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Elucidating the Role of the α7 Nicotinic Receptor in the Etiology of Schizophrenia.

Michelle Johnson Chandley

East Tennessee State University

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Elucidating the Role of the α7 Subunit of the Nicotinic Receptor in the Etiology of Schizophrenia

A dissertation presented to
the faculty of the Department of Anatomy and Cell Biology and the Department of Psychiatry

East Tennessee State University

In partial fulfillment
of the requirements for the degree
Doctor of Biomedical Science

by

Michelle J. Chandley

December 2008

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Keywords: autoantibody, schizophrenia, alpha7 subunit nicotinic receptor, CREB
ABSTRACT

Elucidating the Role of the $\alpha_7$ Nicotinic Receptor in the Etiology of Schizophrenia

by

Michelle J. Chandley

The $\alpha_7$ subunit of the nicotinic receptor, a ligand gated ion channel with an affinity for nicotine, has long been implicated in the pathophysiology of schizophrenia due to the extremely high rate of smoking within the patient population. However, the exact role of the receptor has never fully been determined. In the following studies, various functions the receptor may assume in disease state are evaluated. There is a strong relationship between the immune system and schizophrenia, with the $\alpha_7$ subunit possibly serving as the link between the two. One of the following studies looks at the possibility of the receptor functioning as antigen in an autoimmune response. Blood sera of schizophrenic patients, as well as controls, were analyzed for the presence of antibodies to the $\alpha_7$ subunit of the nicotinic receptor. A sensitive ligand-based assay revealed schizophrenic patients could possess a pathogenic level of antibody that may exacerbate the degenerative nature of the disease, allowing for the possibility that receptor antibodies may serve as a contributing factor in the etiology of the disorder in at least a subset of patients. In other studies, the expression of the $\alpha_7$ receptor was investigated. Recombinant $\alpha_7$ receptor production has eluded researchers in non-mammalian species and this was the focus of our initial studies. In general, the lack of sufficient molecular recombinant techniques utilizing the receptor makes characterization of the $\alpha_7$
receptor and it’s specific protein interactions difficult to evaluate. The regulatory mechanisms of the nicotinic receptor α7 subunit production and receptor formation have yet to be completely elucidated. Results in this investigation found a relationship between a functional CRE-element in the promoter region.
DEDICATION

This dissertation is dedicated to my nephew Lee Herman Chandley, III whose battle with autism inspired me to actively join in the fight for new information in improving the prognosis for all those afflicted with mental illness.
ACKNOWLEDGEMENTS

First, I would like to express my deepest appreciation to my chair, Professor Barney Miller. He has graciously provided me with every opportunity necessary to fully develop my professional education in many areas.

I would like to thank my committee members, Dr. Ron Baisden, Dr. Antonio Rusinol, Dr. Greg Ordway, and Dr. Merry Miller for all the time they have invested in me, especially through insurmountable work schedules of their own.

I would also like to thank everyone involved in the professional development of all the students in the Biomedical Science Program, but in particular Beverly Sherwood and Professor Mitchell Robinson for their tireless devotion to us.

Also, my sincere gratitude goes to two very special people whose immediate guidance and help has made this a wonderful journey. Tracy Wilson, with her expertise and determination, has taught me so much about the technical side of science, as well as made day-to-day life in the laboratory a pleasure. Christine Newell, whose inspiration through her own journey, has guided me in many of the choices I have made.

I would especially like to thank my mother, grandmother, and everyone who helped to ensure my family has been taken care of during those extra times I have had to be at the laboratory or at a computer screen.

Last, but certainly not least, I want to acknowledge my Lord and Savior, Jesus Christ, who has blessed me with all His gifts to make a difference in the world today.
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CHAPTER 1
INTRODUCTION

The search for biological basis of schizophrenia has filled many scientists with more questions than answers at the end of their studies. A prominent figure in the initial move from the psychoanalytical dogma that dominated early schizophrenia research, Dr. Fred Plum, referred to the disease as the graveyard of the neuroscientist. It is the inability to make specific molecular distinctions in a symptomatically indistinct disorder that has left many demanding more information\(^1\). Recent scientific discoveries, particularly in the human genome, are finally contributing to the identification of unique biological factors and their relationships that seemingly contribute to the neuropathophysiology of schizophrenia. It is the purpose of the present study to look at a particular molecule, the $\alpha_7$ subunit of the nicotinic receptor, and elucidate how it may participate in the disease. There are two distinctly different avenues explored in this investigation regarding the receptor. The first avenue examines protein interactions that may inhibit proper functioning of the receptor. The second avenue examines the transcriptional regulation of the receptor subunit, which is currently uncharacterized.

**Background**

Schizophrenia affects up to 1\% of the world’s population. In the United States, the estimated prevalence of schizophrenia is 1 in 125 individuals. To put this in better perspective, a city consisting of one million people will have at least
8000 suffering from schizophrenia. According to a recent report on Medscape Today, Dr. Natalya Weber of the Walter Reed Army Institute released the following information, located in Table 1, at the American Psychiatric Association’s annual meeting that used analyses obtained from 5.7 million US National Hospital Discharge Surveys (abstract NR4-073, May 5, 2008):

Table 1. Percent of discharged patients diagnosed with schizophrenia. This table illustrates the statistics associated with individuals who had a diagnosis of schizophrenia upon hospital release.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Rate of Schizophrenia</th>
</tr>
</thead>
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<tr>
<td>Schizophrenia overall</td>
<td>1.0%</td>
</tr>
<tr>
<td>Males</td>
<td>1.3%</td>
</tr>
<tr>
<td>Females</td>
<td>0.8%</td>
</tr>
<tr>
<td>African American</td>
<td>1.8%</td>
</tr>
<tr>
<td>Caucasian</td>
<td>0.9%</td>
</tr>
<tr>
<td>Other race</td>
<td>1.0%</td>
</tr>
<tr>
<td>US region</td>
<td></td>
</tr>
<tr>
<td>North</td>
<td>1.5%</td>
</tr>
<tr>
<td>Midwest</td>
<td>1.0%</td>
</tr>
<tr>
<td>South/West</td>
<td>0.8%</td>
</tr>
</tbody>
</table>

Also, Dr. Weber’s group examined the prior 24 years and discovered the average hospital stay for someone with schizophrenia is twice as long as the general population at 12.5 days.
Hospitalization of schizophrenic patients is just one example of the debilitating nature of the disease that contributes to a heavy financial burden to not only the individual but to the public as well. Other examples are initially lost wages or services, disability payments, costly therapeutic management, and eventual long-term care. In 2002, it was estimated the cost of schizophrenia exceeded $62 billion dollars that year, with almost half going to direct care cost \(^2\). Schizophrenia is generally regarded as the most expensive disorder worldwide. Many argue the current amount of research dollars designated for schizophrenia does not match the importance the disease occupies financially in everyday life. With schizophrenia’s effects immediately felt by a large number of people, it is extremely important for current research to identify any biological components that could contribute to a disease with such an ill-fated patient prognosis.

**Epidemiology**

The epidemiology behind schizophrenia is most interesting. Men experience their first psychotic episode around 25 years of age, while women are closer to 30 years of age. More literature regarding early onset schizophrenia is becoming available, with most researchers citing lack of diagnosing criteria regarding childhood symptoms as the main culprit in correctly assessing schizophrenia. But it is generally accepted that disease manifestation occurs in early adulthood. A common misconception is that the disease affects men and women equally. However, a recent meta-analysis assessed 31 independent review articles and found a significant difference of 1.4 males for every female\(^3\).
The disease also varies from country to country, illustrating strong genetic influences\(^3\). Family history, genes, stressful life events, birth complications, infection, influenza, autoimmunity, ethnicity, and cannabis use are some of the more well-known risk factors associated with schizophrenia [as reviewed most recently by Messias\(^4\)]. Along with genetic markers, another factor of particular interest to this study is the relationship to influenza. An individual whose mother experienced influenza during the second trimester of pregnancy appear to be at a 2-3 times higher risk for developing schizophrenia than the general population\(^5,6\). Season of birth is also considered a slight risk factor, with winter babies illustrating a higher rate of the disease\(^7\). This could be explained by the occurrence of the second trimester when influenza risk is the greatest\(^8\). It is quite possible neuro-developmental dysfunctions may have happened during this exposure implicating specific biological mechanisms, in particular neuronal signaling pathways and immune system functions. These alterations could contribute to the unique manifestation of the disease in early adulthood when the deficient biological mechanisms could no longer be compensated for. The fascinating epidemiology behind the disease illustrates the participation of specific biological factors or their distinct relationships that have yet to be identified in schizophrenia.

Diagnosis

Understanding the clinical characteristics that attempt to distinguish schizophrenia from other closely related mental disorders is important in the
ability to form specific biological connections. The clinical identification of schizophrenia is not all that different than it was 50 years ago. A patient is interviewed by a clinician, who through the patient’s responses begins to dissect the core issues and makes a clinical diagnosis. It is not all that uncommon for the initial diagnosis to be changed, due to the complex nature of subjectively identifying mental disorders. A clinical diagnosis of schizophrenia involves the manifestation of negative and positive symptoms, even in some cases alternating between the two. Negative symptoms involve a loss of the ability to perform everyday functions. Affective flattening, one of the more common symptoms of schizophrenia, is a reduction in responsiveness by the individual, noted by poor eye contact and reduced facial expressions. Alogia is a lack of speech, characterized by the inability, not the unwillingness, to speak. Avolition is the inability to express interest in any persistent activity. Positive symptoms are the exacerbation or alteration in normal functions including: delusions, disorganized thinking, grossly disorganized behavior, catatonic motor behaviors, and sensory hallucinations, including auditory hallucinations that are by far the most common symptom of schizophrenia. According to the DSM-IV, a schizophrenia diagnosis involves the following criteria. First, two of the five symptoms must occur for the majority of a one-month period, unless delusions are characterized as bizarre, then this may be sufficient alone. Second, any functional area, such as work, home, or education, must be moderately to severely impaired. Last, the signs of general unusual behavior must be evident for at least six months. Stahl adheres to a more stringent diagnosis, including cognitive, aggressive, and depressive
symptoms, calling for a separate assessment of these areas during patient
evaluation (Stahl, 2nd edition).

Paranoid, Disorganized, Catatonic, Undifferentiated, and Residual are all
specific subsets under the general heading of schizophrenia, with each
possessing individual characteristics that warrant a more distinct identification of
the disease. One year following initial diagnosis, patients fall into one of the
following divisions: episodic with inter-episodic residual or negative symptoms,
episodic with no inter-episodic symptoms, single episode in partial remission,
single episode in full remission, or unspecified pattern (DSM-IV, 4th edition). This
multifaceted diagnosing criteria illustrates the complexity in distinguishing mental
disorders in general. The pathophysiology involved in the etiology of
schizophrenia is likely to be similarly complex.

The Purpose of the \( \alpha_7 \) Subunit of the Nicotinic Receptor in Schizophrenia

The nicotinic receptor \( \alpha_7 \) subunit (nACHR\( \alpha_7 \)) is a molecule of particular
interest in schizophrenia. Nicotinic receptors are ligand-gated ion channels that
participate in neurotransmission and cell signaling within the body. Nicotinic
receptor ion channels form from the intertwining of five identical or different
receptor subunits (Figure 1). The subunits consist of \( \alpha_2 \) through \( \alpha_10 \) and \( \beta_2 \)
through \( \beta_4 \). The \( \alpha \)-subunits carry the specific components involved in specific
acetylcholine ligand binding, while the \( \beta \)-subunits contain complementary
components that do not possess ligand binding capability. The known
pentameric channels formed from nicotinic subunits are identified in the brain as shown in Table 1.2. The α7 subunit is not only confined to CNS expression,

![Diagram of nicotinic receptor channels](image)

Figure 1. Representation of nicotinic receptor channels. A homomeric nicotinic receptor ion channel consists of five identical α subunits that contain five acetylcholine binding sites, denoted by stars. A heteromeric or multimeric ion channel consist of acetylcholine binding α units and beta complement units in various configurations, shown here is an (α4β2) receptor.
Table 2. Nicotinic receptors. This a list of the functional nicotinic pentameric channels formed from receptor subunits and their location in the brain. Gotti and Clementi, 2006.

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Subunits</th>
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<tr>
<td>Hippocampus</td>
<td>α7, α4β2, α4β2δ5, α3β4</td>
</tr>
<tr>
<td>Medial habenula</td>
<td>α7, α4β2, α3β4, α3β3β4</td>
</tr>
<tr>
<td>Pineal gland</td>
<td>α7, α3β4</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>α7, α4β2, α3β4, α3β2</td>
</tr>
<tr>
<td>Locus Coeruleus</td>
<td>α3β4, α6β2β3</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>α7, α4β2, α3β2</td>
</tr>
<tr>
<td>Raphe nuclei</td>
<td>α4β2</td>
</tr>
<tr>
<td>Interpeduncular n.</td>
<td>α7, α4β2, α2β2, α3β3β4</td>
</tr>
<tr>
<td>Substantia nigra, VTA</td>
<td>α7, α4β2, α4β2α5, α3β4, α6β2β3</td>
</tr>
<tr>
<td>Thalamus</td>
<td>α4β2</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>α7, α4β2</td>
</tr>
<tr>
<td>Amygdala</td>
<td>α7, α4β2</td>
</tr>
<tr>
<td>Striatum</td>
<td>α4β2, α4β2α5, α6β2β3, α6α4β2β3</td>
</tr>
<tr>
<td>Olfactory Bulb</td>
<td>α7, α4β2</td>
</tr>
<tr>
<td>Cortex</td>
<td>α7, α4β2, α4β2α5</td>
</tr>
</tbody>
</table>

it has been found expressed in blood leukocytes and endothelial cells. In the immune system, it participates directly in cell signaling events that result in activation of the anti-inflammatory pathway, while suppressing the pro-inflammatory pathway.

Figure 2. Two groups of nicotinic receptors. The initial separation of the nicotinic receptors was based on their functional ion channel properties. The α7 receptor functions as a calcium channel, while the other receptors, for example α4β2, function as sodium and potassium channels.
The α7 nicotinic receptor distinguishes itself from the other nicotinic receptors in two important areas, function and pharmacology. The receptors formed from the α7 subunits participate in cell signaling as ion channels that result in a cellular increase in the permeability to calcium, differing from the other known nicotinic receptor ion channels that increase sodium and potassium permeability (Figure 2). The nicotinic receptor family directly participates in neurotransmission, and this functional difference is meant to distinguish the modulation of the different neurotransmitters. The α7 subunit has been thought to be pharmacologically distinct from the other subunits due to the ability to bind bungarotoxin, a toxin found in cobra venom (Figure 3). This was how initial classification of nicotinic receptor subunits was divided, those receptors that exhibited bungarotoxin binding and those that did not. However, recent evidence has shown that bungarotoxin has the ability to bind the α8 (found only in chick retina to date), α9, and α10 subunits (both subunits found in human pituitary gland and inner ear)\textsuperscript{13}. Also, the receptor does not respond to the antagonist mecamylamine, which blocks the other known nicotinic receptors\textsuperscript{14}.

Figure 3. Division within receptor families usually occurs based on ligand affinity. Ligand binding assays utilizing nicotine affinity initially divided the nicotinic receptors into separate categories: low, moderate (Mod) and high. Bungarotoxin affinity (αBTX) exhibited only by the α7 nicotinic receptors receptors was used to further distinguish this particular group of receptors.

A special point of interest is the lack of complete information regarding the nicotinic receptor family. It is noted in the latest review by Dr. Clementi (one of the pioneers in nicotinic receptor research) that the list of identified functional receptor ion channels in the human brain is far from complete\textsuperscript{15}. Affirming this statement are reports that the α7 subunit may exist not only in homomeric ion channels of five identical subunits but also in α7 multimeric ion channels as well. Although this idea is not generally accepted, there is a moderate amount of literature to support the possibility--especially in the chick retina and rat hippocampal interneurons where functional α7 receptors behave
pharmacologically different from homomeric counterparts\textsuperscript{16-18}. This illustrates that technical issues must be taken into account when precisely identifying the $\alpha_7$ receptor from other nicotinic receptors in current molecular investigations.

Many of the identified nicotinic receptor subunits have been implicated in the neuropathology of a variety of diseases. The $\alpha_4\beta_2$ receptor has altered expression in Alzheimer’s disease\textsuperscript{19}. The $\alpha_6$, $\beta_2$, $\alpha_7$, and $\alpha_4$ subunits are all being evaluated in association with Parkinson’s disease\textsuperscript{20,21}. Nicotine has been reported to be a mood stabilizer in depression and to increase attention in ADHD\textsuperscript{22,23}. Four initial findings in the schizophrenic patient population immediately implicate the nAChR$\alpha_7$ as important molecules involved in the neuropathology of the disease\textsuperscript{24}.

One of the first factors noticed associating nicotinic receptors with schizophrenia was the high incidence of smoking in the patient population. More than 90\% of the schizophrenic patient population use nicotine in some form\textsuperscript{25}. This implicates the nicotinic receptor family immediately. All the known functional nicotinic receptors are divided based on their selective ligand affinity to nicotine in combination with other selective agents, such as bungarotoxin as discussed previously. Nicotine binds the homomeric $\alpha_7$ receptor with low affinity; however, with chronic nicotine treatment an up-regulation of the functional $\alpha_7$ receptors has been shown in neuronal cells\textsuperscript{26}. Schizophrenic patients could potentially be self-medicating with nicotine, although the molecular basis has never been completely identified due to the relatively poor knowledge of neuronal mechanisms in general of the nicotinic receptors.
Another factor involves the α7 receptor’s relationship to abnormal auditory gating, one of the few physiological alterations found in schizophrenia. Abnormal auditory gating is one of the few measurable phenotypic traits that takes advantage of this abnormal reaction to stimulus by comparing subject responses in corresponding stimuli. This physiological response in schizophrenia is called the P50 abnormal gating phenomenon. The P50 wave is an electrically positive evoked potential that occurs 50 ms following a response to paired auditory stimulus. The measurement is conducted using electroencephalogram readings of the P50 wave amplitudes. The subject is administered an auditory stimulus, the initial amplitude measured, then immediately exposed to a second auditory stimulus, and the P50 amplitude is measured 50 ms later. The ratio of the initial potential and the P50 response is evaluated, which is referred to as P50 inhibition27. In normal individuals, the ratio is well below 0.5, but in the schizophrenic population the ratio is above the 0.5 ratio. This means wave amplitudes are becoming closer in size supporting a patient response to all environmental stimuli instead of effectively focusing on a single stimulus or being able to “filter” distractive environmental sounds 28. The biological basis of proper P50 inhibition is directly linked to neurons in the hippocampal region. Only the α7 receptor antagonist, α-bungarotoxin, was able to disrupt P50 inhibition, while antagonists for other nicotinic receptors had no effect29. Nicotine has a low affinity for the α7 receptor but was sufficient to normalize the P50 inhibition in schizophrenia without interference from
mecamylamine, a known antagonist for all nicotinic receptors except the α7 receptor, as summarized in Figure 4.14,29

A third factor implicates the α7 subunit through genetic linkage studies that evaluated genotypes in the families of schizophrenic patients with deficient auditory gating. Dr. Freedman’s group evaluated nine schizophrenic patients and their first order relatives, showing the P50 auditory gating deficit is genetically linked to α7 nicotinic receptor locus on chromosome 15q1424. There were several studies following this initial finding that also found α7 nicotinic receptor genetic linkage to specific populations, including those of African American and Northern European American decent30,31. A meta-analysis conducted in 2002 evaluated 18 separate studies to find a significant correlation
between the α7 locus and schizophrenia. It is worth pointing out that several investigations involving Asian lineage have not found genetic linkage of the receptor to schizophrenia. The genetic linkage of the α7 subunit to certain cultural populations stresses the importance of genetic susceptibility that may put someone at risk for developing the disease. However, schizophrenia has always been considered a multifactorial illness that involves the interactions between genetic components and environmental contributions that may eventually evolve into disease state for some patients.

A fourth factor involves α7 receptor expression in schizophrenia. Ligand binding assays using α-bungarotoxin, an α7 receptor antagonist, in autopsy tissue suggest the amount of this ion channel is significantly reduced in the brains of schizophrenics when compared to controls, particularly in the frontal cortex and hippocampal regions. Messenger RNA studies are much less clear, with conflicting reports regarding the actual number of α7 subunit transcripts in schizophrenics. However, a recent report introduced novel splice variants of the α7 subunit mRNA and the changes in expression regarding each variant in different areas of the brain in schizophrenia. Although the function of these α7 transcription variants is unknown, abnormal transcription levels of them in the brains of schizophrenics may contribute to deviant receptor expression or functional alterations in channel activity. Taken together, these four factors suggest the α7 receptor may have a role in schizophrenia (Figure 5).
Figure 5. Four factors implicating the α7 receptor in the neuropathology of Schizophrenia. Smoking, the P50 inhibition (ratio of the measurements of P50 amplitudes), genetic linkage to chromosome 15 locus 14, and altered receptor expression link the α7 receptor to the biological basis of schizophrenia.

Etiological Hypothesis of Schizophrenia

The complete biological basis of schizophrenia has remained elusive through the years. The advent of better molecular tools has resulted in more information. Schizophrenia does not appear to be the result of one particular causative factor, but rather, it is the result of complex relationships between a variety of biological interactions, some of which may involve the α7 nicotinic receptor. Briefly discussed below are several of the more informative etiologies that contribute to the neuropathophysiology of schizophrenia and their general relationship to the nAChRα7.
Dysregulated Immune System

Schizophrenia was initially thought to be an autoimmune disorder based on the similarities shared with onset and progression of other autoimmune diseases. They both manifest into disease later in teens or early adulthood, possibly triggered by an outside contributing factor such as a virus. Autoimmune disorders and schizophrenia both tend to have periods where the disease is worse at certain times intermingled with periods of remission. Autoimmunity results from a person’s immune system to produce an abnormal response to his or her own tissue. Schizophrenic patients exhibit a dysregulated immune response as well. More specifically, there are increased cytokine levels, particularly IL-6 and IL-2 found in schizophrenics. According to another study, there is a 45% increase in the development of schizophrenia if any other autoimmune disease is noted in family history. It has been found abnormal T-cell activity can contribute to the cognitive deficiencies noted in schizophrenia. Also, immunosuppressive therapy along with anti-psychotic treatment significantly improves disease prognosis. It is hypothesized that a portion of patients with schizophrenia may have autoantibodies for specific proteins accounting for the dysregulated immune system observed, such that autoimmunity contributes to the development of this disorder in a portion of the patient population.

The function of the α7 nicotinic receptor in inflammation has just recently become of interest. For many years, the receptor family was thought to be located only in the CNS. Recently, the α7 receptor was identified on leukocytes
and found to participate directly in activation of the anti-inflammatory pathway, while suppressing the pro-inflammatory pathway, as reviewed most recently by Gallowhitch-Puerta\textsuperscript{12}. These findings stimulated a closer look at inflammation in the CNS associated with psychiatric illness. The CNS mononuclear phagocytes, called microglia, serve as the primary cell population for the brain’s inflammatory response. It was found that microglia activation was regulated by $\alpha_7$ receptors\textsuperscript{44}. The $\alpha_7$ receptor is essential in the proper cell signaling events in the CNS as well as peripheral immunity. An alteration in receptor expression or function could result in the dysregulated immune response displayed in schizophrenia.

\textit{Dopaminergic Hypothesis}

The dopaminergic pathway probably has the longest history of association with schizophrenia. In fact, dopamine activity has been isolated to particular pathways that could specifically contribute to the symptoms exhibited by schizophrenics. It has long been shown that stimulants that increase dopamine receptor activation can produce or worsen psychotic episodes\textsuperscript{45}. The relationship was further defined in schizophrenia, where an increase in dopamine through the mesolimbic pathway exacerbates positive symptoms, but if dopamine is decreased positive symptoms are diminished\textsuperscript{46-48}. However, in the mesocortical pathway dopamine deficiency results in an exaggeration of negative symptoms\textsuperscript{49}. Antipsychotic-induced loss of dopamine activity in the nigrostriatal pathway results in movement deficits exhibited by schizophrenics\textsuperscript{50,51}. The precise role of dopamine in schizophrenia has yet to be thoroughly elucidated,
but the involvement of the neurotransmitter’s pathways in the illness has much support\textsuperscript{52}. Currently, the most effective pharmacological treatment in schizophrenia is dopamine receptor antagonists.

The relationship between nicotinic receptors and dopamine release is far from complete, but there are elements that can be stated with confidence. The $\alpha_4\beta_2$ heteromeric nicotinic receptor responds to acetylcholine to participate directly in the release of dopamine throughout the brain. Glutamate, which responds to $\alpha_7$ receptor activation, is thought to facilitate dopamine release as well\textsuperscript{53}. These primary and secondary associations with dopamine establish the importance of nicotinic receptors as a whole in dopaminergic pathways (Figure 6). Any disruption of proper nicotinic receptor function will have an effect on dopamine modulation that could result in the symptoms associated with schizophrenia that were mentioned in the previous paragraph.
Figure 6. Potential effects Acetylcholine (ACh) has on Dopamine (DA) and Glutamate (Glu) Release. The neurotransmitter, ACh, directly facilitates the release of both glutamate and dopamine but also participates in dopamine release indirectly through glutamate binding to dopamine neurons (adapted from Wonnacott)\textsuperscript{53}.

**Cellular Neurodegeneration**

Those suffering from schizophrenia demonstrate a progressive worsening throughout their lifetime if they do not enter remission. Even before diagnosis, there is a gradual progression toward psychosis that has a measurable level of reliability that could be used to predict those individuals who may develop schizophrenia\textsuperscript{54}. This would seem to reinforce a neurodegenerative basis behind the disease. Inefficient neurotransmission resulting in reduced neuroprotection
has been proposed to explain the gradual downhill progression exhibited by schizophrenics (Stahl, 2\textsuperscript{nd} edition). The coordinated actions of inhibitory and excitatory amino acids are of particular interest in this area. Glutamate release is thought to be under the direct control of presynaptic $\alpha_7$ nicotinic receptor in the cerebrum and cerebellum, while the hippocampus neurotransmitter release is under the control of complex secondary interactions with the receptor that may involve other neurotransmitters\textsuperscript{13,55,56}. Activation of the receptor results in the release of calcium stores within glutaminergic neurons facilitating neurotransmitter release\textsuperscript{57}. The release of the inhibitory neurotransmitter, $\gamma$-aminobutyric acid (GABA) in the hippocampus falls under the control of the $\alpha_7$ nicotinic receptors\textsuperscript{58}. Dysregulation of GABA release has been associated with schizophrenia, supported by a loss of a GABAergic interneurons\textsuperscript{59,60}. Loss in $\alpha_7$ receptor function or regulation disrupts precise neurotransmitter communication that could result in molecular neurodegeneration in specific areas of the brain explaining the clinical progression of schizophrenia.

\textit{Environmental Factors}

Environmental triggers dominated early etiological studies of the disease, while molecular methods emerged with scientific evidence to support a biological basis. Discussed below are epidemiological findings that support an intertwining of both biological and environmental causes in the etiology of schizophrenia. Strong substantial evidence of an non-genetic influence is exhibited through the concordance level between monozygotic twins, which was found in early studies
Stressful life events have been postulated to contribute to the beginning of schizophrenia or to episodic relapses. Walker and coworkers reviewed a stress-sensitive pathway, the hypothalamic pituitary adrenal axis (HPA), and found activation of the stress hormone cortisol results in an exacerbation of psychotic symptoms in schizophrenia animal models. Maternal exposure to toxins or microorganisms during gestation could be another environmental contribution resulting in the neuronal deficits manifesting in schizophrenia. A study in a Netherland population during the Germany invasion of 1940 revealed that offspring of women pregnant at this time exhibited higher incidences of the disease. The specific role of the α7 receptor in the previous examples is unknown. However, studies of fluoride exposure with SHSY5Y neuronal cells and animal models resulted in a decline of expressed α7 nicotinic receptors. This is direct evidence that toxin exposure could significantly impact the appropriate function of the α7 nicotinic receptor. Taken together, this information suggests that environmental influences on specific molecular mechanisms could be actively contributing to the manifestation or worsening of schizophrenia through particular signaling molecules, for example the α7 receptors.

*Genetic Contributions*

Although schizophrenia has been demonstrated as a multifactorial illness with a plethora of environmental risk factors, the genetic susceptibility of schizophrenia cannot be overlooked. Dominating the current research literature
in schizophrenia are genetic linkage studies that have identified possible
candidate genes involved in the disease. Potential genes are examined through
a variety of methods, including mass ChIP arrays and single nucleotide
polymorphism identification. These types of studies have introduced many
possible targets that may contribute to the symptoms shown by schizophrenic
patients. It has been shown that nearly every chromosome exhibited some
degree of genetic linkage to schizophrenia, suggesting a high degree of
complexity in the disease. Meta-analyses by Lewis et al. looked at
systematically prioritizing the more heavily studied chromosomal regions relating
to schizophrenia. There are many distinct genes that have been identified with
a statistically significant association with schizophrenia. However, their
participation in the pathogenesis of schizophrenia is only now being investigated.
The α7 nicotinic receptor subunit is one of those target genes that meets both a
statistical criterion and a physiological deficit that can be associated with the
disease.

Specific Aims

The α7 receptor has emerged as a molecule highly implicated in the
neuropathophysiology of schizophrenia. The receptor displays potential
involvement with the dopaminergic, neurodegenerative, and autoimmune
etiological theories of schizophrenia as well as displays direct genetic linkage to
the disease. Schizophrenia has always been considered a multiplex disease that
could have a plethora of different causative factors. It may be that the α7
receptor could be the part of the puzzle that clarifies otherwise poorly understood
neuronal relationships found in the disease. It is the purpose of the present studies to further characterize the role of the receptor in schizophrenia through two entirely different avenues. Improper function or amount of the α7 receptor could contribute to the biological relevance of the receptor in schizophrenia.

The function of the α7 receptor is extremely important in proper neuronal processes as discussed previously. Disrupted neurotransmission is likely at the center of the neuropathology of schizophrenia. When combined with the putative immune dysregulation noted in the neuropathology of the disease, it is conceivable that the schizophrenic may be producing its own nAChR ligand, for example in the form of an antibody. The neurotransmitter and immune deficiencies could be explained by the presence of an auto-antibody that disrupted proper α7 receptor functioning. The ligand-gated ion channel properties of the α7 receptor that delicately control cellular communication could be subject to interference through an auto-antibody binding event (Figure 7).

![Figure 7. Potential auto-antibody effects on the neuron. The effects of an antibody specific for the α7 receptor could be antagonist-like (top nAChRα7 receptor), where the channel would be blocked, or agonist-like, where the channel would be found in a constant open state (bottom nAChRα7 receptor).](image-url)
could involve two potential outcomes. The antibody could block proper receptor function, resulting in an antagonist-like event. Or the antibody could act as a positive allosteric modulator, where the channel would be in the open conformation. As stated previously, the exact role of the α7 receptor in neuronal processes is poorly understood, so to state the outcome of antibody binding events on receptor function is speculation at this point but will be examined further in Chapter Five.

The second avenue explored in these studies examines the regulatory mechanisms that control the endogenous amount of functional receptor. The events that control α7 receptor subunit expression are not well known. In fact, only one transcription factor in the human α7 subunit has been characterized to date. Bungarotoxin binding assays reveal a significantly lower amount of functional α7 receptor in the brains of schizophrenics. Also reported are lower expression levels of receptor subunit mRNA expressed in lymphocytes. Diminished receptor expression would directly influence proper neuronal functioning that would be dependent on the cell type and location in the human brain. This lack of translational product could be due to inefficient transcription of the α7 subunit. Subunit concentration could directly affect cellular events that eventually conclude in the formation of functional homomeric or heteromeric α7 receptors. Without significant amounts of α7 receptor, the presynaptic signaling events that directly control glutamate and GABA release would be diminished. Also, the α7 control of dopamine via secondary signaling from glutamate would
be compromised as well. However, the role of α7 receptors specifically in dopamine transmission is unknown at this time and the effects it may have could not be stated confidently, but the complexity of the crosstalk between neurotransmitters is well-known. Consequences from the loss of efficient neurotransmission involving one transmitter would have a ripple effect on many separate neurotransmission events (Figure 8). This ability to communicate between neurons could be severely compromised by the inefficient transcription of one receptor subunit that resulted in a loss of functional receptor.

Figure 8. Neurotransmitters affected by diminished α7 receptor expression. The neurotransmitters glutamate and γ-aminobutyric acid (GABA) would be directly affected by decreased α7 receptor function or expression. However, the ripple effect on overall neurotransmission could disrupt other events involving dopamine (DA), acetylcholine (ACh), norepinephrine (NE), and serotonin (5-HT) modulation.

Therefore, the specific aims of this study were: 1) to develop the recombinant production of the α7 nicotinic receptor in a variety of expression
systems and to determine the integrity of the product; 2) to determine if there are auto-antibodies to the nAChRα7 by analyzing schizophrenic patient and control blood serum; and 3) to functionally characterize the cyclic AMP response element in the promoter region of the α7 subunit gene and identify any involvement in the transcriptional regulation of the receptor.
CHAPTER 2
MOLECULAR ADVANCES AND DISCOVERIES REGARDING THE α7 SUBUNIT OF THE NICOTINIC RECEPTOR

Background

Nicotinic receptors (nAChR) belong to a large family of ligand-gated ion channels responsible for cellular communication. These receptors are composed of five subunits that consist of either homomeric or heteromeric combination of alpha (α) or beta (β) subunits. Currently, the known subunits are α2 through α10 and β2 through β4. The acetylcholine nicotinic α7 subunit (nAChRα7) receptor is composed of five identical α7 subunits that form a ligand-gated ion channel. The α7 receptor was first discovered based on the ability to bind to α-bungarotoxin, a deadly snake toxin that attacks the central nervous system of victims.

History and Location of the α7 Nicotinic Receptor

The α7 nicotinic receptor was originally thought to be expressed only as a neuronal receptor in the CNS. The regions in the brain found to have high expression levels are the nucleus reticularis of the thalamus, the lateral and medial geniculate bodies, the basilar pontine nucleus, the horizontal limb of the diagonal band of Broca, the nucleus basalis of Meynert, and the inferior olivary nucleus68. More recently, the α7 nicotinic receptor has been identified in peripheral tissues such as endothelial cells (ECs), bronchial epithelial cells, skin keratinocytes, and vascular smooth muscle9-11,69. The tissue variation of the
nAChRα7 receptor would indicate the intense regulation the gene is under in order to achieve such distinct expression patterns\textsuperscript{68}.

The role of the receptor in each tissue is increasingly becoming clear. In the CNS, the receptor is responsible for participating in signal transduction at a post-synaptic and pre-synaptic level crucial to early developmental\textsuperscript{70}. The nAChRα7 receptor appears to work in four distinct manners in the CNS: postsynaptic fast transmission, axonal transmission, increase in intracellular Ca\textsuperscript{2+}, and opening of voltage-gated channels\textsuperscript{71}. The receptor also exhibits a high permeability to Ca\textsuperscript{2+}, which could result in activation of secondary messenger systems or cell death\textsuperscript{72,73}. In the immune response, nAChRα7 has not only been shown to participate in cell proliferation but more specifically in the support of B-lymphocyte survival through IgM and CD40 expression\textsuperscript{74}. The nAChRα7 receptors found in skin keratinocytes are responsible for aiding in cell cycle regulation and participation in setting up the epithelium barrier\textsuperscript{75}.

**Pathophysiology of the nAChRα7 Receptor**

The pathophysiology of the nAChRα7 receptor has been scrutinized heavily in the past few years. It is increasingly becoming a more popular candidate gene in mental disorders such as schizophrenia. The strong correlation between nicotine usage and schizophrenia lends support to the further examination of the relationship between the nicotinic receptor and disease. The inability to filter background stimuli known as the P50 gating deficit and the insufficiency in following visual stimuli are measurable traits in
schizophrenia corrected by nicotine administration\textsuperscript{76,77}. Genetic linkage to schizophrenia involving the CHRNA7 chromosome has been debatable, but mounting evidence concludes it is a viable candidate\textsuperscript{78-82}. The nAChR\textalpha{}7 receptor expression has been shown to be decreased in neuronal cells of schizophrenics, as well in peripheral blood cells\textsuperscript{35,83}. Age-related diseases such as Alzheimer’s (AD) and Parkinson’s (PD) have examined altered expression levels of the receptor, with AD demonstrating a detailed involvement between the receptor and β-amyloid accumulation\textsuperscript{84-86}. Major depressive disorder and bipolar disease research is beginning to characterize nAChR\textalpha{}7 involvement as well\textsuperscript{36,87}. With the emerging notoriety the nAChR\textalpha{}7 receptor is gaining, this review attempts to look at the molecular information and recombinant tools available to study the receptor.

\textit{Molecular Biology of the nAChR\textalpha{}7 Receptor}

\textit{Protein Structure of the nAChR\textalpha{}7 Receptor}

Each \textalpha{}7 subunit of the nicotinic receptor, illustrated in Figure 9, consists of a 502 amino acid molecule with four trans-membrane spanning regions, a large extracellular amino terminal domain, and an intracellular loop between membrane three and membrane four\textsuperscript{88}.
Figure 9. The α7 nicotinic receptor protein. This figure is a representation of the four membrane spanning regions and dual cysteine bridges found in the amino portion of the receptor. Each ion channel consists of five identical receptors, with ligand-binding occurring in the amino portion of the receptor.

Extremely selective ligand binding occurs at the extracellular amino terminal of the receptor\(^8^9\). It could be a single amino acid between membrane regions two and three is responsible for the gating response that occurs upon ligand binding, more specifically interactions with N-terminal cysteine loops could be directly responsible for communicating these changes\(^9^0,9^1\). The presence of highly conserved dual cysteine (C150 and C164) residues in the nAChRα7 extracellular region are necessary to form properly assembled receptors\(^9^1\). The nAChRα7 amino acid formation can vary greatly between species (Table 3). Within species, the crucial ligand-gated extracellular portion and pore lining region
exhibit sequence similarity throughout the mammalian species. However, once one moves to insects, sequence sharing becomes much less evident, especially in the extracellular and intracellular signaling domains.

Table 3. Sequence homology of the α7 nicotinic receptor. This table illustrates the sequence homology of the α7 nicotinic receptor between species and within specie, when compared to the human nAChRα7 receptor.

<table>
<thead>
<tr>
<th>Species</th>
<th>Gi accession number</th>
<th>% identities</th>
</tr>
</thead>
<tbody>
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<td>27806239</td>
</tr>
<tr>
<td>Mouse</td>
<td>gi</td>
<td>156766082</td>
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<td>Rat</td>
<td>gi</td>
<td>144922602</td>
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<tr>
<td>Chick</td>
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<td>Zebrafish</td>
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<tr>
<td>Cat flea</td>
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</tr>
<tr>
<td>Tobacco budworm subunit-2</td>
<td>gi</td>
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<td>gi</td>
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</tr>
<tr>
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<td>213218</td>
</tr>
</tbody>
</table>

The nAChRα7 does not share high sequence similarity within the alpha subunit family, mainly in the amino terminal region, thus expressing greater diversity in ligand binding (this is discussed more in Chapter 3). Interestingly, the coding transcript for the nAChRα7 gene expressed by T-cells is not completely identical to the neuronal subunit, exhibiting 99.6 % homology and presenting with distinct physiological differences.92

**Genetic Location**

The gene encoding nAChRα7 (CHRNA7) is located on chromosome 15q13.93 Physical mapping of the gene indicates the full-length receptor could be
greater than 75 Kb in length, contains 10 exons, and is subjected to formation of splice variants. There is also the existence of a duplicated form of exon 5-10 that is found 1 million bases from the full-length CHRNA7. This CHRNA7 duplicate is found to have an association with a 2-bp deletion found in bipolar disorder.

In mice knock-out studies of the nAChRα7 receptor, the animals do exhibit loss of bungarotoxin binding and fast synaptic currents. However, phenotypically they seem unaltered except in reproductive ability and neonatal development/survival. Knock-out studies of the receptor have warranted further scrutiny due to incomplete loss of immunoreactivity of the receptor; however, this could be explained by the presence of the CHRNA7 duplicate or antibody selectivity. But when anti-sense knockdown studies of the receptor were conducted, changes in sensory motor gating and spatial learning were noted. Knock-in mice created were toxic neonately, but when allele strength was diminished the animals were behaviorally modified to show an extreme hypersensitivity to nicotine.

Transcription of CHRNA7 Gene

With the mounting data concerning transcription factors in general and the use of computer generated models, the identification of regulatory elements that control the CHRNA7 is still quite infantile. The presence of a CNS receptor in peripheral tissues makes the picture even less clear. Also, it has been shown the CHRNA7 promoter acts in a cell-type specific fashion. There is very
little known about specific regulatory elements of the CHRNA7 gene. Table 4 lists the relatively few factors characterized in animal systems.

Table 4. Transcription factors known for the AChRα7 receptor. This is the known published data regarding transcription factors that act directly on the α7 nicotinic receptor promoter.

<table>
<thead>
<tr>
<th>Species</th>
<th>Transcription factor</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow</td>
<td>Egr-1</td>
<td>Carrasco-Serrano et al.(^\text{104})</td>
</tr>
<tr>
<td>Rat</td>
<td>Egr-1, sp1, sp3</td>
<td>Nagavarapu et al.(^\text{105})</td>
</tr>
<tr>
<td>Mouse muscle</td>
<td>USF1, Egr-1</td>
<td>Campos-Caro et al.(^\text{106})</td>
</tr>
<tr>
<td>Mouse lung</td>
<td>TTF-1</td>
<td>Reynold and Hoidal(^\text{107})</td>
</tr>
</tbody>
</table>

The essential promoter region for the human CHRNA7 was established as the first 392 base pairs of the promoter from the transcriptional start site. It was also found by computer analysis to have many potential transcription factor binding sites within the proximal promoter region as well as possess potential regulatory elements up-stream\(^\text{108}\).

Post-translational Modifications to nAChRα7 Receptor

Translational modifications of the nAChRα7 subunit are essential to not only cell-surface expression of the receptor but to the functional response exhibited by the receptor. The addition of three asparagine residues to the extracellular portion of the nAChRα7 receptor subunit has been shown to be essential in the proper folding of the receptor in order to achieve appropriate functionality on the cell-surface when expressed in mammalian cells and oocytes\(^\text{109}\). The importance of glycosylation involving the receptor is represented by the mongoose and cobra, with both animals exhibiting an immunity to the
toxicity of α-bungarotoxin most likely due to an extra glycosylation site in the receptor\textsuperscript{110,111}. Palmitoylation of the receptor occurs in the endoplasmic reticulum before distribution and is essential in the formation of the bungarotoxin binding ability exhibited by the nAChRα7 subunit\textsuperscript{112}. Although it is known to be involved in cellular communication, the exact role of palmitoylation in proteins has remained elusive, but with the discovery of the enzymes participating in this modification more information should reveal the exact function in nAChRα7 subunits\textsuperscript{113}. Phosphorylation, like glycosylation, is not involved in cell-surface expression, yet Tyr-386 and Tyr-442 in the intracytoplasmic loop are phosphorylated and contribute to the proper cellular responses exhibited by the receptor\textsuperscript{114}. In summary, the mature nAChRα7 subunit is expressed as a precursor peptide of 502 amino acids, with a signal sequence of 19 amino acids and undergoes several specific modifications that determine the exact location in which it is deposited and the proper functioning it exhibits in a species-dependent cell-specific manner.

\textit{Assembly of nAChRα7 Receptors}

In light of recent evidence, the direct participation of proteins in the final processing stages of subunit formation is beginning to unravel some of the mystery surrounding the ion channel’s construction. One protein found to participate directly in receptor assembly is RIC-3, resistance to inhibitors of cholinesterase [most recently reviewed by Millar 2008\textsuperscript{115}]. RIC-3 has been shown to directly bind unprocessed nAChRα7 subunit and is required to
participate in receptor subunit folding as a molecular chaperone\textsuperscript{116}. In the absence of RIC-3, little nAChRα7 ligand binding can be detected, where the presence of RIC-3 significantly up-regulates production of the functional receptor\textsuperscript{117}. Immunoprecipitation identification using nAChRα7 revealed that RIC-3 indeed binds directly to the subunit in the cytoplasmic ER\textsuperscript{118}. Illustrating the importance of the protein in α7 receptor formation are molecular studies where RIC-3 mutations resulted in decreased expression of the \textit{c. elegans} nicotinic receptor subunit LEV-1\textsuperscript{119}.

The function of RIC-3 is essential in the formation of functional α7 homomeric ion channels. However, the accessory proteins that participate in heteromeric ion channels have not yet been identified. Initial studies of nicotinic receptors previously only characterized this one nicotinic receptor that used the α7 subunit. However, recent data using bovine adrenal chromaffin cells indicates a relationship between α3 and α5 nicotinic receptor subunits shown by co-immunoprecipitation of the receptors with nAChRα7, indicating the possible existence of heteromeric α7 nicotinic receptors\textsuperscript{120}. This recent discovery in bovine cells gives strong support to previous findings that identified functional heteromeric receptors in rat and chick tissue as well\textsuperscript{18,121}. The formation of homomeric and heteromeric α7 nicotinic receptors would justify the notable inconsistencies observed with identification of the nAChRα7 subunit by antibody and radioligand binding.
Pharmacological Significance of the nAChRα7 Receptor

The nAChRα7 receptor has endured considerable scrutiny as a viable candidate for pharmacological therapeutic treatment. This is largely in part due to the implications relayed through the smoking populations in such diseases as Alzheimer’s and schizophrenia. In schizophrenia, the use of nicotine appears to serve as a “self-medicating” behavior that alleviates the inability to filter background stimulus (known as gating deficits) seen in patients\textsuperscript{122,123}. Generally, it has been found that nicotine can improve cognitive functions such as working memory in schizophrenic patient populations\textsuperscript{124}. When nicotine is administered with typical antipsychotics used to treat mental illness, there is an improvement in cognitive deficits\textsuperscript{125}. Nicotine up-regulates nAChRα7 expression acutely, as verified with experiments in cells and animal models making it a potential target for therapeutic benefit\textsuperscript{91,126,127}.

The prototype nAChRα7 agonist DMXB-A (3-2,4-dimethoxybenzylidene anabaseine) has undergone clinical trials and has been found to normalize the gating deficit seen in schizophrenic patients as well as increase other cognitive functions\textsuperscript{128,129}. In animal trials, the nAChRα7 partial agonist, SSR180711, has reversed cognitive inefficiencies found in methyllycaconitine (an nAChRα7 antagonist) administered rats as well as improved anti-depressant behaviors possibly by inducing c-Fos production in the prefrontal cortex and the shell of the nucleus accumbens\textsuperscript{130,131}. Several other compounds targeting the nAChRα7 are in the developmental phase from the pharmaceutical companies Targacept,
AstraZeneca, and Pfizer, which further strengthens the argument of possible therapeutic benefits.

An alternative approach targeting the therapeutic potential of nAChRα7 is the use of Positive Allosteric Modulators (PAMs), molecules. Receptor binding events involving PAMs are able to influence the transition between channel states that would in turn control the cell surface activity of the nAChRα7 receptors [recently reviewed by Bertrand and Gopalakrishnan132, Faghih et al.133]. Ivermectin and 5-hydroxindole are two well-characterized PAMs that have been shown to modulate energy kinetics to produce better effects from nAChRα7 agonists134,135. Most recently, the PAM galantamine has been used in clinical trials to supplement nAChRα7 agonist treatment in the therapeutic management of schizophrenia with mixed results136,137. The idea of manipulating nAChRα7 activity by the use of therapeutic agents is a very promising area regarding the improved prognosis of mental illness.

Recombinant Expression of the nAChRα7

The limited availability of the human nAChRα7 due to the costly and intricate purification procedures from neuronal tissue has not only slowed results but led researchers to embark in the development of recombinant expression methods for the receptor. Because relatively little is known about transcriptional regulation and translational modifications in combination with the cell-type and species-specific expression, the search for an expression system for this receptor has proven to be a trial and error effort. In our laboratory many attempts
have been used in an effort to develop or improve nAChRα7 receptor expression systems. Insect cells, sf9, were used as host cells in trying to obtain the entire nAChRα7 receptor. Also, cell free protein synthesis with a variety of expression strategies was employed in an attempt to circumvent the problems encountered using the receptor. In the following, the most common recombinant expression systems are reviewed for their success in nAChRα7 expression.

*Expression of nAChRα7 in Bacteria Expression Systems*

Bacterial cell expression can harbor the production of the nicotinic receptor α subunit using techniques specifically designed to overcome the negative elements of the system. Using a trpE fusion vector pATH2, variable fragments from amino acids 166 to 315 of the electric eel nicotinic receptor α subunit were first expressed in HB101 bacterial cells to characterize ligand binding of acetylcholine and alpha-bungarotoxin to the receptor\textsuperscript{138,139}. A full-length extracellular construct of amino acids 1-209 of the eel nicotinic receptor α subunit receptor was later expressed using a pET 3a vector in the bacterial cells BL21 as an inclusion body (100-200mg/Liter) that could be renatured and exhibited the ligand binding properties of intact receptors\textsuperscript{140}. With success expressing the amino portion of the torpedo nicotinic receptor, it was found to be replicated using the mammalian α7 nicotinic receptor. A protein over-expressing system was developed constructing a fusion protein that consisted of the N-terminal portion of the rat nAChRα7 (amino acids 1-196), a linker, and maltose binding protein that was transformed into XL1 Blue E.coli cells and production
occurred at 5 mg/Liter of culture$^{141}$. The human extracellular portion of the nAChRa7 receptor (amino acids 1-205) could be engineered by fusion to the maltose binding protein via the pMAL-c2X vector inserted into E. coli strain BL21, which was found to produce a soluble protein at 35mg/Liter$^{142}$.

**Expression of nAChRa7 in Yeast Expression Systems**

Yeast systems provide the necessary cellular organization of eukaryotic organisms but exist in single cell entities that are easily maintained and can be manipulated to support genetic alterations. The yeast strain, Saccharomyces cerevisiae, was able to support α-subunit expression of the Torpedo californica full-length nicotinic receptor at 1% of all membrane proteins present, which indicates sufficient translational modifications are made for proper membrane insertion$^{143}$. Later, it was found that modifications to the hydrophobic Cys-loop in the amino terminal of the nAChRa7 receptor led to the production of a soluble form of the first 209 amino acids in *Pichia pastoris* yeast cells, which were suitable for structural and physiological studies$^{144,145}$.

**Expression of nAChRa7 in Insect Expression Systems**

Using insect cells to express mammalian proteins offers several advantages: proper folding, less stringent growth conditions, and many proteins report post-translational modifications identical to that of mammalian expression systems. The insect cells, Sf9, were used to express the muscular alpha subunit from bovine using a viral vector, requiring a rigorous isolation process using a
zwitterionic detergent in conjunction with many chromatography steps to produce a milligram quantity of the receptor\textsuperscript{146}. Chick neuronal nicotinic acetylcholine receptor α subunit was also successfully expressed using a baculoviral expression system in Sf9 cells, but at very low levels. However, with human nAChRa7 it would appear the insect cell system is not conducive for recombinant expression of neuronal nAChRa7. It is reported that upon transfection with the CHRNA7 gene, the sf9 cells exhibited variable mRNA expression of a single transcript but did not yield functional ligand binding protein, suggesting that the insect cell system does not adequately support the post-translation packaging required for proper nAChRa7 expression\textsuperscript{147}. This would appear to be supported by our own studies using a baculovirus system in Sf9 cells where human nAChRa7 was not detected (data not shown). It is worth noting that those working with the insect nAChRa7 are still asking for development of better molecular tools to explore this receptor, even after many years of searching\textsuperscript{148}.

\textit{Expression of nAChRa7 in Mammalian Systems}

The human nAChRa7 seems to only be properly expressed in its entirety in a mammalian expression system. Several human neuronal cell lines have been shown to express the nAChRa7 endogenously, leading them to be viable candidates for an over-expression system for the receptors, but these cells have exhibited variable results when trying to use them to genetically engineer the receptor. Rat CHRNA7 was introduced into nine different mammalian cell lines (the first three are non-neuronal): CHO, COS-7, HEK-293, N1E-115, NCB20,
Neuro-2A, NG108-15, PC12, and SHSY5Y cells, with only the latter two exhibiting increased bungaroxin binding receptors on the cell surface\textsuperscript{149}. A supplemental study also revealed that rat PC12 and human SHSY5Y cells have been shown to express functional nAChR\(\alpha_7\) receptors with or without mutations, while the rat pituitary GH3 cells exhibited variable expression when just the mRNA transcripts for the receptor were analyzed\textsuperscript{147,150}. This illustrates the precise cell-specific processing that is required for proper expression of the receptor. However, human neuronal cell lines do not serve well as an over-expression protein system, which led researchers to look at other cells that could effectively express the receptor at more desirable protein levels. The rat fibroblast cells NIH-3T3 did not serve as a viable system exhibiting variable expression levels of rat nAChR\(\alpha_7\)\textsuperscript{147}. A chimeric protein consisting of the N-terminal human nAChR\(\alpha_7\) fused to the mouse C-terminal of the serotonin 5HT3 receptor was developed in HEK293 cells to assess pharmacological profiles of novel agents to further understanding of the receptor\textsuperscript{151}. It was also discovered that human CHRNA7 can be functionally expressed in HEK293 cells only when co-expressed with a human protein known as RIC-3, allowing this system to serve as a possible, robust expression environment for nAChR\(\alpha_7\)\textsuperscript{118}. The null-nAChR\(\alpha_7\) epithelial cell line, SH-EP1, exhibits a vigorous expression setting that can be genetically manipulated to produce the human nAChR\(\alpha_7\) receptor as a complete, functional native ion channel that can serve as an ideal model for molecular studies regarding the receptor\textsuperscript{152-154}. Several expression systems for nAChR\(\alpha_7\) using mammalian cells are now in place that can be used as high-
throughput screening tools in the quest for better understanding regarding the complex nature of this receptor.

Expression of nAChRα7 in Cell-free Systems

Cell-free expression systems are available for researchers to use a simple, quick, and sometimes fairly cost-efficient means to protein expression when compared to their cell system counterparts. This expression environment has been explored concerning nAChRα7. The TNT™ quick coupled system from Promega used rabbit reticulocytes and expression machinery for insertion of fully formed glycosylated chick nAChRα7 into lipid bilayers that exhibited proper channel formation. Our lab was unable to express full-length human nAChRα7 using the TNT system but was able to express the first 202 amino acids of the extracellular portion as identified by autoradiography and Western blotting (data not shown). To ensure proper post-translational modifications are made to the recombinantly expressed receptor, the insect cell-free Easyxpress™ system was also used to produce the extracellular portion of nAChRα7. Protein was created but not to a level that would render it economically valuable to use as an over-expression system by our lab (data not shown). A cell-free system can effectively produce fragmented peptides of the α7 receptor; however, like the other expression systems, it appears the nature of the entire nAChRα7 requires cell-specific factors that facilitate proper modifications that can result in functional receptors.
Expression of nAChRα7 in Systems Utilizing RIC-3

With the discovery of RIC-3 as a chaperone protein directly participating in nAChα7 expression, recombinant production using this protein has now begun to emerge. Xenopus expression was the first system to use recombinant nAChRα7 production upon discovery of RIC-3 to validate the relationship between chaperone and receptor\textsuperscript{118,156}. Several mammalian cell lines have been tested for the ability to efficiently produce the nAChRα7 receptor, including HEK293, Kidney ts201, and CHO-K1\textsuperscript{118,157,158}. Most recently, insect RIC-3 homologues have been investigated in an attempt to uncover the reason behind the inefficiency of insect cells as an expression system for the nAChRα7 receptor\textsuperscript{157}. The presence of RIC-3 has opened avenues to develop potential nAChRα7 over-expressing systems in an effort to provide the tools necessary in identifying the precise role the receptor occupies in the pathophysiology of various mental disorders.

Advances in the research and development using the nAChRα7 subunit have initiated the development of this frontier in the study of mental illness. The important role the receptor is assuming in the patho-physiology of various diseases, coupled with the emerging pharmacological significance exhibited, is making the complete understanding of the receptor vitally important. The information garnered by the various cell-specific or cell-free systems expressing the receptor is aiding in the development of a more complete picture of what could be groundbreaking therapeutic treatment in mental illness.
CHAPTER 3
INCREASED SERA ANTIBODIES FOR THE $\alpha_7$ SUBUNIT OF THE NICOTINIC RECEPTOR IN SCHIZOPHRENIA

Background

Insight regarding the complex etiology of schizophrenia may lie in one of its most well-known symptoms, auditory hallucinations (AH). One abnormality that has been described in schizophrenia, that may contribute to AH, is inefficient auditory gating, which is the ability to filter background stimuli and focus on a single auditory stimulus. The $\alpha_7$ subunit of the acetylcholine nicotinic receptor ($\alpha_7$nAChR), which has low affinity binding to nicotine, has been associated with normal auditory gating$^{76}$. Involvement of the nicotinic receptor family in schizophrenia is suggested by increased tobacco use in the diagnosed patient population, which is between 85% and 90%; this rate is more than triple the normal use and could indicate a self-medication with nicotine$^{159}$. Further investigations revealed neuronal nAChR$\alpha_7$ had diminished protein expression in schizophrenic patients as shown with postmortem ligand binding studies$^{37,160}$. Messenger RNA studies have failed to conclusively support this, identifying post-translational mechanisms or other factors as the culprit in the abnormal levels seen in patients$^{36,37}$. Genetic linkage studies identify the receptor as a possible candidate gene in schizophrenia$^{161}$. The emerging molecular and genetic data regarding the nAChR$\alpha_7$ suggests a direct involvement of the receptor in the neuropathology of schizophrenia.
The nAChRα7 is a ligand-gated ion channel directly involved in the cell signaling events that participate in the control of the anti-inflammatory pathway. Early research noted morphological changes in lymphocytes, altered B and T cell expression, and increased cytokine circulation in schizophrenic patients. A clinical study with chronic schizophrenics who were administered a cytokine inhibitor along with their antipsychotic regimen improved significantly better than those who were not. Also in schizophrenia, there is evidence found through patient family history evaluations that family members tend to develop other autoimmune disorders. There have also been reports of the existence for sera antibodies to a muscarinic subunit of the nicotinic receptor in patients with schizophrenia. The evidence suggests an autoimmune mechanism may contribute to the schizophrenia, leading to our hypothesis that schizophrenic patients possess autoantibodies for the nAChRα7. The goal of this study is to analyze schizophrenic patient blood serum for antibodies exclusive to the nAChRα7 receptor.

**Materials and Methods**

**Patients**

This study was approved by the Institutional Review Board at East Tennessee State University. Patients were required to submit written informed consent for any information and blood extraction. A clinical diagnosis was determined upon evaluation based on patient history, therapeutic analysis, and completion of the Structural Clinical Interview from the DSM-IV axis of diagnosing.
psychiatric illness (SCID). A total of 21 patients were included in the study and were classified as having schizophrenia.

**ELISA Using Recombinantly Expressed α7 Protein**

The Enzyme-linked immunosorbent assay (ELISA) was used according to the following method. Polyvinyl 96-well microtiter plates were coated with 50 ng of recombinant α7 nicotinic receptor (Abnova, Taipei City, Taiwan) produced in wheat germ extract diluted the appropriate concentration in Ringer’s buffer (25 mM Tris pH 7.2, 150 mM NaCl, 5.8 mM KCl, 1.8 mM CaCl₂, 1.3 mM MgCl₂, .1 mg/ml sodium azide) and incubated overnight at 4ºC. The plates were blocked with tris-buffered saline (TBS) containing 3% bovine serum albumin (Sigma, St. Louis, MO) at room temperature for 3 hours. After one wash with TBS-T (.05%), the antigen was incubated with N-terminal α7 antibody at 1:75 dilution (Abcam, Cambridge, MA), overnight at 4ºC. Following one wash with TBS-T (.05%), 200 µl/well of horseradish peroxidase conjugated goat anti-rabbit IgG (1:1000 dilution) (Southern Biotech, Birmingham, AL) was added to plates and incubated for 4 hours at room temperature. After five washes with TBS-T (.05%), the complexes formed in each well were incubated with 150 µl of 1-step ABTS (Pierce, Rockford, IL) for 20 minutes, after which 100 µl of 1% SDS was added to each well. The absorbance values were read at 405 nm using a Biorad 450 microplate reader (Hercules, CA). Protein binding was assessed using the N-term antibody from Abcam as a positive plate control. The serum anti-α7 antibody levels were determined using the same experimental parameters as the
control antibody. After antigen binding and plate blocking was complete, serum from controls and schizophrenic patients diluted at 1:500 in TBS-3% BSA were incubated overnight at 4°C. Optimal sera binding was initially determined by using a dilution curve of patient sera initially ranging from 1:2, 1:10, 1:50, 1:100, 1:500, 1:1000, and 1:10,000 in TBS-3% BSA. Each patient and control serum was subjected to two dilution samples for verification. After wash with TBS-T (.05%), 150 µl of horseradish peroxidase conjugated goat antihuman IgG (1:1000 dilution) (Southern Biotech, Birmingham, AL) in TBS-3% BSA was added to wells and incubated for 4 hours at room temperature and completed as with the positive N-terminal antibody above.

**Determining Antibody Activities**

In order to express a similar distribution, the antibody index was calculated in the following manner. The nAChRα7 antibody index was determined as percent control of the anti-nAChRα7 polyclonal antibody positive control served as a plate standardization and expressed in the following manner:

\[
\text{Relative optical density of sample} \times 100 \div \text{Relative optical density of positive control}
\]

Standard deviation from the mean value was determined for healthy control population. Assay was conducted in triplicate in two separate experiments and indices analyzed in their relationship to more or less than two standard deviations from the mean. The relationship between patient/control age and antibody indices also used these antibody scores. Four different statistical analysis were
employed to determine statistical significance to control mean, t-test, Fisher’s exact test for probability, CHI determination, and a Dunnett probability analysis.

Results

Serum anti-nAChRα7 antibody indices were higher in some of the patients diagnosed with schizophrenia than in the control sample population. There were five patients with a value above +/- 2SD above healthy controls (Figure 10). Overall, 23% of the patient population exhibited anti-nAChRα7 antibodies based on CHI square analysis. However, it should be noted when other statistical analysis were conducted, the findings were close to the accepted level of significance in each one of them: t-test p=.08, Fisher’s exact test p=.06, and Dunnett analysis p=.06.
Figure 10. Antibodies to the nAChRα7 receptor found in Schizophrenia vs. healthy controls. A Chi square analysis was formulated to compare the activity between the control and patient population. The line exhibits +/- 2 S.D. levels from the control mean. Indices above this are considered positive for nAChRα7 antibodies. Significance was determined at p<.05, where the Chi square value was .03.
Figure 11. The linear comparison of antibody titer to age in the patient Population Compared to Controls. Both regression coefficient and ANOVA analysis was used to test the relationship between age and titer. Anti-nAChRα7 antibodies correlate with age according to ANOVA analysis with a p-value <.009.
There were significant differences in the two populations with regard to age and anti-α7 receptor antibodies. The patient group was found to significantly exhibit a strong correlation between age and antibody index, where as the control group exhibited no significant correlation between nAChRα7 antibody in age-matched controls (Figure 11). Shown in Table 5 are the clinical characteristics in the patients who had higher activities of nAChRα7 antibodies, as shown with similar age-matched controls.

Table 5. Clinical characteristics. These are the clinical characteristics of four of the five schizophrenic patients with elevated α7 receptor (nAChRα7) levels, as well as the clinical characteristics of close age-matched controls.

<table>
<thead>
<tr>
<th>Patient Number/Diagnosis</th>
<th>Age/Sex</th>
<th>Other known factors</th>
<th>Antibody index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/SCH</td>
<td>60/M</td>
<td>Depression</td>
<td>682</td>
</tr>
<tr>
<td>2/SCH</td>
<td>61/F</td>
<td>-</td>
<td>637</td>
</tr>
<tr>
<td>3/SCH</td>
<td>46/M</td>
<td>Anxiety</td>
<td>488</td>
</tr>
<tr>
<td>4/SCH</td>
<td>50/F</td>
<td>-</td>
<td>486</td>
</tr>
<tr>
<td>5/CON</td>
<td>61/F</td>
<td>-</td>
<td>321</td>
</tr>
<tr>
<td>6/CON</td>
<td>71/M</td>
<td>-</td>
<td>292</td>
</tr>
<tr>
<td>7/CON</td>
<td>47/M</td>
<td>Depression</td>
<td>211</td>
</tr>
<tr>
<td>8/CON</td>
<td>43/F</td>
<td>-</td>
<td>373</td>
</tr>
</tbody>
</table>

Discussion

In this study, the sera of patients with schizophrenia were examined for antibodies targeting the nAChRα7 subunit. It was found there are elevated levels in the patient population when compared to a non-Schizophrenic control population. The information indicates a pathogenic level of nAChRα7 antibody may actively contribute to the etiological basis of the disease in at least a subset
of patients. Even more interesting is the increased levels of antibody with regard to age, which mirrors the neurodegenerative nature of the disease.

The nAChRα7 receptors are extremely important in the modulation of glutamate and GABA neurotransmitter release and antibodies specific to the receptor may interfere with those events. Also, sera antibodies for the receptor may result in targeting the cell for degradation by the immune system, which would certainly justify the down-regulation of the receptor that has been found by ligand binding assays in the brains of schizophrenics\textsuperscript{35,83}. The findings in this study also contribute to understanding of nAChRα7 role in the immune system. This acetylcholine receptor, once thought to be confined only to the central nervous system, has now been identified in epithelial cells and in blood leukocytes\textsuperscript{69,167}. The presence of the receptor on leukocytes has provoked investigations into the relationship with the immune system. It was found that nAChRα7 receptor activation participates directly in restoring the delicate balance between the pro-inflammatory and the anti-inflammatory pathways. Signaling by the receptor prevents nuclear translocation of NFkB, a cytokine regulatory transcription element, that effectively silences the pro-inflammatory pathway. Simultaneously, the nAChRα7 activates the JAK/STAT pathway, initiating the anti-inflammatory response\textsuperscript{12}. If nAChRα7 receptor activation were reduced by antibody binding events, a loss in control of these inflammatory molecules would result as exhibited in the immune system of some schizophrenic patients\textsuperscript{12}. 
If receptor function can be altered by the presence of auto-antibodies in a subgroup of patients with schizophrenia, then pharmacological manipulation targeting the receptor could be productive in disease management. A clinical trial with DMX-B, a nAChRa7 agonist, found cognitive improvements in schizophrenic patients\textsuperscript{128}. More recently, the combined therapy of CDP-choline (nAChRa7 agonist) and galantamine (nAChRa7 positive allosteric modulator) conducted in a 12-week trial of 6 schizophrenic patients was found to lessen the severity of the disease\textsuperscript{136}. Currently, pharmaceutical companies Pfizer, Astra-Zeneca, Pharmacia, Targacept, and Sanofi-Adventis all have nicotinic agonists in development\textsuperscript{131,168}. Along with development of synthetic molecules is the use of immunotherapy with peptide fragments of the nAChRa7, resulting in the induction of spatial memory in an Alzheimer’s animal model indicating a novel treatment for cognitive deficits in schizophrenia\textsuperscript{169}. By establishing a relationship between the α7nAChR, auto-antibodies, and schizophrenia, a causative factor could be identified and better therapeutic interventions designed for a specific target in a manageable subset of patients.
CHAPTER 4
FUNCTIONAL CHARACTERIZATION OF THE CRE HALF-ELEMENT FOUND IN THE PROMOTER REGION OF THE NICOTINIC RECEPTOR \(\alpha_7\) SUBUNIT

Background

Regulation of human gene expression has been the subject of intense scrutiny when it comes to identifying the mechanisms behind neurotrophic responses to external stimuli. There are hundreds of different transcription regulatory proteins known, with the average gene requiring two dozen factors to form one transcriptional complex. The number of proteins required for one transcriptional complex multiplied by the different complexes necessary to participate in the precise expression pattern exhibited by a specific gene results in an exponential number of scenarios. Knowledge regarding gene expression will continue to grow as the protein interactions with the human genome are identified. One of the most widely investigated regulatory elements in neuronal processes has been the cAMP-response element binding protein (CREB) family of transcription factors. CREB has been shown to be involved in neuronal development, circadian rhythm regulation, learning and memory, neurotrophic responses in the cell, and precursor proliferation\(^{170}\). The transcription factor CREB binds specific DNA sequences known as cyclic-AMP response elements (CRE). In the human genome, there have been 10,447 full CREs and 740,390 half CREs identified in an approximate 3300 bp range relative to potential
transcription start sites\textsuperscript{171}. Zhang and associates used three different algorithms to reduce/refine the above projection to 4,084 putative CREB target genes\textsuperscript{172}.

Because of the growing relationship between CREB-regulated gene expression and neuronal processes, research has greatly intensified to establish this pathway as a therapeutic target. Antidepressant treatment results in an up-regulation of CREB, supporting the hypothesis that CREB-driven regulation of target genes contribute to neuronal plasticity and neurogenesis that result in behavioral effects\textsuperscript{173}. Treatment with lithium for mood disorders results in neuronal survival via an up-regulation of CREB\textsuperscript{174}. Prognosis following brain insult markedly improves following treatment with agents such as somvastatin due in part to neurogenesis stimulated through increased CREB phosphorylation\textsuperscript{175}. Tardito and associates specifically note that CREB-dependent gene transcription not only relies on a particular pharmacological agent but also the specific neuronal area targeted\textsuperscript{176}. Nicotine illicitis a neuroprotective effect via CREB resulting in improvement in long-term potentiation (LTP) and spatial memory in the CA1 area of the hippocampus\textsuperscript{177}. The regulatory element CREB has a complex role in regulating many of the genes involved in proper neuronal function.

Post-mortem studies have shown a decrease in the α7 nicotinic receptor in brains of diagnosed schizophrenic patients\textsuperscript{178}. Pharmacological studies have demonstrated that treatment of animal subjects with agonists specific to the α7 subunit of the nicotinic receptor (nAChRα7) produced enhanced LTP resulting in a decline in cognitive defects, in particular with those associated with
schizophrenia\textsuperscript{179,180}. The proximal promoter region of the α7 subunit has a potential CRE site identified by computer analysis. This could be a functional regulatory element supported by the finding that null-CREB mice exhibit a decreased level of receptor\textsuperscript{181}. Based on this information, we hypothesize nAChRα7 expression could fall under CREB control, either in the initial transcription complex formation or as a signaling mechanism during the translation process. This study aims to test this hypothesis by exploring what role, if any, the half-CRE element found in the promoter of the nAChRα7 occupies in the transcriptional regulation of nAChRα7 receptors.

\textit{Materials and Methods}

\textit{Cell Culture}

Human neuroblastoma cell line SHSY5Y cells were grown in Ham’s F12 modified Eagle’s media supplemented with 15% fetal bovine serum and 1% non-essential amino acids all from Sigma Chemical Company (St. Louis, MO) in an atmosphere containing 5% CO\textsubscript{2} in air at 37°C. Human recombinant CREB was purchased from Prospec (Rhehovet, Isreal).

\textit{EMSA (Electromobility shift assay)}

A 21-bp complimentary oligonucleotide, 5’ GCGGGG\textsc{ACTGTCACG} TGGAGA 3’, and a 45-bp complimentary oligonucleotide 5’ ACGGGG GCGGGG\textsc{CTCGTCACG} TGGAGA GCGGGG CCGCGGGGGCC3’, as well as
mutated α7 promoter regions (Figure 13A), containing the putative CRE motif of the α7 promoter were subjected to EMSA using a Gel shift assay kit according to the manufacturer’s instructions (Promega, Madison, WI). Also used was positive control CRE motif oligonucleotide (Promega, Madison, WI). Oligonucleotides were 5’ end-labeled with a radioactive phosphorus, and Protein-DNA complexes were separated on a 4% non-denaturing PAGE gel. Competition assay used 50X unlabeled probe. Antibody assays used 1 µg of polyclonal anti-CREB antibody (Rockland, Gilbertsville, PA) and anti-Sterol Response Element Binding Protein (Santa Cruz, CA).

Construction of Reporter Plasmids

The promoter sequence from the α7 subunit of the nicotinic receptor containing the suspected transcription factor motif was created using PCR amplification of genomic DNA isolated from human brain (Biochain, Hayward, CA) using the following primers: sense 5’AGA ACG CAA GGG AGA GGT AGA 3’ and anti-sense 5’ GTT GAG TCC CGG AGC TGC AGC GA 3’. The 392-bp fragment was then cloned into TOPO pCR2.1 vector (Invitrogen, Carlsbad, CA) via Kpn I and Xho I restriction sites resulting in the pCR2.1/α7 vector. The promoter was subsequently removed from this vector and subcloned into the pGL3-basic luciferase reporter vector (Promega, Madison, WI) by Kpn I and Xho I sites resulting in pGL3-α7. Nucleotides within the promoter -151 to -172 were then subjected to site mutagenesis using the Quikchange kit (Stratagene, Santa
Clara, CA) following manufacturer’s instructions, resulting in the pGL3-α7 vector. All DNA was verified by sequencing.

Transfections and Reporter Gene Assays

SHSY5Y were plated in a 96-well plate at a density of 10,000-15,000 cells per well. The following day, cells were transfected with pGL3-b, pGL3-positive control, pGL3-α7, and pGL3-M α7 vectors in triplicate using the FuGene HD kit for lipotransfection following manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). Firefly luciferase was normalized to the activity of renilla luciferase by co-transfecting pRL-SV40 vector. Cells were harvested at 24, 48, 72, and 96 hours for reporter gene assays. Cells were lysed and luciferase assayed using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). Cells in some experiments were treated with DMSO vehicle or 10 µM forskolin (4, 12, and 24 hours). In other experiments, cells were transfected with 1 ug total of pGL3-α7 control, pGL3-α7 control + shuttle DNA, pGL3-α7 + pCMV-CREB, pGL3- α7 + pCMV-KCREB. Dominant negative CREB vectors were purchased from Clontech (Mountain View, CA).

Chromatin Immunoprecipitation

SHSY5Y cells were incubated with 25 µM of forskolin (Sigma, St. Louis, MO) for 20 minutes. Cells were cross-linked for 10 minutes using 1% formaldehyde. Cross-linking was then stopped using glycine. Cells were manually harvested and pelleted. Cells were lysed using detergent and dounce homogenizing.
Nuclei were then collected and resuspended in shearing buffer then sonicated 10 times for 10 seconds on ice. Sheared chromatin samples were centrifuged at 14,000 rpm at 4°C. Chromatin samples were then pre-cleared for 2 hours at 4°C using 80 µl of 50% Protein-G sepharose (Pierce, Rockford, IL), 250 µl of sheared chromatin, 250 µl of immunoprecipitation buffer, 5 µl of protease inhibitor cocktail (Sigma), 1 µg/ul BSA (Fisher Sci), and 4 µg/ml poly dl-dc-poly-dl-dc (Sigma, St. Louis, MO). Pre-cleared samples were then incubated with polyclonal CREB-antibody, or anti-mouse antibody, overnight. Antibody complexes were then recovered by adding 100 µl of Protein G-sepharose beads and incubated 4 hours at 4°C with rotation. Bead complexes were then washed 4 times with immunoprecipitation wash buffer. Chromatin was then eluted using an SDS solution. The eluates were digested with 10 mg/ml RNAase and 0.3 M NaCl overnight to reverse crosslinks and digest RNA. Samples were then digested with 0.1 mg/ml Proteinase K and cleaned using the Wizard SV kit according to manufacturer’s instructions. DNA was then amplified by PCR using α7 promoter primers, 5’AGAACGCAA GGGAGAGGTAGA 3’ sense, 5’GTTGAGTCCCCGAGCTGCAGCGA 3’antisense gel purified, and verification made by DNA sequencing. Subsequent experiments were conducted utilizing the ActiveMotif ChIP-it Express with Enzymatic Shearing following manufacturer’s instructions (Carlsbad, CA). PCR products were subjected to verification by DNA sequencing.
Western Blot

Western blot was performed with anti-CREB antibody (Rockland, Gilbertsville, PA). α7 oligonucleotides were used and incubated with SHSY5Y nuclear protein as in EMSA. Nuclear lysate was isolated by incubating 5 x10^6 cells with 2 ml of the following: 10 mM Hepes, 10 mM KCl, .1 mM EDTA, .0004% Igepal, antiproteolytics (DTT, Pepstatin, Leupeptin, PMSF) directly in 150 mm plate and subjected to manual harvest. After which the cells were disrupted by sonication and nuclei were pelleted by centrifugation for 3 minutes at 14,000 x g. Nuclei were then suspended in 20 mM Hepes, .4M NaCl, 1mM EDTA, antiproteolytics (DTT, Pepstatin A, Leupeptin, PMSF) vigorously mixed for 2 hours at 4°C, and centrifuged at 14,000 x g for 5 minutes to recover nuclear proteins. Protein/DNA oligo complexes were separated on a 4% polyacrylamide gel. Proteins were transferred to nitrocellulose and nonspecific sites blocked using 5% milk. The blot was incubated with 1/1000 dilution of p43 anti-CREB (Rockland, Gilbertsville, PA) and then with 1/20,000 anti-rabbit. The blot was washed three times with TBS-T .05% for 10 minutes and developed with Pico Supersignal (Pierce, Rockland, IL).

RNA Extraction and end point RT-PCR

RNA was extracted from SHSY5Y cells using the RNAqueous micro (Ambion, Austin, TX) according to manufacturer’s instructions. In some experiments the SHSY5Y cells were treated with DMSO mock, 10 µM forskolin for 4, 12, and 24 hours or transfected with pCMV-CREB, pCMV-KCREB and empty vector
harvested after 24 hours. RNA was reverse transcribed into single strand cDNA, and RT-PCR were carried out using the following primers specific for nAChRα7, sense 5’ CGCCACATTCCACACTAAC 3’, and antisense 5’ ACC TTT CAC TCC TCT TGC C 3’. Each reaction contained 20 pmol 5’, 3’ primer, 5 units Taq, 10µM DNTP’s, 10X reaction buffer, varying degrees of template DNA conducted at 95°C for 5 minutes, followed by 30 cycles of 94°C, 61°C, and 72°C. Primers for β-actin were used as a control under same conditions with a cycle number of 24, sense 5’ TCA CCC ACA CTG TGC CCA TCT ACG A 3’ and antisense 5’ CAG CGG AAC CGC TCA TTG CCA ATG G 3’.

Results

Ectopic CREB Protein does Stimulate Expression of nAChRα7

Messenger RNA was used to analyze the expression of the nAChRα7 receptor in SHSY5Y cells that were subjected to a raise in the level of ectopic CREB. The mRNA level of α7 was significantly higher in SHSY5Y cells that were transfected with pCMV-CREB vector that expresses wild-type CREB than were control levels. Cells transfected with the pCMV-KCREB were found to have lower levels of the receptor (Figure 12A, lane 4). Figure 12B shows forskolin treatment, which induces phosphorylated CREB, did not significantly affect the production of nAChRα7 transcripts. This study is focused on the validation of the half-CRE element conserved in the human nAChRα7 promoter.
Figure 12. Potential regulation of nAChRα7 expression by CREB. In panel A total RNA was extracted from untreated SHSY5Y cells (control), cells transfected with pGL3-b vector (mock), pCMV-CREB (wild type CREB), and pCMV-KCREB (CREB mutant) and expression of mRNA was analyzed by RT-PCR using primers found in materials and methods. β-actin was used as control for cDNA input, PCR was performed, and virtual gel analyzed using Agilent 2100 software. Panel B shows various treatments involving SHSY5Y cells, where total RNA was extracted from untreated cells, cells treated with 10 µM forskolin (FSK) for 12 hours. β-actin was used as control for cDNA input, PCR was performed, and virtual gel analyzed using Agilent 2100 software. Asterisk * denotes significance p<.05, assay conducted in triplicate.

**Binding of CREB to α7 Subunit of the Nicotinic Promoter**

The suspected CRE-element is not a concensus CRE binding sequence with the perfect palindrome TGACGTCA; instead, it takes on a half-element form which has been proven to be sufficient enough to bind CREB and initiate transcription (Table 6).
Table 6. Functional half-CRE elements. Genes found to exhibit CREB protein binding to the non-concensus DNA CRE half-element (CGTCA)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phox2A</td>
<td>Proneural transcription element</td>
<td>182</td>
</tr>
<tr>
<td>Oatp2</td>
<td>Anion transport protein</td>
<td>183</td>
</tr>
<tr>
<td>UCP1</td>
<td>Mitochondrial uncoupling protein</td>
<td>184</td>
</tr>
<tr>
<td>ppN/OFQ</td>
<td>Prepronociception gene</td>
<td>185</td>
</tr>
<tr>
<td>EF-2</td>
<td>Peptide chain elongation</td>
<td>186</td>
</tr>
</tbody>
</table>

Various nAChRα7 oligonucleotides were constructed, with mutations introduced to the crucial CG that is essential for CREB binding (Figure 13A). Using nuclear proteins extracted from SHSY5Y cells, the nAChRα7 oligonucleotides formed four different complexes in the EMSA. Introduction of mutations in the crucial CG regions were found to completely abolish one of the complexes (Figure 13B). Findings were replicated using recombinant CREB protein, where anti-CREB antibody validated a unique CREB interaction by creating a super shift in the banding pattern (Figure 13C). To indicate specificity, α7 subunit promoter (denoted in figure by α7) complexes were subjected to competition assays using 50-fold excess of unlabeled oligonucleotide with varying CRE sequences, with only the concensus CRE-element introduced into the α7 subunit promoter (cα7) found effective enough to compete for binding to the radioactive α7 CRE-element (Figure 13C). The flanking sequences are not solely responsible for the bound complexes because the crucial CG-mutated α7 subunit promoter (mα7) with intact α7 subunit CRE half element flanking regions were found to ineffectively compete for binding to the radiolabeled α7 promoter (Figure 13D). This indicates that the flanking regions could contribute to protein stability, but the crucial CG
nucleotides present in the CRE half-element are an important factor in the stabilization process of complex formation.

Oligonucleotide used in the following

cc 5’ agagattcgctGAGTCAagagctag 3’
α7 -184 5’acggggcggggcggggtCTGACAgtggagaggccgggggc3’ -138
ma7 -184 5’acggggcggggcggggtTAGTCAgtggagaggccgggggc3’ -138
cα7 -184 5’acggggcggggcggggtGAGTCAgtggagaggccgggggc3’ -138
sα7 -172 5’ gcgggggtGTCAtgtggaga 3’ -151

B

![Image of gel electrophoresis results showing CREB complex and unbound oligo for rCREBhu and SHSY5Y cells]
Figure 13. EMSA assays illustrating CREB binding to the CHRNA7 promoter. Lanes 1-4 in panel A are different CRE-like element oligonucleotides radiolabeled with P\textsuperscript{32}ATP incubated with rCREB\textsubscript{hu} protein separated on a 4% nondenaturing polyacrylamide gel. Lanes 5-8 are the CRE oligonucleotides incubated with SHSY5Y nuclear lysates. Lanes 1 and 5 are the consensus CRE element purchased from promega. Lanes 2 and 6 are the CHRNA7 promoter with the CRE half-element. Lanes 3 and 7 are the CHRNA7 promoter with a mutation in the crucial CG in the CRE element. Lanes 4 and 8 are the CHRNA7 promoter with a consensus CRE-element insert. (B) EMSA supershift assay using anti-CREB antibodies. Lane 1 is CHRNA7 radio-labeled oligonucleotide incubated with rCREB\textsubscript{hu} protein in the presence of anti-CREB antibody. Lane 2 is radiolabeled concensus CRE oligonucleotide (Promega) incubated with rCREB\textsubscript{hu} protein with anti-CREB antibody. Lane 3 is radiolabeled oligonucleotide incubated with rCREB\textsubscript{hu} protein, no antibody. Lane 4 is free P\textsuperscript{32} labeled CHRNA7 probe. Panel C is competition assays using radio-labeled CHRNA7 oligonucleotide complexes formed with rCREB\textsubscript{hu} protein in which 100-fold excess of indicated unlabeled probe was introduced to the reaction.

Specific CREB binding to the CRE half-site of the CHRNA7 promoter in vitro was explored through the use of SHSY5Y neuroblastoma cells that endogenously produce the α7 receptor. Nuclear lysate from SHSY5Y cells was incubated with the unlabeled oligonucleotides used in the previous gel shift assays shown above. Complexes formed between nuclear protein and α7 oligonucleotides were separated on a polyacrylamide gel and then subjected to Western blotting using anti-CREB antibodies. The consensus CRE element probe and the α7 probes were positively identified by anti-CREB antibodies in complexes formed with CREB, while the nonspecific (sp1) and mutated α7 probe
failed to be associated with CREB (Figure 14A). To further illustrate CREB specificity, oligonucleotides of the α7 CRE half-element, mutated α7 CRE-element, and a consensus CRE-element were labeled with biotin and used as bait in SHSY5Y cell lysate to probe for CREB binding as indicated in Figure 14B. Only the mutated α7 CRE element failed to be extracted using the biotin-avidin interaction. These findings were validated invivo where immunoprecipitation with anti-CREB antibody was used to extract chromatin fragments that had been chemically cross-linked to nuclear protein. Chromatin/DNA complexes were isolated directly using anti-CREB antibodies, upon which PCR amplification, using primers specific for the proximal promoter region of the α7, was able to positive identify CREB binding to the half-CRE element (Figure 14C).
Figure 14. The in vitro and in vivo identification of CREB protein bound to the CHRNA7 CRE half-element in the promoter region. Panel A) Protein/DNA complexes were separated on 4% acrylamide gel, then probed with CREB p43 antibody (Rockland, MD). Lane 1 is consensus CRE-element (Promega), Lane 2, α7, is CHRNA7 promoter CRE-like element, Lane 3, mα7, is CHRNA7 promoter CRE half-element with crucial CG mutation, Lane 4 is Sp1 oligonucleotide (Promega, Madison WI), Lane 5 is protein only without DNA. Panel B is the identification of CREB binding using oligonucleotides labeled with biotin, incubated with SHSY5Y nuclear lysate and captured using avidin coated magnetic beads (Invitrogen, Carlsbad, CA). Complexes were resolved on a 10% SDS polyacrylamide gel and probed using anti-CREB antibodies (Santa Cruz, CA). Lane 1 is CHRNA7 promoter, lane 2 is mutated CHRNA7, and lane 3 is CHRNA7 promoter with consensus CRE insert. Panel C is chromatin from SHSY5Y cells immunoprecipitated using anti-CREB antibodies. SHSY5Y cell protein was cross-linked to DNA using a 1% formaldehyde solution, DNA was extracted and subjected to PCR using primers for the CHRNA7 essential promoter. PCR amplification in lane 1 uses DNA isolated from crosslinked chromatin using CREB antibodies trial 1, lane 2 uses SHSY5Y cDNA, lane 3 is negative control mock immunoprecipitation, lane 4 is DNA isolated from crosslinked chromatin using CREB antibodies trial 2, lane 5 is DNA isolated from cross-linked chromatin using CREB antibodies trial 3, and lane 6 uses DNA from pGL3 with CHRNA7 promoter insert as positive control.
Functionality of CRE Half-element in the CHRNA7 Promoter

To explore the functionality of the CRE half-element present in the CHRNA7 promoter, a luciferase reporter gene assay was used. A 392 base pair fragment, denoted as the essential promoter, of the CHRNA7 promoter (-1 to -392) was inserted into the pGL3-b-Luc vector to produce pGL3-α7. This vector was transfected into SHSY5Y neuronal cells and luciferase activity was measured. This part of the promoter had previously been examined in this manner and found to exhibit transcriptional activity. To begin to explore a more specific relationship with CREB, mutations were introduced into the crucial CG base pairs of the suspected CRE half-element of the pGL3-α7 luciferase construct and pGL3-mα7 luciferase activity was compared in the two vectors. When transfected in SHSY5Y cells, the CG mutated promoter exhibited significantly lower expression levels than the wild-type α7 promoter (Figure 15).
Figure 15. Functionality of the CRE half-element is evaluated by luciferase reporter gene assays. This is the functional analysis of point mutations in the CHRNA7 promoter by a luciferase reporter gene assay. Neuronal cells, SHSY5Y, were transfected with different plasmid constructs to assess the function of the α7 promoter. Striped bars represent transfections with the luciferase vector pGL3 under the control of the α7 promoter. Dark bars represent transfections with the luciferase vector pGL3 under the control of a mutated α7 mutated promoter in which a crucial CG in the CRE-like region was mutated to a AT. Promoter activity was assessed by measuring firefly luciferase activity and expressing in percent control of renilla luciferase. The FugeneHD transfection reagent was used per 2 µg of pGL3-DNA per 15,000 cells, co-transfected with 0.2 µg of pRL-SV40. All units were expressed as percent control to the protein produced by the pRL-SV40 vector.

To look at the detailed relationship CREB plays in expression of receptor, the CHRNA7 constructs used in the luciferase reporter gene assays were co-transfected with either pCMV-CREB (expresses wild-type human CREB) or pCMV-KCREB (forms dominant-negative heterodimer with CREB). The data revealed that expression of the wild-type α7 promoter is upregulated substantially when in the presence of overexpressed wild-type CREB. In the presence of the
dominant repressor KCREB, which acts to form a dimmer with CREB that effectively blocks the ability to bind CRE elements, transcriptional activity was reduced in comparison to wild type CREB. These results show the α7 promoter is affected by CREB binding. However, the α7 promoter, where the crucial CG for CREB binding was mutated, behaved in the same manner (Figure 16). This indicates the α7 CRE half-element investigated here is not solely responsible for the increase in CREB controlled transcription of the α7 subunit.

Figure 16. Dominant-Negative CREB mutants affect α7 transcription. SHSY5Y neuronal cells were transfected with pGL3α7 (under wild type α7 promoter) or pGL3-ma7 (containing a crucial CG mutation to CRE element) vector. The striped bar in each time frame exhibits activity where promoter was co-transfected with wild-type CREB. The dark bar is luciferase activity when pGL3-α7 co-transfected with pCMV-KCREB. Promoter activity was assayed by measuring firefly luciferase activity. All bars represent the mean +/- SD assayed in triplicate.

Further investigation involved forskolin treatment SHSY5Y neuronal cells transfected with either pGL3α7 or pGL3-ma7. The data indicate that treatment
did not initiate in transcription of the wild-type alpha7 promoter; rather, it resulted in a down-regulation of the promoter after a 24 hour treatment (Figure 17). However, the mutant vector did not respond in the same manner to forskolin treatment. This reveals the crucial mutation to the CG in the CRE-like element does alter production of the promoter in response to PKA activated CREB. The firefly luciferase results clearly show that CREB is directly involved in the production of α7 subunit, but it may be an interaction of CREB heterodimers that function as a repressor at this particular CRE-element.

Figure 17. Invivo functional analysis of the α7 subunit promoter using luciferase reporter gene assay. The essential promoter -1 to -392 sufficient for transcription of the α7 subunit was inserted into the pGL3-b reporter plasmid as represented by light bars (pGL3-a7). The α7 promoter with a crucial CG mutation (pGL3-ma7) in the CRE half-element was inserted into the pGL3-b reporter plasmid shown by dark bars. The vectors were co-transfected with the reporter plasmid pRL-SV40 into SHSY5Y cells and subjected to DMSO vehicle or forskolin (FSK) treatment. Cells were harvested at 48 hours as described in materials and methods. Renilla luciferase produced from the pRL-SV40 vector was used to normalize firefly luciferase and expressed as Relative Firefly luciferase activity.
Discussion

To date, there are many missing elements concerning the mechanisms involved in the actual transcription of the gene. When the essential promoter region was identified by sequence excising experiments, very few elements were immediately denoted as potential regulatory modulators\(^{108}\). Previously, the rat \(\alpha_7\) promoter was characterized, reporting that the CRE-like element was not conserved in this species, even though the entire receptor exhibited high homology\(^{105}\). Further complicating the understanding of the transcriptional control of the gene is the existence of alternative splice variants referred to as CHRNA7 variants\(^{94,95}\). In an attempt to contribute to the clarity of transcription, this is the first report to our knowledge to characterize the CRE-like element in the human promoter. Consistent with earlier findings, our data support chip arrays citing CREB association with the promoter results in down-regulation of the receptor after forskolin treatment\(^{172,187}\). We have shown CREB binding to the promoter can be manifested in controlled conditions as well as in a native neuronal environment; yet, results suggest that receptor subunit gene expression is the result of interactions between proteins that may or may not always directly bind the \(\alpha_7\) CRE half-element. It would appear that secondary signaling events due to CREB activation results in the production of \(\alpha_7\) subunit, yet those same events ultimately result in restoring a balance of receptor.

Treatment with 10 uM forskolin for 24 hours did result in diminished gene production as indicated by diminished promoter activity as shown in luciferase reporter gene assays. This supports previous studies using forskolin treatment
that resulted in significantly lowering mRNA levels of the α7 receptor\textsuperscript{172,187}. The experimental evidence found here indicates the PKA pathway could be responsible for the loss in promoter activity resulting in diminished α7 subunit mRNA transcripts. This brings into question the affinity for the DNA binding site for members within the leucine zipper family that could share a high degree of homology for this particular sequence of nucleotides. This is a particularly large family of transcription factors consisting of CREB, ATFs, CREMs, and CBP. It should be noted the palindromic consensus CRE-element can not only bind CREB but CREB heterodimers or CREM proteins (cAMP-responsive element modulators)\textsuperscript{188}. Also, it has been demonstrated DNA binding heterodimers consisting of an activator and repressor can be responsible for repressing transcription due to the inability to efficiently bind with co-activators to form mediator complexes\textsuperscript{189}. The occupancy of the CRE-element by either homodimer or heterodimer elements acting as repressors could be dependent on the activating signals via the PKA pathway resulting in down-regulation of the gene. It has even been postulated that this dimerization and eventual binding to DNA can occur regardless of phosphorylation state of the CREB protein resulting in an inhibition of cAMP responsiveness that would corroborate our data concerning the α7 receptor\textsuperscript{190}.

Of particular interest from the CREB family is the inducible cyclic AMP early repressor (ICER). It is a splice variant from the CREM gene and is the only isoform known to function as an inducible repressor. ICER has the ability to form heterodimers with other members of the CREB/CREM family and inhibit gene
expression\textsuperscript{191,192}. In our studies up-regulation of wild type CREB does induce transcription of the gene via the promoter, but not at the CRE half element as shown by the mutated α7 promoter. However, levels of mRNA transcripts are diminished by forskolin treatment on the wild type α7 promoter, but levels are relatively unchanged in the α7 mutated promoter. Taken together, this illustrates that the CRE half-element investigated does not function as the activating site but as the repression site when bound to activated CREB. It has been shown in previous studies that ICER can be induced in particular by forskolin treatment to inhibit cAMP responsiveness. This indicates the alpha7 subunit promoter CRE half-element could be under the direct control of an early response repression control mechanism via the PKA pathway. Illustrated in Figure 18 are two potential CREB pathways resulting in very different transcriptional control outcomes.
Figure 18. Possible CREB pathways associated with α7 expression. A schematic flow chart representing the possible signaling pathways that CREB activation could be involved in self-regulated gene expression using interactions between CREB and CREM isomers.

Control mechanisms involving the CRE-element could contribute to the precise expression pattern exhibited by the gene. Receptor identification in human was limited to the central nervous system, although it has been recently identified in leukocytes, endothelial cells (ECs), bronchial epithelial cells, skin keratinocytes, and vascular smooth muscle. In particular, regions in the brain found to have high expression levels are the nucleus reticularis of the thalamus, the lateral and medial geniculate bodies, the basilar pontine nucleus, the horizontal limb of the diagonal band of Broca, the nucleus basalis of Meynert,
and the inferior olivary nucleus. Recently, functional differences are found to exist between leukocyte α7 receptors and CNS α7 receptors, indicating processing at some level is responsible for functional changes. The α7 receptors in the CNS are involved cell signaling through direct calcium permeability, while those in leukocytes function as secondary messengers signaling via co-expression with a T-cell receptor/CD3 protein. If functional differences are prevalent based on tissue specific expression patterns, the issue remains to find what control mechanisms are directly responsible for this. Further studies remain to not only further understand the overall transcriptional control of the promoter but to also determine if the CRE-half element characterized in this study participates in tissue specific expression.
CHAPTER 5
CONCLUDING REMARKS

The chemical and biological abnormalities found in schizophrenia are the intense focus of much current research associated with schizophrenia as well as in these studies. The molecular differences with regard to the α7 receptor observed in schizophrenia were of particular interest to this investigation. Diminished α7 receptor functioning is one of the few known deficient molecular mechanisms associated with a subset of patients in schizophrenia. This finding could be the result of an autoimmune response or dysfunctional regulatory mechanisms in a group of patients affected with the disorder. The results of the present research should help us to better understand the role of the α7 receptor as a potential contributing factor in the complex etiological mechanisms that contribute to the pathophysiology observed in schizophrenia.

Recombinant Expression of the α7 Nicotinic Receptor

In the first study, the recombinant production of the receptor was investigated. Regulation of the α7 gene undergoes extreme control in order to exhibit the complex pattern of expression associated with it. Resolving the essential elements involved in receptor production is essential to understanding the pathophysiology the receptor displays in schizophrenia as well as creating a source of the receptor for experimental studies. The α7 subunit is a four transmembrane region protein, with large extracellular and intracellular components when tethered together with four more subunits form a homomeric
ligand-gated ion channel. The receptor is hydrophobic, and this feature likely contributes to the difficulty in its isolation. It is possible the concentration of subunit directly affects receptor ion channel formation. Next, the post-translational processing is dominated by signaling and chaperoning proteins to properly transport the product to the membrane. Also, there are additional tethering proteins found in the membrane to anchor the receptor correctly that affect expression. Recently, proteins have been found to co-express with the nAChRα7 that may participate in specific cell signaling events, which further complicates recombinantly producing the receptor (see Chapter 2). The complex sequence of events required to form functional channels results in many areas that could be potentially deficient in current molecular recombinant methods. These factors severely hampered efforts to recombinantly express the receptor. Insect cells were initially used based on their ability to properly add post-translational modifications, initiate correct protein folding, exhibit high levels of protein expression, and survival in low-stringent conditions. Unfortunately, the complexity of nAChRα7 receptor expression could not be overcome in this system. Attempts to produce the entire recombinant receptor in high-yield insect cells using a viral vector were unsuccessful. This was not surprising, due to the lack of any literature successfully citing recombinant α7 receptor production in insect cells. Concurrent with insect cell expression, attempts to express the entire receptor in commercial systems were used. The rabbit reticulocyte expression system from Promega and the insect cell system from Qiagen were both employed in full-length α7 subunit expression without success. With the
formation of entire receptor not an option, the next suitable idea was to express pieces of the receptor. The first 200 amino acids make up the extracellular portion of the α7 subunit. This was the only portion of the subunit that was successfully expressed in the commercial systems but was not successfully produced in the insect system chosen (Figure 19). Further investigation by expression of distinct parts of the receptor in indicate the membrane region, along with the carboxylic terminal, cannot undergo the proper modifications needed for production in any system other than a mammalian system. However, the knowledge gained from these attempts will be instrumental in future studies to elucidate the potential biological mechanisms needed in completing the picture regarding nAChRα7 expression, noted in Chapter 2. But until the precise regulatory mechanisms controlling the α7 receptor expression are identified, mammalian cells and frog oocytes are the only options in recombinant expression of the functional α7 receptor.
Figure 19. Recombinant α7 protein. The α7 recombinant subunit is a four-transmembrane spanning protein. The arrow pointing to the ellipse is the only portion of the receptor that was recombinantly produced, the first 204 amino acids.

Autoimmunity in Schizophrenia

The second study investigated the presence of antibodies in the schizophrenic patient population that could be responsible for impaired neuronal functioning. The success of this study was contingent on the ability to produce recombinant α7 subunit in its full-length form with an identifying marker or tag that could be used in an ELISA method (see Chapter 3 methods). Only the extracellular portion was available based on the previous experiments. However, a commercially produced recombinant truncated version of the protein, with more amino acids of the subunit, became available during our efforts. Used in the previous experiments, as shown in Chapter 3, was a partial α7 subunit (318 amino acids) expressed in wheat germ extract. This was the best available source of the receptor, with a portion of the transmembrane region included in expression. The protein was produced in a wheat germ expression system. This
made it possible to adjust for anti-human reactivity in the wheat germ molecules. There was specific binding to indicate the subunit can be differentiated through bungarotoxin and antibody identification as an α7 subunit. The results of the experiments, where the truncated α7 subunit was used, indicated there may be an antibody specific for the α7 subunit, seen in at least of a subset of patients affected with schizophrenia (Figure 20).

![ELISA assay](image)

Figure 20. ELISA assay utilized to determine sera IgG levels against α7 receptor in Schizophrenics. ELISA assay determined differences in Sera IgG levels specific for the α7 receptor in schizophrenic patients and controls.

The presence of an antibody could contribute in many different ways to disease neuropathology. Initially, the antibody may be functioning as the body’s own antagonist. The functional α7 receptor is responsible for cell signaling involved in the propagation of action potentials, ultimately ending in modulation of neurotransmitter release. If an antibody were to bind to the receptor, the initial effects could impede ligand binding or ion movement through the channel, both events impairing α7 signaling events that would occur downstream. This would compromise neurotransmitter release or interactions. The mechanisms behind
α7 receptor controlled neurotransmission are poorly understood, so it would be hard to identify specific potential effects at this time. Myasthenias Gravis is an example of an autoimmune disorder acting in this manner. Self-produced antibodies block the acetylcholine receptors found in muscle inhibiting acetylcholine binding that results in muscle weakness, particularly of the facial muscles. Also, an antibody binding event may target the cell for destruction. The patient’s immune system could be inducing cellular degradation of the target tissue. An example of this is found in multiple sclerosis. Antibodies bind specifically myelin basic protein as well as other oligodendrocyte proteins. This event leads to the eventual loss of oligodendrocytes, resulting in demyelination of axons in the CNS. In schizophrenia, such an antibody event would explain the reduced brain volume noted in the disease. An antibody specific to the α7 receptor could result in a loss of a particular cell population. There are specific deficiencies noted in GABA functioning that indicates potential loss of hippocampal neurons noted in schizophrenia. The neurons are responsible for the release of the inhibitory neurotransmitter GABA. The α7 receptors role in GABA modulation is not well-known, but receptor activation by acetylcholine does participate directly in release of the neurotransmitter. Alternatively, an antibody could be acting as an allosteric modulator in which the α7 receptor was in a constant state of operation. The channel would allow more calcium signaling events to occur resulting in an over-activation of the cell. This would fit the glutamate excitotoxicity hypothesis in schizophrenia. This theory states in schizophrenic brains there is constant glutamate release that literally excites cells
to death, resulting in slow neurodegeneration (Stahl, 2nd edition). Lastly, an antibody specific for the $\alpha_7$ receptor could be present during prenatal or early childhood neuronal development. The antibody could be interfering with the delicate neuronal processes occurring at this time, resulting in the cognitive deficits noted later in the disease.

However, the validity of these findings will always be questioned until a completely pure source of the entire receptor can become available. The experimental data obtained only used 60% of the $\alpha_7$ subunit to probe human sera for antibody binding (Figure 21). Human $\alpha_7$ receptor from native cells is not a good source at this time. Extraction of the nAChR$\alpha_7$ from brain or neuronal cells with current methods leads to a very impure source of the receptor. This source of receptor leads to high amounts of suspected nonspecific interactions with human sera that cannot be controlled. To clarify, there is the same amount of antibody or more antibody binding in cells that do not endogenously produce the receptor than those containing $\alpha_7$ receptor (Figure 22). Nicotinic receptors of the nAChR$\alpha_3$ heteromeric type, tubulins, and other cytoskeletal proteins have an affinity for the compounds used to isolate and purify recombinant nAChR$\alpha_7$. Selective ligands that were used to initially characterize recombinant $\alpha_7$ receptors in early studies behave pharmacologically different in native systems$^{92}$. This questions the selectivity of the $\alpha_7$ receptor purified from brain tissue as a true source of the receptor, further casting doubt on identification of an antibody specific for the receptor. Also, the $\alpha_7$ receptor is no longer thought to be a homomeric channel. The $\alpha_7$ subunit could be forming ion channels with $\alpha_3$
subunits\textsuperscript{120}. The antibody identified in this particular study only explores binding to a portion of a recombinant subunit, not to a functional $\alpha_7$ homomeric nicotinic receptor. This antibody should be validated in future studies to a pure source of the $\alpha_7$ receptor.

Figure 21. Antibody specificity to recombinant $\alpha_7$ receptor amino acid sequence. The antibody binding is specific to only the truncated $\alpha_7$ subunit that was produced recombinantly in wheat germ extract and verified by Western blotting. The green boxes are the membrane spanning regions of the subunit. The truncated version did not possess the intracellular portion or the last transmembrane region. The subunit did not form functional receptor, as shown by the circles.

The results from the patient sera evaluation do suggest an increase in antibody specific for the truncated $\alpha_7$ subunit with age in the schizophrenic population. If a more suitable source of the $\alpha_7$ receptor became available, better
evaluations of environmental triggers, pharmacological treatments, symptom manifestation, and disease susceptibility could be evaluated through α7 antibody titer levels. The patho-physiology of nAChRα7 is implicated in many other diseases, such as Alzheimer’s disease and autism. It may be possible to use this assay in those diseases as well to probe for an antibody contribution in their disease physiology. Future studies are necessary to support the results obtained, but this work certainly opens the door for ideas to be revisited concerning an auto-immune basis for schizophrenia, which has generally been disregarded in recent years.

Figure 22. SHEP1 and SHEP1-pCEPa7 cells in α7 autoantibody evaluation. This is a sample sera dilution curve that represents the amount of nonspecific binding that occurred when human serum antibodies were incubated with membrane fractions from the epithelial cells, SHEP1, that were null α7 (light bars) or epithelial cells, SHEP1α7, that recombinantly produced α7 (blue bars).
Transcriptional Regulation of the α7 Receptor

There are many factors that affect proper expression of functional α7 receptors in human cells. Subunit concentration and specific molecular chaperone proteins were two of those factors mentioned previously. The expression of the α7 subunit is crucial in understanding receptor pathophysiology in schizophrenia. The precise mechanisms that result in a functional α7 receptor are poorly understood. This led to the final investigation that examines transcriptional regulation of the α7 receptor subunit. The study identified a functional transcriptional cyclic AMP response element (CRE) in the promoter of the α7 subunit gene that was previously uncharacterized. The CRE half element was shown to sufficiently bind the transcription factor in both in-vitro and in-vivo studies. Protein interactions with the CRE half-element directly affect transcription of the receptor that was shown through luciferase reporter gene assays. Overall, it was found the protein binding events with the α7 CRE-half element involves a complex interaction between multimeric CREB dimer proteins that could act in a negative feed-back regulatory manner in the transcription of the α7 subunit (Figure 23).
Figure 23. Potential interactions between CREB isomers and the nAChRα7 promoter. The interaction between cyclic AMP response element binding protein (CREB) and other proteins determine transcriptional events of the α7 gene. CREB dimers may result in activation of the α7 gene, while heterodimers formed with inducible cyclic AMP early repressor (ICER) would repress transcription.

The transcription of the α7 subunit is extremely important in functional receptor formation. Receptor subunit concentration may play a role in proper receptor expression. The transcription of different nicotinic receptor subunit genes involves a complex interaction of control elements that have yet to be identified. This transcription factor CREB and the possible association with repressor elements may be a distinct regulatory mechanism of the α7 subunit. The α7 receptor has been thought to induce phosphorylation of the CREB transcription factor upon ligand binding, although this has not been well-supported. It would then be plausible to consider that the effects of this event may induce a positive and negative regulatory mechanism, which is different than
previously thought. Initially, ectopic CREB expression allows the receptor to be expressed at functional levels; even upon up-regulation of wild type CREB there is an increase of alpha7 subunit gene expression (Chapter 4). But at a certain level CREB phosphorylation begins to participate as a repressor to effectively control the levels of α7 receptor after induction of the PKA pathway. This has been demonstrated in other neurotransmitter receptors, most notably in the GABRA1 receptor where an interaction between CREB homodimers and heterodimers control receptor subunit expression 194.

Any upset of this regulatory mechanism could result in inefficient subunit production and ultimate loss in functional receptor. Dr. Sherry Leonard and her associates have shown functional polymorphisms in the promoter region affect α7 receptor subunit transcription. These nucleotide changes have been implicated in the auditory gating deficit found in schizophrenia. Two of the functional SNPs are found on either side of the α7 subunit CRE half-element108. Future studies could evaluate the effect of the identified SNPs on proper CREB transcriptional complex formation. Also, it is important to establish the exact signaling mechanisms involved in α7 activation of CREB that results in α7 subunit gene repression through direct binding to the CRE half-element found in the promoter region. These studies could focus on the Calmodulin-CREB pathway and further define the precise relationship to nAChRα7 expression. Evaluation of the association of CREB with the α7 receptor indicates the need to explore the receptor’s role in secondary cell signaling events. This opens all
sorts of venues to explore concerning the exact role the receptor occupies in neuronal as well as peripheral signaling.

In summary, the findings here indicate the α7 nicotinic receptor does play an important role in the pathophysiology of schizophrenia. These studies support the general notion that the improper functioning of the nAChRα7 receptor, whether by aberrant transcription or dysfunctional physiology by auto-antibody binding, could contribute the neurophysiology of the disease, at least in a percentage of patients. The advent of better molecular tools by which to study the physiology of the nAChRα7 receptor and the release of more information regarding the specific function of the receptor will not only further our understanding of the precise mechanisms the receptor occupies in schizophrenia but hopefully lead to the development of a better prognosis overall.


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