EFFECTS OF BASAL FOREBRAIN STIMULATION ON THE DISTRIBUTION OF CHOLINERGIC RECEPTORS IN THE SENSORIMOTOR CORTEX OF RAT BRAIN

by

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B.S. in Bioengineering, Yıldız Technical University, 2014

Submitted to the Institute of Biomedical Engineering
in partial fulfillment of the requirements
for the degree of
Master of Science
in
Biomedical Engineering

Boğaziçi University
2019

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ACKNOWLEDGMENTS

Firstly, I would like to express my sincere gratitude to my advisor Prof. Burak Güçlü for the continuous support of my master study and related research, for his endless patience, high motivation, and immense knowledge.

I would like to thank to each member of Taclab. I am proud to be a part of it. I have the most supportive lab mates Bige Vardar, İpek Karakuş, Deniz Kılınç, Sevgi Öztürk who always trying to rise my motivation and emphatic attitude during my research.

My heartfelt, special thanks go to Andaç Kılıçkap for moral, emotional and unconditional supports and motivational speeches in every stage of my research since we met.

Finally, I must express my most profound gratitude to my parents for supporting me emotionally and spiritually throughout writing this thesis and my entire life.

This work was supported by Boğaziçi University Research Fund Grant no: 17XP2 to Prof. Dr. Burak Güçlü.

ACADEMIC ETHICS AND INTEGRITY STATEMENT

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ABSTRACT

EFFECTS OF BASAL FOREBRAIN STIMULATION ON THE DISTRIBUTION OF CHOLINERGIC RECEPTORS IN THE SENSORIMOTOR CORTEX OF RAT BRAIN

In this thesis, the effects of basal forebrain stimulation on the distirbution of α4 and α7 type nicotinic acetylcholine receptors were studied in three different brain areas: primary motor cortex, hindlimb somatosensory cortex and barrel field somatosensory cortex. Basal forebrain, the main source of cholinergic inputs, was electrically stimulated (for each trial: 0.5-ms bipolar pulses with 50 µA, at 100 Hz for 0.5 s). In total, the experiment was carried out with 12000 pulses and it took 4 hours. After stimulation, transcardially perfusion was applied, and their brains were used for immunofluorescence staining. According to hemisphere in which the stimulation was applied and histological sections were obtained, the animals were separated into three experimental groups: control (no stimulation, n=7), ipsilateral (same hemisphere as stimulation, n=7) and contralateral (opposite hemisphere of stimulation, n=7). The results show that basal forebrain stimulation has a significant effect on the distribution of only α7 type nicotinic acetylcholine receptor. Ipsilateral and contralateral groups are statistically different from control group but there is no difference between ipsilateral and contralateral experimental groups. Specifically the number of receptor α7 complexes and their density of receptor complexes (normalized with layer thicknesses) were increased significantly with respect to control. Additionally, the number of α7 type receptor complexes and the density in primary motor cortex were mostly lower than those in the hindlimb area and barrel field of primary somatosensory cortex. A similar finding was found for only α4 type receptor count but not for the density. Overall, this thesis shows anatomical evience for cholinergic modulation of somatosensory system. The results may be important for understanding attentional processes, and neuropsychiatric diseases which affect them.

Keywords: Somatosensory, Cholinergic system, Attention, Nicotinic acetylcholine receptor.

ÖZET

BAZAL ÖNBEYİN UYARIMININ SIÇAN BEYNİ SENSORİMOTOR KORTEKSTEKİ KOLİNERJİK RESEPTÖR DAĞILIMINA ETKİSİ

Bu tez çalışmasında, bazal ön beyin uyarımının üç farklı korteks bölgesindeki (birincil motor korteks, birincil beden duyusu korteksinin arka bacak ve bıyık alanları) α4 ve α7 nikotinik asetilkolin reseptör dağılımını nasıl etkilediği çalışılmıştır. Kolinerjik girdilerin ana merkezi olan bazal ön beyin elektriksel olarak uyarılmıştır (her deneme için: 0.5-ms'lik bipolar darbe (50 µA), frekans 100 Hz). Toplam olarak, deney 12000 darbe ile yapılmış olup, 4 saat sürmüştür. Uyarım sonrasında, hayvanlar transkardiyal olarak perfüze edilmiş, ve beyinleri immunofloresan boyama uygulaması için çıkarılmıştır. Bazal ön beyin uyarımının hangi yarımküreye uygulandığına, ve boyama kesitlerinin hangi yarımküreden alındığına bağlı olarak hayvanlar üç deneysel gruba ayrılmıştır: kontrol (uyarım yok, n=7), uyarım ile aynı yarımküre (n=7) ve uyarım ile farklı yarımküre (n=7). Sonuçlar bazal ön beyin uyarımının sadece α7 nikotinik reseptör alt tipi dağılımı üzerinde anlamlı bir etkisi olduğunu göstermektedir. Uyarım ile aynı yarımküre ve farklı yarımküre grupları kontrol grubundan farklı çıkmış ancak uyarı ile aynı yarımküre ile farklı yarımküre olan gruplar arasında bir fark gözlenmemiştir. α7 reseptör kompleks sayısında ve yoğunluğunda kontrol grubu ile karşılaştırıldığında anlamlı derecede artış gözlenmiştir. Ek olarak, α7 reseptör kompleks sayısı ve yoğunluğu korteks bölgeleri arasında birincil motor korteksinde diğer tüm alanlardan daha az olduğu görülmüştür. Benzer bulgular α4 tipi için sadece reseptör kompleks sayısı açısından bulunmuştur. Sonuç olarak, bu çalışma beden duyusu korteksinde kolinerjik modülasyonuna anatomik bulgular sunmuştur. Elde edilen sonuçlar dikkat mekanizmalarının ve ilgili nöropsikiyatrik hastalıkların bu mekanizmaları nasıl etkilediğinin anlaşılmasında önemli veri sağlayabilir.

Anahtar Sözcükler: Beden duyusu, Kolinerjik sistem, Dikkat, Nikotinik asetilkolin reseptör.

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LIST OF SYMBOLS

Ca^{2+}	Calcium ion
Cl^{-}	Chlorine ion
h	Hour
Hz	Herts
K^{+}	Potassium ion
μ	Micro
Na^{+}	Sodium ion

LIST OF ABBREVIATIONS

ACh	Acetylcholine
$AChRs$	Acetylcholine receptors
AD	Alzheimer's disease
$ADHD$	Attention deficit hyperactivity disorder
BF	Basal forebrain
IP	Intraperitoneally
LDT	Laterodorsal pontine tegmentum
$M1$	Primary motor cortex
$mAChRs$	Muscarinic acetylcholine receptors
$nAChRs$	Nicotinic acetylcholine receptors
$NBPFA$	Neutral buffered paraformaldehyde
NGS	Normal goat serum
NBM	Nucleus basalis of Meynert
PB	Phosphate buffer
$PBTx$	PB that contains TritonX-100
$PBTxg$	PBTx in NGS
PDD	Parkinson's disease
PPT	Pedunculopontine tegmental nucleus
$S1$	Primary somatosensory cortex
$S1HL$	Primary somatosensory hindlimb area
$S1BF$	Primary somatosensory barrel field
SI	Substantia innominata
vDB	Vertical limbs of the diagonal band of Broca

1. INTRODUCTION

1.1 Motivation

At any given moment, brain receives thousands of inputs from environment although it cannot process all inputs. Therefore, there should be a mechanism that selects some inputs and eliminates others. This mechanism is called attention. Attention can be accepted as one of the cognitive functions and cholinergic system is the most important component of it. Cholinergic system modulates brain functions and activities by controlling neurotransmitter release. The acetylcholine (ACh) that is the main component of the cholinergic system is the one of the first molecules discovered as a neurotransmitter, thus its function in the brain has not been fully understood. Abnormalities in the cholinergic system mostly depends on changes in ACh levels and receptor expression. These changes associate with several neurodegenerative and cognitive diseases such as Alzheimer's disease (AD), Parkinson's disease (PDD) and attention deficit hyperactivity disorder (ADHD). The most of attention research focuses on mechanisms of selection within sensory modalities, like vision, audition and touch, but still there are several questions that should be answered, such as which cortical areas are essential for attention when there is a tactile stimulus.

It is still not clear how cholinergic effects are mediated while processing information in the neocortex. We attempt to clarify that by studying the distribution of cholinergic receptor subtypes in different layers in sensorimotor cortex areas after basal forebrain stimulation, the main source of cholinergic inputs. Our study will potentially contribute to how the cholinergic inputs into these areas in the cortex affect the responses of neurons and cholinergic circuitry. Based on the current knowledge, this will be the quantitative study which investigates the relationship between the basal forebrain stimulation and sensorimotor cortex in the brain at receptor level of $\alpha 4$ and $\alpha 7$ type of cholinergic receptors.

1.2 Hypothesis

The aim of this study was to investigate the effects of basal forebrain stimulation on the distribution of nicotinic acetylcholine receptors in the different brain regions specifically, primary somatosensory cortex (hindlimb and barrel areas) and motor cortex.

According to the current knowledge, we expected that there will be a change in cholinergic receptor expression after basal forebrain stimulation. The hypothesis of this thesis was that the expression of nicotinic acetylcholine receptors would change on primary somatosensory and motor cortex areas due to the basal forebrain stimulation.

1.3 Outline

In the first chapter, motivation and hypothesis of the study was presented. In the second chapter, information about attention and cholinergic system was given in general, such as structure of acetylcholine receptors, their role in attention and their localization in cortex, structure of neocortex, and basal forebrain structure and its role in cholinergic system. In chapter 3, materials and methods of this study were explained. The results of the experiments were presented in the chapter 4. In the last chapter, the general conclusions were presented and the results were discussed with limitations and future work.

2. BACKGROUND

2.1 Attention and Cholinergic System

Cholinergic system might be the most significant modulatory neurotransmitter system in the brain and controls activities that depend on several cognitive functions such as attention, learning and memory [1, 2, 3, 4, 5].

Cholinergic system projections can be categorized into two groups: the basal forebrain (BF) cholinergic system and the brainstem cholinergic system. The BF cholinergic system cells are located in four different regions which are nucleus basalis of Meynert (NBM), the vertical limbs of the diagonal band of Broca (vDB), the medial septal nucleus (MS) and substantia innominata (SI). These regions send their projections to neocortex, entorhinal cortex, hippocampus, amygdala and olfactory bulb while the brainstem cholinergic system with its cells located in the pedunculopontine tegmental nucleus (PPT) and laterodorsal pontine tegmentum (LDT). Their projections are sent to the thalamus and BF (Figure 2.1) [6, 2, 7, 8].

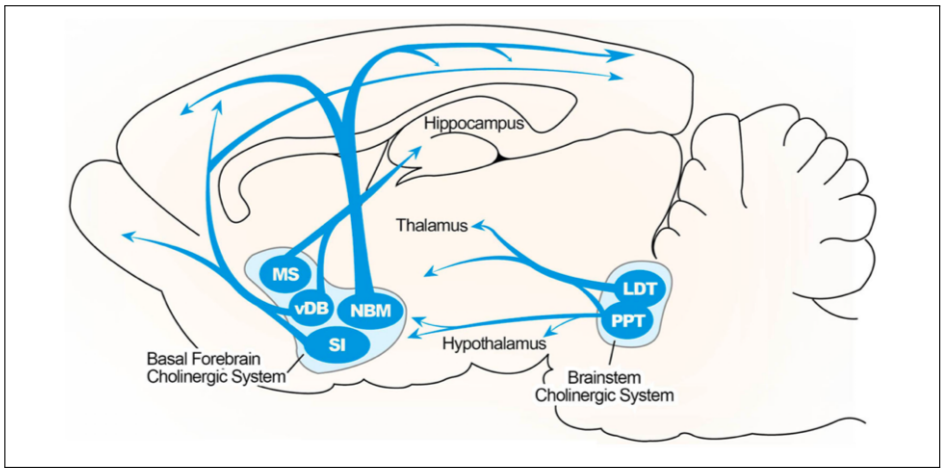


Figure 2.1 The overview of the cholinergic pathways in the rat brain [9].

In general, the anatomical organization of cortical cholinergic fibers shows a widespread, uniform innervation of cortical areas and also cortical layers [10]. Al-

though the density of cholinergic fibers differs, the distribution of cholinergic terminals in the cortex shows that all cortical areas and layers contain these fibers [11, 10].

Cholinergic influences on attention can be associated with performance on attention tasks. Additionally, cholinergic neural transmission between primary and secondary sensory regions can regulate stimulus processing by affecting signal-to-noise ratio, information flow, and response selectivity [12, 1].

In order to understand sensory processing, identifying the direction of the information flow is an important step. There are three directions which information transfer can occur. The first one is feedforward or bottom-up processing. The second one is feedback or top-down processing and the other is lateral processing which refers to horizontal connection between different brain areas [12, 6, 13].

Spence [14], Sarter et al. [15], and Himmelheber et al. [16] tried to understand how the sensory information was processed in the brain and they showed that the cholinergic system contributes to attention by enhancing the process of sensory inputs. Enhancement of input processing occurs with two not only different but also interacting mechanisms in cholinergic system. One of them is bottom-up which is merely mediated by external inputs via sensory systems. The other, top-down, is mediated by knowledge based on changes in signal detection through prefrontal modulation of cholinergic inputs (Figure 2.2) [17, 3, 10, 18]. These two processes direct the attentive focus control [19].

Top-down processes are knowledge driven mechanisms which improve the neuronal processing of relevant sensory inputs. On the other hand, bottom-up processes are operated by the characteristics of the stimulus. In many situations, bottom-up and top-down interact to reach the optimal attentional performance [5, 18, 4, 20]. Cortical cholinergic activity includes complex interactions between bottom-up and top-down mechanisms and mediates improvement of input processing at cellular level and also attentional performance at behavioral level [3].

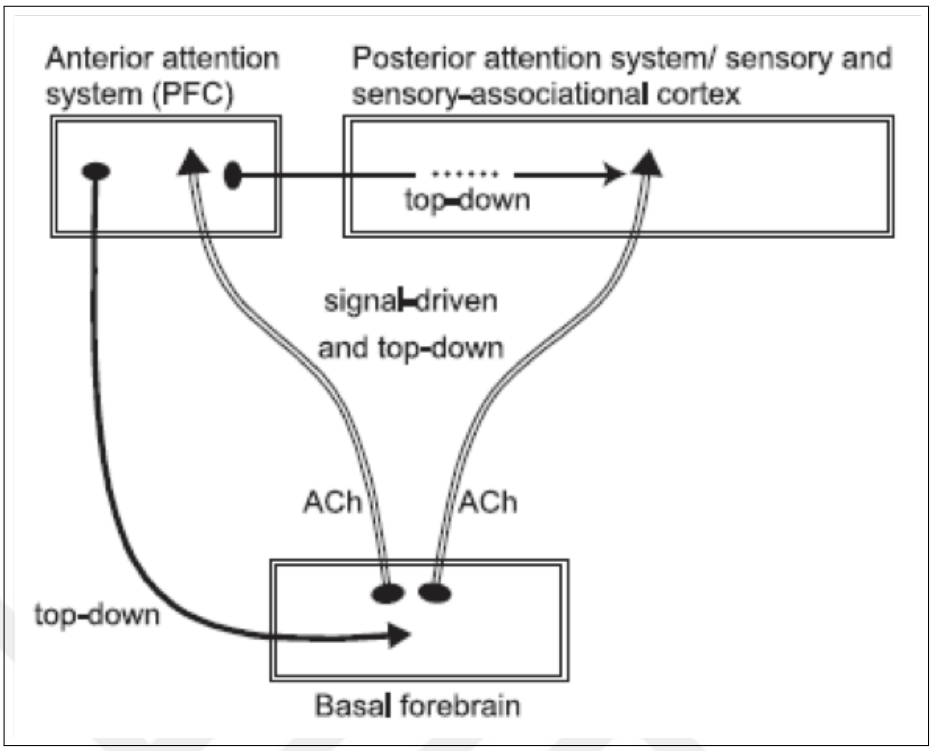


Figure 2.2 Schematic illustration of top-down cholinergic modulation [3].

2.2 The Role of Acetylcholine in Attention

ACh which is the main component of the cholinergic system has a significancy in several cognitive functions such as attention, learning and memory [21, 22]. Although small number of cells produce ACh that is a key neurotransmitter, its effects are widely distributed in the brain [6]. ACh manages attention in two ways: adapting to environmental stimuli and reduces responses to ongoing stimuli or increasing responses to ongoing stimuli [23, 24]. Previous studies suggest the hypothesis that cortical ACh mediates the detection, selection and the processing of sensory information by increasing the responsiveness of cortical neurons to other afferent inputs while suppressing other inputs [25, 10, 26, 27]. The enhancement of sensory input processing affects stimulus intensity [3].

Parikh et al. [28] by using cyclic voltammetry technique shows that the attention required events resulted in ACh release in the frontal cortex and increased levels of ACh when attentional effort was required. [28, 29].

Klinkenberg et al. [21], Rasmusson et al. [30] and Sarter et al. [31] studied about the cortical cholinergic input system, generally concentrated on the diffusion of cholinergic projections. They suggest that levels of extracellular ACh which alter slowly mediate different cognitive functions. The ability of to reach extra synaptic space instead of remaining synaptic cleft is considered to be the main issue for understanding cholinergic system [21, 30, 31].

Source of the information which can be top-down or bottom-up can affect the response of heterogeneously distributed ACh in the brain. For instance, in somatosensory cortex, acetylcholine receptors (AChRs) suppress or facilitate excitatory or inhibitory inputs that come from cortico-cortical connections. Conversely, excitatory thalamocortical inputs are not suppressed or facilitated by AChRs [3].

Sarter et al. [15, 3], Parikh et al. [22] suggest that two different acetylcholine receptor subtypes have different roles in cognitive processes, and it can be stated that the mediation of intrinsic connections (top-down) which can be excitatory or inhibitory are via muscarinic acetylcholine receptors (mAChRs). On the other hand, sensory input processing (bottom-up) is regulated by nicotinic acetylcholine receptors (nAChRs) [26]. Lesioning is one of the techniques which is used by many researchers in order to understand the relationship between the cholinergic neurons which release acetylcholine and increased attentional performance. However, lesions might affect all neurons containing glutamatergic or GABAergic receptors. Therefore, this makes it difficult to understand the role of ACh in the cholinergic system. This is solved with the development of cholinergic immunotoxin 192-IgG-saporin which selectively binds to AChRs [12, 21].

Sarter et al. [15] and Mirza et al. [32] to understand the role of different subtypes of AChRs in cholinergic modulation applied antagonists. Their results suggest that both acetylcholine receptor subtypes are necessary for attention. The presence of two different antagonists which have specifity for nAChRs and mAChRs suggest that both nicotinic and muscarinic receptor subtypes have an importance at different stages of information processing. The first one may be important in the early stages of

stimulus while the other is important in later stages of response selection [16, 32, 15].

On the other hand, Herrero et al. [33] performed a study with non-human primates(*Macaca mulatta*) to investigate ACh application effects on attentional performance. Its results show that attentional modulation can be increased by applying low doses of ACh and in order to understand which subtype contributes to increased attentional performance, nicotinic and muscarinic antagonists are applied. The result suggested that muscarinic antagonist suppresses increased attentional performance while nicotinic antagonist has no effect [33].

2.3 Structure of acetylcholine receptors

ACh is widely distributed in the brain and it can be the accepted modulator instead of neurotransmitter regulating information processing because its concentration alters significantly in one region of the brain to another. ACh consists of two groups which are synthesized from choline and acetyl group by acetyl-coenzyme A. The ACh is synthesized in the axonal terminals and catabolized by the choline acetyltransferase [34, 35, 36].

2.3.1 Muscarinic acetylcholine receptors

ACh can alter the excitability and firing properties of cortical pyramidal neurons by activating muscarinic acetylcholine receptors. According to the current knowledge of muscarinic-induced cortical processing, there are two main models: muscarinic signaling enables the cortex to depend on information coming from the sensory organs, or muscarinic signaling enables efficient filtering of information [36].

Application of cholinergic antagonists show that receptive field properties can be changed in sensory areas, especially somatosensory and auditory cortex [37, 38]. The discovery of the muscarinic acetylcholine receptor and its binding sites are based on the study about physiological effects of mushroom. At present, pharmacological, immunological, biochemical and molecular biology studies demonstrate that there are

five different subtypes for mAChRs [38, 36].

The mAChRs are the member of large family of receptors called G-coupled metabotropic receptors. There are five identified subtypes of muscarinic receptors (M1-M5) in mice while rats have four of them; M1-M4 [39, 34]. These receptor subtypes have different regional localization. The M1 receptors are present in cortex and striatum while the M2 receptors locate predominantly in cortex and basal forebrain. M3 subtype is reported in the basolateral and central amygdala and hippocampus. On the other, the M4 receptors are found in neostriatum and olfactory bulb [40, 33, 41].

Besides this general idea about the distribution of mAChRs, the M1, M2 and M4 localize differently in the cortex that has a six-layered structure. Immunostaining studies show that the distribution of mAChRs in the cortex is characterized by the laminar distribution. Predominantly, layer V pyramidal neurons contain these receptors and they project apical dendrites and branches to the layer I and II. In addition, less mAChRs is shown that layer II, III and VI and the least in the layer IV [6, 40, 42, 43, 36].

mAChRs can be located both postsynaptically and presynaptically positions, whereas presynaptic function is limited with M2 type. Therefore, all types can change excitability of neurons and release various neurotransmitters but only M2 type receptors show cholinergic feedback control over ACh release [29]. For the postsynaptic position, they can be on the soma or apical dendrites. Postsynaptic labeled mAChRs are mainly concentrated in layer IV. ACh increases the depolarization of the neuron in the cortex by activation of mAChRs postsynaptically located. The presynaptically located mAChRs which can be on the terminal of the neuron modulate the release of ACh itself [44, 36].

In the nervous system, mAChR activation has primary effects on K^+, Ca^{2+} cation channels by increasing input resistance of cells, and excitability. However, Na^+ and Cl^- channels also may be affected [36].

2.3.2 Nicotinic acetylcholine receptors

In order to understand the role of nAChRs in sensorimotor cortex, more information are required such as different subtypes of nAChRs, their respond to ACh or psychoactive drugs, location of them in areas, layers and synaptic cleft [45]. nAChRs which consist of five subunits centered around a pore are ligand gated ion channel play a role in many sensory-cognitive functions. Its structure can be described as a combination of α and β subunits called a heteromeric receptor or five α subunits as a homomeric receptor. Each heteromeric nAChR has two binding sites, and homomeric receptors have five binding sites. There are nine α subunits (α2-10) and three β subunits (β2-4) distributed across different nicotinic receptors subtypes [46, 47, 48, 49] (Figure 2.3). Besides the subunit variability, α7 and α4β2 are widely expressed subtypes in the mammalian brain [50, 51, 52].

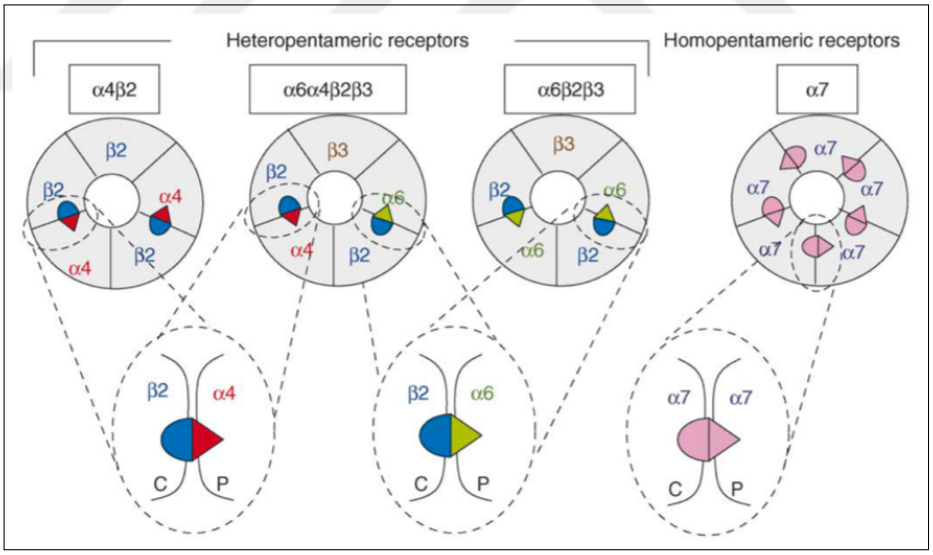


Figure 2.3 Illustration of homomeric and heteromeric structure [47].

nAChRs which are controled by an extracellular signaling molecule, they regulate the flow of ions across the cell membrane [53]. The ion channel depolarizes the cell membrane and the excitability increases due to the net influx of cations [53, 54, 55]. Nicotinic modulation of neurotransmitter release vary according to subtypes, and this

variablity can be different within brain areas [56, 24, 57].

nAChRs consist of extracellular amino terminal domain that has the ACh binding site and also called cys-loop, four hydrophobic transmembrane domains and a long cytoplasmic loop, and other shorter loops connecting the domains. [58, 46, 59, 60] (Figure 2.4).

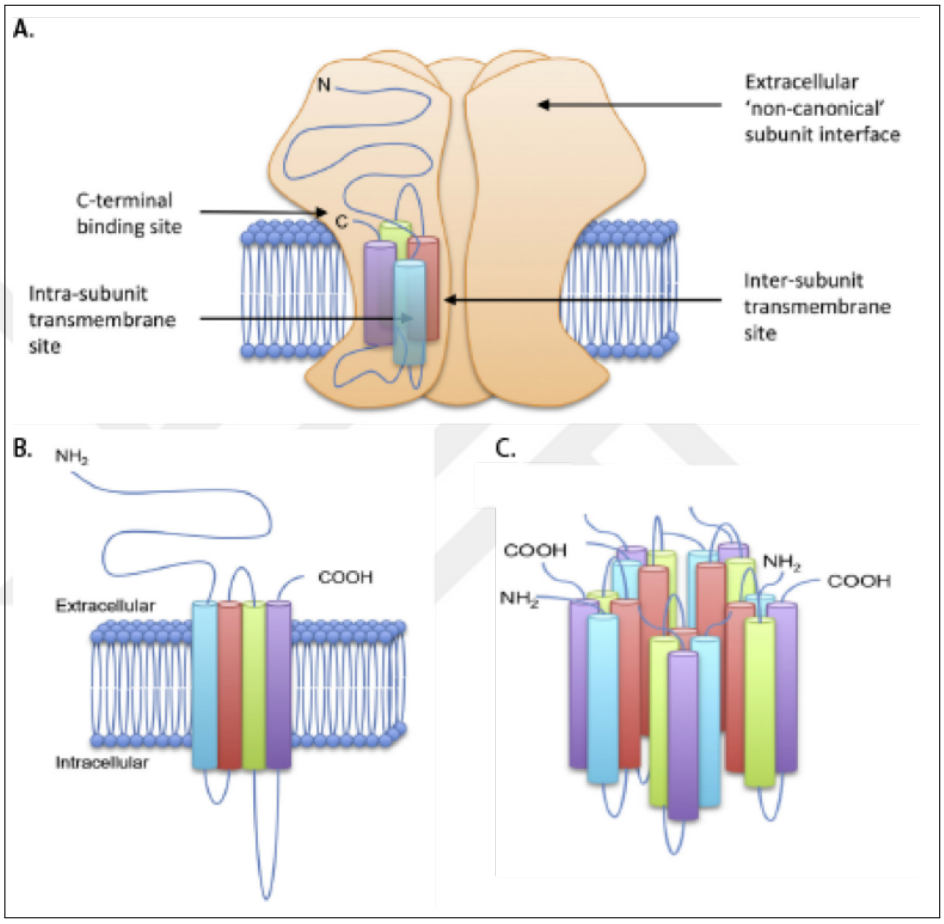


Figure 2.4 Structure of nAChRs [58].

The contribution of nAChRs to cognitive functions such as attention can be understood better by specifying the distribution of different subtypes of receptor. Ligands like the frog toxin epibatidine or the snake toxin α-bungarotoxin are used for these purposes in the previous studies [49, 61, 60]. Additionally, [^{3}H] epibatidine or [^{125}I] epibatidine can be used to label heteromeric nAChRs which have high affinity, while [^{125}I] α-bungarotoxin was used to label α7-containing homomeric nAChRs which have low affinity [62].

There is also another mapping technique which is the α-bungarotoxin sensitive or insensitive. α-bungarotoxin sensitive receptors might be homomeric or heteromeric but α-bungarotoxin insensitive receptors only can be heteromeric receptors [63, 46, 48].

nAChRs can be found in three possible conformational states. These are the closed state at rest, the open state, and the desensitized state. With binding of ACh or other agonists, nAChRs change their conformation from closed to open state for several milliseconds. Then following state of receptor is desensitized which means unresponsive to agonists for a very brief moment. The rate of changes between conformational states can affected from many factors like subunit composition. When the receptor is open, nAChR current consists of sodium, potassium and calcium which make an important contribution [64, 65]. nAChR's permeability is not limited with just sodium and potassium ions; they are permeable to calcium ions and this permeability can depend on receptor subtypes. For example, α7 homomeric nAChRs have high calcium permeability and rapid activation while α4β2 receptors have lower calcium permeability [66, 64, 67, 46].

nAChRs can have both presynaptic and postsynaptic localization [68, 56, 35]. According to their localization, their effects to the cognitive processes are different. Firstly, they can be found on dendrites and cell bodies presynaptically. Presynaptically localized nAChRs can modify synaptic transmission by regulating the amount of both excitatory and inhibitory neurotransmitter release [69, 70]. In the post synaptic location, nAChRs are responsible for rapid excitation [71, 63]. Additionally, nAChRs can be located on extra-synaptic sites so they can change cell excitability on post synaptic cells and neurotransmitter release at presynaptic sites [46, 53, 35].

Generally, these receptors are located presynaptically on thalamocortical terminals in layer III and IV [72]. The other receptor location is on axon terminals which means postsynaptically. nAChR open state is modulated by external calcium ions that have an effect on cholinergic synaptic currents. Additionally, the calcium influx through these receptors is enough to activate calcium dependent chloride and potassium channels. nAChRs also mediate rapid excitation of postsynaptic neurons [45, 70, 73, 48].

According to our current knowledge, nAChRs are widely and nonuniformly distributed in the brain; the most expressed subtypes are α7 and α4β2. Homomeric α7 subunit receptors are mainly expressed in the cortex, hippocampus and subcortical

limbic regions while they are expressed less in the thalamic regions and basal ganglia. On the other, α4β2 can be almost all brain regions but mainly they are expressed in the cortex, striatum and cerebellum [46, 47, 74, 75, 76] (Figure 2.5).

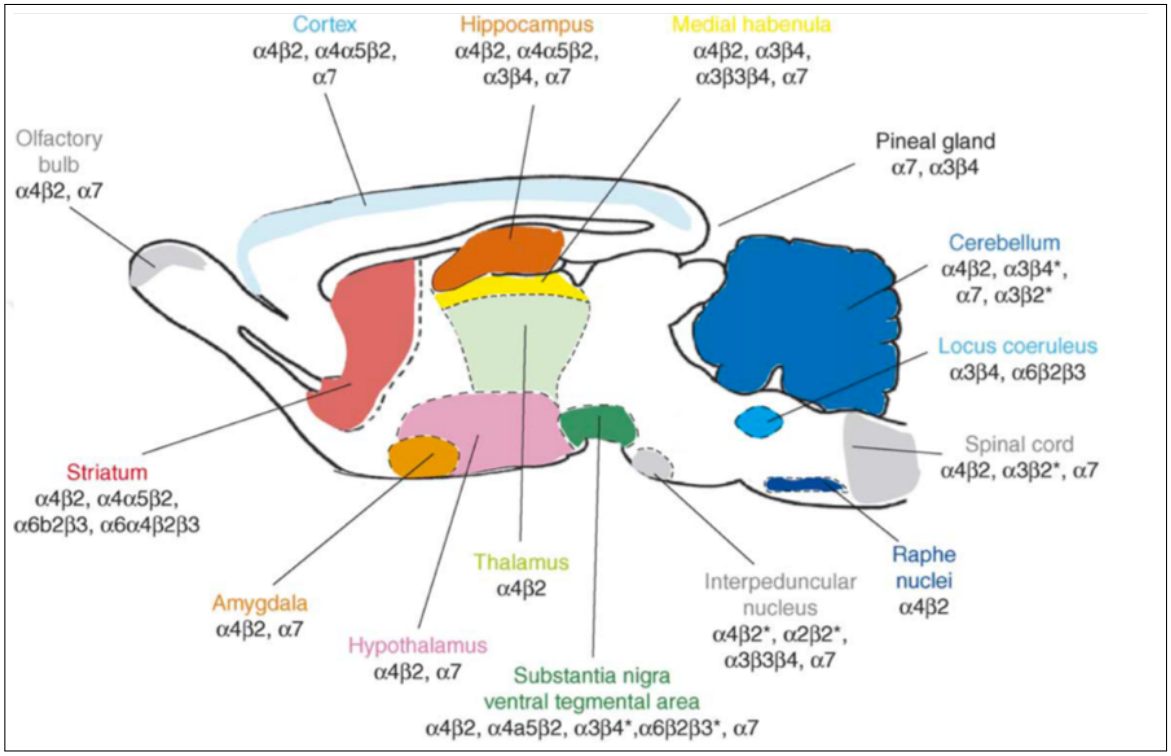


Figure 2.5 Distribution of different nAChR subtypes in the rat brain [60].

In the cortex, nAChRs are found mostly in deep layers with high density. These are layer IV where the inputs came from thalamus and layer V/VI [77, 78]. Also, they can be found in supragranular layers in inhibitory cells, including all layer I interneurons. In sensory cortical areas, the inputs that come from the thalamus enter the layer IV of the cortex in bottom-up processing. Layer IV neurons project to the upper layers. Layer V conveys the information to the deepest layer which is the layer VI. The deepest layer sends inputs back to the superficial layers [6, 45, 15, 65].

2.4 Structure of Neocortex

It is considered that high cognitive functions develop with the evolution of the neocortex in mammals. However, neocortices of different mammalian species can be significantly different in terms of shape, size, and neuron number. In order to understand how brain works and neocortex structure models of neocortical development depend on studies of the mouse and rat because their neocortex have many common features in all mammals, in general including a six-layered organization [79, 80].

The neocortex is organized into columns and layers. Behind its approximately 2.5 mm thickness (on avarage in mammals), there are thousands of cell types, a lot of layers, and complex connectivities. The connections between cells shows a columnar flow of information within some layers of cortex [81, 82].

Columnar cortical circuits conduct afferent input conveying "bottom-up" and "top-down" information. Bottom-up inputs arrive from mostly the thalamus, which gets inputs from sensory systems and the spinal cord, cerebellum etc. Top-down input arises primarily from association areas of the cerebral cortex and secondary thalamic areas [83]. These complex input-output relationships depend on layer organization of cortical neurons; each layer contains different inputs and outputs. Most of the neocortex contains six layers, defined from the external layers of the cortex to the white matter with roman numerals [84, 85] (Figure 2.6).

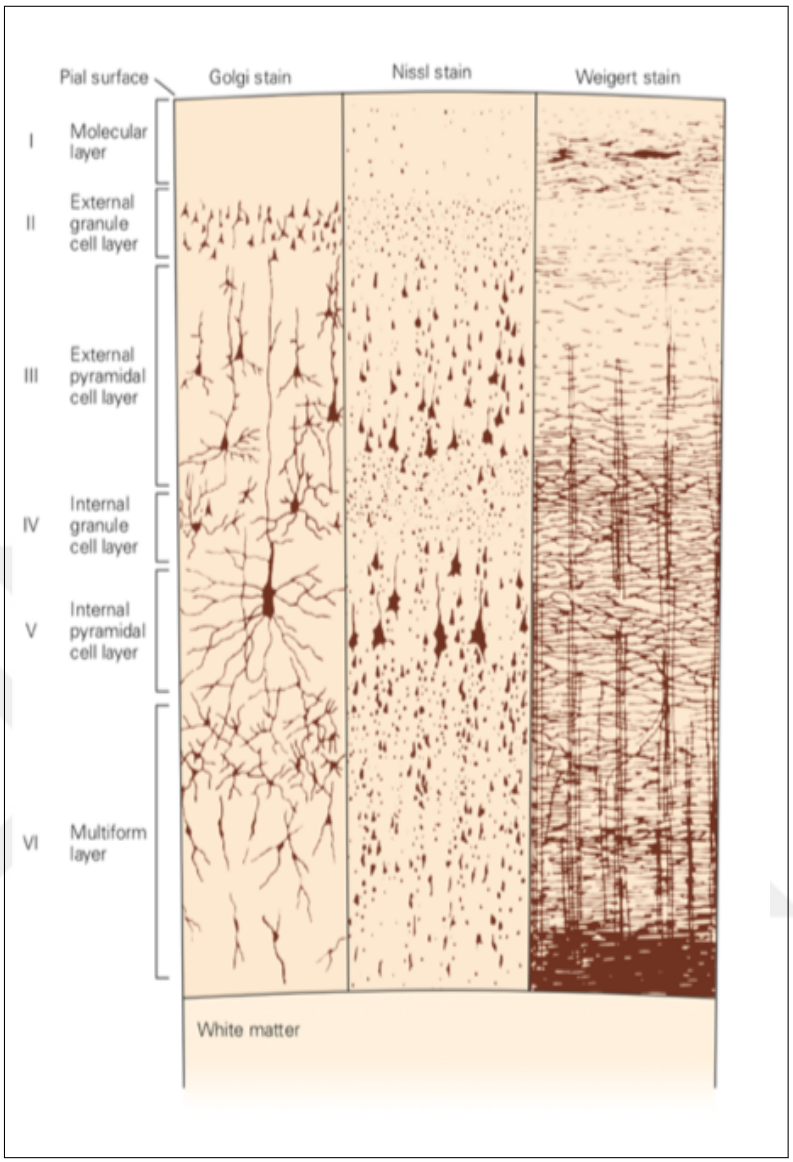


Figure 2.6 The neurons of neocortex [84].

Layer I, called as the molecular layer, comprises of the dendrites and axons of cells found in deeper layers. Layer II, the external granular cell layer, is one of two layers that contain small spherical neurons. Layer III is called the external pyramidal cell layer that has moderate and large sized pyramidal neurons. Additionally, there are some granular neurons. Afferent fibers comes from thalamus and cortico-cortical connections reach layer III and I. Layer IV called the internal granular cell layer, contains a large number of small spherical neurons. It is the main layer that sensory input comes from the thalamus. The next layer, the internal pyramidal cell layer, contains

mainly pyramidal cells that are larger than cells found in layer III. Pyramidal neurons in this layer form major output pathways of the cortex, project their axons to other cortical areas. The neurons in layer VI are fairly heterogeneous and so it is called the polymorphic or multi-form layer [86, 84, 87].

Excitatory neurons make up approximately 80% of the total neuron population of the neocortex. The first group of excitatory neurons consist of spiny stellate neurons which are found on only in the layer IV and generally responsible for conveying thalamocortical inputs. Additionally, their axons make connection with basal dendrites of pyramidal neurons in layer III and IV. The second class of excitatory neurons include pyramidal neurons. Their projections go into the corpus callosum. The third class of excitatory neurons are pyramidal neurons that locate in layer V. They project to deep subcortical areas such as the spinal cord and brainstem. The final group of excitatory neurons are corticothalamic pyramidal neurons mainly found in layer VI. Their apical tufts may branch within layer IV. Therefore, in the same time they can receive thalamocortical connections with their apical tufts and their basal dendrites can receive inputs from layer V pyramidal neurons [83, 82, 87] (Figure 2.7).

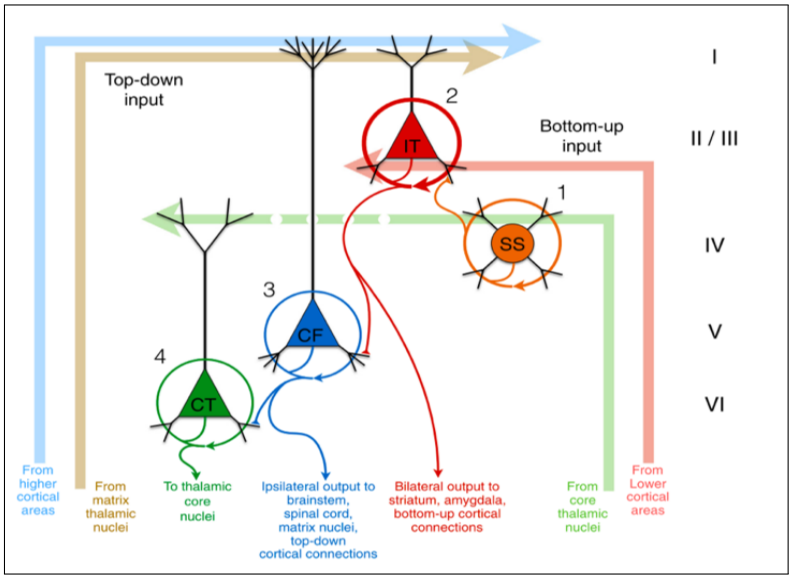


Figure 2.7 Excitatory cortical circuitry in primary sensory cortex (1) spiny stellate neuron (2) intratelencephalic neuron (3) corticofugal neuron (4) corticothalamic neuron [83].

2.5 Basal Forebrain and Its Role in Cholinergic System

Cholinergic neurons originate from four regions. These include the brainstem, thalamic nuclei, the striatum and the basal forebrain nuclei which are the major sources of cholinergic projections to neocortex [19].

The cholinergic system which originated in the BF has a importance for increasing bottom-up sensory input to the cortex and cortical coding by decreasing noise correlations [88, 89].

BF can be defined as a continuation of subcortical neurons that project to neocortical and other brain areas. It has several different types of neurons that are cholinergic, non-cholinergic projection neurons and interneurons which contribute to different ascending or descending pathways. BF cholinergic system that can be considered as a main component of top-down processes makes contribution to many cortical processes such as attention, learning and memory [90, 91, 4, 92, 93]. Previous studies demonstrate that BF cholinergic system contributes to attention in two ways. The first is managing detection process which can be defined as altering attention from ongoing activities to awareness of stimuli. The other is the activation of cue-associated response [94, 95, 96, 22].

The cell bodies of the BF cholinergic neurons are categorized in four different groups: MS nucleus which project mainly to the hippocampus, vDB (both vertical and horizontal domains) projecting to the hippocampus and cingulate cortex, NBM that project primarily to the olfactory bulb and entorhinal cortex, and SI projecting neocortex and amygdala [19, 22, 91, 97, 93] (Figure 2.8).

The nucleus basalis of the basal forebrain which is the main component of the neuromodulatory system targets the cortex. ACh is released in the cortex by neurons whose cell bodies are located in the BF, so the nucleus basalis is accepted as a main source of cortical ACh [98, 99]. ACh release increases via stimulation of the nucleus basalis and it can change neuronal excitability and synaptic efficacy, signal-noise ratio, and regulate sensory processing [100, 101, 102, 90, 103]. Previous studies show that in order to understand neurons's excitability paired nucleus basalis and sensory stimulation should be applied. As a result of these kinds of paired stimulation, cortical firing properties vary between cortical layers (increase in layer IV, V and VI, and decrease

in layer II/III) [19, 100, 101, 96, 22] (Figure 2.9).

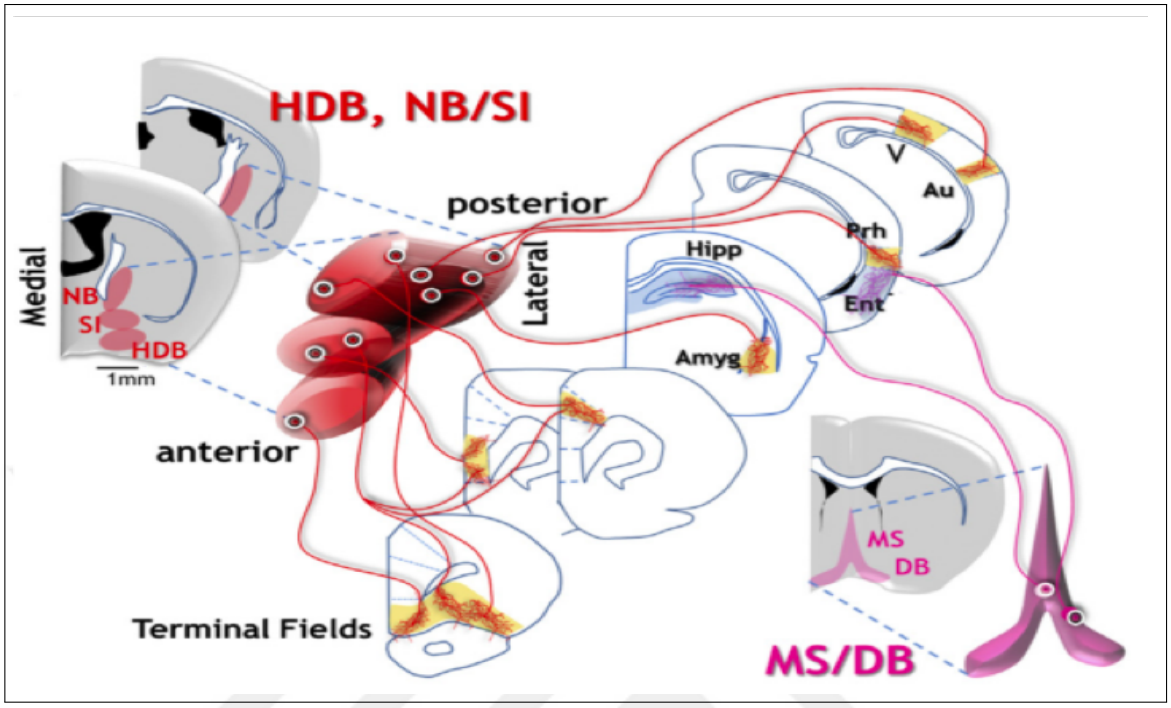


Figure 2.8 Basal forebrain nuclei [19].

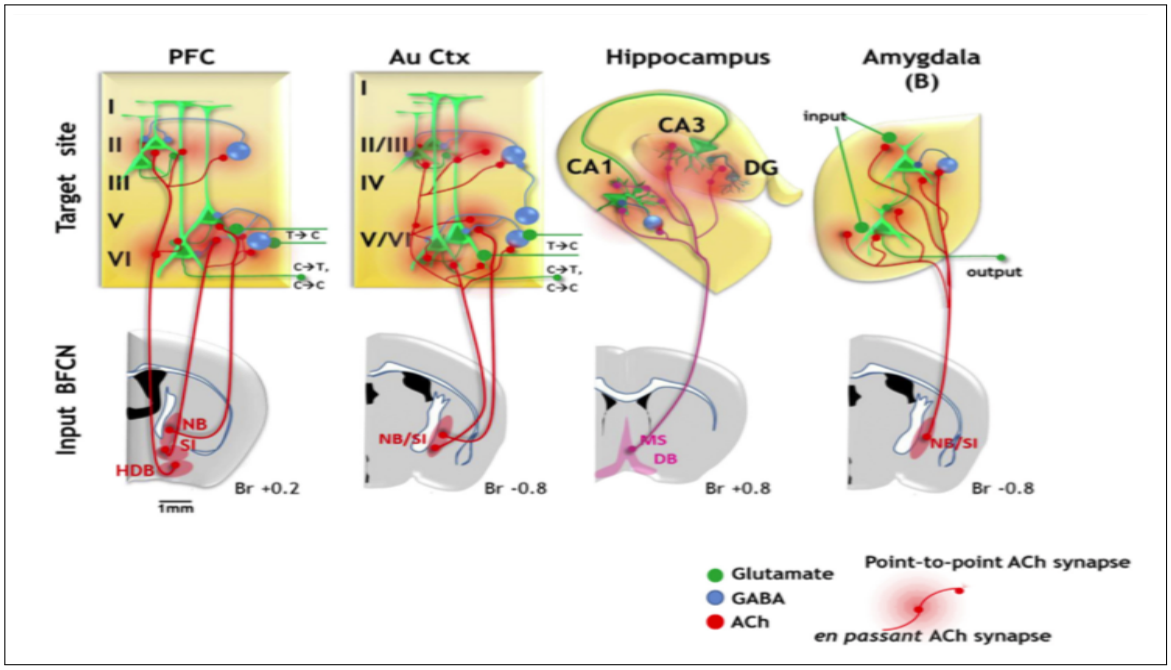


Figure 2.9 Basal forebrain[19].

Additionally, paired stimulation of basal forebrain and sensory is not enough to distinguish which acetylcholine receptor type affects neuronal excitability, signal-noise ratio, and sensory processing. In order to be aware of this discrimination, muscarinic or nicotinic antagonists should be applied [90, 93].

3. MATERIALS and METHODS

3.1 Animals and Husbandry

All experiments were approved by Boğaziçi University Institutional Local Ethics Committee for the Use of Animals in Experiments (BÜHADYEK). In this study, 4 adult female Wistar albino rats were used for Nissl staining and 20 adult Wistar albino rats (7 male and 13 female) were used for immunocytochemistry procedures. The animals were parted from another study which is neural spike responses in somatosensory cortex.

3.2 Electrophysiology

Intraperitoneally (IP) administration of ketamine (65 mg/kg) and xylazine (10 mg/kg) is used for anesthesia before the electrophysiological study of another researcher. Rectal temperature was checked and kept at 37 °C by using heating pad. The general condition of the rat was checked periodically as well as the state of anesthesia by controlling palpebral and pedal reflexes. To maintain the appropriate level of anesthesia, additional 1/3 dose of injection of anesthesia was given when found necessary. The head was fixed with a stereotaxic frame and a craniotomy was performed to place stimulation electrode either in right or left hemisphere. After this step, rats were ready for BF stimulation.

Basal forebrain stereotaxic coordinates, and motor cortex coordinates which correspond to hindlimb area, and barrel field were shown in figure 3.1 and figure 3.2, respectively. For histological procedures, brain sections were cut between 0(bregma) - (-2) stereotaxic coordinates.

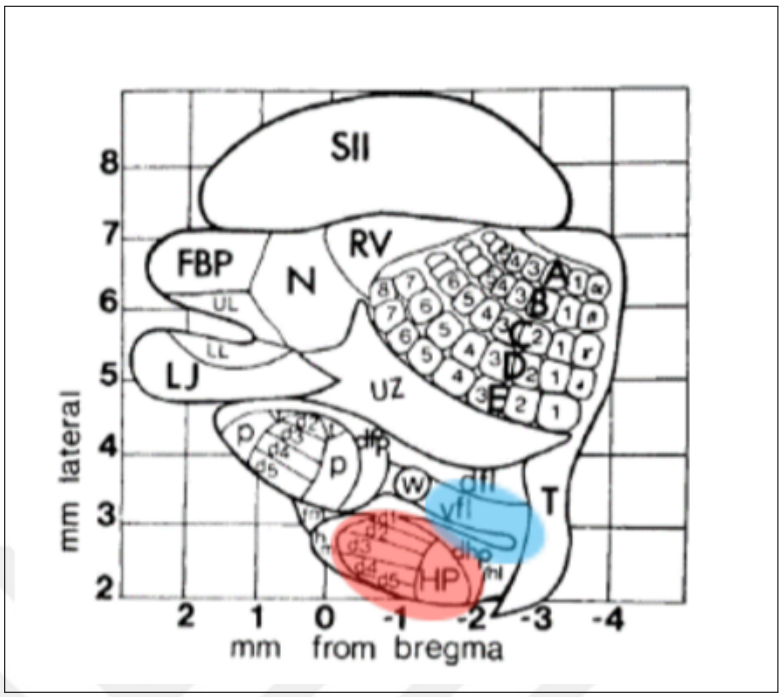


Figure 3.1 Stereotaxic coordinates, red circle shows the area representing the hindlimb area of SI cortex for recording whereas blue circle gives location of NB area for electrical stimulation of BF [104].

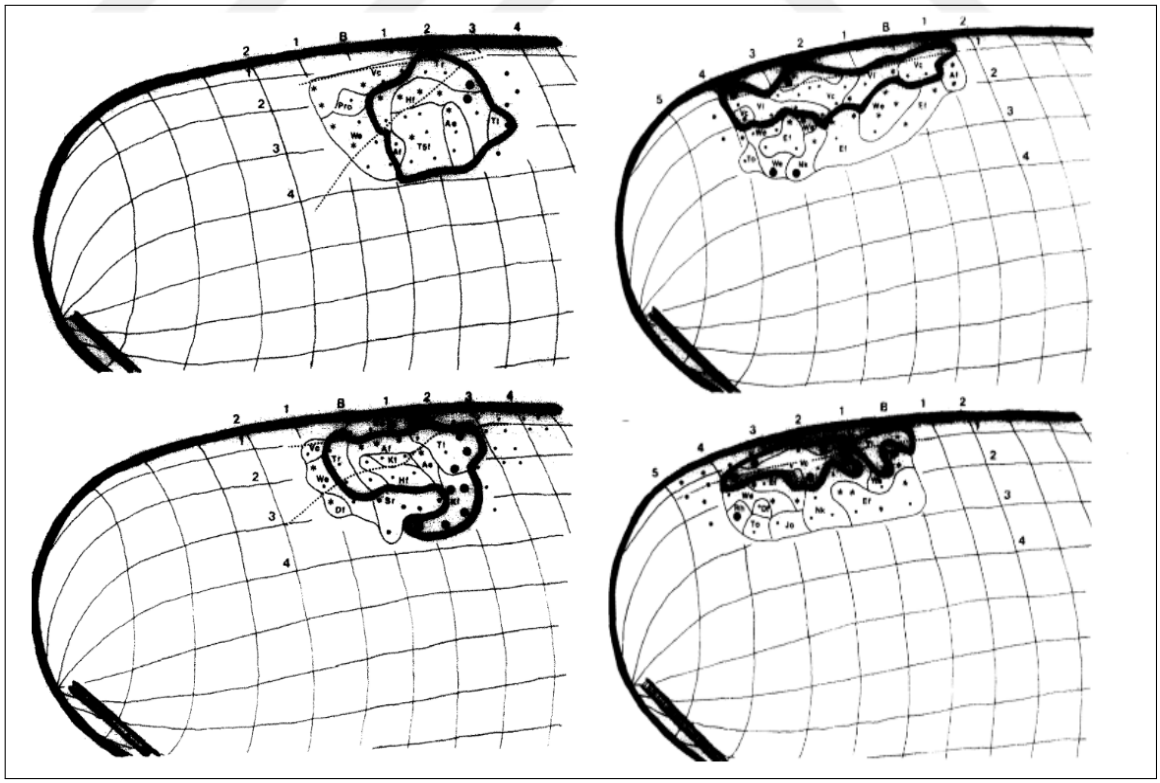


Figure 3.2 Two separate stimulation mapping experiments (right column shows barrel field representing motor cortex and left column shows hindlimb area) [105].

BF stimulation electrode was inserted into Nucleus basalis of Meynert to a depth of 8.2 mm from the cortical surface at 2.3 mm posterior to bregma and 2.9 mm lateral from the midline according to the rat brain stereotaxic coordinates in the atlas of Paxinos and Watson [104] as shown in figure 3.3. According to the hemisphere that was stimulated experimental groups were created as such control, ipsilateral and contralateral. Control group received no stimulation. Ipsilateral group received stimulation in the same hemisphere for which histological analysis was performed whereas contralateral group was stimulated in the opposite hemisphere.

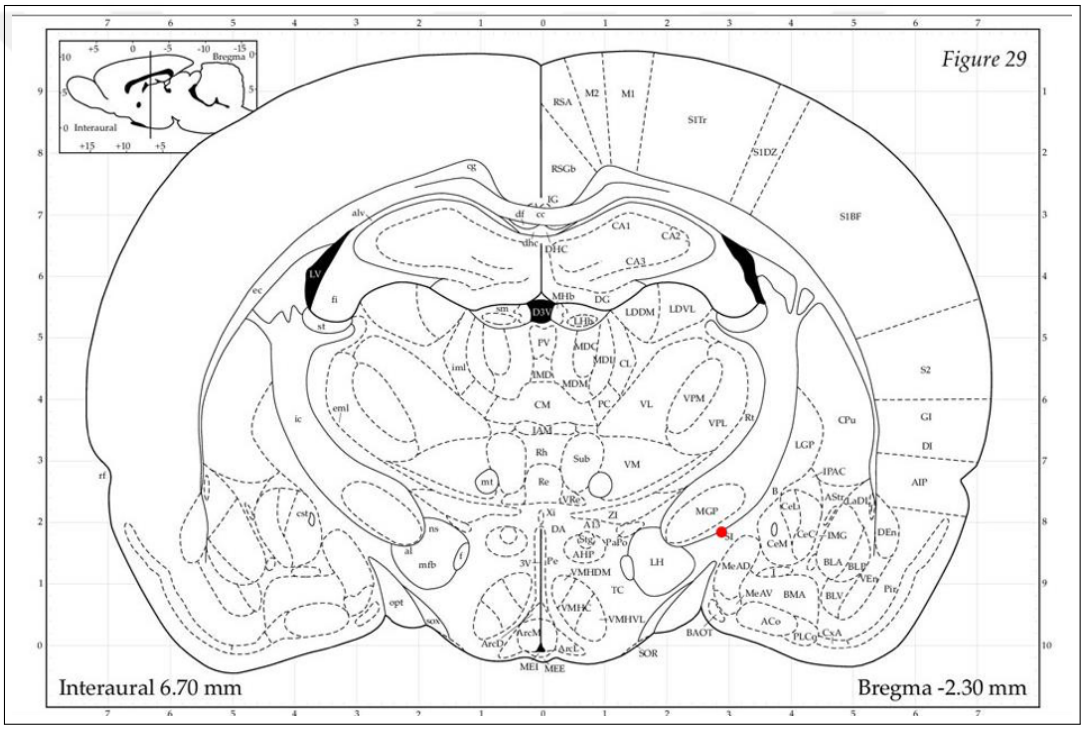


Figure 3.3 NBM neurons are located at SI region of Brain (shown as red dot) [104].

BF stimulation parameters were 12000 pulses in total, and each bipolar current pulses with amplitude 50 µA, 50 pulses were applied at 100Hz. After BF stimulation, each rat was perfused for histological procedures.

3.3 Histological Procedures

3.3.1 Nissl Staining

In order to understand general six-layered cortex structure in the brain, Nissl staining was performed. Nissl staining is a specific method which gives a general idea about cortical organization of the brain. In order to determine the nucleic acid content of cells like Nissl substances (rRNA in rough endoplasmic reticulum) and nucleolus Nissl staining protocol was applied.

Before the Nissl staining protocol, brain sections (50 µm) were cut coronally through the anterior portion of the brain by using vibrating blade microtome (Leica VT1000S vibratome) in phosphate buffer (PB). Gelatin-coated slides that each one contains four sections were used for section mounting. These slides were transferred to stainless-steel slide racks (20 slides/rack) after leaving them at room temperature to dry for about 12-18h and then they will be stained with Cresyl Violet. In the Nissl staining protocol, the racks followed these steps: Dehydrate/demyelinate/rehydrate sections by alcohol (EtOH) series, rinsing with dH_2O staining with Cresyl Violet Acetate solution, destainig with acetic acid, dehydration again and cleaning with xylene.

3.3.2 Immunocytochemistry

Standard immunofluorescence protocol was applied to coronal sections (thickness: 50 µm). 12 sections were prepared in order to investigate the specific brain areas. 6 of them were chosen for α4 nACh receptor subtype and rest of them were used for α7 nACh receptor subtype. The 1^{st} sections were selected for DAPI staining for both α4 and α7 nACh receptor subtypes. From 2^{nd}-4^{th} sections were used for immunofluorescence protocol and the avarage of them were used for the analysis. For secondary antibody control 5^{th} was chosen therefore the secondary antibody was not added to sections. The 6^{th} was chosen for primary antibody control therefore the primary antibody was not applied.

To localize α4/α7 nACh receptor complexes on the neurons of specified sensorimotor cortex areas, for the α4 nACh receptor subtype Rabbit polyclonal anti-nAChRα4 (sc-5591) was chosen as primary antibody and for the α7 nACh receptor subtype, Rabbit polyclonal anti-nAChRα7 (sc-5544) was chosen. As a secondary antibody, Goat anti-rabbit IgG (H+L) with Alexa Fluor 594 (ThermoFisher Scientific: A-11012) was chosen for both subtypes. Its excitation and emission wavelength are 591 nm and 614 nm respectively (Figure 3.4).

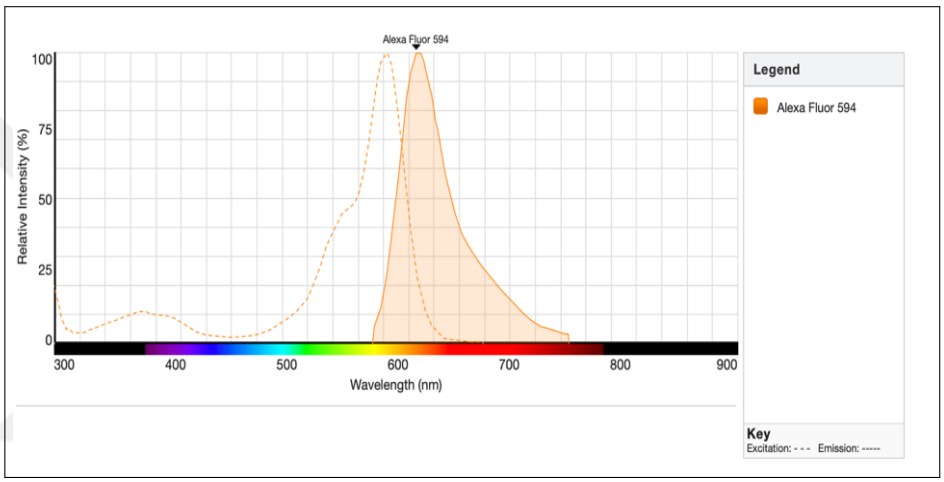
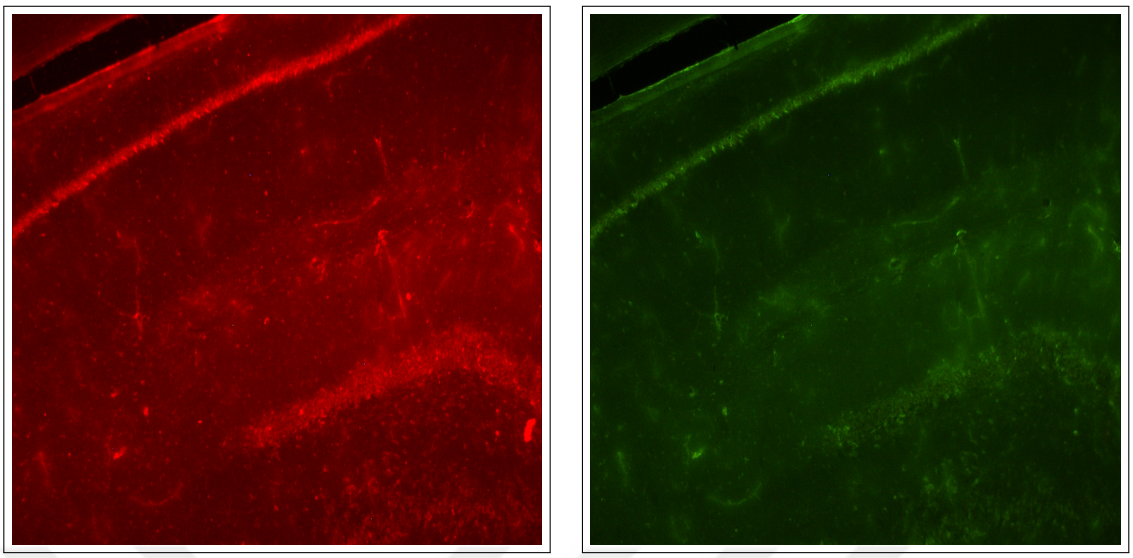


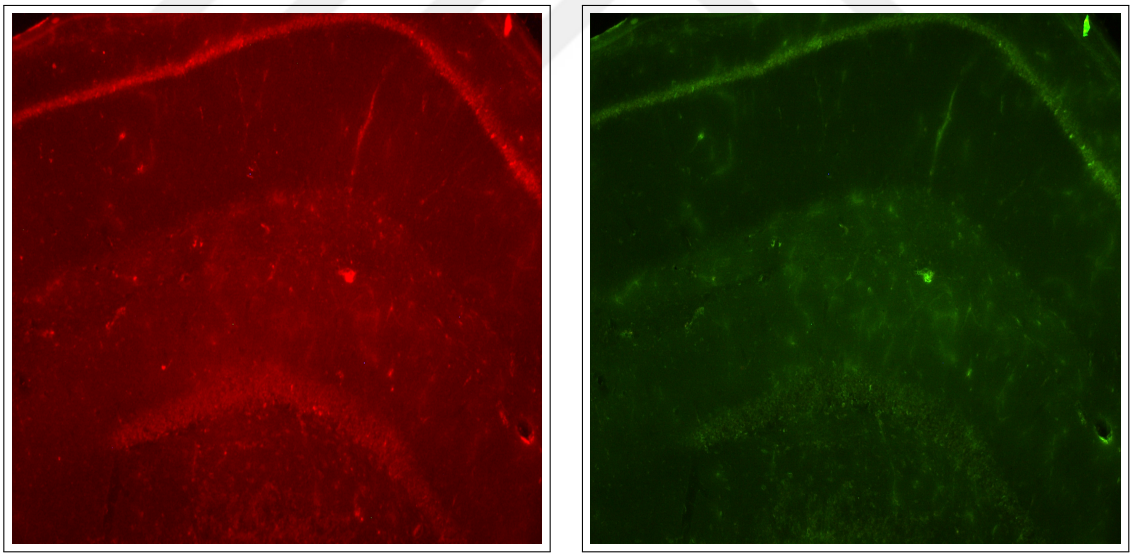
Figure 3.4 Goat anti-rabbit IgG (H+L) with Alexa Fluor 594(ThermoFisher Scientific: A-11012) excitation and emission wavelength.

In addition to primary and secondary antibody controls, we run positive control in our experiments in order to confirm that α4 and α7 antibody stainings were accurate and reliable. Positive control means sections contains the target molecule in its known location. In our experiments specificities were checked with sections from hippocampus and cerebellum regions in the rat brain. Whole hippocampal regions were immunoreactive for both α4 and α7 subtypes but the most reactive side was CA1 region [53, 49, 61]. Our positive control staining for both subtypes confirmed this specificity (For α7 subtype Figure 3.5 and for α4 subtype Figure 3.6).



(a) (b)

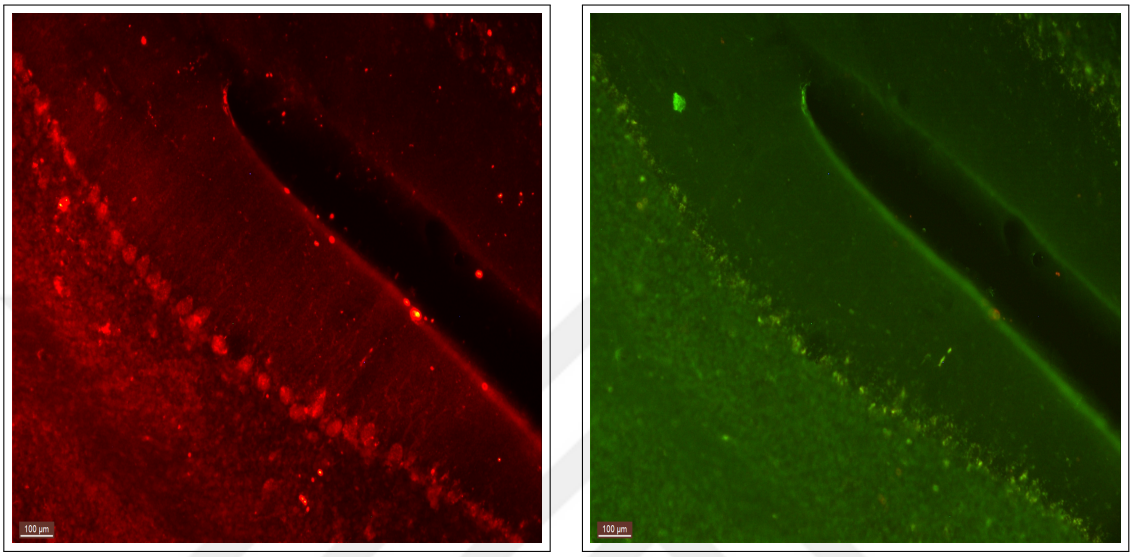
Figure 3.5 (a) α7 staining from CA1 region of hippocampus viewed in Leica filter cube: N2.1. (b) α7 staining from CA1 region of hippocampus viewed in Leica filter cube: I3.



(a) (b)

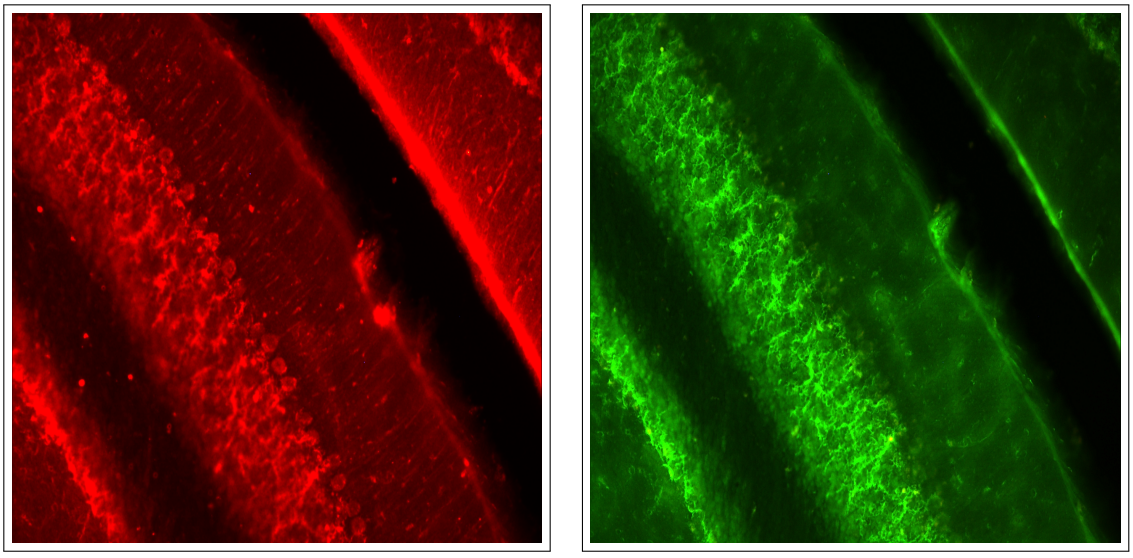
Figure 3.6 (a) α4 staining from CA1 region of hippocampus viewed in Leica filter cube: N2.1. (b) α4 staining from CA1 region of hippocampus viewed in Leica filter cube: I3.

In cerebellum, α4 and α7 subtypes showed immunoreactivity mostly in Purkinje layer. Additionally, granular layer also showed reactivity for both subtypes [106, 77, 107]. Our data confirmed these studies for both α4 and α7 specificity for Purkinje layer (For α7 subtype Figure 3.7 and for α4 subtype Figure 3.8).



(a) (b)

Figure 3.7 (a) α7 staining from cerebellum viewed in Leica filter cube: N2.1. (b) α7 staining from cerebellum viewed in Leica filter cube: I3.



(a) (b)

Figure 3.8 (a) α4 staining from cerebellum viewed in Leica filter cube: N2.1. (b) α4 staining from cerebellum viewed in Leica filter cube: I3.

Firstly in the post fixation step, brain was expected to sank in the neutral buffered paraformaldehyde (NBPFA)/Sucrose solution (20% sucrose in 4% paraformaldehyde). PB that contains Triton X-100 (PBTx) which is a detergent for permeabilizing the tissue membrane was used for washing the brain 6 times.Then the brain was sectioned by using vibrating blade microtome (Leica VT1000S vibratome). Sections were rinsed again with PBTx in the first row by using a multi-well container (tissue culture plate). Then the sections (from 2^{nd} to 5^{th}) were rinsed with PBTx with 10% normal goat serum (NGS) (PBTxg) for an hour. This step called as a blocking step. After that, 150 µl primary antibody solution (diluted 1:50 in PBTx) was added on them and incubated with for 24-48 hours in the refrigerator at 4 °C. PBTx was used for washing the sections 3 times (each 30 min). Then, 150 µl secondary antibody solution (diluted 1:50 in PBTxg) was added on the sections for 12-24 hours in the refrigerator at 4 °C in the dark. The sections were rinsed 3 times (with at least 30 min/rinse) in different rows each time that contain 1.5 mL fresh PB. 4mM sodium carbonate solution was applied in order to reduce background staining to all sections 2 times (each 15 min) as a final step. Then, gelatin-coated slides were used for the placement of the sections. As mounting medium fluoroshield (Sigma-Aldrich) was used . Slides were kept away from light and stored in the refrigerator.

3.4 Counter Staining for Immunofluorescence

Layer thicknesses and the total cell number in the primary somatosensory hindlimb (S1HL), primary somatosensory barrel field (S1BF) and primary motor cortex (M1) were determined on sections stained with DAPI dihydrochloride (Sigma D9542). DAPI is a blue fluorescent nucleic acid stain that stains double-stranded DNA structure. Binding of DAPI to DNA enhances fluorescence. The fluorescence is directly related with the amount of DNA in the cell.

It was applied to 1^{st} section of both α4 and α7 nACh receptor subtypes. Its excitation and emission wavelength are 358 nm and 461 nm respectively (Figure 3.9). Thus, the cells stained with DAPI were visualized under UV light (Leica filter cube: A).

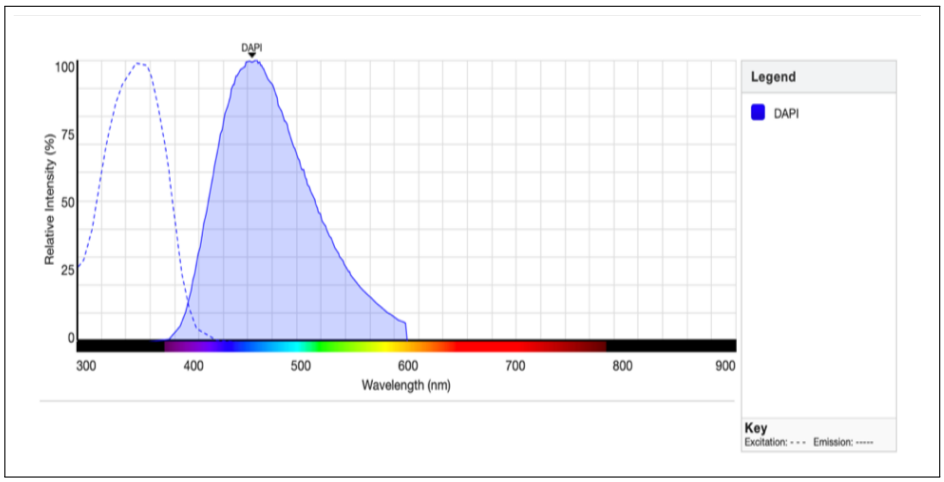


Figure 3.9 DAPI dihydrochloride (Sigma D9542) excitation and emission wavelength.

1^{st} sections were incubated with 150 µl DAPI solution for 1-5 minutes. Then samples were rinsed 3 times with fresh PB (15-30 min/rinse). As a mounting medium Fluoroshield (Sigma-Aldrich) was used.

3.5 Cell Counting

Boundaries of the brain regions (M1, S1HL, S1BF), were determined with a normalization procedure because of the fixation differences and variability between subjects. Normalization procedure was applied according to Paxinos & Watson atlas. Following equation was used to calculate the area boundaries. According to the Paxinos & Watson atlas, hemispheric width was calculated the average (7.4 mm) of starting point that includes three interested areas together (bregma -0.36, 7.2 mm) and end point that includes three interested areas together (bregma -2.28, 7.6 mm). Then, based on midline distance, for each area boundaries were calculated; 1.8 mm, 2.9 mm and 5.8 mm for M1, S1HL and S1BF, respectively. These calculations were used for to normalize measured subject midline distance and for each area. For instance;

For M1

Hemispheric width in atlas (7.4 mm) $\bar{M}$idline distance in atlas for M1 (1.8 mm)
Measured hemispheric width in subject (HW) Normalized midline distance for M1 (x)

x= HW*1.8/7.4

600 µm of the counting area was identified by drawing lines on images. Annotations and measurements were completed on the Leica LAS software. Autofluorescence in the tissues were detected by using filter cube (I3 (excitation filter BP 450-490 nm and long pass filter LP 515 nm for emission)). In order to detect immunostaining the filter N2.1 (excitation filter BP 515-560 nm and long pass filter LP 590 nm for emission) was used. The Leica software was used for all measuring and determining steps. Fiji (Ver:2.0.0-rc-69/1.52i) was used to count the receptor complexes and cells. Images were captured at 20x magnification to count receptor complexes. Digitized images were processed via changing brightness and contrast levels in order to enhance the visibility of cells and receptor complexes. Approximately twice as bright cells and receptors from the background were counted. In immunofluorescence, only cells and receptor complexes labeled with the fluorophore were marked in filter N2.1. If the cells and receptor complexes are apparent in both filter cube (N2.1 and I3) they are accepted as an autofluorescence.

3.6 Statistical Analysis

3.6.1 Nissl stained data

Based on cytoarchitecture reference, cortical layers were identified. The thicknesses of the layers of three cortical areas (M1, S1HL and S1BF) were determined in Leica software. Mean, standard error and standard deviation were calculated for each layer thickness in a given cortical area. One-way ANOVA was carried out to determine how the total cortical thickness change between the areas and two-way ANOVA was

conducted to understand the effects of factors (layer and cortical area). All statistical analysis were done by using SPSS Ver 25 (IBM Corp., Armonk, NY, USA). For any significant interactions post hoc t-tests were studied.

3.6.2 Immunostained data

The average layer thicknesses (T) from DAPI stained sections that fit with Nissl layer boundaries results were used to optimize layer thicknesses in immunostained receptor complexes counted. The number of counted α4 and α7 receptor complexes were defined as N within interested cortical layer and area. D which can be formulated as D = N / T, represented the density of α4 and α7 receptor complexes. C =N/Ntot was normalized number of α4 and α7 nACh receptor complex numbers in a layer based on the total number of cells. Ntot is the total number of cells in a given cortical layer and area which is stained with DAPI sections. Statistical analyses were performed by repeated measures ANOVA on three dependent variables: number of labeled receptor complexes (N), density of receptor complexes (D) and number of labeled receptor complexes were normalized by number of total cells (C). Between subject factor was experimental condition (control, ipsilateral and contralateral) and within subject factors were cortical area (M1, S1HL, S1BF), nicotinic receptor subtypes (α4vsα7)and cortical layer (I, II, III, IV, V, VI).

4. RESULTS

4.1 Nissl Stained Data

Brain sections from four Wistar Albino rats were used for measurements and the results were shown in the Table 4.1. The thickness of each layer was determined according to the avarage of the four measurements. Table 4.1 represent avarages of subjects and standard errors. The layer boundaries were found for three brain areas M1, S1HL and S1BF (Figure 4.1). Additionally, total thicknesses were calculated.

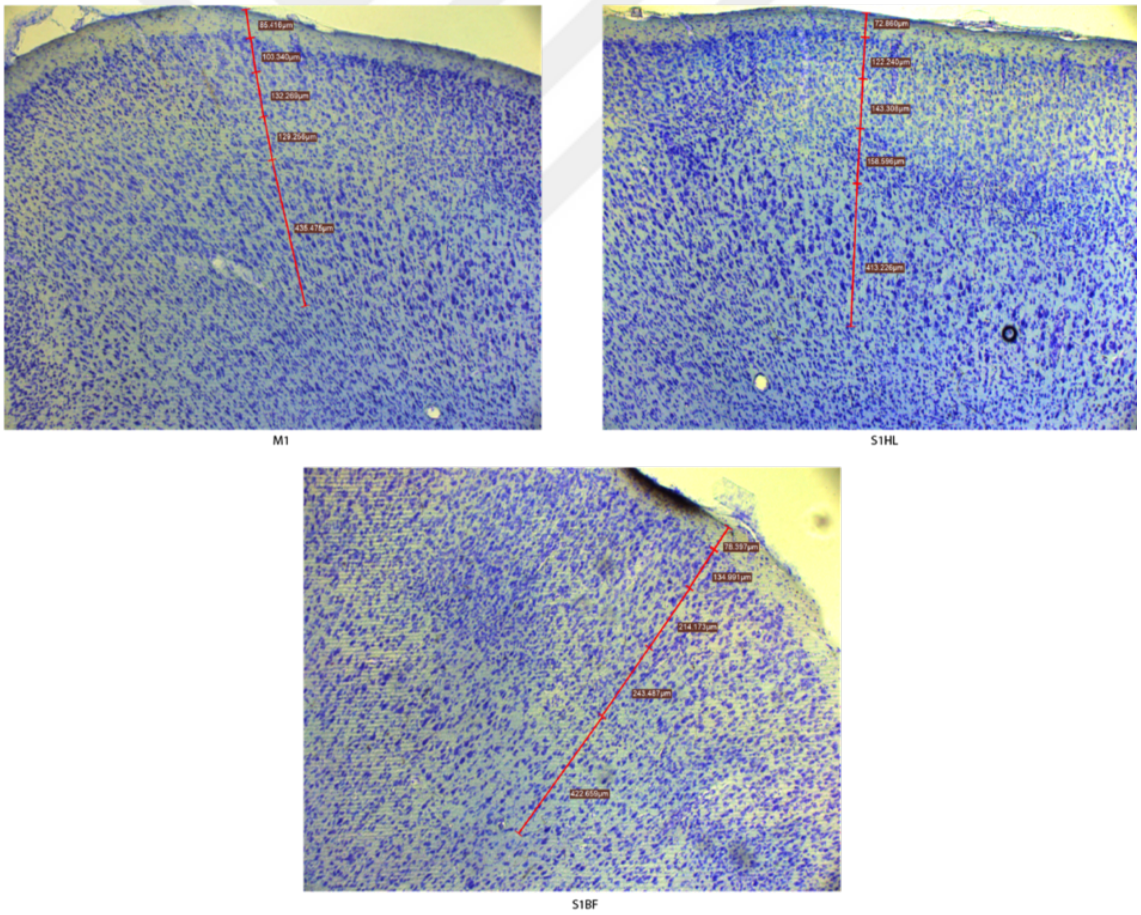


Figure 4.1 Nissl staining samples from M1, S1HL and S1BF in Neocortex, respectively.

One-way ANOVA result shows that, interested brain areas seems similar (p=0.11) (Figure 4.2). The total thickness of M1 is 1428.57 ± 77.48 µm, while of those in S1HL and S1BF are 1636.79 ± 52.57 µm and 1488.23 ± 58.15 µm, respectively (Table 4.1).

Table 4.1
Layer thicknesses from three different cortical areas.

LAYER THICKNESS (µm) Mean ± SEM	S1HL	S1BF	M1
I	69.867 ± ±7.56	75.46 ± ±3.93	82.54 ± ±3.45
II	118.88 ± ±1.89	135.74 ± ±2.58	107.04 ± ±1.73
III	136.18 ± ±5.81	181.99 ± ±15.10	123.65 ± ±2.63
IV	176.64 ± ±1.89	205.35 ± ±19.10	117.48 ± ±2.84
V	371.13 ± ±26.01	392.38 ± ±6.38	431.08 ± ±18.15
VI	555.86 ± ±43.54	664.36 ± ±29.39	626.42 ± ±39.15
Total cortex thickness	1428.57 ± ±77.48	1636.79 ± ±52.57	1488.23 ± ±58.15

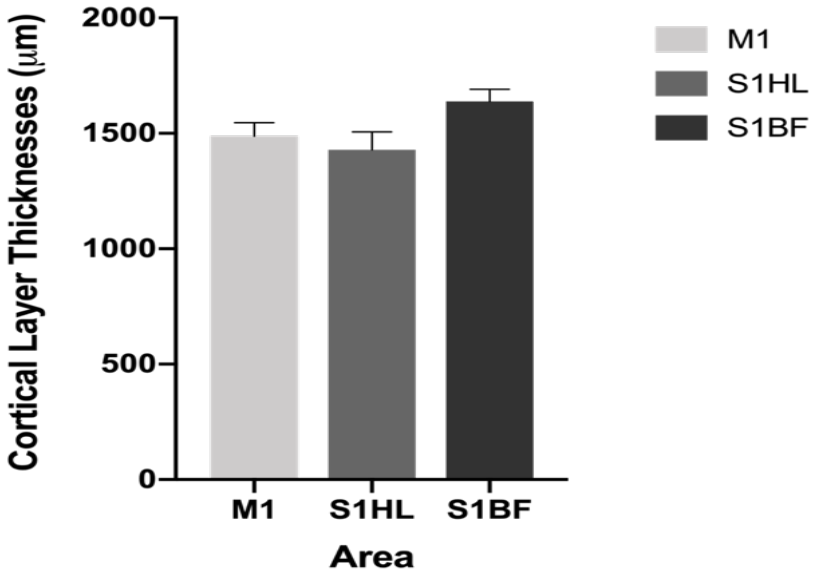


Figure 4.2 Layer thicknesses from three different cortical areas.

According to the two-way ANOVA results, layer and cortical area interaction was found significant. ($p<0.005$). In order to show interaction between layers and cortical areas, paired post-hoc t-test was carried out on the layers. The post hoc t-test results shows that brain area had not effect on layers I, V and VI (Figure 4.3). However, the area had significant effec on layers II, III, IV ($p<0.05$). Layers II, III and IV are thicker in S1BF when considered other two areas. On the other hand, S1HL has lower thickness in layer V when considered S1BF and M1 (Figure 4.3).

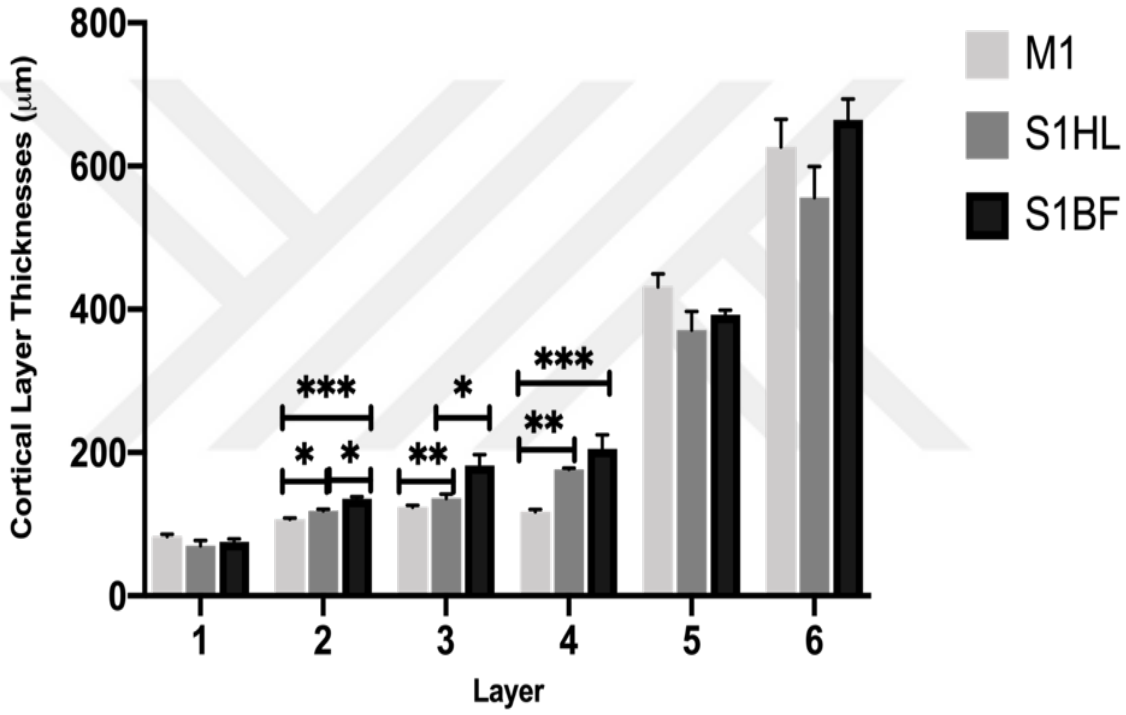


Figure 4.3 The thickness of layers in interested cortical areas (Error bars are indicated in SEM, p-values denotes $* < 0.05$, $** < 0.005$ and $***<0.001$).

4.2 Immunostained Data

α4 and α7 nACh receptor complexes number and total number of cells in each layer were counted. Layer boundaries were determined by DAPI staining and checked with Nissl staining results. For each area of interest (M1, S1HL and S1BF), one slides for DAPI staining and three slides for α4 and α7 nACh receptor complexes staining

were used. DAPI and α4 and α7 staining samples were shown in figures below for both (Figure 4.4 and Figure 4.5) and (Figure 4.6 and 4.7, Figure 4.8 and 4.9 respectively). Average number of α4 and α7 receptor complexes in a layer (N), density of α4 and α7 reseptor complexes with respect to layer thickness (D) and normalized α4 and α7 per total number of cells (C) are given for α4 in Table 4.2 and for α7 in Table 4.3.

Table 4.2
N, D and C values for α4 nAChRs.

N (♯)	Layer I	Layer II	Layer III	Layer IV	Layer V	Layer VI
M1	109.02	226.25	267.75	220.10	774.83	1070.68
S1HL	115.61	271.67	339.48	372.96	675.05	1057.65
S1BF	105.29	303.58	346.90	393.58	651.58	1029.44
D (♯/μ m)	Layer I	Layer II	Layer III	Layer IV	Layer V	Layer VI
M1	1.10	1.98	2.01	1.98	1.51	1.57
S1HL	1.06	2.09	2.20	2.15	1.61	1.64
S1BF	1.27	1.93	1.82	1.93	1.73	1.62
C (♯/♯ tot)	Layer I	Layer II	Layer III	Layer IV	Layer V	Layer VI
M1	0.81	0.84	0.93	0.93	0.95	1.01
S1HL	0.91	0.84	0.86	0.93	0.95	0.98
S1BF	0.90	0.77	0.87	0.80	0.85	0.95

Table 4.3
N, D and C values for α7 nAChRs.

N (♯)	Layer I	Layer II	Layer III	Layer IV	Layer V	Layer VI
M1	96.60	260.32	303.86	224.32	772.36	1056.19
S1HL	109.13	284.85	329.02	398.15	725.39	1070.98
S1BF	105.38	329.79	373.68	478.56	675.83	1059.06
D (♯/μ m)	Layer I	Layer II	Layer III	Layer IV	Layer V	Layer VI
M1	0.99	2.26	2.26	1.96	1.51	1.54
S1HL	1.00	2.23	2.16	2.23	1.71	1.68
S1BF	1.20	2.12	2.01	2.29	1.76	1.67
C (♯/♯ tot)	Layer I	Layer II	Layer III	Layer IV	Layer V	Layer VI
M1	0.93	0.77	0.85	0.95	1.02	1.03
S1HL	0.94	0.89	0.89	0.90	0.97	0.98
S1BF	0.97	0.83	0.85	0.77	0.90	0.96

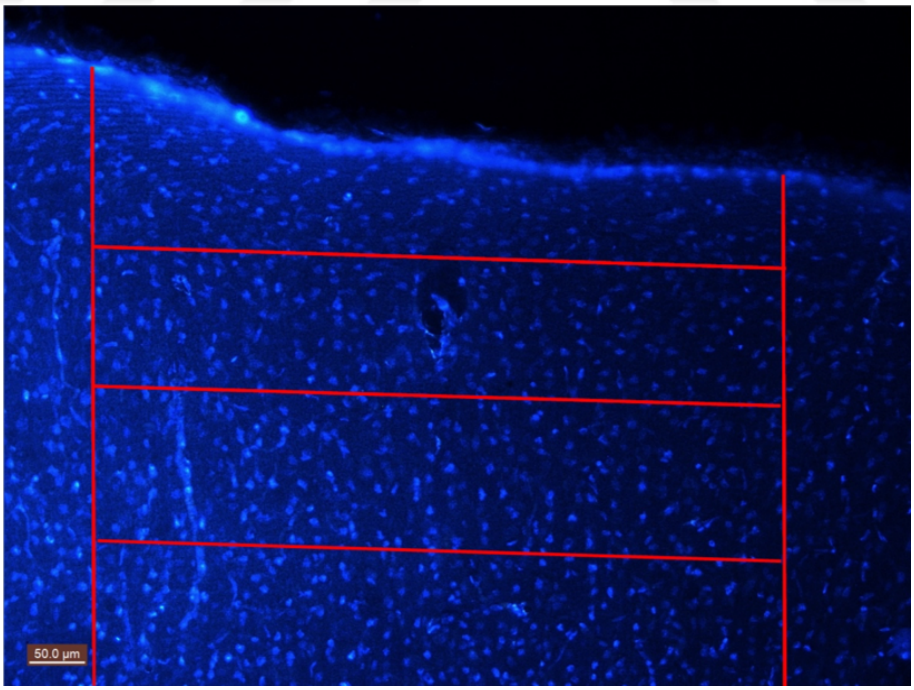


Figure 4.4 DAPI staining for counting the total number of cells in S1HL layer I-II-III. Horizontal red lines indicate layer boundaries. (Rat 21-slide 2-Magnification x20).

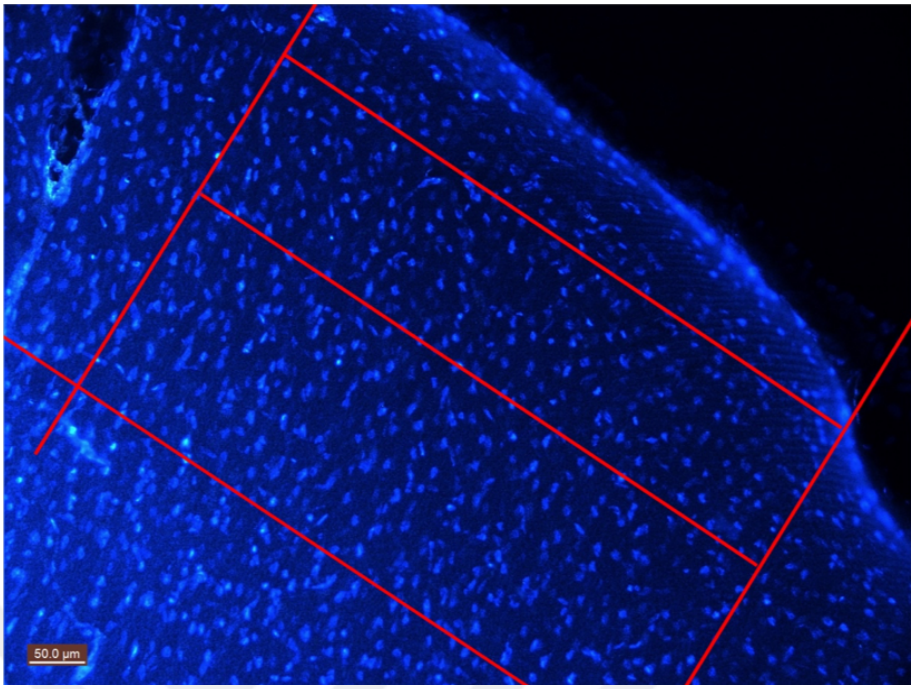


Figure 4.5 DAPI staining for counting the total number of cells in S1BF layer I-II-III. Horizontal red lines indicate layer boundaries. (Rat 21-slide 2-Magnification x20).

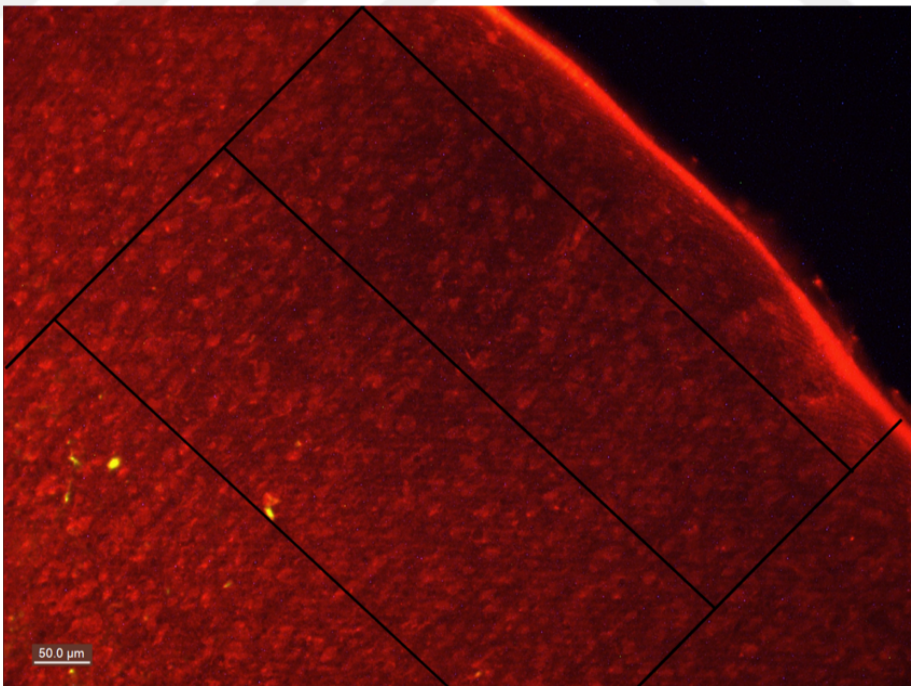


Figure 4.6 α4 nAChRs staining for counting the total number of receptors in S1BF layer I-II-III. Horizontal black lines indicate layer boundaries. (Rat 263-slide 2-Magnification x20).

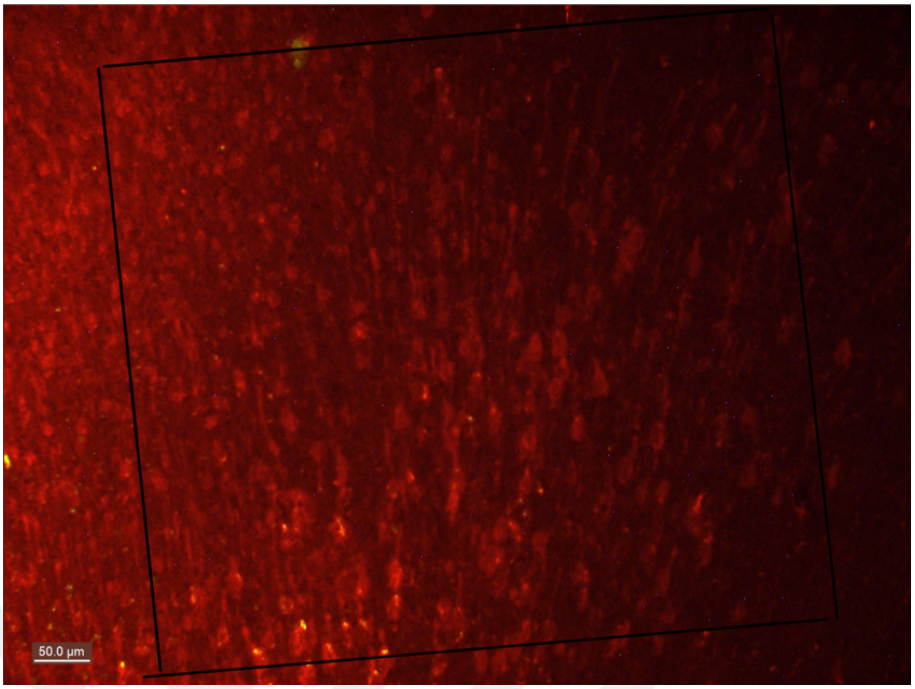


Figure 4.7 α4 nAChRs staining for counting the total number of receptors in M1 layer V. Horizontal black lines indicate layer boundaries. (Rat 263-slide 2-Magnification x20).

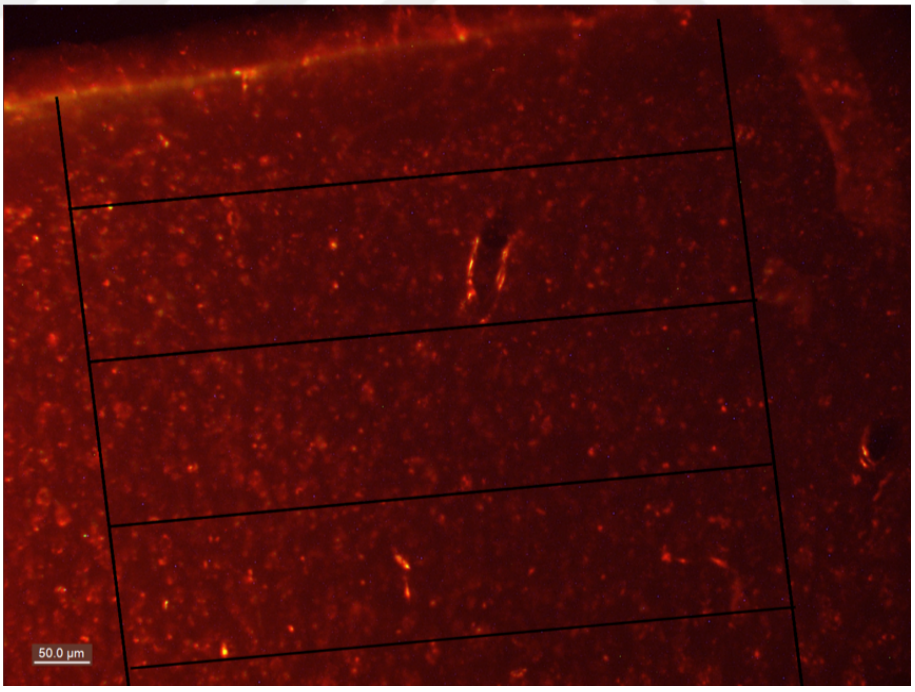


Figure 4.8 α7 nAChRs staining for counting the total number of receptors in S1HL layer I-II-III. Horizontal black lines indicate layer boundaries. (Rat 263-slide 2-Magnification x20).

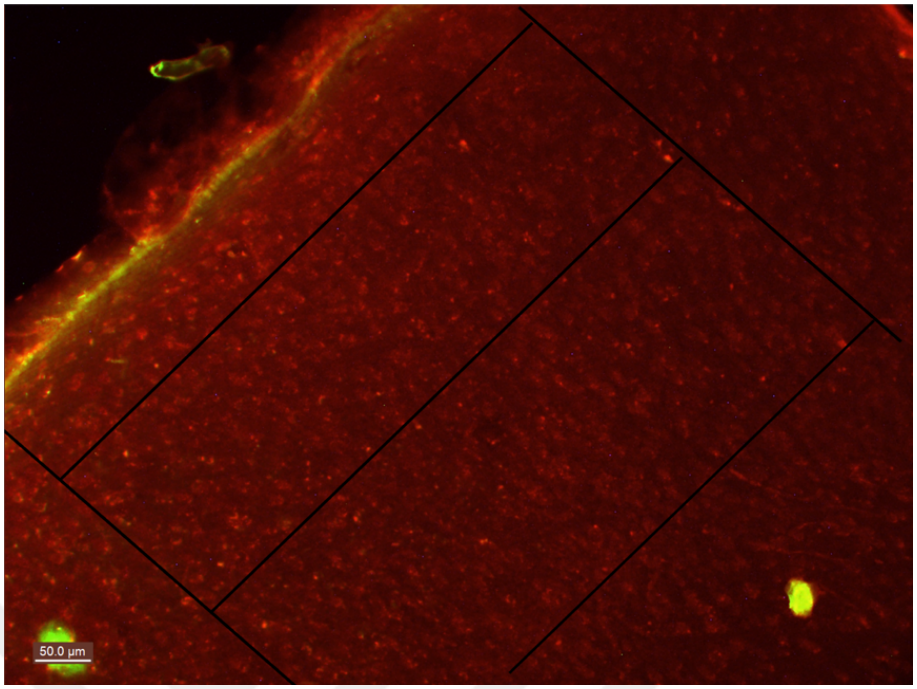
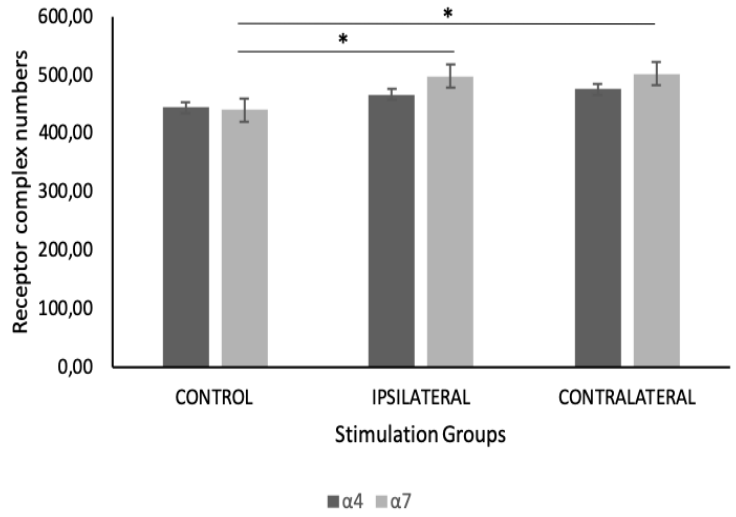


Figure 4.9 α7 nAChRs staining for counting the total number of receptors in S1BF layer I-II-III. Horizontal black lines indicate layer boundaries. (Rat 263-slide 2-Magnification x20).

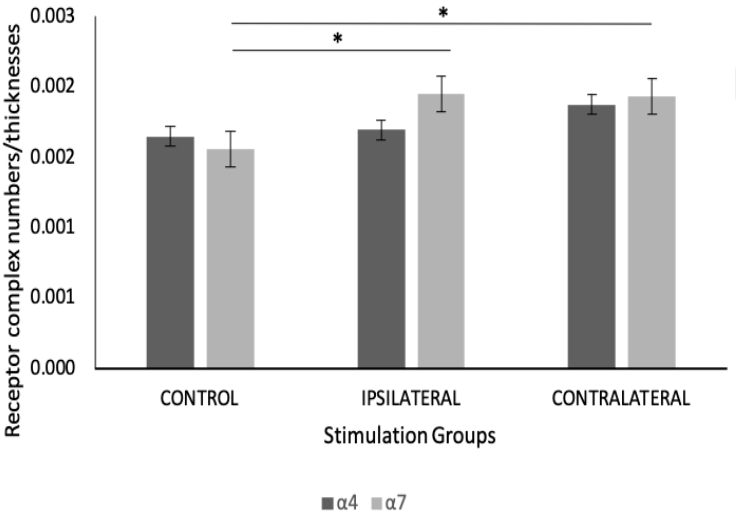
According to the repeated measures of Anova that between and within subjects were stated above, there are strong layer, area, α4vsα7 and stimulation effects separately for both N and D ($p<0.005$ and $p<0.05$, respectively). Layer-stimulation interaction was found only for D ($p=0.002$). α4vsα7-stimulation effect also was found for both N and D ($p=0.028$ and $p<0.001$, respectively). Although there is a strong interaction between area and layer for both for N and D ($p<0.001$), area and α4vsα7 interaction was found significant for only N ($p=0.008$). Post-hoc tests for stimulation condition show that control group is different from ipsilateral and contralateral groups for N and D (N, $p=0.022$ and 0.05; D, $p=$ 0.007 and 0.008, respectively). However, there is no difference between ipsilateral and contralateral stimulation groups for both N and D. Between the area groups, M1 is different from S1HL for N and D ($p<0.001$ and $p=0.002$, respectively). On the other hand, M1 is different from S1BF for N ($p<0.001$).

In order to obtain further results about relationship between α4 and α7, α4vsα7 was excluded from within subject factors and same statistical analyses were done for α4 and α7 separately. The main difference between two receptor subtypes is that stim-

ulation condition has a significance for only α7 receptor subtype. Although ipsilateral and contralateral stimulation groups seem similar, control group is different from them for both N and D ($p<0.001$) (Figure 4.10).



(a)



(b)

Figure 4.10 (a) N and stimulation relationship for α4 and α7; (b) D and stimulation relationship for α4 and α7 (Error bars are indicated in SEM, p-values denotes $* < 0.05$).

Also, area differences were examined according to the stimulation groups for both α4 and α7 separately (Figure 4.11). There is a difference between M1-S1HL, and S1HL-S1BF areas for α4 when considered D ($p<0.001$ and $p<0.05$, respectively). On

the other hand, M1 is different from other areas for α4 when considered N ($p<0.001$). For α7 receptor subtype, area differences are the same for N and D. M1 is different from other cortical areas ($p<0.001$).

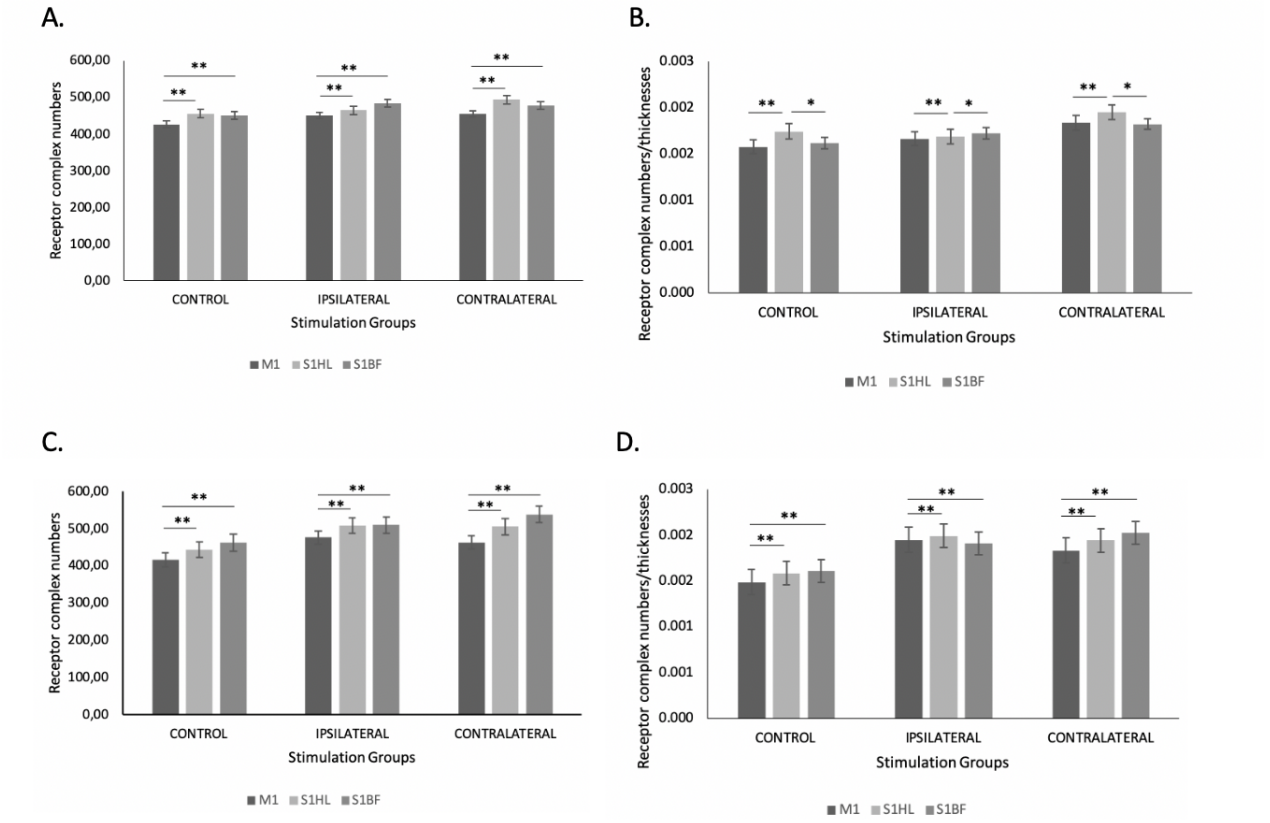


Figure 4.11 A: Stimulation relationship α4 for N value, B: relationship α4 for D value, C: Stimulation relationship α7 for N value, D: Stimulation relationship α4 for D value (Error bars are indicated in SEM, p-values denotes $*<0.05$, $**<0.001$).

When layer differences examined according to each interested area for N and D values, although α4 receptor complex numbers seem to like increase from superficial layers to deep layers for N values, after N values normalized with layer thicknesses, there are relatively linear change between layers. For α4 receptor subtype, layer I, V and VI are different from other layers according to N values in three different stimulation condition ($p<0.001$). On the other hand, according to D values, layer I is different

from other layers, layer II and III are different from layer I, V and VI, and layer V is also different layer I, II, III and IV ($p<0.001$) (Figure 4.12).

For α7 receptor subtype, according to the N values, each layer is different from the rest ($p<0.001$). According to the D values, layer II, III and IV are similar but layer I, V and VI are different from the others ($p<0.001$) (Figure 4.13).

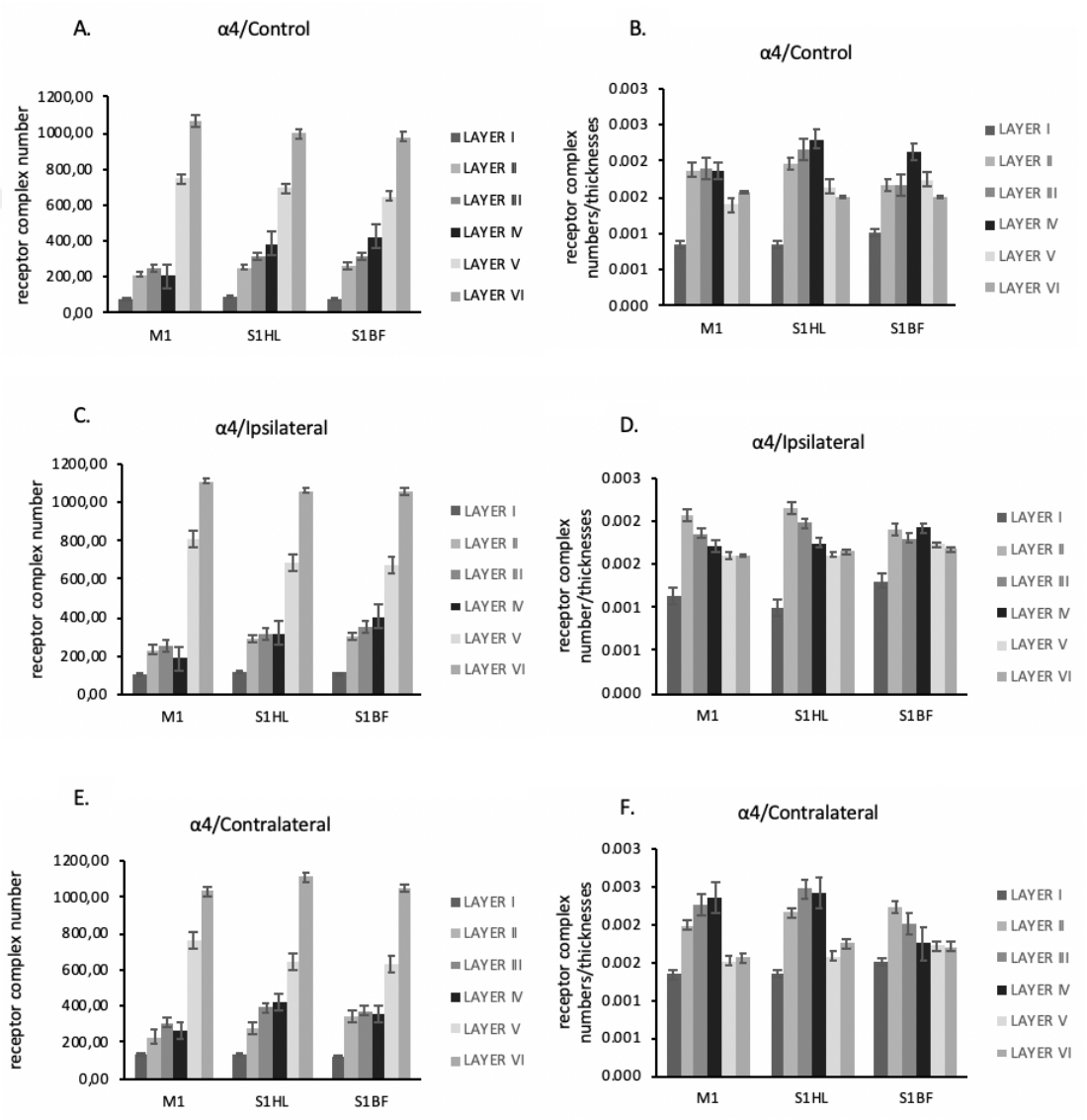


Figure 4.12 A: α4 Layer differences according to the N for control group, B: α4 Layer differences according to the D for control group, C: α4 Layer differences according to the N for ipsilateral group, D: α4 Layer differences according to the D for ipsilateral group, E: α4 Layer differences according to the N for contralateral group, F: α4 Layer differences according to the D for contralateral group.

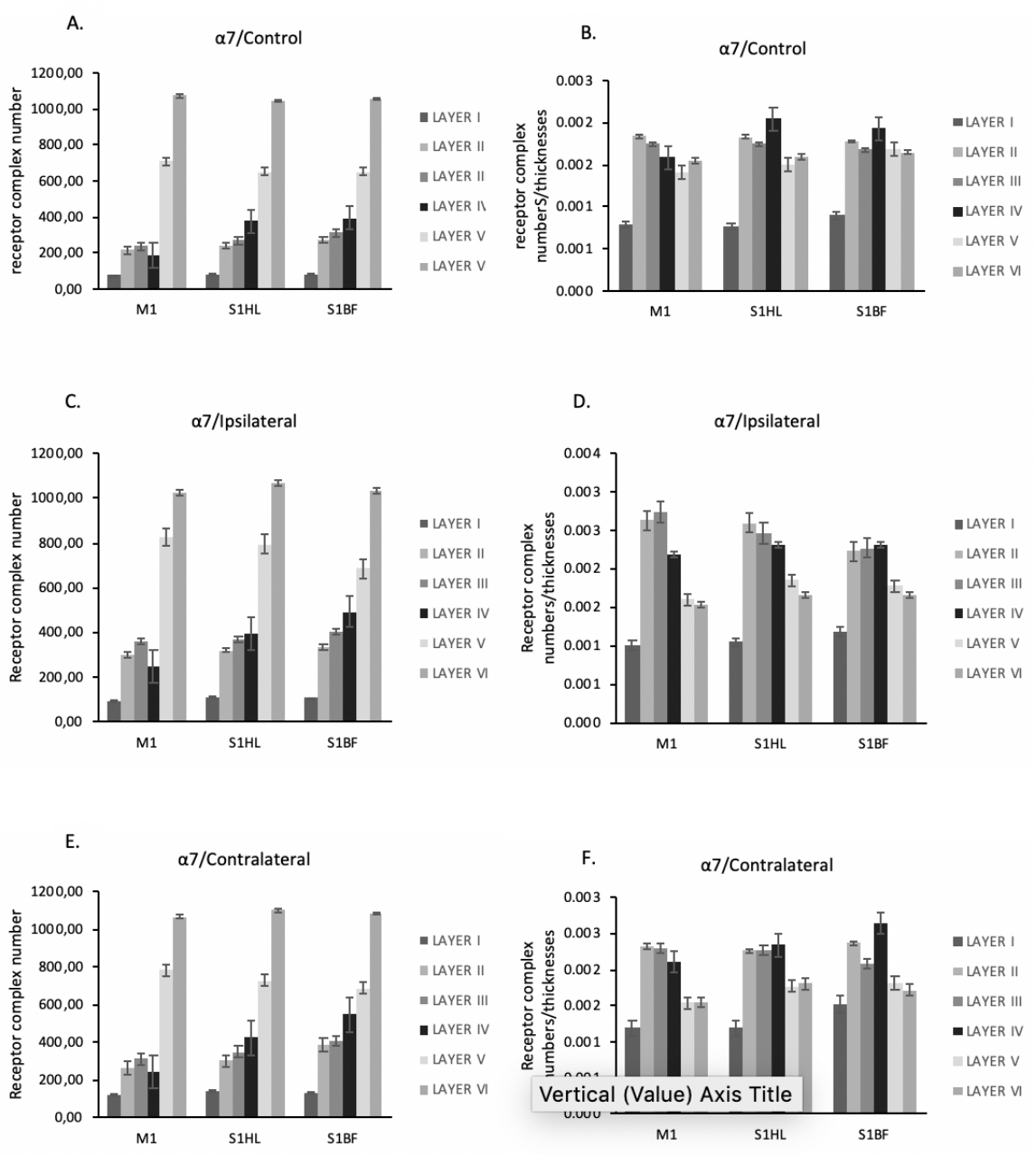


Figure 4.13 A: α7 Layer differences according to the N for control group, B: α7 Layer differences according to the D for control group, C: α7 Layer differences according to the N for ipsilateral group, D: α7 Layer differences according to the D for ipsilateral group, E: α7 Layer differences according to the N for contralateral group, F: α7 Layer differences according to the D for contralateral group.

5. DISCUSSION

5.1 General Conclusion

There are two interacting attentional mechanisms which are bottom-up and top-down and there is no profound knowledge about how these mechanisms work together. In order to understand these two mechanisms, behavioral experiments which are required attentional tasks and modulation of sensory feedbacks. However, experiments like this do not give us information about subtypes of nicotinic acetylcholine receptor expression in the sensory cortex. In the literature about attention, there is still unknown areas regarding specific cholinergic system and attentional mechanisms in the cortex.

In order to compare acetylcholine mechanisms in different S1 areas in cortex, six layered cortex structure was identified in this study. Results suggest that there are strong layer-area interactions. Layer I, V and VI do not vary depending on the areas. On the other hand, layer II, III and IV change significantly in different S1 areas. S1BF area has the thickest II, III and IV layers. On the other hand, layer V has the lowest thickness in three S1 areas.

Besides, BF's several cognitive functions, it has a massive effect on sensory processing. It controls release of ACh in sensory cortices and increases excitability of neurons and modifies selectivity. In this thesis, we showed that BF stimulation modulate responses of S1 based on layer and area differences. The results are: (i) BF stimulation affects specifically α7 nAChR distribution in cortical layers in S1. This differences in expression between subtypes can be resulting from different networks in the cortex. (ii) Besides layer differences, three regions of S1 are affected differently from BF stimulation. In general, the density of receptor complex M1-S1HL, and M1-S1BF are different from each other for α4 subtype. On the contrary, M1 is different from other cortical areas when numbers of receptor complex for α4 subtype are considered. For α7 subtype, M1 is different from other areas for both number and density of receptor complex.

5.2 Comparison with Previous Literature

Metherate et al. [45] and Sihver et al. [108] studied the localization of nAChR subtypes in the cerebral cortex. Their results showed that in primary somatosensory cortex, middle layers specifically layer III and IV were the most dense layers for α4. On the contrary in primary motor cortex layer III and V had high density. Our data for α4 subtype in control group was consistent with these results. For D value, just layer III had the higher density from other layers.

Dominguez et al. [67] and Broide et al. [63] carried out similar study for α7 subtype and they showed that the most dense layers were III and IV in primary somatosensory cortex. Tribollet et al. [49] and Fuchs et al. [109] showed layer IV had the highest density specifically in barrel field. Our data for α7 subtype in control group supported these findings for D value. For barrel field, layer IV had the highest bar in graphs and layer III and IV were the most dense layers in cortex regions.

Much attention studies focus on the BF cholinergic system because its important role of increasing bottom-up sensory input to the cortex, cortical coding, cortico-cortical connections and reliability [110, 111, 112, 113, 114, 115, 116, 93]. Many researcher groups tried to understand how BF mediates sensory inputs in different cortex areas.

Metherate and Ashe [113] studied effects of BF stimulation on thalamocortical transmission. They used intracellular and extracellular recording technique in auditory cortex, and they administered atropine sulfate in the same the after BF stimulated. Their results show that BF cholinergic neurons can alter neocortical functions.Goard and Dan [101] are tried to understand the role of BF neuromodulation in sensory perception. In order to understand the effects of nucleus basalis activation on the sensory responses of cortical neurons they recorded from visual cortex while stimulating nucleus basalis. They suggest that decorrelation between cortical neurons occurs due to the nucleus basalis stimulation. Simşlarly, Mercado et al. [96] examined the changes in neuronal responses in rats by the presentation of a complex sound by stimulating BF at the same time. They obtained recordings from the auditory cortex. They showed that auditory cortex was 2-5 greater responsive to complex sounds in paired stimulation rats than in native rats. Their findings show that BF stimulation can change selectivity in the auditory cortex. Based on these studies, it can be said that BF stimulation

can change cholinergic regulations in positive way. Our data for both α4 and α7 were increased in the same trend.

Chaves-Coira et al. [117] tried to answer how BF madiates the neuronal activity on both hemispheres in sensory areas. They found that the cholinergic system conducts both hemsiphere in order to increase a stimulus that can be originated on either side. Additional to the previous literature, also they showed that NBM showed merely ipsilateral projections to the cortex. Therefore, they suggest that sensory cortex of pyramidal neurons mostly in layer V target contralateral sensory cortex via the corpus callosum.

5.3 Limitations

Firstly, it is known that anesthetic substances mainly suppress brain activity and bottom-up and top-down attentional mechanisms. Stimulation location that is nucleus basalis receives inputs from subcortical regions to mediate bottom-up mechanism and prefrontal cortex to mediate top-down mechanism. Top down modulated BF stimulation in this study can be affected from anesthesia. Therefore current study need to be repeated in awake animals.

Noncholinergic and some GABAergic neurons also are located in BF and project to cortex. They also have caused activation which influence anatomical results presented here. According to the literature, only 1/3 of projections of BF are cholinergic. Therefore, it is difficult to separate the cholinergic projections and other projections from BF. When the BF is stimulated, GABAergic projections may show inhibitory effect on primary somatosensory cortex.

Other issue is damage of the stimulation electrode to the cortex. The stimulation electrode should be implanted with an angle to the BF because S1 and BF are on the same line vertically. There is a possibility of damage to the related cortex areas during removal of stimulation electrode.

5.4 Future Work

More information about cholinergic system and how it is mediated is important for the treatment of diseases such as ADHD, AD. Many studies show that the decrease of the cholinergic neurons in BF cause AD.

Identifying brain structures and circuits involved in attention and how cholinergic system is mediated is not easy. In this study we found that nicotinic receptors have heterogenous distribution and specific subtypes are modulated by BF stimulation. However, in order to see more detailed view of cholinergic modulation, using specific agonist and antagonists by microinjection technique would give us more information. The application of different agents to the cortex could help to understand the properties of the specific neurons responding to each drug in each layer. This information also contributes to understand cortico-cortical and horizontal connection within specific areas.

6. LIST of PUBLICATIONS PRODUCED FROM THE THESIS

Effects of basal forebrain stimulation on the distribution of nicotinic acetylcholine receptors with α4 and α7 subunits in the somatosensory and motor cortex of rat brain, Devlet, B., B. Vardar, B. Babür, and B. Güçlü, in National Neuroscience Congress Turkey, (Trabzon, Turkey), 2019

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