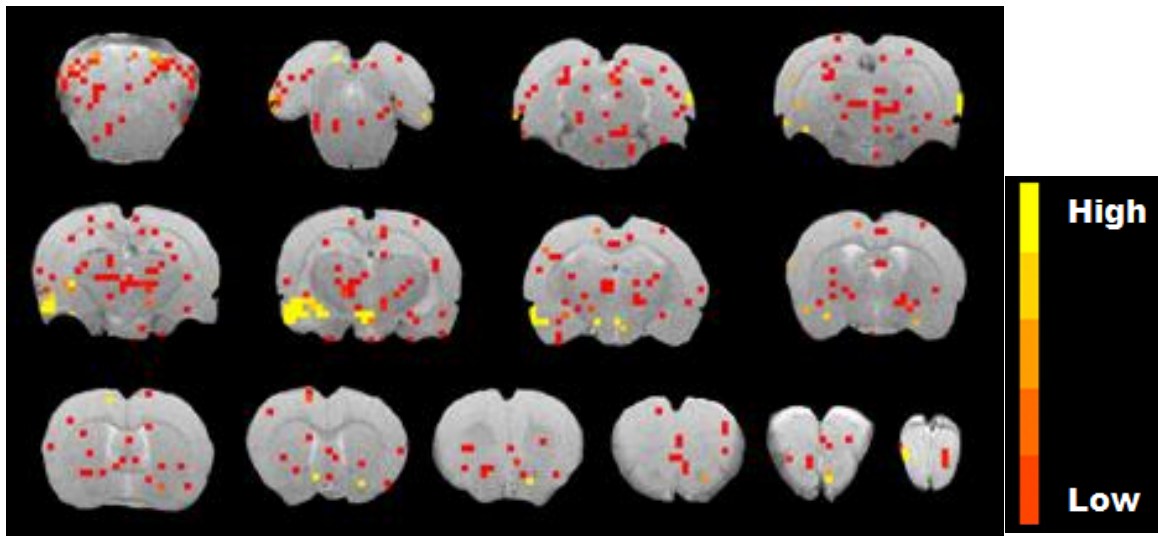


Figure 23 - Composite brain activation in the saline (control) group

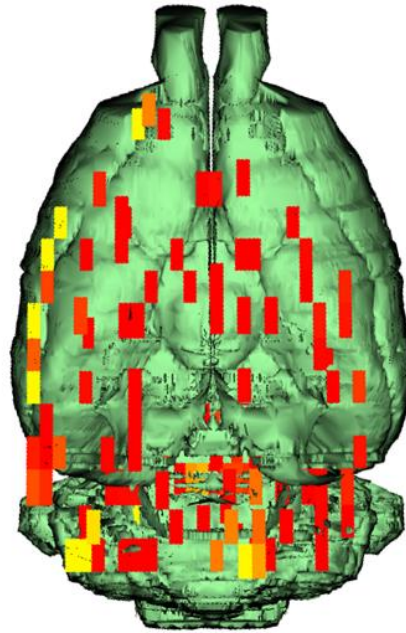


Figure 24 – Composite result of brain activation in the nicotine group

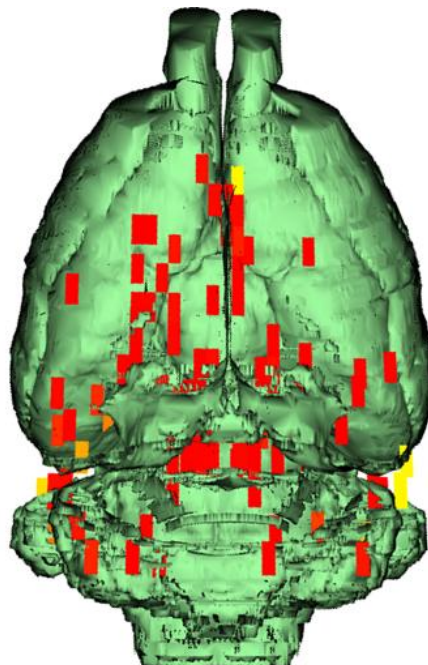


Figure 25 – Composite result of brain activation in control group

The full length time response of all regions of interest (ROIs) is shown in figure 26 to 32.

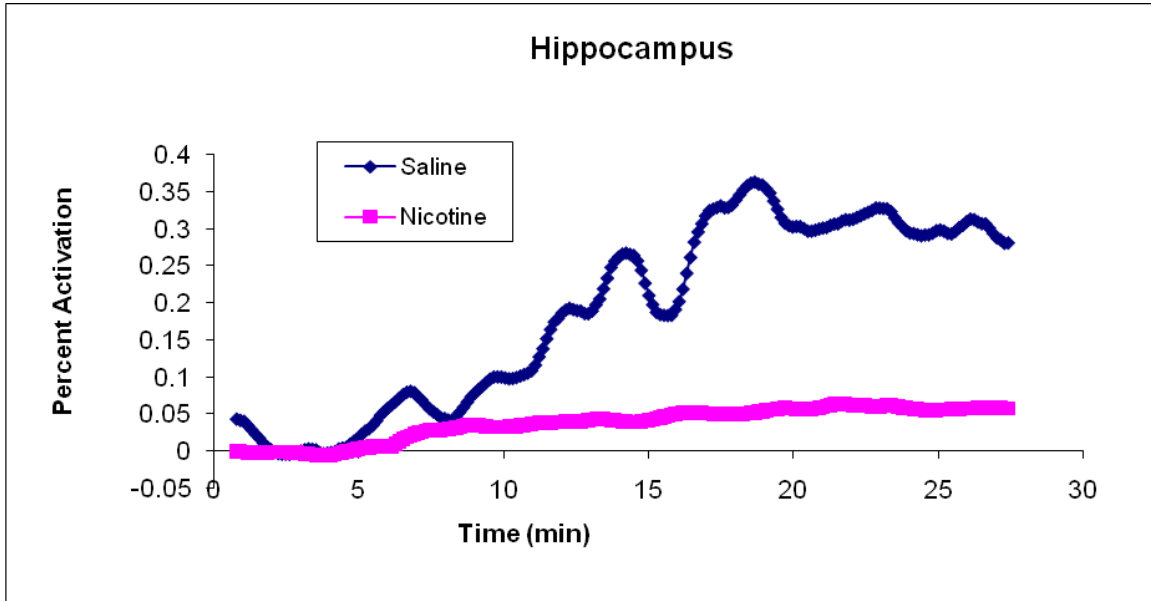


Figure 26 – Percent Activation in Hippocampus

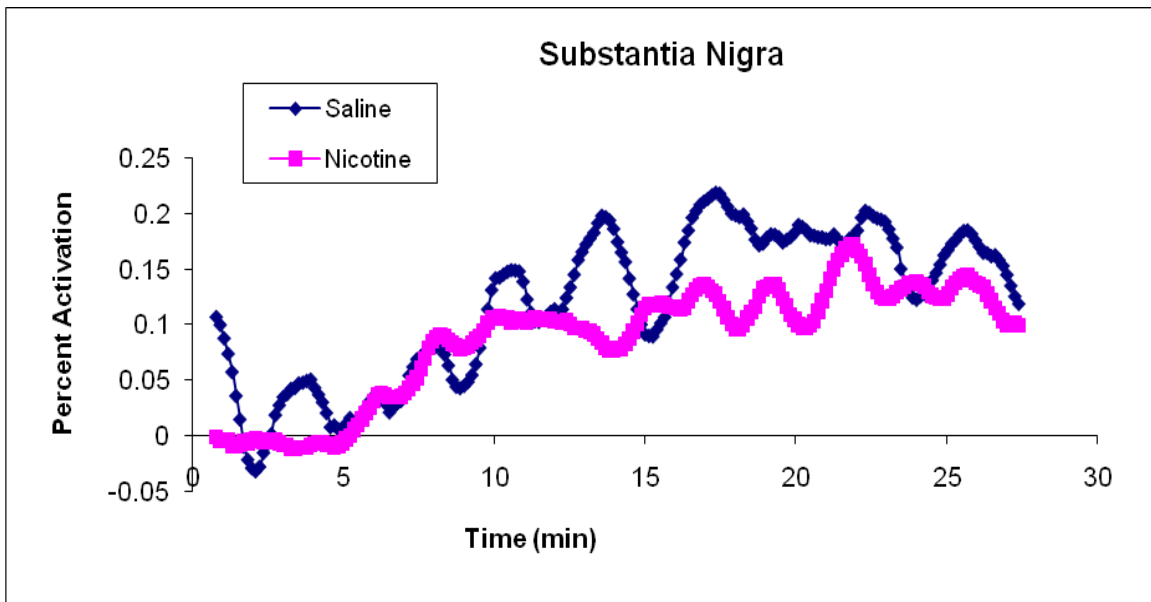


Figure 27 – Percent Activation in Substantia Nigra

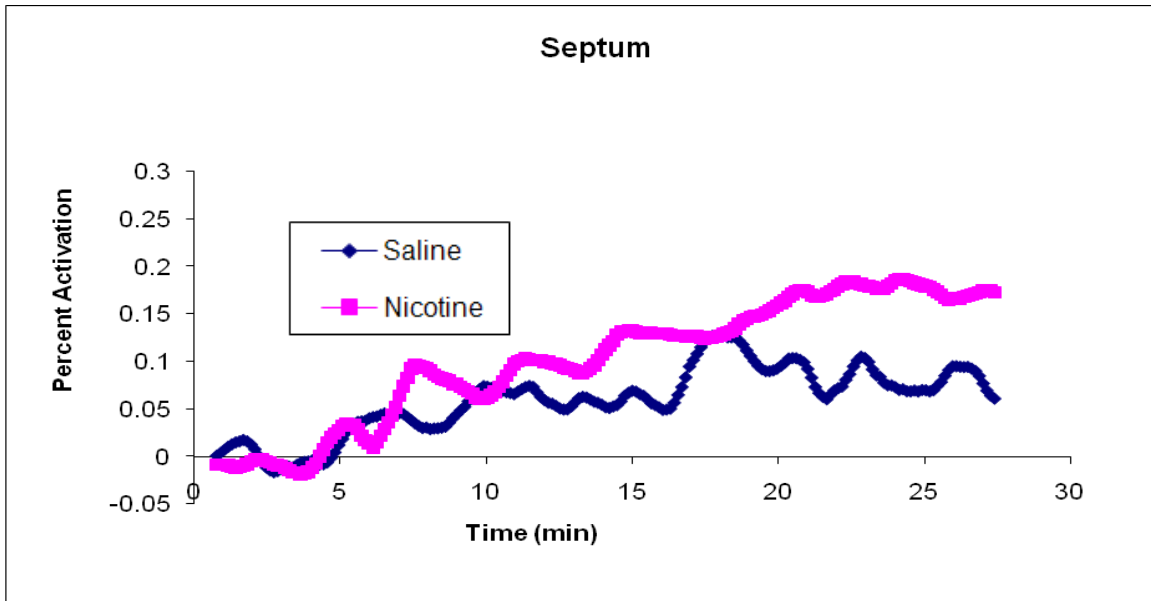


Figure 28 – Percent Activation in Septum

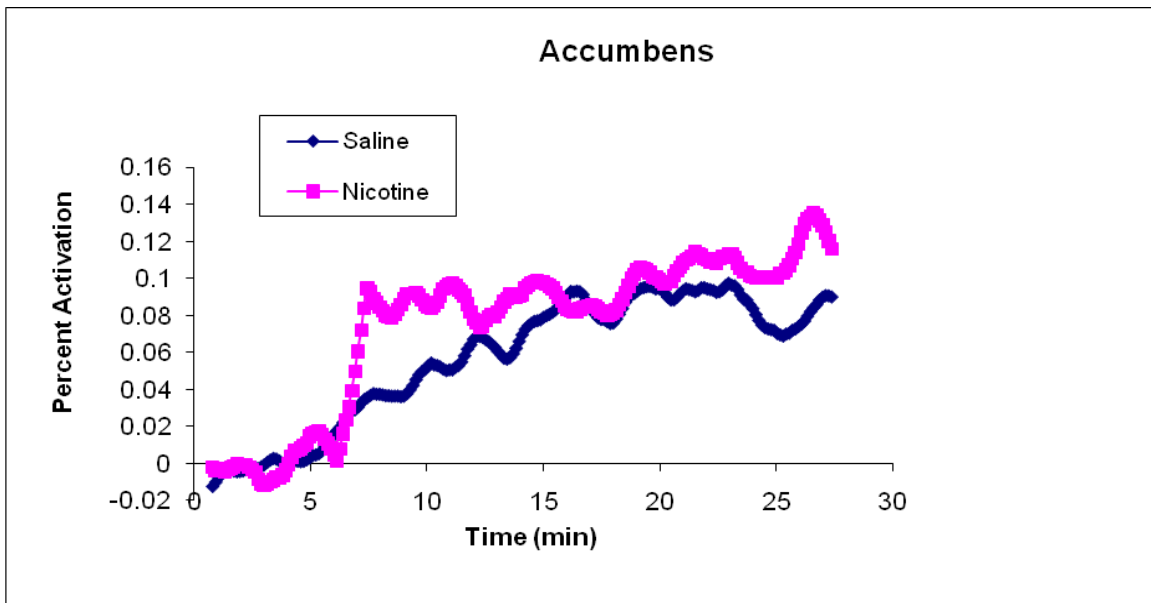


Figure 29 – Percent Activation in Accumbens

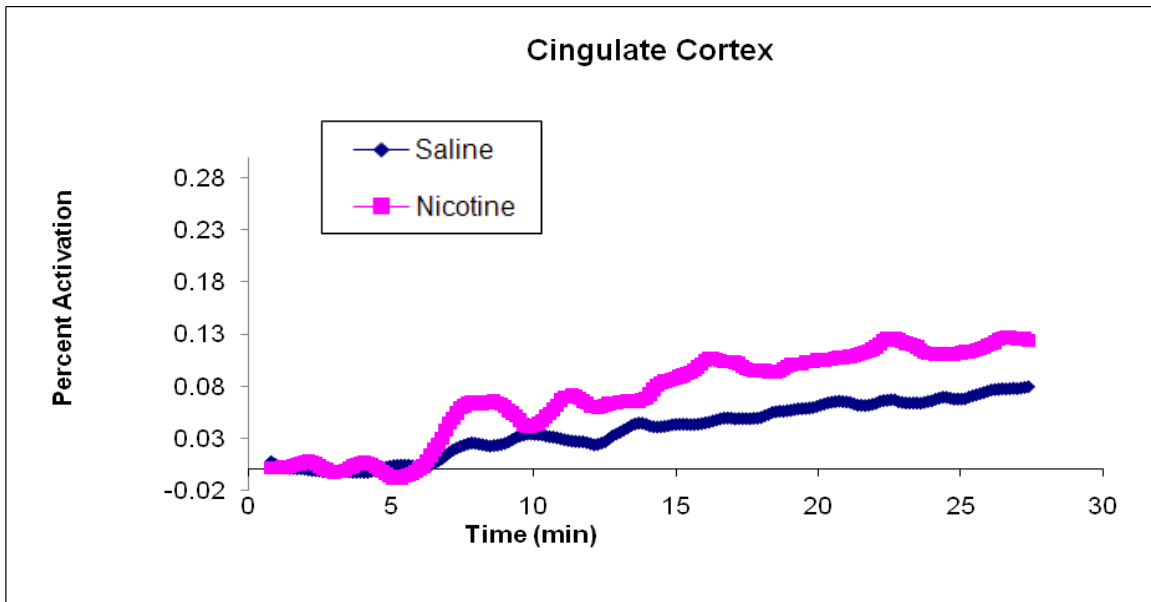


Figure 30- Percent Activation in Cingulate Cortex

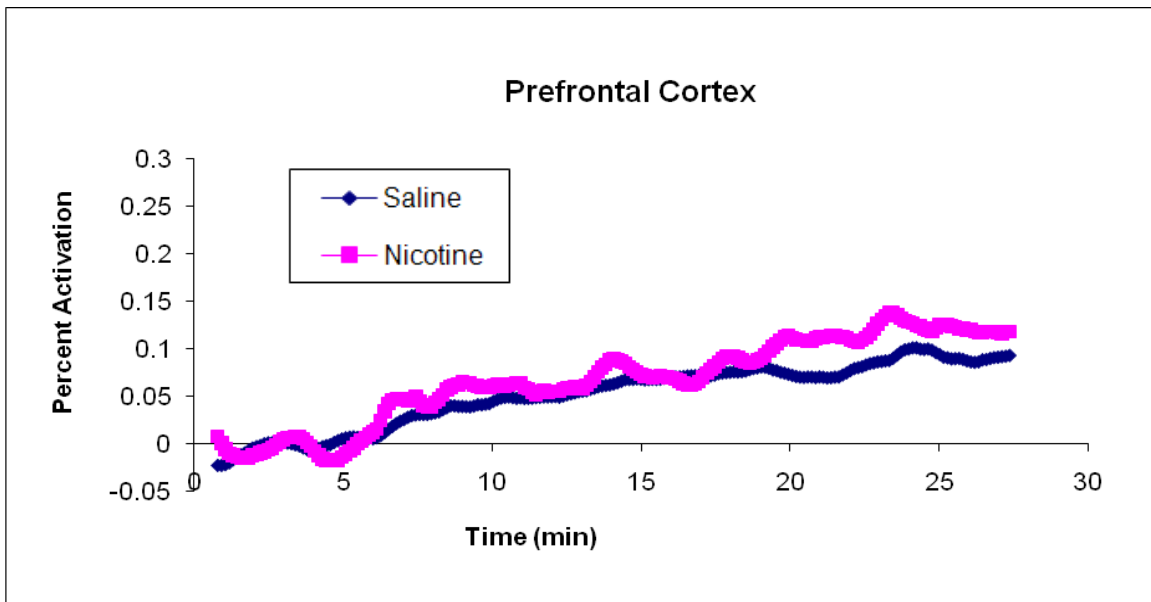


Figure 31 - Percent Activation in Prefrontal Cortex

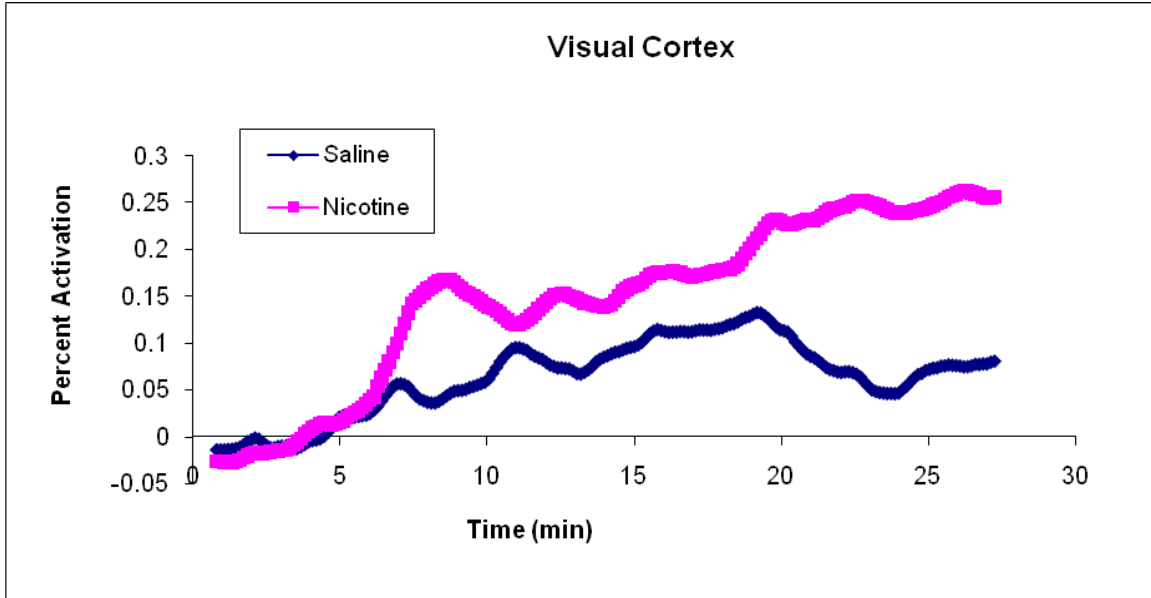


Figure 32 – Percent Activation in Visual Cortex

CHAPTER 7 - ANALYSIS AND DISCUSSION

Behavioral Study

The data show that the distance traveled by the subjects in the experimental group showed a gradual increase during the first three days followed by a steady plateau achieved through day 6th. The data demonstrates that nicotine sensitization is achieved by day 6th. The control group on the other hand does not follow this trend.

Looking at the refractory period results, we can also observe that the locomotion activities of the saline and nicotine groups are not significantly different during the 12 hour period immediately after injection on day 6. The locomotor activity in the experimental group is still inhibited and lower than the response on day 6. Since the rats are not fully responding to the stimulus, we can conclude that they are within the refractory period of nicotine. The 12-hour point within the refractory period was chosen for the fMRI study.

fMRI Study

The functional data show a close overall correlation between the nicotine and saline activation over the 30 minutes of stimulation period. Most of the data falls within 0.4% activation for both saline and nicotine. Zhixhin et al. 2008, reported activation between -0.077% and 0.431% in the ROIs of control subjects receiving saline during regular sensitization. Activation levels of 3.03% to 4.23% were recorded in ROIs of nicotine sensitized rats during regular dose.

When these fMRI results are compared with this previous study, it is clear that the regions of brain that have response in the past show a significantly lower activation reaction to the stimuli. While figure 21 and 22 show visual representation of the

activation in the brain to be greater in the nicotine rats, the statistical analysis demonstrates that this activation is not significantly different.

However there are areas, such as the visual cortex in figure 32 where the nicotine activation is clearly greater than the saline activation. Areas like the prefrontal cortex, accumbens and cingulated cortex seem to have a very tight activation for both groups, showing that there is no difference in activation. The hippocampus however seems to have relatively large response activation from the saline rats and the lowest response from the nicotine group. This result is not expected and overall not in agreement with the rest of the ROIs sensitive to nicotine addiction.

CHAPTER 8 - CONCLUSIONS

The Activation from nicotine and saline were not significantly different during the refractory period. The fMRI study is consistent with the behavioral results. The areas that are normally excited by nicotine are relatively dormant during the refractory period and have the same response as saline. These results coupled with the studies of drug reactions beyond the refractory period can be used in the development of pharmaceuticals that are aimed at specific regions of the brain or at determining targeted drug delivery schedules.

CHAPTER 9 - RECOMMENDATIONS

In order to improve this study, to further analyze the refractory period of nicotine within the normal rat model, and to produce data that will aid in the exploration of nicotine addiction a few measures must be taken.

The behavioral study tested for the presence of the refractory period within the first 12 hours after the regular sensitization does. This was half way between two consecutive regular doses. This provided us a window into the refractory period but did not fully uncover the duration of the refractory period. By exposing the subjects to challenge does throughout the 24 hour period a more precise duration of the refractory period can be observed.

Once the refractory period is identified in full, fMRI imaging should be conducted at the very beginning and the end of the refractory period. This will provide a greater window to the recovery of the receptors within the brain tissue. This will result in a greater understanding of different levels of addiction and can give greater insight to time schedules of pharmaceuticals.

Lastly, if the resources allow, using a larger sample size will result in more significant and precise data. This will also allow further testing of the refractory period in the behavioral study. It is also recommended that in future analysis, motion and drift compensation softwares be used along with MIVA and MATLAB.

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