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CHARACTERIZATION OF A RAT NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR α7 SUBUNIT GENE PROMOTER AND ANALYSIS OF ITS CELL TYPE SPECIFIC EXPRESSION

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By
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*****

The Ohio State University
2002

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ABSTRACT

Nicotinic Acetylcholine Receptors (nAChRs) belong to a family of ligand gated ion channels that include GABA<sub>A</sub>, glycine, 5-HT3, and NMDA type glutamate receptors. The nAChRs are widely distributed in a vast majority of organisms from nematode to humans. They are cation channels and are classified as muscle and neuronal based on their location at the neuromuscular junction and in nervous tissue respectively. The primary function of nAChRs is to modulate synaptic transmission.

A vast variety of receptors, formed by the assembly of different combinations of receptor subunit α and β proteins, fall under the umbrella of neuronal nAChRs. Each receptor is made up of five subunits and based on the type of subunits that come together to form the receptor, will exhibit subtle to vast differences in receptor properties.

One neuronal nAChR subtype is competitively inhibited by a snake venom toxin α-bungarotoxin (α-
This subtype is widely distributed in the central and autonomic nervous system and is shown to be composed uniformly of α7 subunits. Since this α7 nAChR has been implicated in several important functions, such as, synaptic transmission, pre-synaptic control of neurotransmitter release, cell growth, neurite outgrowth, apoptosis, and neuronal development, an understanding of the regulation of the precise and timely expression of this subunit is highly warranted.

This document describes the identification and characterization of a promoter for the rat neuronal nicotinic acetylcholine receptor α7 subunit gene. A 178bp fragment from the 5' flanking region to the α7 gene was sufficient to drive expression of a reporter construct in PC12 cells and was termed as the minimal promoter. Within this minimal promoter, two sequences were identified that were shown to be important in regulating basal expression of the gene. One sequence was a GC rich region at -172 to the translational start site, which when mutated increased the expression of the reporter construct several fold. This indicated the presence of a negative regulatory element at this position that was shown to bind as yet unidentified nuclear proteins. The other sequence, an E-box, at -116
relative to the translational start site, was shown to bind
transcription factor USF-1 and positively regulated gene
expression. A GC-rich site directly adjacent to the E-box
was shown to bind Egr-1, Sp1, and Sp3. Thus several
transcription factors bind in close proximity to regulate
expression of the α7 gene indicating an interactive role
for each in the control of expression.

The 178bp minimal promoter was neuronal cell type
specific as it expressed in neuronal cells like PC12,
Neuro2A, and SN17 and not in non-neuronal cell types such
as L6 and HEK. During the study of the sequences
responsible for the cell specificity of the promoter, a
139bp sequence directly downstream to the 178bp promoter
was identified as having no promoter activity on its own in
neuronal cells, but when included downstream with the
minimal promoter, served to increase the strength of the
minimal promoter. This 139bp element increased the promoter
strength of any construct that showed promoter activity
and, interestingly, caused the loss of cell specific
expression behavior of the minimal promoter as the
combination of the two led to the expression of the
reporter construct in non-neuronal cells as well.
Dedicated to my parents and friends
ACKNOWLEDGMENTS

I would like to thank my adviser, Dr. R. Thomas Boyd for making Graduate School a most wonderful experience. His guidance, patience and most of all his personality has benefited and impacted me immensely.

I would like to acknowledge the support of my colleagues Usha Nagavarapu, Susan McKay, and Jeffrey Zirger.

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<tr>
<td>Ach</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>nAChR</td>
<td>nicotinic acetylcholine receptor</td>
</tr>
<tr>
<td>α-BgT</td>
<td>α-Bungarotoxin</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>Kb</td>
<td>kilo base pair</td>
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Chapter 1

Introduction

Overview

Acetylcholine (ACh), which mediates cholinergic transmission, was the first identified neurotransmitter. Since then it was demonstrated that receptors for cholinergic transmission could be divided into two groups based on the pharmacological sensitivity to muscarine (muscarinic receptors) or nicotine (nicotinic Receptors). Nicotinic acetylcholine receptors (nAChRs), best studied at the vertebrate neuromuscular junction and the fish electric organ, were the first ion-channels to be purified and cloned (Hille, 1992) and have been for a long time, a model for ligand-gated ion channels.
nAChRs belong to a super family of ligand-gated ion channels that includes the GABA<sub>A</sub>, 5-HT<sub>3</sub>, glycine, and NMDA glutamate receptors. The members of this family share several structural features. The nAChRs can be classified as muscle or neuronal depending upon their position at either the neuromuscular junction (muscle) or in the nervous system (neuronal). The receptors are cation specific, are activated by acetylcholine (ACh) and nicotine, mediate neurotransmission, and are widely distributed in organisms from nematode to humans (Gerschenfield, 1973; Darlison et al., 1993; Fleming et al., 1993; Leech and Sattelle, 1993). The neuronal nAChRs are present in the central and autonomic nervous system, adrenal medulla, PC12 cells and chromaffin cells. They are also situated in cholinergic, dopaminergic and glutaminergic areas of the brain (Ochoa et al., 1994). Neuronal nAChRs have important functions in neurotransmitter release, cognition, memory, muscle contraction, development and have been implicated in several diseases such as Alzheimer’s, Parkinson’s, tobacco abuse, and Tourette’s syndrome.
Muscle nicotinic receptors

The muscle nAChRs are located on the post-synaptic side of the vertebrate neuromuscular junction. Here they mediate muscle contraction in response to ACh released by the motor neuron in the synaptic cleft. The receptors in the electric organ of the electric ray *Torpedo californica* are extensively characterized. The electric organ is a rich source for nAChRs as it is made up of muscle cells that have lost their contractibility and have several nerve innervations per synapse as opposed to just one in regular muscle cells (Changeux, 1990). Electron crystallographic studies of *Torpedo* electric organ nAChRs reveal a pentagonal array of subunits organized like barrel staves around a central channel (Unwin 1993, 1995). The subunits are termed as α1, β1, γ/ε, and δ and are organized in a stoichiometry of \((\alpha_1)_2\beta_1\gamma_\delta\) in the adult form and of \((\alpha_1)_2\beta_1\gamma_\delta\) in the embryonic form. The whole receptor is about 290 kD with the average size of the subunits being around 50-60 kD (Stroud et al., 1990).
Each subunit has a large N-terminal extracellular domain of about 220 amino acids that has sequences, which determine the proper assembly of the subunits. The extracellular domain is followed by three closely spaced, highly conserved putative transmembrane regions (TM1-TM3) making up the next 90 amino acids (Figure 1). There exists a large cytoplasmic domain between TM3 and TM4 that varies the most in sequence between different subunits and between different species for the same subunit, is about 120-140 amino acids, and has consensus sites for phosphorylation. Phosphorylation of these sites leads to increased rate of desensitization of the receptors (Huganir and Greengard, 1990). Finally the C-terminus is made up of a fourth putative transmembrane domain (TM4) of about 20 amino acids and a short 10-20 amino acid extracellular domain.

The α1 subunit is called the ligand-binding subunit although the ligand-binding pocket is formed at the interface of the γ subunit with one α1 subunit, and the δ subunit with the other α1 subunit (Figure 2). Thus two molecules of the agonist are required to activate this channel. The two agonist binding sites differ in binding
properties due to the difference of the subunits that combine to form them (Prince and Sine, 1998).

The α1 subunit has a characteristic disulfide bond between two vicinal cysteines located at position 192 and 193 from the N-terminal end that is lacking in the other subunits. This region is part of the ligand-binding site (Kao et al., 1984; Stroud et al., 1990). The extracellular region of the α1 subunits is the main immunogenic region with pathological significance in myasthenia gravis (MG) (Tzartos et al., 1991).

The central cation channel is made by the assembly of the TM2 domains of each of the five subunits making up the receptor. (Claudio et al., 1983; Noda et al., 1983; Stroud et al., 1990; Changeux et al., 1992; Lester, 1992; Unwin, 1993). In the absence of the agonist the TM2 domains assemble to form the gate of the channel near the center of the plasma membrane (Unwin 1989, 1993). The amino acids lining the pore determine the ion selectivity for the receptor. In nAChRs the pore is lined with amino acids that allow the gating of cations. By changing only three channel-lining amino acids to those characteristic of GABA_A and glycine receptors, the ion selectivity of the receptor switches from cationic to anionic (Galzi et
al., 1992). Negatively charged amino acids at the mouth of the pore also help to concentrate the cations at the surface (Imoto et al., 1988; Galzi et al., 1992).

Upon binding of the agonist to the receptor there occurs a conformational change in the receptor resulting in the opening of the channel and the inflow of cations such as sodium, potassium and calcium. The gate of the channel closes when the agonist is depleted from the synaptic cleft or, if it remains bound, the receptor undergoes a change in conformation to a desensitized state in which the channel is closed (Stroud et al., 1990; Unwin, 1993; Karlin and Akabas, 1995). The muscle nAChRs are competitively inhibited by α-bungarotoxin (α-BgT), a snake venom toxin resulting in muscle paralysis (Dunn et al., 1993).

Disorders linked to the muscle nAChRs include myasthenia gravis in which autoimmune antibodies are developed against the α1 subunit. The antibody cross-links the receptors on the post-synaptic side resulting in internalization and degradation of the receptors. This results in reduced number of active receptors at the neuromuscular junction.
Neuronal nicotinic acetylcholine receptors

The neuronal nicotinic receptors differ from their muscle counterparts in their variety. Neuronal nicotinic receptors are not a single entity, but rather there are many different subtypes constructed from a variety of nicotinic subunit combinations. This structural diversity and the presynaptic, axonal, and postsynaptic locations of nicotinic receptors contribute to the varied roles these receptors play in the central nervous system. The different subtypes of the neuronal receptors produced by the combination of different subunits can differ in affinities to agonists, drugs and toxins along with differences in cation permeability. Nicotinic receptors are distributed to influence many neurotransmitter systems at more than one location, and the broad yet sparse cholinergic innervation throughout the central nervous system ensures that nAChRs are important modulators of neuronal excitability (Dani, JA, 2001).
Figure 1. View of the nicotinic acetylcholine receptor subunit.

Schematic representation as the subunit appears in the membrane. Each subunit has 4 transmembrane regions termed TM1-TM4. A large cytoplasmic loop separates TM3 and TM4. The N-terminal extracellular loop is large and forms the ligand binding pocket. All subunits have a disulfide bond between cysteines placed about 15 amino acids apart corresponding to position 128 and 142 of the muscle α1 subunit. The α subunits have a disulfide link between two adjacent cysteines that form part of the ligand binding pockets. All non-α subunits lack the vicinal cysteine residues. The TM2 region of each subunit forms the lining of the ion channel and the amino acids in TM2 are responsible for regulating the type of ions that can flow through the channel.
Figure 1

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Figure 2. Putative subunit arrangements around the central cation channel of some major nAChR subtypes.

ACh binding sites are depicted at the appropriate interfaces between subunits.
Figure 2

Muscle AChR

α4β2 neuronal AChR

α3βα5 Neuronal AChR

α7, α8, α9 AChR
Structural features of the nAChRs such as the pentameric organization, four transmembrane orientation of each subunit, formation of agonist binding sites between interfaces of subunits are conserved in the neuronal type. However neuronal nAChRs have tremendous variety as there exist several subtypes of such receptors as opposed to two (embryonic and adult) of the muscle nAChRs. Each subtype is formed by different combination of subunits resulting in receptors with subtle to marked differences in pharmacology, kinetics and receptor properties. Neuronal nAChRs gate more Ca\textsuperscript{2+} than their muscle counterparts exerting influences on certain cellular processes (Bertrand et al., 1993, Rogers and Dani, 1995).

**Subunits of neuronal nAChRs**

Neuronal nAChRs are made up of different $\alpha$ (agonist-binding) and $\beta$ (structural) subunits. To date nine $\alpha$ subunits ($\alpha_2-\alpha_{10}$) and three $\beta$ subunits ($\beta_2-\beta_4$) have been
identified (Lindstrom et al., 1987; Goldman et al., 1987; Wada et al., 1988; Nef et al., 1988; Boulter et al., 1986, 1987, 1990; Schoepfer et al., 1988, 1990; Couturier et al., 1990a; Luetje et al., 1990; Seguela et al., 1993; Elgoyhen et al., 1994; Deneris et al., 1988, 1989; Duvoisin et al., 1989; Sgard F et al, 2002). Each of these subunit proteins are encoded by different genes. The nAChR subunits are believed to have evolved from a primordial homopentameric receptor through a series of gene duplications (LeNovère et al., 1995). The α subunits possess a pair of extracellular vicinal cysteines characteristic of the muscle α1 subunit. The β subunits do not have the vicinal cysteine motif. Oocyte expression studies have shown that the β and the α2-α6 subunits do not form functional receptors when expressed on their own. The α7-α9 subunits however can form receptors when expressed alone indicating that they form homopentameric receptors. All pairwise combinations of α2, α3, α4, and α6 with β2 and β4 yield functional nAChRs. α5 and β3 are similar in that they have to be combined with another α and β subunit to form receptors. Thus there are three types of neuronal nAChR subunits: those that assemble
into homomeric structures (α7-α9), those that need to be heterodimeric, and those that are parts of heterotrimeric structures (α5, β3) (Figure 2).

The neuronal nAChR subunit genes are about 50% homologous to muscle nAChR genes within the same species. The α2, α3, α4, α5, and α6 subunit genes are about 40-60% similar to each other (LeNovere, 1995). The α7, and α8 subunits are related to each other but are fairly different from other neuronal α subunits (Sargent, 1993). The amino acid sequences of the β subunits differ from each other and from that of the α subunits, with the exception of α5 and β3, which are quite similar to each other (Figure 3. A).

**Diverse properties of the neuronal nAChR subtypes**

Various neuronal nAChR can be assembled by the combination of different α and β subunits. Not all of the possible combinations of subunits form functional receptors. However a large number of native nAChRs exist in the nervous system as a result of the wide variety of subunits from which the cell can select to form receptors. As shown in Figure 3B, through expression of
different combinations of subunits in oocytes, the different receptors can vary subtly or remarkable in ligand binding selectivity, antagonists, channel open time, ion selectivity, permeability and desensitization rates. The $\alpha_7$ receptor has a very short open time indicating fast desensitization as compared to other receptors. Also the permeability of Ca$^{+2}$ through $\alpha_7$ receptors is about 12 times that through other nAChR receptors. Since too much Ca$^{+2}$ is toxic to the cells, it goes to explain the reason for quick desensitization of the $\alpha_7$ receptors.

This table also describes the affinity of various receptors to ACh and nicotine. The receptor with the highest affinity for nicotine is the $\alpha_4\beta_2$ receptor. The antagonist $\alpha$-bungarotoxin ($\alpha$-Bgt) is selective for $\alpha_7$ receptors.

**Distribution of neuronal nAChRs**

Neuronal nAChRs are widely distributed in the central and peripheral nervous system. $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_7$, $\beta_2$ and $\beta_4$ mRNAs have been identified in rat sympathetic and chick ciliary ganglia (Mandelzys et al., 1994; Zoli et
In trigeminal ganglia, a type of sensory ganglia, high expression of α3 and β4 subunits is seen indicating that these subunits form a common type of receptor (Flores et al., 1996). In addition to these subunits expression of the α4, α6, α7, and β2 subunits is also seen.

In rat, high affinity nAChR sites revealed by [3H](-)
nicotine, are abundant in selective areas of the cerebral cortex (predominantly layers III & IV), thalamus, interpeduncular nucleus and the superior colliculus, but are of low to moderate abundance in the hippocampus and hypothalamus.

The second class of sites, labeled by [125I] α-BgT, are enriched in the hippocampus, hypothalamus and layers I and VI of the cerebral cortex (Alkondon and Albuquerque, 1993) and are thought to be made up of predominantly α7 homopentameric receptors.
Figure 3. nAChR subunit cladogram and receptor subtype properties

A) A depiction of the evolutionary tree for the different neuronal nicotinic acetylcholine receptor subunits. An important point to notice is that the α7 and α8 subunits were the earliest to be evolved and that all other subunits were derived from it by a series of processes such as gene duplications.

B) A comparison of the diverse properties of several nicotinic acetylcholine receptor subtypes. From "Neuronal Nicotinic Receptors: Pharmacology and Therapeutics Opportunities". Edited by Stephen P. Arneric and Jorge D. Brioni.
Figure 3
In situ hybridization assays have demonstrated that the α4 subunit mRNA is expressed strongly in a number of areas including the thalamus, deeper layers of the cerebral cortex, ventral tegmental area (VTA), the medial habenula and substantia nigra (SN) pars compacta (Goldman et al., 1987; Wada et al., 1989; Dinley-Miller and Patrick, 1992; Zoli et al., 1995) and that α3 is expressed strongly in the locus coeruleus (LC), habenula, and interpeduncular nucleus. These subunits are also expressed in peripheral tissue, with being expressed in autonomic and sensory ganglia (Boyd et al., 1988; 1991; Rogers et al., 1992; Goldman et al., 1986; Wada et al., 1989; Morris et al., 1990).

The α2 subunit has a much more restricted pattern of expression, with mRNA being expressed at high levels only in parts of the interpeduncular nucleus (Wada et al., 1989; Daubas et al., 1990).

α7 message is particularly high in the hippocampal formation, interpeduncular nucleus, amygdala and cerebellum, which correlates well with the high level of α-BgT binding this region (Seguela et al., 1993;
Dominguez del Taro et al., 1994; Broide et al., 1995, 1996).

α8 has not been found in rat and the distribution of α9 appears restricted to skin, tongue, cochlea and sensory tissue (Hiel H et al., 2000). The presence of α9 subunits in nAChRs in cholinergically innervated outer hair cells of the cochlea may explain the unusual cholinergic pharmacology found in this tissue (Elgoyhen et al., 1994).

Among the β subunits, β2 is widely expressed, whereas expression of β4 and β3 is more variable. β4 message is highly expressed in a number of areas in which substantial α3 message is found, including the habenula, area postrema, and LC (Duvoisin et al., 1989; Flores et al., 1992; Dinley-Miller and Patrick, 1992). One notable exception to this pattern is that substantial expression of α3 message is found in DA neurons in the SN, whereas β4 message is absent (Klink et al., 2001).

β3 message appears to be prominent in brainstem catecholaminergic areas such as SN and LC (Roztocil et al., 1998). Similarities in the distributions of α6 and β3 message suggest that these two subunits might be
important in the nAChR-mediated effects on catecholamine release (Roztocil et al., 1998), although the lack of pharmacological tools selective for nAChR subtypes containing these subunits has restricted investigation of the pharmacology of this effect.

Basic Function of nicotinic receptors

Upon binding ACh, the nAChR ion channel is stabilized in the open conformation for several milliseconds. Then the open pore of the receptor/channel closes to a resting state or closes to a desensitized state that is unresponsive to ACh or other agonists for many milliseconds or more. While open, nAChRs conduct cations, which can cause a local depolarization of the membrane and produce an intracellular ionic signal. Although sodium and potassium carry most of the nAChR current, calcium can also make a significant contribution (Castro and Albuquerque 1995; Decker and Dani 1990; Dani and Mayer 1995; Seguela et al 1993; Vernino et al 1992, 1994). Calcium entry through nAChRs can be biologically important and is different from calcium influx mediated by voltage-gated calcium channels or by the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors. Both
voltage-gated calcium channels and NMDA receptors require membrane depolarization to pass current freely. At hyperpolarized (negative) potentials, voltage-gated calcium channels generally do not open and NMDA receptors are blocked by extracellular magnesium ions. Nicotinic nAChRs do open and pass current freely at negative potentials that provide a strong voltage force driving cations into the cell. Thus, calcium currents mediated by nAChRs can have different voltage dependence than is seen for other calcium-permeable ion channels. Also, incoming calcium has a different spatial distribution that depends on the location of nAChRs on the cell surface.

Ligand-binding studies indicate that two major types of functional nAChRs in the brain are formed by the heteromeric assembly of \( \alpha 4\beta 2 \) subunits (the high-affinity nicotine-binding sites), and the homomeric assembly of \( \alpha 7 \) subunits (the majority of the \( \alpha \)-bungarotoxin binding sites). More recent evidence indicates that other subunits can co-assemble in vivo. For physiological studies, the selectivity of pharmacological agents has largely provided the basis for the characterization of nAChR subtypes, although recent studies have begun to use single-cell RT-PCR and gene knockouts.
Nicotinic mechanisms in the CNS

The most widely seen synaptic function of nAChRs in the CNS is to mediate fast synaptic transmission and to modulate the release of practically every known neurotransmitter including glutamate and GABA (Albuquerque et al 1997; Alkondon et al 1997; Gray et al 1996; Guo et al 1998; Jones et al 1999; Li et al 1998; McGehee et al 1995; McGehee and Role 1995; Radcliffe and Dani 1998; Radcliffe et al 1999; Role and Berg, 1996; Wonnacott, 1997). Exogenous application of nicotinic agonists can increase and antagonists can decrease the release of ACh, dopamine, norepinephrine, GABA, glutamate and serotonin.

Nicotinic receptors also have roles in synaptic plasticity and neuronal development (Broide and Leslie, 1999; Role and Berg, 1996).

Nicotinic cholinergic systems in the brain have been implicated in several aspects of some important mental diseases such as Alzheimer's disease (AD), attention-
deficit/hyperactivity disorder (ADHD), Tourette’s syndrome, and schizophrenia. Both clinical and animal studies have indicated a role for central nicotinic systems in cognition, learning and memory (Samuels and Davis, 1998; Wonnacott, 1997).

**Role of α7 nAChRs**

The α7 nAChR subunit mRNA is expressed in different regions of the brain during development in a transient manner. This suggests rigorous control of its transcription during various stages of embryonic and post-natal development. Studies have been performed to determine the timing and level of α7 expression at different periods of development in rat, mouse, chick and humans. The expression of the α7 receptor corresponds to the expression of α7 binding sites in the brain indicating that these toxin-binding proteins are made up of the α7 subunit proteins. The α7 sensitive nAChRs have been implicated in several biological activities that include:
A) In rat thalamocortical development: Distribution of α7 mRNA expression was seen in the developing rat sensory cortex and thalamus as early as embryonic day 13 (E13). The α7 mRNA was markedly increased during the late pre-natal period, which persisted till the first post-natal week, followed by a decline in adulthood. This transient and distinct pattern of distribution of the α7 neuronal nicotinic receptor coincides with the major phase of thalamocortical development, and implicates that it may play a major role in the development of cortical circuitry and its plasticity. (Broide, R.S. et al., 1995).

B) Neurite retraction: Activation of α7 nAChRs in chick ciliary ganglia can cause neurite retraction. This is likely to result due to the subsequent Ca²⁺ influx through these receptors resulting in membrane depolarization leading to opening of voltage gated Ca²⁺ channels. The resulting increase in intracellular Ca²⁺ causes neurite retraction. This process of neurite retraction can be blocked with α7 nAChR antagonists like α-Bgt and MLA. Thus the α7 nAChRs may be involved in remodeling of the neurite morphology in the ganglion (Pugh, P.C. and Berg, D.K., 1994).
C) **Antinociception:** In mice dose-dependent antinociceptive effects were seen with the α7 agonist choline after spinal and supraspinal injection using the tail-flick test. Furthermore, α7 antagonists MLA and α-BgT significantly blocked the effects of choline. (Damaj MI et al., 2000).

D) **Mediating cell growth:** Nicotine increases cell number in small cell lung carcinoma and this effect is mediated through an interaction at the nicotinic α-bungarotoxin sensitive (α7) receptor population. These results suggest that the α-bungarotoxin site may be involved in modulating proliferative responses in neuroendocrine derived SCLC cells (Quik, M et al., 1994).

E) **Cell death:** α7-AChR activation by low doses of nicotine results in apoptotic cell death of both primary and immortalized hippocampal progenitor cells. The activation of α7-AChRs by nicotine results in the induction of the tumor suppressor protein p53 and the cdk inhibitor p21. The cytotoxic effect of nicotine is dependent on extracellular calcium levels and is probably attributable to the poor ability of undifferentiated progenitors to buffer calcium loads (Berger, F. et al., 1998).
F) Pre-synaptic control of neurotransmitter release: Nanomolar concentrations of nicotine enhanced both glutamatergic, serotonergic, GABAergic, and cholinergic synaptic transmission by activation of presynaptic nAChRs that increased presynaptic intracellular Ca\(^2\) concentration through α7 nAChRs (McGehee, D.S. et al., 1995).
CHAPTER 2

METHODS

Northern Analysis

RNA was purified from PC12, L6, HEK, COS-1, Neuro2A and SN17 cells using Trizol (Invitrogen, Inc.) or Gentra Pure Script (Gentra Systems). Northern blot analysis was performed using 1% (w/v) agarose gels containing 7.4% (v/v) formaldehyde in 20 mM MOPS, 1 mM EDTA, and 5 mM sodium acetate at pH 7.0. 10 μg of RNA was run on each lane of the gel. After electrophoresis, the RNA was transferred to GeneScreen Plus (NEN) in 10 x SSC according to the manufacturer's instructions. Rat α3, α4, β2, β4, and α7 cDNAs were
labeled with [α-32P] dCTP using the Prime-It RmT random primer labeling kit (Stratagene). The 32P-labeled probe was hybridized to the GeneScreen Plus membrane in 5 × SSPE, 50% deionized formamide, 5 × Denhardt’s solution, 1% SDS, 10% dextran sulfate, and 100 µg/ml salmon sperm DNA at 42 °C. The filters were washed in 2 × SSPE at room temperature, 2 × SSPE, 2% SDS at 65 °C and in 0.1 × SSPE for 45 min, 0.1% SDS at room temperature for 15 min. The blots were exposed to x-ray (Kodak XAR-5) film at −70 °C with an intensifying screen. A Molecular Dynamics PhosphorImager was also used to quantify the signal intensities in each lane.

**Cell Culture:**

Rat pheochromocytoma cells (PC12), COS-1 cells and L6 myoblasts were cultured in RPMI 1640 supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum (all from Invitrogen, Inc.). The medium also included 100-units/ml penicillin/streptomycin (Invitrogen, Inc.). Cells were maintained in a humidified incubator at 37 °C with a 5% CO2 and 95% air atmosphere. The PC12 cells were a subclone designated N21 and were a gift from Dr.
Richard Burry, Department of Neuroscience, The Ohio State University College of Medicine. The L6 myoblasts were a gift from Dr. Tony Young, Ohio State University Neurobiotechnology Center, The Ohio State University. Drosophila melanogaster Schneider cells (SL2 cells) were a gift from Dr. Mark Seeger, Ohio State University Neurobiotechnology Center. The SL2 cells were maintained in Schneider medium supplemented with 10% fetal calf serum (both from Invitrogen, Inc.) at 25 °C. Neuro2A and SN17 mouse neuroblastomas were a gift from Dr. Paul D. Gardner, Medical School, University of Massachusetts, Wooster, Massachusetts. Mouse Neuro2A cells were cultured in Minimum Essential Medium (Invitrogen) containing 10% fetal calf serum (Invitrogen), and 100-μg/ml streptomycin, 100 units/ml Penicillin at 37°C in humidified atmosphere with 5% CO₂. SN17 cells were grown in DMEM supplemented with 10% fetal calf serum (Invitrogen), and 100-μg/ml streptomycin, 100 units/ml Penicillin at 37°C in humidified atmosphere with 5% CO₂.
Transfection

PC12, L6, HEK, Neuro2A, and SN17 cells were transfected at 60% confluency in 60 mm dishes with 3 μg of each α7 promoter-luciferase construct DNA and 0.1 μg of pRL-TK vector DNA (where Renilla luciferase reporter gene expression is under the control of the Thymidine Kinase Promoter) (Promega) (internal control for transfection efficiency) using LipofectAMINE (Invitrogen, Inc.) with a ratio of 1:10 of DNA to LipofectAMINE in serum free RPMI 1640 medium. All transfections were done in duplicate dishes with several experiments for each group of hybrid constructs. The day following transfection the medium was replaced with serum containing RPMI 1640 medium. 2 days after transfection, extracts were harvested and luciferase activity measured using the Promega Dual Luciferase Assay Kit, which allows for analyzing both Firefly and Renilla luciferase in the same tube. Firefly luciferase activities were normalized to the Renilla luciferase activities present in the extracts.
Expression Of Sp1 and Sp3 in Insect Cells:

The expression plasmids pPACSp1 and pPACuSP3 (kindly provided by Dr. Guntram Suske, Klinikum Der Philipps-Universitat At Marburg) were transfected into SL2 cells as follows. 1 day prior to transfection, the cells were plated onto 60-mm tissue culture dishes at a density of $4 \times 10^6$ cells/plate. Cells were transfected with 4 μg of plasmid DNA using LipofectAMINE in Schneider medium without serum. 12 h after the addition of DNA, the cells were placed in Schneider medium supplemented with 10% fetal calf serum at 25 °C. 24 h later the cells were washed twice with phosphate-buffered saline, and nuclear extracts were isolated. Briefly, the cells were washed twice in phosphate-buffered saline, pelleted, and resuspended in 400 μl of cold buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, and 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT and 0.5 mM phenylmethysulfonyl fluoride. Cells were lysed by adding 25 μl of 10% Nonidet P-40, and the crude nuclei were pelleted. The nuclei were resuspended in 100 μl of buffer containing 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 1 mM phenylmethysulfonyl fluoride. The tube was rocked
vigorously at 4 °C for 15 min. The nuclear extracts were obtained by centrifugation and stored at -70 °C.

**Electrophoretic Mobility Shift Assays and antibody supershifting:**

PC12 nuclear extracts were prepared as described above. The protein concentrations of the nuclear extracts were determined using the Bio-Rad protein assay reagent. Double stranded oligonucleotides were end labeled with [³²P] ATP using T4 kinase (U. S. Biochemical Corporation). For binding reactions 20,000-25,000 cpm of ³²P end-labeled oligonucleotide was incubated with 5-10 µg of PC12 or SL2 nuclear extract in a 10-µl volume containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 1 mM MgCl₂, 4% glycerol, and 0.5 µg of poly (dI-dC)·poly (dI-dC). For some experiments, the incubation buffer contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 10% glycerol, 5 µg of bovine serum albumin, and 2 µg of poly (dA-dT)·poly (dA-dT). For some of the Spl and Sp3 antibody supershifting experiments a buffer containing 20 mM HEPES (pH 7.9), 100 mM KCl, 2 mM DTT,
1 mM EDTA, 20% glycerol, 0.01% Nonidet P-40, and 0.5 μg/μl bovine serum albumin was used. For competition, unlabeled oligonucleotides were added at 100-fold molar excess to each reaction for 10 min prior to the addition of the labeled probe.

After incubation at 4 °C for 30 min, the complexes were separated on 4% polyacrylamide gels (80:1, acrylamide: bisacrylamide) at room temperature in 0.5 × Tris borate buffer. Gel supershift assays were performed in the same way except that before incubation of the probes with nuclear extracts, 2.0 μl of TransCruz™ gel supershift antibody (100 μg/ml) was added to the reaction mixture and incubated for 1 h on ice. The Spl, USF1, and USF2 supershifting antibodies were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). The gels were dried onto a Whatman 3MM paper, and were exposed to x-ray (Kodak XAR-5) film at -70 °C with an intensifying screen.

Some of the EMSA using an anti-Egr-1 antibody were performed using the Nushift Kit (Geneka) according to the manufacturer's instructions. Briefly, the nuclear extract

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was incubated with the binding buffer and stabilizing solution on ice for 20 minutes. The rabbit Egr-1 polyclonal antibody was included in supershifting reactions. Radioactively end-labeled probe was added and incubated further for another 20 min on ice. The reactions were loaded onto a 5% acrylamide: bisacrylamide gel (19:1) and electrophoresed in TGE buffer at 15 V/cm at 4 °C. The gels were exposed to x-ray film (Hyperfilm MP- Amersham) for 16-24 h.
Oligonucleotides Used for EMSA:

**USF consensus**  
5'-CACCCGGTCACGTGGCCTACACC-3'

**Egr consensus**  
5'-GGATCCAGCGGGGGCGAGCGGGGGCGA-3'

**Spl consensus**  
5'-ATTCGATCGGGGCGGGGCGAGC-3'

**A**  
5'-AGTTCCCGGGGGCGGCCACGG-3'

**B**  
5'-CTGTCACGTGTGGGGGCGCG-3'

**C**  
5'-CTGTCACGTGTGGGGGCGCGCCGCGC-3'

**C-m**  
5'-CTGTCACGTGTGGGGGCGCGCCGCGG-3'

**D**  
5'-TGTGGGGGCGGCGCCGCGGGCTGC-3'

Table 1
Point Mutations:

Point mutations were made in the 178-bp BstYI-SstII fragment using the QuikChange site-directed mutagenesis kit (Stratagene). Briefly, complementary mutagenic oligonucleotide primers (detailed below), containing the desired mutation were annealed to the 178-bp BstYI-SstII luciferase construct plasmid and polymerase chain reaction with Pfu Turbo DNA polymerase used to synthesize a new mutant plasmid. The sequence changes introduced are in bold and underlined. The E-box mutant plasmid was used with the mutant GC oligonucleotides to create the double mutant. The non-mutated parental plasmid DNAs were then digested with DpnI, and new mutant plasmid DNA transformed into XL-1 Blue supercompetent cells.
Oligonucleotides Used for Point Mutations:

**GC mutant**

5' -GGATGCAGTTCCCTAATCGGCCACGGAGCTGCACGGC-3'

5' -GCCGTGCAGCTCCGTGGCCGATTAGGGAACTGCATCC-3'

**E-box mutant**

5' -CGCCCCCACAATAGACAGCGCGCTCCCGTCTCG-3'

5' -CGAGACGGGAGCGCGCTGTCTATTGTGGGGCG-3'

**Egr-1 mutant**

5' -CTGTACGTGTGAAAGCGCGCCGCGC-3'

5' -GCCGCGGCCGCCTTTACACGTGACAG-3'

**Table 2**
Generation of Stable Bovine \( \alpha_3 \) nAChR Subunit Expressing Cos-1 Cell Line:

Cos-1 cells were cultured and transfected as described above with the pCDNA 3.1 vector (Invitrogen) expressing the bovine \( \alpha_3 \) subunit cDNA. The bovine \( \alpha_3 \) cDNA was provided by Dr. Manuel Criado, which was then cloned into the pCDNA 3.1 vector. Two days after transfection the medium was supplemented with 500 \( \mu \)g/ml of G418 (Invitrogen). The cells were cultured for 2-3 weeks with G418 during which time the desired cells would be split depending upon their confluence. Northern blot analysis was done to determine if the selected cells could express the \( \alpha_3 \) mRNA at the end of three weeks.
RT-PCR

Total RNA isolated from PC12, Neuro2A, SN17, L6 and HEK were individually reverse transcribed using the SUPERSCRIPT first strand synthesis system (Invitrogen) according to the manufacturers instructions, using the random primer method. The first strand cDNA thus obtained was further amplified by PCR using the PCR primers described below following instructions from the PROMEGA Readymix for PCR kit. The products were then run on a 1% agarose gel in TBE buffer.
Oligonucleotides used for RT-PCR

PRIMERS FOR β2

5’: atc ccc tgc gta ctc atc ac
3’: cac gct agt gac gat gga ga

PRIMERS FOR β4

5’: ttc cat cgt cac cac tgt gt
3’: cga tga agc tga cgc cct ct

PRIMERS FOR α3

5’: tgt ctc agc tgg tga agg tg
3’: atg gga agt agg tca cgt cg

PRIMERS FOR α4

5’: atc ctg aca tca cct acg cc
3’: gtg aag agc agg tac t cg cc

Table 3
Analysis of mRNA Half-Life

PC12 cells were grown to 60-70% confluency (usually a day after splitting), treated with 1 µg/ml actinomycin D (Fisher Scientific) in DMSO. A set of PC12 cells were also treated with 50 ng/ml NGF. Total RNA was isolated at various time points from both the NGF untreated and treated cells using the Gentra RNA isolation kit. The various RNA samples from different time points of both the sets of cells were then blotted and subjected to Northern Analysis. The blot was probed with a Random Primer generated $^{32}$P-labelled α7 probe. Similarly, it was later probed with a GAPDH probe to measure loading efficiency. The intensity of the signals produced was measured using a PhosphorImager and quantified. The readings were plotted as a ratio of the signal generated by the α7 probe to that generated by the GAPDH probe against the various time intervals tested. A best fit line was generated to fit the readings and the slope of this line was indicative of the rate of decay of the α7 mRNA.
CHAPTER 3

RESULTS AND DISCUSSION

Characterization of a Rat Neuronal Nicotinic Acetylcholine Receptor α7 Promoter

Background and Preliminary Data

The α7 subtype of neuronal nicotinic acetylcholine receptors (nAChRs) is one of the major subtypes of nAChRs found in the nervous system. They are composed of five α7 subunit proteins and have high permeability to Ca$^{2+}$ on par with the NMDA type of glutamate receptors. They desensitize rapidly and are inhibited by α-bungarotoxin, which sets them apart from other nAChRs. The α7 receptors
are expressed at presynaptic nerve terminals and also around the synapse (Lindstrom, J., 1997). They have been implicated to have several important functions in neuronal development, cell growth (Quik et al., 1994) neurotransmitter release control (McGehee et al., 1995), cell death and mediating synaptic transmission. Their expression pattern varies at various stages of development of the organism suggesting that tight regulatory mechanisms exist, which control $\alpha7$ receptor expression in the nervous system. Regulation of one gene i.e. the $\alpha7$ subunit gene can allow for control of expression of the widely expressed $\alpha7$ receptor since the receptor is composed of five identical subunits. This is in contrast with other nAChRs (with the exception of $\alpha8$ and $\alpha9$), which require expression of at least two genes (one $\alpha$ and one $\beta$) for the formation of a receptor. Thus the control of timing and location of expression of the $\alpha7$ subunit gene is of considerable significance.

Transcription of a gene is perhaps one of the most crucial rate-limiting steps of gene expression and sets the distribution and abundance of a gene product in any given tissue (Mandel and McKinnon, 1993). Thus, the aims of this study were to identify the regulatory elements,
which control the transcription of the α7 subunit gene along with the transcription factors that bind to them to drive transcription.

The main types of DNA regulatory elements that control gene expression are the promoters, enhancers, and silencers (Blackwood and Kadonaga, 1998). All genes require at least one promoter in order to position and orient the RNA polymerase complex near the start site of transcription. Many genes have an A-T rich sequence termed the TATA box, which binds to the TATA binding protein and acts to recruit other factors including RNA polymerase II (Orphanides et al., 1996). Many neuronal genes, however, including most neuronal nAChR genes (with the exception of the β3 gene), lack this TATA box. Therefore, the pol II complex must recognize alternative sequences upstream of these genes in order to select a start site.

The α7 gene promoter has been studied in chick (Matter-Sadzinski et al., 1992) and bovine chromaffin cells (Criado et al., 1997). There were certain similarities in the promoters between the two organisms. For instance, they both lacked the consensus TATA or CCAAT boxes, the region of the gene immediately upstream
of the transcription start sites was highly GC rich, the promoter sequences had about 60% sequence similarity, and both promoter sequences had several consensus Spl transcription factor binding sites. The chick α7 promoter included the sequence in the -406/-188 region relative to the initiator codon, and confers tissue and stage specific expression of the gene at the time of neuronal differentiation (Matter-Sadzinski et al., 1992). In contrast to the two transcription start sites in chick, the bovine promoter initiates transcription at a single site located less than 100-bp upstream of the initiator codon. This region has several consensus Spl, Egr-1, Myc-Max, and E-box motifs, which are sequences recognized by the above mentioned transcription factors. There are three GC-rich boxes and an E-box in the bovine promoter, which are essential for its basal activity. Transcription factors such as the zinc finger factor, Egr-1 and the basic helix-loop-helix factors USF1 and USF2, and Spl have been implicated in regulating the basal activity of the bovine promoter.

Our laboratory had previously isolated a 2.3 Kb Kpn I rat genomic clone, which contained the 5’ upstream region of the rat α7 subunit gene along with the first
two exons and the intron between them. In addition the 3' region of this clone is part of the intron between exons 2 and 3 (Figure 4). The two major transcription start sites set ~30 nucleotides away from each other, were determined by RNAse protection assays (Nagavarapu, U. et al., 2001) and are indicated in Figure 4 by arrows. The exons are underlined and the translation start site (ATG) is in bold. No consensus TATA or CCAAT boxes were present and the region flanking the first exon on the 5' end was extremely GC-rich especially the region 100 bp upstream of the translational start site, which was 82% GC-rich. The upstream region of the α7 gene identified in other organisms such as bovine (Criado, M et al., 1997), human (Gault, J et al., 1998), and chick (Matter-Sadzinski, L. et al., 1992) also have similar high GC-rich regions and all of them lacked TATA or CCAAT boxes. It is generally believed that in TATA-less promoters, and in the absence of functionally equivalent structures such as the initiator, the recruitment of the TATA box-binding protein and consequent formation of the preinitiation complex may be mediated by Spl (Emili, A. et al., 1994). However, the specific functional role and contribution of a given Spl site in this kind of promoter is often
unpredictable. For instance, in the bovine α5 nicotinic receptor subunit gene promoter, multiple functionally equivalent Sp1 sites cooperate to ensure basal functions (Campos-Caro, A., et al, 1999), whereas the rat α3 promoter contains a unique Sp1 site whose mutation does not abolish the transcriptional activity of the regulatory region (Yang, X. et al, 1995).
Figure 4. Sequence of the 5′-region of the rat nAChR α7-subunit gene.

A 2.3-kb Kpn I fragment present in several clones isolated from a rat genomic library was shown to contain exons 1 and 2 of the α7 gene. The Kpn I fragment was sequenced using a combination of the Sanger method and automated sequencing with an Applied Biosystems model 373A. Restriction sites used for subsequent clonings are labeled and underlined (thick lines). The downstream Sst II site is noted with an asterisk (*). Exons 1 and 2 are underlined. Approximate transcriptional start sites are noted by arrows. The ATG translation start is underlined and in bold.
Figure 4

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Identification of an α7 gene promoter within the 2.3 kb Kpn I fragment

To identify the regions that had promoter activity upstream of the α7 gene, several constructs were created from the 2.3-Kb Kpn I fragment and cloned into the pGL2-Basic plasmid. The constructs were the 1-Kb Kpn I- BstY I, the 1.3-Kb Kpn I-Sst II, 178-bp BstY I-Sst II, 319-bp BstY I-Sst II*, and the 139-bp Sst II-Sst II* fragments (Figure 6) cloned into the reporter plasmid pGL2-Basic (figure 5). This reporter plasmid lacks any promoter element of its own and allows for the cloning of DNA elements suspected of having promoter activity upstream of a reporter firefly luciferase gene. If the cloned fragments have reporter activity, they will drive expression of the luciferase gene once transfected into the cells of interest, provided the gene in question is normally expressed in that cell line. The amount of luciferase enzyme produced can be quantified and acts as a measure of the promoter strength of the DNA fragment being assayed.
For this study the constructs were transfected into rat pheochromocytoma cells (PC12 cells). These cells are derived from a rat adrenal tumor and are excellent as a model to study nAChRs. They express several nAChRs subunits such as α3, α4, α5, α7, β2, and β4. Upon the addition of nerve growth factor (NGF), PC12 cells stop dividing, become electrically excitable and develop into a phenotype similar to that of sympathetic neurons.

Results of transfection of the various constructs into PC12 cells are shown in Figure 6. The luciferase activities produced by the different constructs were compared to that produced by the pGL2-Control (Figure 5). This plasmid is similar to pGL2-Basic except that it has a SV40 promoter and enhancer elements resulting in strong luciferase expression in many types of mammalian cells. Thus the promoter strength of the different constructs were compared to a strong ubiquitous promoter. All luciferase activities were normalized to Renilla-luciferase activities derived from a plasmid pRL-TK (Figure 5), which was co-transfected with the constructs to serve as an internal control for transfection efficiencies. This plasmid has a type of luciferase from
the Sea pansy Renilla that uses a different substrate to generate luminescence.

The 1-kb Kpn I-BstY I fragment had no significant promoter activity indicating that it may not be essential for promoter activity. All other fragments had significant promoter activity. The 1.3-kb Kpn I-Sst II, 178-bp BstY I-Sst II, and the 319-bp BstY I-Sst II* fragments had 42%, 52% and 81% promoter activity relative to the SV40 promoter. All three of these fragments include either one or both of the transcription start sites. Thus the addition of the 139-bp BstY I-Sst II* fragment to the 178-bp BstY I-Sst II fragment caused a ~30% increase in promoter activity.
Figure 5 Maps of reporter plasmids used for promoter analysis.

The different DNA fragments were cloned into the promoterless pGL2-Basic plasmid upstream of the luciferase gene and transfected into PC12 cells. The luciferase activities from these constructs were compared to that produced by the ubiquitous SV40 promoter in the pGL2-Control plasmid. The pRL-TK plasmid was cotransfected with the different constructs as a measure of transfection efficiency. It has a Renilla luciferase gene driven by a constitutively active Thymidine Kinase (TK) promoter.
Figure 5
The smallest fragment with significant promoter activity was the 178-bp BstY I-Sst II piece (Figures 6,7). All the constructs, which included this fragment, had significant promoter activity, which could probably explain the lack of activity in the 1-kb Kpn I-BstY I fragment. Also the difference in the levels of activity between the 2.3-kb Kpn I fragment and the 1.3 kb Kpn I-BstY I fragment indicates that there may be silencer elements in the regions not included in the latter. It is possible that other promoters might exist for the α7 gene upstream of the 2.3-Kb Kpn I fragment. However our studies were restricted to this piece.
Figure 6 Identification of an α7 gene promoter within the 2.3-kb Kpn I fragment

A) The various reporter constructs that were created are depicted. Each fragment from the region 5′ to the gene was cloned into the vector pGL2-Basic upstream from a luciferase reporter gene.

B) Results of the reporter assays for each construct relative to the SV40 promoter are shown.
Figure 6
The shortest sequence with promoter activity is detailed here. All constructs that had this 178-bp sequence had significant promoter activity. This sequence was defined as the minimal rat α7 nAChR subunit gene promoter. The GC-rich boxes and the E-boxes are shown.
BstY I
ACTGGATCTG GAAATTGTTA TGGTGTGAGT ACAGGCACA TTTGATAGCA
GGAGCAAGTA GCTTGGGGTC AGAGACCATC AACAAGCTGC GCAGGGATGC

GC-box
AGTTCCCGGG GCGGCACGG AGCTGCACGG CCCAGCGAGC GCCCGCGAGA

E-box GC-box
CGGGAGCGCG CTGTCAAGTG TGGGCGCGCG CGCGCG

Sst II

Figure 7

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Functional Analysis of Specific Sequences in the α7 Promoter

The 178-bp BstY I-Sst II minimal promoter was further analyzed to assess the role of each regulatory element contained within the fragment. As shown in figure 7, this region of the promoter has a consensus E-box (CACGTG) and several GC-rich boxes. The E-box is a binding site for some helix-loop-helix type of transcription factors such as USF1, USF2, and Myc-Max. The GC-rich boxes allow for binding of Sp family of transcription factors and also Egr-1. In order to determine in what capacity these elements were involved in controlling α7 gene expression, point mutations were made within these boxes. Mutations were made in the GC-boxes and the E-box to generate mutant constructs. A double mutant was also created in which there was a mutation in both the upstream GC box and the E-box. These were then transfected into PC12 cells as before and assayed. The activities of the mutant constructs were compared to the wild-type constructs.

Mutation in the E-box led a decrease in promoter activity by nearly 40% (Figure 8) as compared to the
wild-type construct. However mutating the upstream GC-box lead to a 2-3-fold increase in activity as compared to the wild-type fragment (Figure 8). This indicates that the E-box plays a positive regulatory role in α7 expression whereas the GC-box has a negative role in expression. The double mutant, in which both the E-box and GC-box were mutated, showed activity similar to that shown by the E-box mutant (a 40% decrease in activity), indicating that the mutation in E-box dominates the other mutation (Figure 8). This implicates the E-box in having a major role in α7 expression, as it does not allow the increase in expression caused by removal of a negative element. Mutating the GC-box downstream to the E-box had no effect on the promoter activity.

There is a significant contrast to the effect of mutation of GC and E-boxes in the bovine counterpart of the rat α7 nAChR gene promoter. In the bovine promoter there exist three GC rich boxes and an E-box. Mutation of an E-box led to a decrease in promoter activity similar to that seen in rat, but there does not exist an element whose mutation leads to an increase in promoter activity. There seems to be a synergy between the GC and E-boxes in the bovine promoter as simultaneous mutation of a GC box

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and E-box led to a further decrease in promoter activity (Carasso-Serrano et al., 1998).

**Binding of PC12 Cell Nuclear Proteins to a GC-box within the α7 Minimal Promoter**

To begin characterization of transcription factors binding to the α7 promoter, electrophoretic mobility shift assays (EMSA) were used to determine where PC12 nuclear extract proteins bound to the minimal 178-bp α7 promoter. Oligonucleotides were designed that corresponded to the various regions within the promoter that were suspected as being important in regulation of expression. These oligonucleotides are shown in Figure 9 and were used in EMSA.
Mutations were made in the GC-box and the E-box (CACGTG) in the α7 promoter contained in the 178-bp BstY I-Sst II fragment. A double mutant for both the GC-box and the E-box sequences was also made. The promoter-luciferase constructs were transfected into PC12 cells, and the normalized luciferase activities present in the extracts were determined as described above. The luciferase activity of the wild-type α7 promoter contained in the wild-type 178-bp BstY I-Sst II fragment was set at 100%.
Figure 8

Relative Luciferase Activity
Figure 9

**Figure 9 Sequence of the α7 promoter.** Oligonucleotides used for EMSA (A-D) are overlined.
Because mutation of the upstream GC-rich sequence led to increased promoter activity, we first used an oligonucleotide containing this sequence from the promoter for EMSA using PC12 extracts. An oligonucleotide (A) containing the GC-rich sequence was shown to produce three retarded complexes when combined with PC12 nuclear extract (Figure 10, lane 2). The upper and lower species were competed by excess unlabeled oligonucleotide A and were determined to be specific (Figure 10, lane 4). The middle band was poorly competed and thought to be nonspecific. Because this GC-rich sequence contains a potential Spl binding site, human recombinant Spl (Promega) was used for EMSA with oligonucleotide A. Recombinant Spl did not retard the mobility of the oligonucleotide A; however, recombinant Spl bound to an oligonucleotide containing a consensus Spl binding site that was used as a positive control (Figure 10, lane 9).
**Figure 10: Binding of PC12 nuclear proteins to a GC-rich sequence in the α7 nAChR gene promoter**

Oligonucleotide A was used for EMSA with lanes 1–5, and a consensus Sp1 oligonucleotide was used for lanes 6–11. PC12 extract was present in lanes 2, 4, 5, 7, 8, and 10. Human recombinant Sp1 was used in lanes 3, 9, and 11. Anti-Sp1 antibodies were used for supershifting in lanes 5, 10, and 11. Unlabeled competitor oligonucleotides were present in 100-fold excess in lanes 4 and 8. Shifted complexes are indicated by the ovals and antibody supershifted complexes by the box.
<table>
<thead>
<tr>
<th>Probe</th>
<th>Oligonucleotide A</th>
<th>Consensus Sp Oligonucleotide</th>
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<tbody>
<tr>
<td>PC12 Extract</td>
<td>•</td>
<td>•</td>
</tr>
<tr>
<td>Human Sp1</td>
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Figure 10

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Antibody supershifting of oligonucleotide A and PC12 nuclear extract with an antibody against Sp1 (Santa Cruz) had no effect (Figure 10, lane 5). The oligonucleotide containing a consensus Sp1 binding site (Promega) produced three retarded complexes with PC12 extract in addition to binding recombinant Sp1 (Figure 10, lane 7). Antibody supershifting with anti-Sp1 antibodies produced additional retardation of the slowest moving complex produced with the PC12 extract and the Sp1 oligonucleotide (Figure 10, lane 10). Nuclear extracts from SL2 cells expressing mouse Sp1 and Sp3 were also used for EMSA with oligonucleotide A and did not produce retarded complexes. Antibody supershifting with anti-Egr-1 antibodies (Geneka) also did not produce a supershift with oligonucleotide A and PC12 nuclear extract. Taken together these results indicate that the GC-rich sequence in the α7 minimal promoter contained in oligonucleotide A binds protein(s) present in PC12 nuclear extracts which are distinct from Sp1, Sp3, or Egr-1.
Binding of USF1 to an E-Box present within the α7 nAChR gene promoter

Binding of PC12 nuclear proteins to a second region of the α7 promoters was also examined. A consensus E-box was present in the α7 promoter, and mutation of this sequence was shown to decrease promoter activity. An oligonucleotide B containing the E-box and surrounding sequences from the α7 promoter was used for EMSA and produced one retarded complex with PC12 nuclear extract (Figure 11, lane 6). Because several transcription factors can potentially bind an E-box, we attempted antibody supershifting with antibodies to several transcription factors. Antibody supershifting with anti-USF1 antibodies revealed that USF1 binds to the α7 E-box (Figure 11, lane 8), although some of the retarded complex was not supershifted. Interestingly, the amount of retarded, nonsupershifted complex appeared to be increased in the presence of anti-USF1 (compare lane 6 with lane 8 in Figure 11). Anti-USF2 antibodies failed to produce a supershifted complex (Figure 11, lane 9). USF2 was also not detected in our PC12 cells using Western analysis. An oligonucleotide containing a
consensus USF binding site (TCACGTG) also produced one retarded complex when incubated with PC12 nuclear extract (Figure 11, lane 2). This band produced with the consensus oligonucleotide was completely supershifted with the same amount of anti-USF1 antiserum (Santa Cruz), which failed to supershift the entire retarded complex produced by oligonucleotide B (Figure 11, lane 4). The failure to shift completely oligonucleotide B with the anti-USF1 antibody could be caused by technical limitations inherent to these types of experiments or was an indication that additional protein(s) bound to oligonucleotide B. However, when USF1, USF2, and Egr-1 antibodies were used together for supershifting with oligonucleotide B, the entire complex was supershifted (Figure 11, Lane 10). Because USF2 by itself did not produce a supershift, the complete supershift could be the result of antibodies to USF1 and Egr-1.
Figure 11  USF1 Binds to an E-box within the 178 bp BstY I-Sst II fragment

A consensus USF1 oligonucleotide was used for EMSA in lanes 1-4. Oligonucleotide B was used for EMSA in lanes 5-10. PC12 extract was present in lanes 2-4 and 6-10. Anti-USF1 antibodies were used for supershifting in lanes 4, 8, and 10; anti-USF2 antibodies in lanes 9 and 10; and anti-Egr-1 antibodies in lanes 7 and 10. Unlabeled competitor oligonucleotide was present in 100-fold excess in lane 3.
Figure 11
Egr-1 binds to a site adjacent to the E-box

Examination of oligonucleotide B revealed a sequence with homology to a potential Egr-1 binding site. To confirm whether both USF1 and Egr-1 bound in this region, antibody supershift analysis was performed using a nuclear extract from 12-O-tetradecanoylphorbol-13-acetate-treated K-562 cells expressing high levels of Egr-1 (Geneka) and oligonucleotide B. K-562 cells also express USF1. One retarded complex is seen using the K-562 extract as seen with PC12 cell extract (Figure 12, lane 1), whereas antibody supershifting with both anti-USF1 and anti-Egr-1 together produced two slower moving complexes (Figure 12, lane 4), confirming that Egr-1 and USF1 were present in the same band. Thus Egr-1 binds to a site near USF1 in the α7 promoter.
Figure 12. Egr-1 binds to a site close to the E-box

Oligonucleotide B (lanes 1-4) was used for EMSA with a nuclear extract from 12-O-tetradecanoylphorbol-13-acetate (TPA)-treated K-562 cells expressing a high level of Egr-1 (lanes 1-4). Unlabeled competitor oligonucleotide B was present in 100-fold excess in lane 2. An antibody to Sp3 was present in lane 3. Antibodies to USF1 and Egr-1 were used together for antibody supershifting (lane 4).
Figure 12

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To study further the region of the α7 promoter where USF1 and Egr-1 bound, two additional oligonucleotides were used for gel shift assays. Oligonucleotide C contained USF1 and Egr-1 binding sites as well as additional 3′-sequence. When used for EMSA with PC12 nuclear extract two strong bands were produced (Figure 13, lane 1), one of which was completely supershifted with the Egr-1 antibody (Figure 13, lane 2). When a second oligonucleotide, C-m, containing a mutation that eliminates the Egr-1 binding site, was used, one strong broad band and a weaker band were produced (Figure 13, lane 3), and no supershift was seen with the Egr-1 antiserum (Figure 13, lane 4). This entire remaining strong band was supershifted with anti-USF1 (Figure 13, lane 6). This confirms the Egr-1 binding to the mutated region and is consistent with the remaining binding to oligonucleotide C-m being the result of USF1. These results using the mutant oligonucleotide confirm that Egr-1 binds to the sequence directly adjacent to USF1 in oligonucleotide C.
Figure 13 USF1 and Egr-1 bind close to each other

EMSA was performed using oligonucleotide C (lanes 1 and 2) and a mutant version of oligonucleotide C (C-m) without the Egr-1 binding site. Nuclear extracts from 12-O-tetradecanoylphorbol-13-acetate-treated K-562 cells expressing a high level of Egr-1 (Geneka) were used in lanes 1-4; PC12 cell nuclear extract was used in lanes 5 and 6. Antibodies to Egr-1 were used in lanes 2 and 4; antiserum to USF1 was used in lane 6. Shifted complexes are indicated by ovals, and antibody supershifted complexes by boxes or triangles.
Figure 13
Spl and Sp3 bind close to the binding site for Egr1

To characterize further this region an additional oligonucleotide (D) was constructed which lacked the nearby E-box (CACGTG). EMSAs with this oligonucleotide and PC12 extract produced one major specific species (Figure 14A, lane 1). This band was not supershifted with anti Egr-1 antibody (Figure 14A, lane 2) as expected. A supershift also was not seen when the 12-O-tetradecanoylphorbol-13-acetate-treated K-562 cell nuclear extract expressing Egr-1 was used in place of the PC12 extract. Although oligonucleotide D contained the entire presumptive Egr-1 binding site, in our hands it did not bind Egr-1 using conditions under which oligonucleotides B and C bound Egr-1. Because oligonucleotide D produced a retarded complex with PC12 extract, some protein presumably bound to oligonucleotide D besides Egr-1. Binding sites for Spl and Egr-1 sometimes overlap, and Egr and Sp family members compete for binding to GC-rich sequences. To determine whether Spl or Sp3 bound to this region, EMSAs were performed using oligonucleotide D with mouse Spl and mouse Sp3 expressed in SL2 cells. Oligonucleotide D produced a
strong band with the Sp1 extract (Figure 14B, lane 2) and a weaker one with the Sp3 extract (Figure 14B, lane 4).

We next determined that Sp1 and Sp3 present in PC12 extracts bound to oligonucleotide D. Using a buffer designed to optimize binding of Sp1 and Sp3, one major and two minor retarded complexes were produced when PC12 nuclear extract was used with oligonucleotide D. Note that only one major band was observed using conditions that optimize Egr-1 binding (Figure 15). This points out the importance of using multiple conditions to study transcription factor binding. The addition of anti-Sp1 antiserum produced a slight supershift of the slowest complex (Figure 15, lane 3). This is consistent with the slight supershift produced when a Sp1 consensus oligonucleotide and anti-Sp1 antiserum were used as a positive control. Anti-Sp3 did not produce a supershift with oligonucleotide D, but the two faster moving complexes disappeared as occurred with the positive control. This result is consistent with Sp1 and Sp3 binding to oligonucleotide D and supports the insect expressed Sp1 and Sp3 binding result.
Interestingly, oligonucleotide C, which contained all of the sequence present in oligonucleotide D except the 3' three nucleotides, did not bind to the mouse Sp1 or Sp3, but bound USF1 and Egr-1.
Figure 14 Spl and Sp3 bind close to the E-box

A) EMSA using oligonucleotide D plus PC12 nuclear extract (lane 1) or PC12 nuclear extract and anti-Egr-1 antibodies (lane 2).

B) EMSA using oligonucleotide D plus control SL2 extract (lanes 1 and 3), EMSA using oligonucleotide D plus SL2 extract expressing mouse Spl (lane 2), EMSA using oligonucleotide D plus SL2 extract expressing mouse Sp3 (lane 4). Nonspecific binding present in insect extracts is indicated by the arrow.
Figure 14
Figure 15: Sp1 and Sp3 from PC12 nuclear extracts also bind close to the E-box

EMSA was performed using PC12 extracts (lanes 1-4, 6-8) and oligonucleotide D (lanes 1-4) or a consensus Sp1 oligonucleotide. Anti-Sp1 antiserum (lanes 3 and 7) or anti-Sp3 antiserum (lanes 4 and 8) was used for supershifting. 100-fold excess of unlabeled oligonucleotide D was used in lane 2; no nuclear extract was present in lane 5. Shifted complexes are indicated by ovals and antibody supershifted complexes by boxes or triangles.
Figure 15
Figure 16: Model showing transcription factor binding to the minimal \( \alpha_7 \) nAChR subunit gene promoter.

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Summary

A promoter for the rat neuronal nicotinic acetylcholine receptor α7 subunit gene in PC12 cells was isolated from a 2.3 kb fragment that had the 2 major transcription start sites and some 5' sequence flanking the gene. The minimal promoter was a 178 bp fragment from the Bst Y I- Sst II (Figure 16). This sequence was highly GC-rich analogous to the chick and bovine α7 promoters. Within this 178 bp region there lay some sequences capable of binding transcription factors. A GC-rich box was identified as a repressor of gene expression and was capable of binding as yet unidentified transcription factors. An E-box capable of binding USF1 served as a positive regulator of gene expression. USF1 probably binds to this sequence as a homodimer as USF2 with which it is capable of forming heterodimers was not found in the PC12 cells used for analysis. Another GC-rich box immediately downstream of the E-box was capable of binding Egr-1, Sp1 and Sp3 indicating interplay between these factors, perhaps by competition, to regulate expression.
A 139 bp region directly downstream of the minimal promoter had no promoter activity on its own. However when combined with the 178 bp region acted to increase the activity of the minimal promoter. Further analysis of this region is required to test if this region acts in a distance or orientation independent manner to increase transcription in order to qualify it as an enhancer.
Chapter 4

Results and Discussion

Cell specificity of the α7 nicotinic acetylcholine receptor subunit gene promoter

Background

The mechanisms that underlie tissue and cell-specific gene expression remain to be elucidated. However, an increasing number of transcription factors (Akazawa et al., 1992; Lee et al., 1995) and DNA elements (Mori et al., 1990; Kraner et al., 1992; Bessis et al., 1993) involved in neuron specific transcription have now been characterized. Most neural specific genes use negative regulatory mechanisms to direct their expression in unique neuronal populations (Mandel and McKinnon, 1993). Expression of the type II sodium channel gene is
restricted to certain subsets of neurons in the rat brain by negative regulation that silences transcription in nonneuronal cells (Kraner et al., 1992; Chong et al., 1995). In addition, the expression of several other neuronal-specific genes, including SCG10 (Mori et al., 1990; Schoenherr and Anderson, 1995), synapsin I (Li et al., 1993), neuron-glia cell adhesion molecule (Kallunki et al., 1995), and choline acetyltransferase (Lonnerberg et al., 1996), are thought to be mediated by the neuron-restrictive silencing element (NRSE) or restrictive element 1 (RE1) (Mori et al., 1990; Kraner et al., 1992).

This type of negative regulation is also seen in neuronal nicotinic acetylcholine receptor subunit gene expression. For example, the mouse β2 nicotinic subunit gene (Bessis et al., 1995) has a neuron-restrictive silencer element (NRSE), and is shown to bind the neuron restrictive silencing factor (NRSF) or repressor element-1 silencing transcription factor (REST). This element and transcription factors binding to it are sufficient to restrict the expression of this gene only to neuronal cells.

The tight neuron specific expression of the rat and chicken α2 nAChR subunit is regulated by a 11 bp motif in
the 5'-flanking region of the gene that is repeated 6 times over (Daubas et al., 1990; Morris et al., 1990; Wada et al., 1992). This 6-time repeat has to be intact to maintain neuron specific gene expression. It is believed that a yet unidentified POU family transcription factor may bind to this domain and confer this restrictive property to these elements (Bessis et al., 1993). It is possible that negative regulation may be a general mechanism of restricting gene expression to subsets of neurons.

Neuron specific expression of the α3 gene seems to be dependent on a downstream regulatory region (DRR), which has binding sites for transcription factors such as NFkB, AP2, and MED-1. This element increases the expression of this gene in neuronal cells four-fold (Fornasari et al., 1997). α3 is also expressed in one non-neuronal tissue, namely the T lymphocyte (Mihovilovic and Roses, 1993). The expression of this gene in T lymphocytes depends on the same basal promoter as neuronal cells, however the DRR is inactive (Battaglioli et al., 1998). This indicates that the DRR is a neuron specific facilitator of transcription.
The α7 nAChR subunit gene is quite different from other subunits in that it is expressed in a wide variety of tissues in the CNS and autonomic nervous system (Britto et al, 1992; Dominguez del Toro et al., 1994; Rubboli et al., 1994; Zhang et al., 1996) as opposed to a restricted expression of some subunits like the β3. In addition the α7 gene is expressed in some selected non-neuronal tissues such as small cell lung carcinoma cell lines (Tarroni et al., 1992), tendon, periosteum and skeletal muscle fibers (Romano et al., 1997a,b).

The α7 subunit also has a complex pattern of developmental regulation. For example, in the developing chicken brain its promoter is highly active in non-differentiated tissues and only later acquires specificity for certain neuronal populations (Matter-Sadzinsky et al., 1992).

**Preliminary Data**

Our laboratory has previously characterized a promoter for the rat neuronal α7 subunit gene (Nagavarapu 94).
et al., 2001). A 178 bp DNA element, upstream of the transcriptional start site, served as a minimal promoter required to drive expression of a reporter gene in PC12 cells. Analysis of this region revealed the binding of several transcription factors such as USF-1, Egr-1, Spl and Sp3 in close proximity. Some yet to be identified factor/s bound to a cis-element that had a repressor effect on α7 expression.

This manuscript describes further analysis of this promoter, along with other constructs spanning various upstream regions of the α7 gene, in an effort to identify elements that confer neuronal specificity to the expression of this gene.

**Identification of a 139 bp element that increases the minimal promoter expression.**

We have previously described the characterization of a rat α7 nicotinic acetylcholine receptor subunit gene promoter (Nagavarapu et al., 2001). A 178 bp BstY I-Sst II fragment within a 2.3-kb Kpn I fragment was found to be the minimal promoter required to drive the expression of a reporter gene in PC12 cells.
This 178bp fragment did not express in rat L6 myoblasts. This cell line does express the muscle type of nAChR but not the neuronal.

Several reporter luciferase constructs in which different fragments from the 2.3 kb Kpn I fragment, spanning varying lengths of the 5' upstream region fused to the luciferase gene were created. These constructs were transfected into N21 and PC12 cells. A 139 bp Sst II-Sst II* fragment had no promoter activity on its own. However when fused to the 178 bp minimal BstY I- Sst II fragment, to generate a 319 bp BstY I-Sst II* construct, it served to increase the promoter activity of the minimal promoter in both N21 and PDG PC12 cells (Figure 17). Addition of the 139 bp fragment downstream to any construct that demonstrated promoter activity seemed to increase the activity of that fragment as shown when it is fused downstream to the 1.3 kb Kpn I-Sst II to generate the 1.4 kb Kpn I-Sst II* construct. This indicates that the 139 bp Sst II-Sst II* fragment has positive effects on the expression of the promoter.
Figure 17 Identification of a 139bp positive element.

A 139bp Bst Y I-Sst II* fragment had no promoter activity on its own but seemed to increase the promoter strengths of all constructs previously shown to have promoter activity. The constructs indicated were transfected into the two types of PC12 cells. The activity of each construct was compared to that produced by an SV40 promoter (pGL2 Control) and plotted.
Figure 17
Cell type specificity of the α7 nAChR promoter constructs

Since the α7 nAChR is expressed predominantly in the nervous system, we were interested in investigating whether the promoter elements had any role to play in regulating the neuronal specific expression of this gene. The various promoter constructs described earlier were transfected into a variety of neuronal and non-neuronal cell lines. Namely the N21 and PDG rat PC12 cells, and the mouse Neuro2A and SN17 neuroblastoma cells constituted the neuronal cell lines whereas the Human Embryonic Kidney cells (HEK) and rat myoblasts (L6) comprised the non-neuronal cell lines. The expression of the various constructs was compared to the expression of the reporter construct driven by a SV40 promoter in each cell line (pGL2 Control). The SV40 promoter is a ubiquitous promoter and expresses in all the cell lines tested, although in varying degrees.

Results of the transfection of the reporter constructs into these cells are summarized in Figure 18. As indicated the 178 bp minimal promoter was neuron specific in that it expressed in all neuronal cell lines, but not in non-neuronal cell lines. It is interesting to
note that this fragment had higher expression than the minimal promoter in Neuro2A cells as compared to the other neuronal cell lines suggestive of the fact that there might be an element within it that promotes its expression in Neuro2A cells. The 139 bp Sst II-Sst II* fragment had little or no expression in the neuronal cell lines and L6 cells. However this fragment displayed considerable promoter activity in HEK cells. This may suggest that the 139 bp fragment does have intrinsic promoter activity, which is silenced in neuronal cell lines.

Most interesting of all were the results from the 319 bp Bst Y I-Sst II* fragment that consisted of the fusion of the 178 bp minimal promoter and the 139 bp enhancer. This fragment had significant promoter activity in all the cell lines it was transfected into. The activity of this fragment was magnified several times as compared to the 178 bp minimal promoter, especially in Neuro2A, SN17, L6 and HEK cells (Figure 18). Not only did the 139 bp fragment act as a positive element but it also caused the expression of the α7 gene promoter constructs in non-neuronal cells causing a loss of neuron specific regulation.
Expression of α7 nAChR mRNA in various cell lines

Since combining the 139 bp fragment to any construct with promoter activity seemed to enhance the expression in all cell lines used for analysis including the non-neuronal cell lines, Northern Blot and RT-PCR analysis were performed to analyze the expression of the α7 nAChR mRNA. α7 mRNA was clearly detected in the N21 and PDG PC12 cells. However, in the rest of the cell lines, except SN17 cells, the α7 mRNA was not detected by Northern analysis (Figure 19A). A more sensitive method, namely RT-PCR was used to detect the presence of α7 nAChR mRNA in the cell lines. Again expression was detected only in the PC12 and SN17 cells (Figure 19B). Thus the α7 nAChR gene has a restricted pattern of expression although some of its promoter constructs are capable of expression in several cell types. Analysis of the sequence of the various fragments used did not reveal a restrictive element like the NRSE. There must exist other elements in the α7 gene surroundings, either upstream or downstream that regulate the tight expression of this gene.

nAChR subunit proteins vary considerably in their expression pattern throughout the nervous system. For
example, the β2 subunit is widely distributed in the central and peripheral nervous system, whereas the α2 subunit is expressed sparsely in highly restricted regions in the brain. Also, the timing of expression of the various subunits can differ during neuronal development suggesting a role for some receptor subtypes in neuronal differentiation and development (Zoli et al., 1995).

Although, mouse neuroblastoma cell lines such as Neuro2A and SN17 were used in the studies mentioned above, no literature was found on the evidence that these cells express nAChR subunits other than β4. Hence, primers were designed to various nAChR subunit genes, using the sequences from published mouse nAChR in GenBank (The mouse β4 sequence was provided by Dr. Jerry Stitzel, University of Michigan, and is not published in GenBank). RT-PCR was done using the RNA from Neuro2A and SN17 cells and the results are shown in Figure 20. This indicates that the Neuro2A and SN17 cells do express other nAChR subunits and can be used as models to study nAChR expression.

The expression of several nAChR subunits has been studied relative to their promoters. The study of
expression regulation of nAChRs will go a long way in understanding their functions and better comprehending neuronal plasticity.

Since neuronal nAChRs are expressed largely in the nervous system, as opposed to their muscle counterparts at the neuromuscular junction, the study of their neuron specific expression gains importance. Especially in disease conditions such as Alzheimer's, in which there is a diminishing of the cholinergic pathway that is linked to the loss of neuronal nAChRs. Understanding the expression of the nAChRs will also help to elucidate the role of the neuronal nAChRs in development and anomalies linked to them in this process.
The various promoter constructs were transfected into neuronal (PC12 N21 and PDG, Neuro2A, SN17) and non-neuronal (L6, HEK) cell lines and assayed. The expression of each reporter construct was first normalized to the expression of a second reporter construct (pRL-TK) to correct for transfection inadequacies. Each normalized reading was then compared to the normalized reading of expression from the pG12-Control plasmid that is driven by an SV40 promoter. This promoter expresses in all the cell lines tested although to a varying degree.
Figure 18

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The expression of several nAChR subunits has been studied relative to their promoters. The study of expression regulation of nAChRs will go a long way in understanding their functions and better comprehending neuronal plasticity.
Figure 19. Expression of the α7 nAChR subunit mRNA in various cell lines as assayed by A) Northern analysis or B) RT-PCR.
Figure 19
Figure 20. Expression of various nAChR subunit genes in Neuro2A and SN17 cell lines as detected by RT-PCR
Summary

Analysis of α7 expression in various neuronal and non-neuronal cell lines was done to investigate the cell specificity of the various promoter constructs. α7 expression was clearly seen by Northern blots and RT-PCR in the two types of PC12 cells and the SN17 cells. No expression of α7 was seen in Neuro2A and the two non-neuronal cell lines L6 and HEK. The 178 bp minimal promoter did not express in the non-neuronal cell lines indicating neuronal cell type specific expression. The 139 bp region directly downstream of the minimal promoter also had no promoter activity in all cell lines tested except for HEK in which it indicated some promoter activity. It is possible that the 139 bp region might be a second promoter which is only active in fibroblasts. Interestingly, combining the 139 bp region with the 178 bp region lead to a complete loss of cell specificity of the minimal promoter as the construct generated by the combination of the two fragments lead to the expression in all types of cell lines tested. This new construct also had promoter activity that was significantly higher than the minimal promoter in all cell lines, a feature seen during the analysis of the constructs in PC12 cells.
Thus the 139 bp region seems to increase the activity of the minimal promoter in all cell lines. The difference in the levels of the promoter activity in different cells might indicate that certain constructs are favored for expression in those cells. As no α7 gene expression was seen in the L6 and HEK cells although the combined 178 and 139 bp fragments showed promoter activity it is possible that there must exist some other elements not included in our scope of study that restrict the expression of this region thus inhibiting the expression of the α7 nAChR gene.
CHAPTER 5

Preparation of purified recombinant large intracellular loop of the bovine neuronal α3 and β4 subunits.

Introduction

Neuronal nAChRs are crucial for the release of catecholamines from sympathetic neurons and the adrenal medulla (chromaffin cells). The agonist (ACh) binds to the nAChRs on the cell surface, leading to receptor channel opening, followed by influx of cations that subsequently leads to a minor membrane depolarization. This depolarization triggers the voltage gated sodium channels leading to further depolarization of the cell membrane now activating the voltage gated Ca²⁺ channels.
The increasing intracellular Ca\(^{2+}\) concentration is a signal for release of catecholamines stored in granules in the cell by exocytosis.

The exact composition of the nAChRs involved in catecholamine release is not known. The existence of several possible subtypes of nAChRs and the lack of highly receptor subtype specific agonist/antagonists/antibodies make the discovery of the composition of the receptors a difficult task.

The adrenal medullary chromaffin cell is a classic neurosecretory cell and is developmentally homologous with sympathetic neurons in their synthesis, storage and secretion of catecholamine and enkephalin neurohormones. The accessibility of bovine adrenal chromaffin cells makes them an excellent model system for the study of vesicle exocytosis in neurons as well as other excitable secretory cells.

To date, the presence of \(\alpha 3, \alpha 7, \alpha 5\) and \(\beta 4\) nAChR subunit genes have been identified in bovine adrenal chromaffin cells (Campos-Caro et al., 1997; Criado, M et al., 1992; Wenger BW., 1997). The major subtype of nAChRs that mediate adrenal secretion have been described as mAb35 nAChRs (the receptor subtype that is recognized by
a monoclonal antibody-mAb35 raised against the muscle nAChR) and are thought to consist of α3, α5 and β4 subunits. However, other nAChR subtypes may also be involved in regulating adrenal secretion since mAb35 nAChRs cannot account for all secretory responses to nicotine (Wenger et al., 1997; Gu et al., 1996).

However the α7 class of nAChRs does not seem to be involved in catecholamine release, as indicated by studies involving complete inhibition of these receptors with α-BgT that did not produce a change in secretion (Wilson and Kirshner, 1977).

To identify the composition of mAb35 nAChRs, subtype specific tools would be necessary. In an attempt to create specific antibodies against the α3 and β4 subunits (those suspected of forming mAb35 sensitive receptor subtypes), the region of the subunits corresponding to the intracellular loop was used as an antigen. This region was selected due to its unique property of having the highest possible sequence divergence among the various subunits. Hence, using the large intracellular loop to generate subunit specific antibodies would most likely be the best strategy. As a means to test the specificity of the antibodies that will be created,
stable COS-1 cell lines expressing the desired subunit, either alone or in combination with other subunits, would be desirable.

As part of the project to study bovine chromaffin cell nAChRs involved in catecholamine secretion, the description of creating the purified proteins corresponding to the bovine α3 and β4 subunit large intracellular region is detailed, along with the generation of a stable COS-1 cell line expressing the bovine α3 nAChR subunit.

**Expression and purification of the bovine α3 and β4 nAChR subunit large intracellular region in bacterial cells.**

The bovine α3 nAChR subunit cDNA was kindly provided by Dr. Manuel Criado. The sequence of the α3 cDNA that was used to recombinantly express and purify from bacteria, is shown in Figure 21. Oligonucleotides for PCR were created flanking the 5’ and 3’ region of this selected region. The oligonucleotides had additional sequences at their ends that would allow cloning of the amplified product by the appropriate restriction digest of the PCR product.
The PCR product of 423bp was restriction digested using BamH I and EcoR I and inserted in the pCALn plasmid (Stratagene) directly downstream to a calmodulin binding protein (CBP) coding region. This plasmid allows the generation of a fusion protein comprising of a calmodulin binding protein (CBP) fused to the recombinant protein of interest on its N-terminal side. This construct was sequenced to ensure the viability of the open reading frame and then transformed in XL1-Blue bacterial expression cells (Stratagene) (Figure 21). After induction of protein expression with IPTG the cells were harvested and assayed for the production of the recombinant protein.

For the process of purification of the recombinant protein from the bacterial extract a calmodulin resin column was used. The bacterial extract was passed through the column in a buffer containing 2 mM Ca$^{+2}$. In the presence of Ca$^{+2}$, CBP binds to the calmodulin. The column was washed several times and then the recombinant protein was eluted using a buffer having 2mM EGTA, which serves to chelate the Ca$^{+2}$ and release the protein from the column.
Unfortunately most of the recombinant protein was formed in inclusion bodies in insoluble form. The inclusion bodies were dissolved by denaturation in a medium containing 8M urea. A property of the CBP, that it maintains its binding ability to calmodulin even in the denatured state in the presence of Ca\(^{2+}\), was used for the purification process. The whole inclusion body solution in 8M urea was passed through the column, followed by several washes with a buffer having 8M urea and Ca\(^{2+}\), and finally elution with a buffer having 2 mM EGTA in 8M urea.

This process lead to the successful purification of the recombinant α3 nAChR subunit intracellular loop (Figure 22). However the protein was in denatured state and had to be renatured. Serial dialysis was carried out in buffers with descending concentrations of urea. However in buffers with urea concentration below 4M, the recombinant protein would revert back to the insoluble form. This solution was given to Dr. Paul Gottlieb, University of Texas, to generate polyclonal antibodies. The same process was carried out to generate the β4 recombinant protein (Figure 22).
Creation of the stably α3 expressing COS-1 cell line.

The bovine α3 nAChR cDNA provided by Dr. Manuel Criado was digested out using EcoRI and ligated into PCDNA 3.1+ vector downstream to a CMV promoter (Figure 21). This vector allows mammalian cell overexpression of the desired gene. In addition, it has a neomycin resistant gene that allows for selection of cells that have taken up the plasmid while killing off the rest.

The α3-PCDNA3.1+ plasmid was sequenced to ensure sequence integrity and then transfected into COS-1 cells. Two days after transfection, the cells were grown in serum supplemented RPMI 1640 medium containing 500 μg/ml G418. Three weeks later the cells were assayed for expression of the α3 nAChR subunit mRNA using Northern Blot analysis, and were shown to be stable expressing the α3 mRNA (Figure 23).
Figure 21. Expression of α3 and β4 nAChR large intracellular loop in bacteria. Sequence of the region of the α3 and β4 nAChR protein recombinantly expressed in bacteria is shown in A) and B) respectively. The pCALn plasmid used for generating the fusion proteins is shown in C). The pcDNA 3.1 plasmid used to generate stable α3 expressing COS-1 cells is shown in D).
Figure 22. Expression and Purification of the large intracellular loop region of the bovine α3 and β4 nAChR subunit from bacterial cells.

A) Expression of α3 and β4 proteins in XL1-Blue cells. Coomassie stained 15% SDS-PAGE Lanes. 2 and 4 indicated uninduced bacterial cells whereas 3 and 5 indicate induced cells expressing α3 and β4 protein respectively.

B) Purified α3 protein (~19 kD) after affinity purification shown on a 15% coomassie stained SDS-PAGE gel.

C) Purified β3 Protein (~19 kD) after affinity purification shown on a 15% coomassie stained SDS-PAGE gel.
Figure 22

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Figure 23. Northern blot analysis of the stable expression of the α3 nAChR subunit mRNA in COS-1 cells.
CHAPTER 6

Effect of Nerve Growth Factor on α7 nAChR gene expression

Introduction

There exists a rigorously controlled cascade of signal transduction events that occur during neuronal differentiation. There is temporal and spatial expression of genes that are involved in the process of neuronal development (Melnikova and Gardner, 2001). Neurotransmitter gated ion channels form a crucial class of such differentially regulated genes, as they are involved in signal generation and propagation in the nervous system.

Nerve growth factor is essential for the survival of cholinergic and sympathetic neurons. NGF binds to its receptors on the cell surface. Two such NGF
receptors exist, one is the high affinity Trk A receptor and the other, the low affinity p75 receptor. Binding of NGF to its receptors initiates several signal transduction cascades, including, among others, ras-dependent mitogen-activated protein kinase (MAPK), phosphoinositide 3-kinase (PI3-K), and the cAMP-dependent protein kinase pathways (Friedman and Greene, 1999). Transcription factors such as Egr-1, AP-1, cAMP responsive element-binding protein, and NF-κB are downstream targets of the signaling by NGF.

Trk A activated pathways seem to be important in neuronal differentiation. Activated TrkA promotes conversion of the membrane-anchored Ras-GDP into Ras-GTP. Ras then cooperates in activation of a serine/threonine kinase, Raf-1, which phosphorylates the dual specificity MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK), which in turn phosphorylates and activates the ERKs, ERK1 and ERK2. This is in conjunction with the PI3 activated pathway as both the Ras-MAPK and PI3 pathways seem to independently activate ERK (York et al., 1998; York et al., 2000). Activation of Erk occurs rapidly through the Ras-MAPK pathway, whereas a sustained activation is mediated by the PI3-kinase pathway (York et
Erk activation is responsible for the NGF responsiveness of PC12 cells (York et al., 1998; York et al., 2000). Erks enter the nucleus, phosphorylate and thus activate existing c-jun and also induce expression of more c-jun, which leads to differentiation (Leppa et al., 1998).

PC12 cells again serve as excellent models in which to study the intricate molecular mechanisms underlying the role of nicotinic acetylcholine receptors in neuronal differentiation. Upon the addition of nerve growth factor (NGF), PC12 cells stop dividing, become electrically excitable, send out neuritis and differentiate into a phenotype not unlike the sympathetic neuron (Dichter et al., 1977; Green and Tishcler, 1982; Halegoua et al., 1992). The different nAChR subunit genes respond to NGF differently based on their expression. Upregulation, and also downregulation of some genes is seen.

**Effect of NGF on the α7 nAChR mRNA level**

In PC12 cells, various laboratories have shown the upregulation of nAChR genes such as α3, α5, α7, β3, and β4 (Henderson et al., 1994; Hu et al., 1994; Nakayama et al. 2000). Recently, studies done on the expression
pattern of the β4 subunit in response to NGF indicate that NGF activated Ras-MAPK and PI3-kinase pathways serve to increase transcription of the β4 gene, whereas the cAMP activated pathway does not seem to have an effect. The target on the β4 gene promoter that NGF exerts its effects on, seems to be an Spl binding site, and is mediated by c-jun interacting with Spl (Hu et al., 1994; Melnikova and Gardner, 2001).

In the N21 variety of PC12 cells used in our laboratory, a different effect on the expression of the α7 nAChR gene is seen. An earlier study indicated an increase in the steady state α7 nAChR mRNA level in PC12 cells following NGF treatment (Henderson et al., 1994; Nakayama et al., 2000). However in the PC12 cells used in our laboratory, a significant decrease in the mRNA levels of α7 is seen following NGF treatment (Figure 24). This contradictory result maybe due to the differences in the subclones of PC12 cells used in the different laboratories and is not an uncommon phenomenon.
Figure 24. Effect of Nerve Growth Factor on the mRNA level of the α7 nAChR subunit.

Northern blot analysis of α7 nAChR message after NGF treatment in PC12 cells. The same blot was probed with cDNAs for Egr-1, Sp1, Sp3 and GAPDH. GAPDH mRNA levels do not seem to be affected by NGF treatment and hence were used to normalize the levels of mRNA in each lane.
Figure 24

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One of the common features of neuronal differentiation of PC12 cells by NGF is the rapid upregulation of immediate early response genes such as those for transcription factors c-fos, c-jun, and Egr-1 (Kendall, G. et al., 1994; Ito, E et al., 1989, 1990). These transcription factors then work to regulate delayed early genes whose products serve to mediate neuronal differentiation. In order to test if NGF stimulation of the N21 subclone of PC12 cells caused a similar response, the same blot that was used earlier was analyzed for the level of the Egr-1. As shown in Figure 24, the level of Egr-1 mRNA was induced several fold over that of the NGF untreated cells. This indicated that the PC12 subclone tested here did respond to NGF in the classical way. The mRNA levels of other transcription factors known to bind to the rat α7 gene promoter such as Sp1 and Sp3 remained unchanged following the NGF treatment.

The upregulation of other nAChR genes by NGF has been shown to be in part due to increase in transcription. Most of the increase in nAChR protein levels following NGF treatment seems to be due to post-translational events. NGF does increase protein stability, decreases protein turnover and increases
translational efficiency thus causing increased amount of protein from the same number of mRNA molecules.

To test the possible NGF mediated upregulation of transcription of the α7 gene at the promoter level, the 178bp luciferase promoter construct was transfected into PC12 cells and the amount of reporter protein produced by the construct in NGF untreated and treated cells was compared. Interestingly, instead of a decrease, NGF treated cells showed a 3-fold increase in promoter activity (Figure 25). This increase was also seen when the 1.3kb promoter construct was used. Thus, NGF seemed to increase the promoter activity of the α7 nAChR gene in PC12 cells indicating an increase in transcription.
Figure 25. Effect of NGF on the expression of the α7 nAChR gene promoter constructs.

NGF caused an increase in promoter activity in two of the constructs studied. The reporter assays were normalized to another reporter (Renilla Luciferase or β-galactosidase) to correct for transfection efficiencies.
In the face of increasing transcription, it is still possible to have low amounts of mRNA if the stability of the message somehow decreases due to NGF treatment. That is, the message becomes unstable and degrades quickly, hence, showing lower amounts of mRNA in Northern analysis.

To test if this was the case in NGF treated PC12 cells, a study was done to measure the α7 message stability. NGF untreated and treated cells were grown in the presence of Actinomycin D and total RNA isolated at various time points. Actinomycin D is a drug that inhibits RNA pol II mediated transcription. Thus, no new mRNA molecules are created and the existing molecules now degrade based upon their half-life, which can be measured by Northern analysis.

Using this study it was shown that upon the addition of NGF the α7 mRNA seemed to degrade quicker (have a shorter half-life) than that in untreated cells (Figure 26). Thus the increased instability of the α7 nACHr mRNA might go to explain the decrease in mRNA levels in spite of the increase in transcription.

Further studies need to be conducted to analyze the target on the α7 gene promoter that is acted upon.
Figure 26

Figure 26. α7 nAChR mRNA stability analysis in NGF untreated and treated PC12 cells.

mRNA isolated from NGF untreated and treated PC12 cells grown in the presence of 1μg/ml Actinomycin D at various time intervals was analyzed by Northern Blot using the α7 cDNA as probe. The signal produced by the α7 message was normalized to that produced by a GAPDH probe at the same time interval and plotted. Signals from untreated cells at the various time points are indicated by the squares whereas those produced by the treated cells are indicated by triangles. The bold and dashed lines indicate the best fit-line to accommodate these points in untreated and treated cells respectively.
Summary

NGF treatment of PC12 cells for 24 hours lead to a decrease in the \( \alpha 7 \) nAChR mRNA levels. This decrease was accompanied by an increase in mRNA levels of Egr-1. mRNA levels of other transcription factors such as Sp1 and Sp3 remained unchanged. NGF treatment of PC12 cells lead to an increase in promoter activity of the minimal promoter indicating an increase in transcription. However the reduced levels of \( \alpha 7 \) mRNA could be due to its reduced mRNA half-life after NGF treatment. Thus although NGF increases \( \alpha 7 \) transcription the message is more unstable leading to quicker degradation resulting in lower levels of \( \alpha 7 \) mRNA.
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