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ELUCIDATING THE TRANSCRIPTIONAL NETWORK
UNDERLYING EXPRESSION OF A NEURONAL NICOTINIC
RECEPTOR GENE

A Dissertation Presented

By

MICHAEL D. SCOFIELD

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

(September 8th, 2010)

INTERDISCIPLINARY GRADUATE PROGRAM

**ELUCIDATING THE TRANSCRIPTIONAL NETWORK UNDERLYING
EXPRESSION OF A NICOTINIC RECEPTOR GENE**

A Dissertation Presented By

Michael D. Scofield

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September 8, 2010

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my mentors, Paul Gardner and Andrew Tapper, who have provided me with the support and direction needed to set the foundation for an independent career. I would also like to thank all the members of the Gardner / Tapper lab for their support and advice, and also for making our lab a great place to work.

I would like to thank my dissertation committee, Daniel Kilpatrick, Anthony Imbalzano, Haley Melikian and Ann Rittenhouse for devoting their time and effort and also for providing valuable insight and advice. In addition, I would like to thank Robert Maue for serving on my committee and also for coming out from Dartmouth Medical School for my defense.

Finally, I would like to acknowledge my friends and family, especially my wife Deidre Scofield for her continued support and undying interest in my research.

ABSTRACT

Neuronal nicotinic acetylcholine receptors (nAChRs) are involved in a plethora of fundamental biological processes ranging from muscle contraction to the formation of memories. The studies described in this work focus on the transcriptional regulation of the CHRN4 gene, which encodes the $\beta 4$ subunit of neuronal nAChRs. We previously identified a regulatory sequence (5'– CCACCCCT –3'), or "CA box", critical for CHRN4 promoter activity *in vitro*. Here I report transcription factor interaction at the CA box along with an *in vivo* analysis of CA box transcriptional activity. My data indicate that Sp1, Sp3, Sox10 and c-Jun interact with the CHRN4 CA box in the context of native chromatin. Using an *in vivo* transgenic approach in mice, I demonstrated that a 2.3-kb fragment of the CHRN4 promoter region, containing the CA box, is capable of directing cell-type specific expression of a reporter gene to many of the brain regions that endogenously express the CHRN4 gene. Site-directed mutagenesis was used to test the hypothesis that the CA box is critical for CHRN4 promoter activity *in vivo*. Transgenic animals were generated in which LacZ expression is driven by a mutant form of the CA box. Reporter gene expression was not detected in any tissue or cell type at ED18.5. Similarly, I observed dramatically reduced reporter gene expression at PD30 when compared to wild type transgenic animals, indicating that the CA box is an important regulatory feature of the CHRN4 promoter. ChIP analysis of brain tissue from mutant transgenic animals demonstrated that CA box mutation results in decreased interaction of the transcription factor Sp1 with the CHRN4 promoter.

I have also investigated transcription factor interaction at the CHRNA4 promoter CT box, (5' – ACCCTCCCCTCCCCTGTAA –3') and demonstrated that hnRNP K interacts with the CHRNA4 promoter in an olfactory bulb derived cell line.

Surprisingly, siRNA experiments demonstrated that hnRNP K knockdown has no impact on CHRNA5, CHRNA3 or CHRNA4 gene expression. Interestingly, knockdown of the transcription factor Pur α results in significant decreases in CHRNA5, CHRNA3 and CHRNA4 mRNA levels. These data indicate that Pur α can act to enhance expression of the clustered CHRNA5, CHRNA3 and CHRNA4 genes. Together, these results contribute to a more thorough understanding of the transcriptional regulatory mechanisms underlying expression of the CHRNA4 as well as the CHRNA5 and CHRNA3 genes, critical components of cholinergic signal transduction pathways in the nervous system.

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LIST OF FREQUENTLY USED ABBREVIATIONS

Abbreviation	Term
A3I5	Transcriptional repressor located in the fifth intron of the CHRNA5 gene
ACh	Acetylcholine
α -Bgtx	α -bungarotoxin
AU1B	Alpha-conotoxin derived from the venom of the predatory sea snail <i>Conus aulicus</i>
β 2m	Beta-2-microglobulin
β -gal	Beta-galactosidase
CA Box	A 5'-CCACCCCT-3' regulatory element
ChIP	Chromatin immunoprecipitation
CHRNB4	Neuronal cholinergic receptor β 4 subunit gene
CHRNA5/A3/B4	The locus that encodes the CHRNA5, CHRNA3 and CHRNB4 genes
CNR4	Conserved non-coding region #4
CNS	Central nervous system
CT Box	A 5'-ACCCTCCCCTCCCCTGTAA-3' regulatory element
DRG	Dorsal root ganglia
ED18.5	Embryonic day 18.5
ETS	E26 transformation-specific sequence
GABA	γ -aminobutyric acid

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GTP	Guanosine triphosphate
GWAS	Genome wide association study
hnRNP K	Heterogeneous nuclear ribonucleoprotein K
IgG	Immunoglobulin G
KO	Knock out
LacZ	Beta-galactosidase
MAR	Matrix attachment region
nAChR	Nicotinic acetylcholine receptor
NGF	Nerve growth factor
NLS	Nuclear localization sequence
NMJ	Neuromuscular junction
PAC	P1-derived artificial chromosome
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PD30	Postnatal day 30
PNS	Peripheral nervous system
qRT-PCR	Real-time quantitative reverse transcription PCR
SCG	Superior cervical ganglion
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNP	Single nucleotide polymorphism

CHAPTER I: INTRODUCTION

Neuronal communication

The transmission of signals through synapses is an essential component of the neuronal circuits responsible for a wide array of physiological functions including memory formation, reward, reinforcement, perception, and emotion (Leisman and Koch, 2009). The discovery of the synapse would not have been possible without the advancements in staining techniques pioneered by the Italian anatomist Camillo Golgi and later refined by the Spanish anatomist Santiago Ramón y Cajal. Using the “reazione nera” or black reaction, Golgi was able to stain the membranes of neuronal tissue with a silver-chromate precipitate (Golgi, 1873). This technique stained the soma, axon and dendrites of a neuron black allowing anatomists to observe the morphological aspects of neurons in stunning detail. However, due to limitations in the staining procedure, myelinated axons were not stained effectively, making the projections of adjacent neurons difficult to resolve. The limitations of Golgi’s staining technique undoubtedly contributed to his sustained defense of Joseph von Gerlach’s reticular hypothesis (von Gerlach, 1871), a widely accepted theory at the time proposing that the brain consists of a large network of directly interconnected cells. Cajal refined Golgi’s silver-chromate staining and also used brain tissue from younger animals in order to minimize the amount of myelinated axons in the preparation. The resulting drawings of neural tissue (Fig. 1.1) clearly demonstrated that neurons are independent elements and not a network of interconnected filaments (Cajal, 1888, 1894).

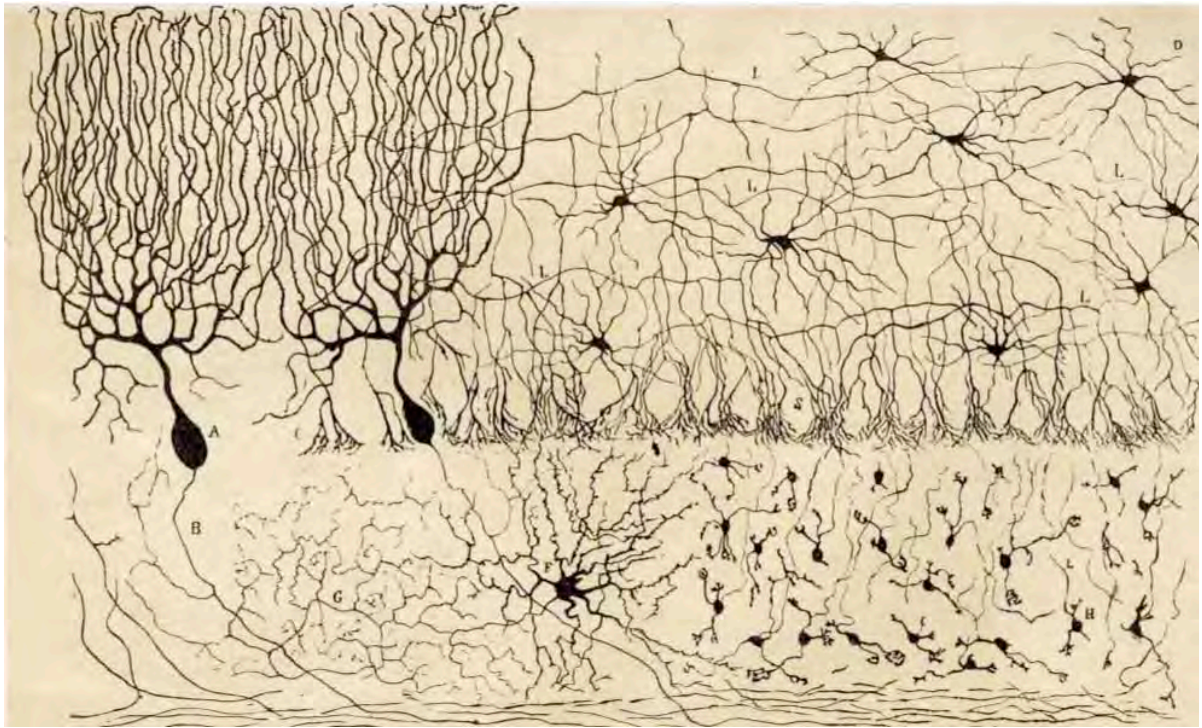


Figure 1.1: Chick cerebellum. Shown here is a drawing of neurons in the chick cerebellum done by Ramón y Cajal (Cajal, 1888; Sotelo, 2003).

Cajal's findings quickly gained the attention of several prominent German anatomists including Wilhelm von Waldeyer and Rudolph Albert von Kölliker, who had both previously been ardent supporters of the reticular hypothesis. Cajal's work also provided the fundamental basis for the "neuron doctrine" proposed by Waldeyer (Waldeyer, 1891) in a seminal review article combining the observations of several scientists including Albert von Kölliker, Camillo Golgi, Franz Nissl, Auguste Forel, Wilhelm His and Santiago Ramón y Cajal. Specifically, the neuron doctrine refuted the reticular hypothesis by stating that neurons are connected to each other at distinct sites of contact (later defined as synapses) and *not* through a cytoplasmic continuity. The neuron doctrine also states that the neuron is the structural and functional unit of the nervous system and has three main parts, the dendrites, soma and axon, with conduction taking place in the direction of dendrite to soma, ending in the arborizations of the axon (Waldeyer, 1891).

The synapse

The term "synapse" was derived from "synaptein" a term coined by the British physiologist Charles S. Sherrington. "Synaptein" is a combination of the Greek words "syn-" meaning together, and "haptein" to clasp (Sherrington, 1906). Although first described histologically by Cajal, the synapse would not be fully resolved until the invention of the electron microscope and the subsequent work of George Palade, Eduardo de Robertis and George Bennett (De Robertis and Bennett, 1955). Electron micrographs of the frog sympathetic ganglia and the neuropile of the

earthworm nerve cord showed for the first time, in high magnification, detailed images of the synaptic ultrastructure. These experiments confirmed the seemingly clairvoyant hypotheses regarding the synapse and neuronal communication made by anti-reticularists like His, Nansen, Forel, and Cajal. Neuronal signals are transmitted through two main types of synapses, the electrical synapse and the chemical synapse (Kandel et al., 2000).

Many scientists in the early 20th century assumed that electrical synaptic transmission was the most likely method of neuronal communication. However, proof of the existence of the electrical synapse would not come until the 1950s with the work of Furshpan and Potter, demonstrating functional electrical synapses in the giant neurons of the crayfish (Furshpan and Potter, 1957, 1959). The electrical synapse is a conductive link formed at a 3.5 nm gap between the presynaptic and postsynaptic cell (Bennett and Zukin, 2004). At the electrical synapse, several receptor proteins span the membranes of both cells, effectively connecting the cytosol of the pre- and postsynaptic cells and allowing for the flow of electrical current. Signal transduction through electrical synapses is characterized by rapid conduction of nerve impulses, bi-directional signal transmission, and lack of inhibitory action (Bennett and Zukin, 2004). Despite the incredibly fast rate of transmission, electrical synapses cannot amplify the magnitude of the incoming signal from the presynaptic cell, *i.e.*, the induced response in the postsynaptic cell is always smaller than the original signal from the presynaptic cell (Kandel et al., 2000).

Signal transduction through chemical synapses is the primary mode of neuronal communication and is essential for the function of the neural circuits responsible for perception and thought (Leisman and Koch, 2009). Unlike the electrical synapse, the chemical synapse is unidirectional, can either be inhibitory or excitatory and can amplify the extent of the incoming signal. The chemical synapse has several key features: first, a presynaptic neuron that when activated releases a chemical messenger called a neurotransmitter into the synaptic cleft; second, a physical space of approximately 20 – 40 nm that separates the pre- and postsynaptic cells through which neurotransmitters diffuse; and finally, receptor elements that recognize the neurotransmitters released from the presynaptic cell and initiate signal transduction in the postsynaptic cell (Fig. 1.2) (Kandel et al., 2000).

Concrete evidence for chemical signal transmission was not uncovered until 1921 when the German pharmacologist Otto Loewi performed his famous experiment using a frog heart nerve-muscle preparation (Loewi, 1921). Loewi dissected two beating frog hearts, the first heart with the vagus nerve still attached and the second extracted on its own. He then placed each heart in a saline solution and then electrically stimulated the vagus nerve on the first heart, resulting in a decrease in heart rate. When Loewi applied some of the liquid surrounding the first heart (“Vagusstoff”) to the second heart, he also observed a reduction in heart rate.

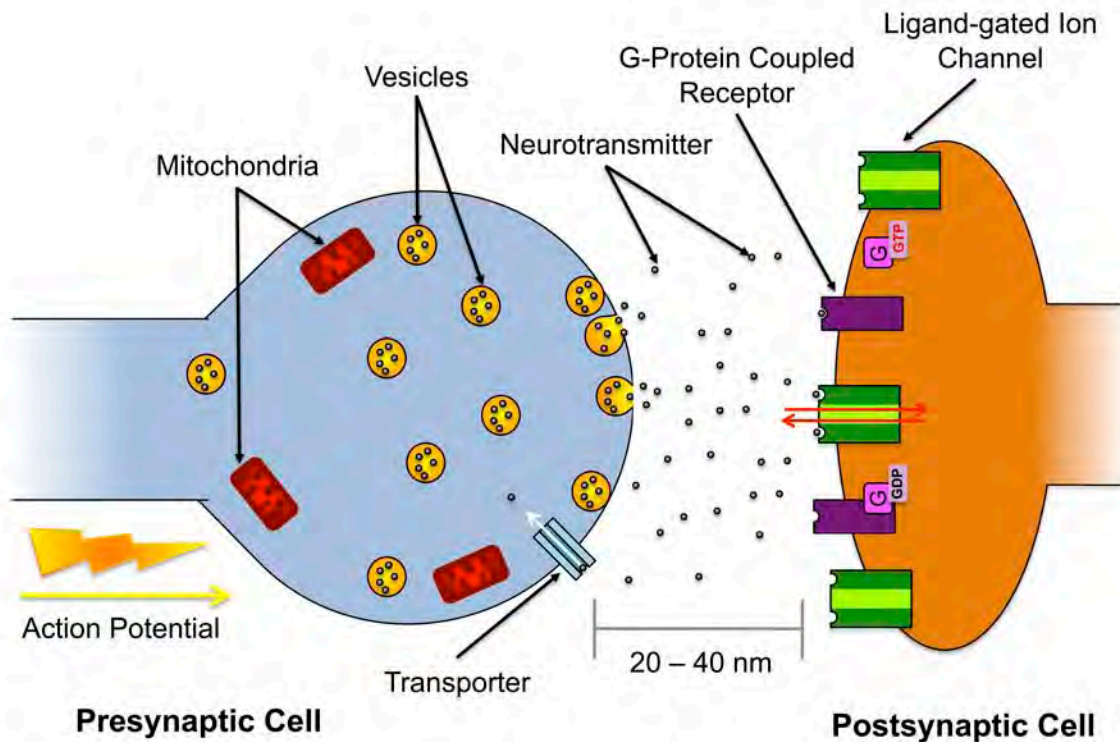


Figure 1.2: The chemical synapse. Schematic representation of the synapse with the presynaptic nerve cell depicted in blue and the postsynaptic cell depicted in orange. In the presynaptic cell, synaptic vesicles are depicted as yellow circles with neurotransmitters depicted as smaller grey circles, mitochondria are depicted as red rectangles. In the postsynaptic cell, ligand-gated ion channels are depicted as green rectangles with flow of ions through activated channels depicted as red arrows. G-protein coupled receptors are depicted as purple rectangles with the inactive G-protein, shown in pink, bound to GDP and the active G-protein bound to GTP (red) disassociated from the G-protein coupled receptor. The direction of the propagation of the action potential is shown under the presynaptic cell as a lightning bolt and a yellow arrow.

These results indicated that a soluble chemical messenger from the vagus nerve was released upon electrical stimulation and that application of this chemical messenger could slow the heart rate of an un-stimulated heart (Loewi, 1921; Loewi and Navratil, 1926).

Additional evidence supporting chemical neurotransmission was provided by Henry Hallett Dale, who suggested that acetylcholine (ACh) may function as chemical neurotransmitter in 1914 (Dale, 1914; Fishman, 1972). Years later while studying the physiological effects of ergot, a fungus that grows on rye and related plants, Dale discovered that the active component of the ergot fungus could profoundly inhibit heart rate when intravenously injected into an anesthetized cat (Dale, 1953). During his experimentation with ergot, he began to notice that the physiological effects elicited by the unknown alkaloid from the fungus appeared to be identical to those elicited by ACh. These results caused Dale to focus his attention on testing the hypothesis that ACh acts as a chemical neurotransmitter. In a series of experiments performed between 1929 and 1936, he was able to demonstrate that ACh is released at nerve endings following electrical stimulation (Dale, 1935) and that application of ACh to muscle tissue resulted in contraction in the absence of electrical stimulation (Dale et al., 1936). Despite the emerging body of evidence supporting the existence of chemical transmission, many scientists still believed that neuronal communication was mediated by electrical synapses. Bernard Katz provided definitive proof of chemical transmission in a series of experiments using intracellular electrodes to study the neuromuscular junction (NMJ), a specialized

synapse formed between motor neurons and muscle fibers. These experiments demonstrated that the fluctuations in membrane potential observed while recording from the muscle fiber were the result of the random release of synaptic vesicles containing ACh from the motor neuron (Fatt and Katz, 1951). Katz later demonstrated that chemical synaptic transmission at the NMJ was unidirectional and that exogenous application of ACh to the NMJ resulted in muscle contraction, similar to what is observed following electrical stimulation of the motor neuron (Katz, 1969). These data confirmed the hypotheses made years earlier by Loewi and Dale regarding ACh and chemical neurotransmission.

Neurotransmitters and their receptors

Neurotransmitters are defined as endogenous chemical messengers that relay, amplify and modulate neuronal signals (Purves et al., 2008). In order for a molecule to be considered a neurotransmitter it must fulfill four specific criteria: first, the compound is synthesized in the neuron; second, it is present in the presynaptic terminal and is released in amounts that are sufficient to exert a defined response in the postsynaptic cell; third, exogenous application of the substance in question at a reasonable concentration must mimic exactly the action of the endogenously released transmitter; finally, a method of inactivation or clearance must exist to remove the chemical from the synaptic cleft after it is released (Kandel et al., 2000). Neurotransmitters can be divided into three main classes; amino acid neurotransmitters, biogenic amines and neuroactive peptides (Kandel et al., 2000).

The most abundant neurotransmitter in the central nervous system (CNS) is the amino acid neurotransmitter glutamate, which like other amino acid transmitters including glycine, γ -aminobutyric acid (GABA), aspartate and serine, can be synthesized at nerve terminals from non-essential amino acids (Purves et al., 2008). Glutamate is the primary excitatory neurotransmitter in the brain. Neurons that use glutamate as a neurotransmitter are located in a vast array of brain regions. GABA is also predominant in the CNS and plays a mainly inhibitory role in the brain, which is likely a consequence of the fact that GABA is the primary neurotransmitter for inhibitory interneurons (Kandel et al., 2000). Amino acid neurotransmitters like glutamate and GABA are responsible for the majority of fast synaptic transmission in the CNS. Disruption of amino acid neurotransmission may contribute to the development of neurological disorders. Specifically, perturbations of inhibitory signaling mediated by GABA in the brain can result in seizures. Furthermore, disruption of GABA signaling has also been implicated in anxiety disorders, premenstrual dysphoric disorder, and schizophrenia (Wong et al., 2003).

The second class of neurotransmitters is the biogenic amines, which include dopamine, epinephrine, norepinephrine and serotonin. These neurotransmitters are small charged molecules derived from amino acids (Purves et al., 2008). Typically, these neurotransmitters are produced in short biosynthetic pathways that are regulated at a single enzymatic step. Mature neurons that participate in biogenic amine neurotransmission typically only express one type of “controlling enzyme” (Kandel et al., 2000). Disruption of this type of neuronal signaling has been

implicated in several neurological and psychiatric disorders and interestingly, modification of biogenic amine signaling is also the mechanism of action for several drugs of abuse including cocaine and methamphetamine (Deadwyler, 2010; Yamamoto et al., 2010).

Unlike amino acid neurotransmitters and biogenic amines, neuroactive peptides such as insulin, substance P and neuropeptide Y cannot be synthesized at nerve terminals and must instead be derived from secretory proteins that are assembled and processed in the cell body. Once processed, these peptides are loaded into vesicles and transported to nerve terminals where, like the other classes of neurotransmitters, they are released into the synaptic cleft following activation of the presynaptic cell. Neuroactive peptide signaling can result in either excitation or inhibition of target cells. Signaling with these molecules has been implicated in regulation of sensory perception and emotions (Katsouni et al., 2009; Pape et al., 2010).

Neurotransmitters evoke a response in the postsynaptic cell by interacting with members of a diverse group of proteins called neurotransmitter receptors. These proteins are embedded in the postsynaptic membrane and recognize specific neurotransmitter molecules. Once activated by the binding of neurotransmitter, these receptor proteins modify the electrical properties of the postsynaptic cell by either opening or closing ion channels. In this manner, the chemical signal mediated by neurotransmission from the presynaptic cell can be converted back into an electrical

signal in the postsynaptic cell. Molecules that interact with a receptor and initiate functional changes in the physiological properties of the postsynaptic cell are called agonists, whereas those that bind competitively or block the interaction of neurotransmitters with their receptors are called antagonists.

At present, neurotransmitter receptors can be classified into two main superfamilies based on their mechanism of action following the binding of the ligand. The first neurotransmitter receptor superfamily consists of ligand-gated ion channels.

Ionotropic receptors allow for the flow of ions through a central pore as a result of the binding of neurotransmitter. The second neurotransmitter receptor superfamily, known as metabotropic receptors, initiates a signal cascade following ligand binding that has a downstream effect on ion permeability. Metabotropic receptors will not be discussed at length in this work. Ligand gated ion channels are typically multimeric structures consisting of two main functional units, an extracellular segment that recognizes the neurotransmitter and a membrane-spanning domain that forms a pore through which ions flow. Upon binding of ligand, the channel protein goes through a conformational change allowing for the flow of ions (Unwin, 1993).

Signaling through these types of receptors occurs rapidly and usually only lasts for a few milliseconds (Purves et al., 2008).

The Cys-loop superfamily

The Cys-loop superfamily constitutes a major class of ligand gated ion channels in mammals and comprises both cationic (acetylcholine (ACh) and 5-

hydroxytryptamine (5-HT₃) receptors) and anionic (GABA and glycine receptors) channels (Connolly and Wafford, 2004). Mature Cys-loop receptors are pentameric structures assembled with the combination of five subunits arranged around a central pore. These receptors can either be homomeric (assembled with 5 copies of a single subunit protein) or heteromeric (assembled with combinations of several subunits). Subunits in the Cys-loop superfamily have an extracellular amino-terminus, four transmembrane domains with a variable intracellular loop between transmembrane segments three and four and an extracellular carboxyl-terminus (Sine and Engel, 2006). The term “Cys-loop” refers to a characteristic sequence of 13 residues linked by covalently bound cysteines forming a closed loop, located in the extracellular amino terminus of each individual receptor subunit protein (Connolly and Wafford, 2004). Cys-loop receptor subunits are constructed on a modular basis, with the extracellular domain forming the agonist binding sites and the transmembrane domain containing the channel gate, which regulates ion selectivity and pore, through which ions flow (Miller and Smart).

Interestingly, the genes encoding of the Cys-loop receptor superfamily subunits are distributed in two monophyletic groups (Connolly and Wafford, 2004), with the subunits that form anionic channels (e.g., GABA_A, and Glycine,) in the first group and the subunits that form cationic channels (e.g., ACh and 5-HT₃ receptors) in the second. In mammals, sequences of six α , three β , three γ , one δ , three ρ , one ϵ , one π and one ψ GABA_A receptor subunits have been reported (Barnard et al., 1998) with four isoforms of an α -subunit (α 1 – α 4) and one variant of a β -subunit (β 1)

reported for Glycine channels (Moss and Smart, 2001). A total of a total of 17 ACh receptor subunits (α 1-10, β 1-4, δ , ϵ and γ) have been identified (Albuquerque et al., 2009) whereas just three 5HT₃ receptor subunits have been reported (5-HT_{3A}, 5-HT_{3B} and 5-HT_{3C}) (Reeves and Lummis, 2002).

Though not part of the Cys-loop superfamily, an additional class of ionotropic channels gated by the excitatory neurotransmitter glutamate also exists (Kandel et al., 2000). Glutamate receptors include members of the N-methyl-D-aspartate (NMDA), α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and kainate receptor classes (Madden, 2002). Like the Cys-loop receptors, glutamate receptors can also be homomeric or heteromeric. However, these receptors are assembled into cation-selective tetramers as opposed to the pentameric organization observed in the Cys-loop receptor family. Furthermore, the structure of the individual glutamate receptor subunits also differs from what is observed in the Cys-loop family. Glutamate receptor subunits have an extracellular N-terminus, three transmembrane domains (TM1, TM3 and TM4), a channel lining re-entrant 'p-loop' located between TM1 and TM3, and an intracellular C-terminus (Traynelis et al., 2010).

Muscle nicotinic receptors

The concept of a receptor protein responsible for the recognition and transmission of a signal from a neuron to a muscle fiber was first hypothesized in the early 20th century by John Newport Langley (Langley, 1905). Langley developed the idea of a

“receptive substance” from the observation that nicotine, the addictive component of tobacco, caused tonic contraction of denervated muscles in the frog, fowl, and toad, which could be blocked by the poison, curare (Langley, 1909b, a, 1914). From these experiments Langely concluded that the “receptive substance of muscle” received stimuli from the nerve cell, and transferred it to the muscle cell, ultimately resulting in contraction (Maehle, 2004). The muscle type nicotinic acetylcholine receptor (nAChR) was the first ligand-gated ion channel protein to be isolated and purified. Using the electric organ of the eel *Electrophorus*, Jean Pierre Changeux and colleagues were able to isolate the receptor protein in 1970. Purification was achieved by exploiting the receptor’s high affinity for α -bungarotoxin (α -Bgtx), a component of the venom of the *Bungaris Multicinctus* snake that competitively and irreversibly binds to the receptor (Changeux et al., 1970). Further experiments demonstrated that the muscle type nicotinic receptor is a membrane spanning allosteric glycoprotein with an approximate molecular weight of 275,000 Da (Weill et al., 1974). Muscle type nicotinic receptors are pentameric structures consisting of five types of polypeptide chains (α , β , γ , δ and ϵ). As discussed above, each muscle nAChR subunit protein contains four putative transmembrane spanning domains deemed M1-M4 (Fig. 1.3 A), with the M2 segment of each subunit in the pentameric receptor contributing to the pore-forming domain of the pentameric receptor (Fig. 1.3 B) (Albuquerque et al., 2009). The individual muscle type nAChR subunits were named in accordance to the speed of their migration in protein gels, with the α subunit migrating the fastest and the δ subunit migrating the slowest (Klett et al., 1973; Karlin, 1974; Heidmann and Changeux, 1978).

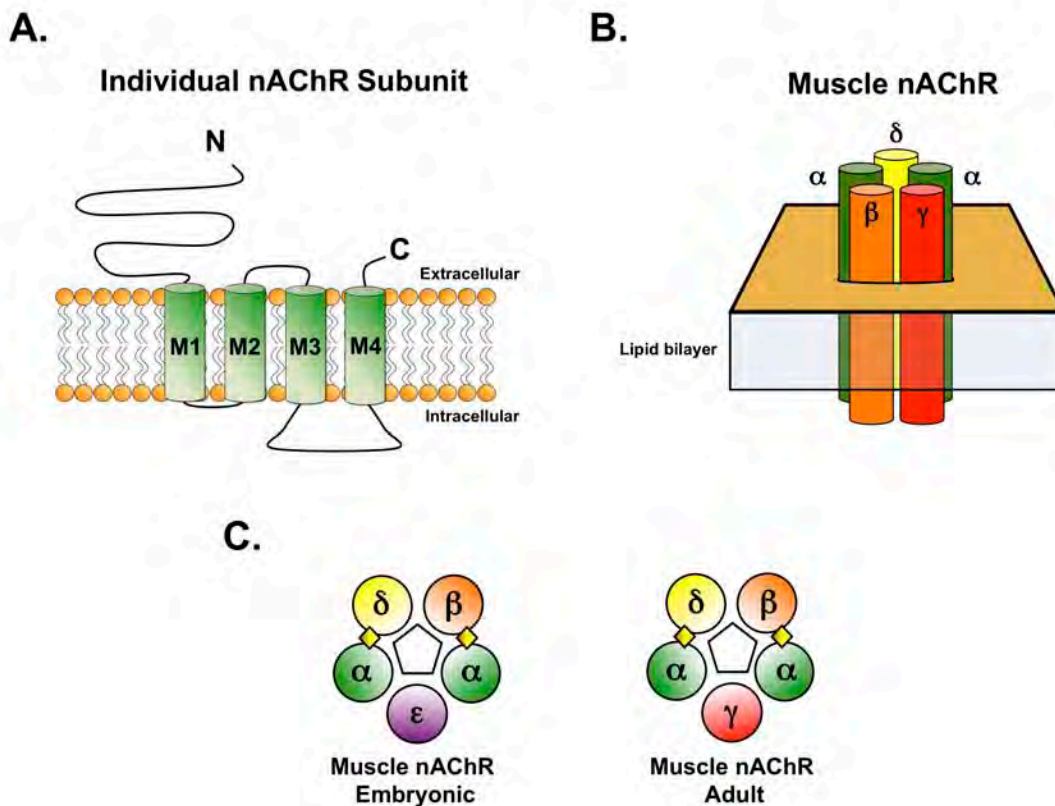


Figure 1.3: Muscle-type nAChR subunits and subtypes. (A) Schematic representation of an individual nAChR subunit protein. The amino and carboxy terminals of the protein are labeled N and C, respectively, with the transmembrane segments labeled M1 – M4. (B) Schematic representation of the pentameric muscle-type nAChR, with individual subunits depicted as colored cylinders. (C) Subunit composition of the muscle-type nAChRs during development (left) and in adulthood (right). Individual receptor subunits are represented as colored circles. Diamonds located between adjacent receptor subunits represent ligand-binding sites while pentagons in the center of each pentamer represent the pore region.

Mature muscle nAChR subunits are assembled in the stoichiometric combination of $\alpha_2\beta\gamma\delta$ early in development and $\alpha_2\beta\epsilon\delta$ at adulthood (Takai et al., 1985; Mishina et al., 1986) (Fig. 1.3 B, C). Each receptor has two ACh (ligand) binding sites, which are located at the interface of an α and a non- α subunit (Fig. 1.3 C). The receptor itself can occupy three distinct functional states: the resting state, where it has low affinity for agonists and the ion channel is closed; the active state, where agonist occupies the ligand binding sites and the channel is open, allowing for the flow of ions; and finally, the desensitized state, where the channel is closed and the receptor is no longer responsive to ligand (Albuquerque et al., 2009).

Muscle type nAChRs are crucial for the transmission of nerve signals to skeletal muscle and in turn underlie the molecular machinery responsible for the operation of the voluntary and involuntary muscle response (Kandel et al., 2000). Disruption of neuronal communication through muscle type nAChRs can result in neuromuscular disorders that effect muscle control like Myasthenia Gravis. In Myasthenia Gravis the observed decline of the voluntary muscle response is a result of the destruction of muscle type nAChRs at the NMJ caused by an abnormal immune response (Lindstrom, 2000b).

Neuronal nicotinic acetylcholine receptors

Evidence for the existence of several functionally distinct classes of nAChRs in neuronal tissue was uncovered during early autoradiography experiments that compared the binding of [^3H] – ACh and [^3H] – Nicotine to [^3H] - α -Bgtx in rodent and

chick brains (Marks and Collins, 1982). These experiments demonstrated that there were nAChRs in nervous tissue and that there appeared to be at least two classes of neuronal nicotinic receptors, those that were sensitive to α -Bgtx and those that were not (Marks and Collins, 1982). Norman and colleagues were the first to purify a neuronal nicotinic receptor protein from the chick optic lobe in 1982, using α -Bgtx as a ligand (Norman et al., 1982). Whiting and Lindstrom purified the first α -Bgtx insensitive receptor from chicken brains in 1986. Purification of the α -Bgtx insensitive receptor protein was achieved with affinity chromatography, using a monoclonal antibody that recognizes the muscle-type nAChR protein (Whiting and Lindstrom, 1986). The α -Bgtx insensitive receptor purified from chick brain had high affinity for nicotine, was resistant to desensitization and was comprised of two subunits. These results demonstrated the existence of nAChRs in the brain that were pharmacologically and functionally distinct from their muscle-type counterparts. While performing a low stringency screen of a cDNA library using the muscle-type nAChR subunit α , Boulter and colleagues isolated a cDNA clone for a neuronal nAChR subunit (Boulter et al., 1986). These experiments set the stage for the discovery of an entire family of neuronal nAChR subunit genes in rapid succession (Heinemann et al., 1990; Gotti and Clementi, 2004; Gotti et al., 2007; Albuquerque et al., 2009).

The neuronal nAChR gene family

Like the muscle-type receptor subunits, each neuronal nAChR gene encodes a protein consisting of an extracellular N-terminus, four transmembrane segments

(M1-M4), a variable intracellular loop between the M3 and M4 transmembrane segments as well as an extracellular C-terminus (Corringer et al., 2000). Similar to what is observed in the muscle receptors, five neuronal nAChR subunits co-assemble to form ligand-gated channels (Cooper et al., 1991; Brejc et al., 2001). However, the diversity of the neuronal nAChR subunit gene family allows for a vast array of subunit combinations, with the biophysical and pharmacological properties of a given receptor subtype determined by subunit composition (McGehee and Role, 1995). At present, 12 members of the nAChR subunit gene family exist including CHRNA2 – CHRNA10 and CHRNB2 – CHRNB4 (Fig. 1.4) (Dani and Bertrand, 2007b; Albuquerque et al., 2009). The α 2 – α 6 subunits can form functional receptors in combination with the β 2 – β 4 subunits while the α 7 – α 10 subunits are capable of forming homomeric nAChRs. Two classes of unique heteromeric receptors have been discovered recently, which are assembled with the combination of the α 9 and α 10 subunits (Elgoyhen et al., 2001; Lustig et al., 2001) or the α 7 and β 2 subunits (Liu et al., 2009b). The functional diversity exhibited by the neuronal nAChR family is a consequence, in large part, of the differential expression of the various subunit genes leading to the incorporation of distinct subunits into mature receptors (Albuquerque et al., 2009). Thus, the incorporation of a particular subunit into a mature receptor is controlled, at least in part, by the transcriptional regulation of the nAChR subunit genes.

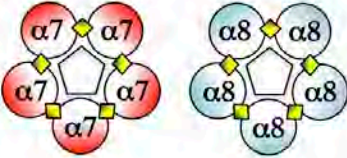
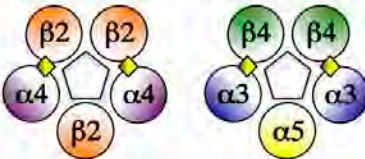
Subtype	Physiology	Main locations
<p data-bbox="402 373 545 401">Homomeric</p>  <p data-bbox="391 611 565 636">e.g., $\alpha 7$ and $\alpha 8$</p>	<p data-bbox="672 411 850 438">α-BgT sensitive</p> <p data-bbox="672 459 829 487">$P_{Ca^{2+}}/P_{Na^{+}} \sim 10$</p> <p data-bbox="672 508 883 535">Nicotine > Cytisine</p> <p data-bbox="672 556 1029 583">Rapid desensitization ($t=20$ ms)</p>	<p data-bbox="1057 411 1365 438">$\alpha 7$: Limbic system, cortex</p> <p data-bbox="1057 459 1338 487">$\alpha 8$: Only found in chick</p> <p data-bbox="1057 508 1382 535">$\alpha 9$: Cochlea, olfactory bulb</p> <p data-bbox="1057 556 1393 583">$\alpha 10$: Cochlear inner hair cells</p>
<p data-bbox="399 684 550 711">Heteromeric</p>  <p data-bbox="345 968 605 995">e.g., $\alpha 4\beta 2$ and $\alpha 5\alpha 3\beta 4$</p>	<p data-bbox="672 716 873 743">α-BgT insensitive</p> <p data-bbox="672 764 841 791">$P_{Ca^{2+}}/P_{Na^{+}} \sim 1-3$</p> <p data-bbox="672 812 938 840">$\beta 2$-containing nAChR:</p> <p data-bbox="672 840 883 867">Cytisine < Nicotine</p> <p data-bbox="672 867 1029 894">Fast desensitization ($t=200$ ms)</p> <p data-bbox="672 947 938 974">$\beta 4$-containing nAChR:</p> <p data-bbox="672 974 883 1001">Cytisine > Nicotine</p> <p data-bbox="672 1001 1029 1029">Slow desensitization ($t=1.2$ s)</p>	<p data-bbox="1057 716 1354 770">$\alpha 5$: Cortex, monoamine-containing nuclei</p> <p data-bbox="1057 791 1393 846">$\alpha 3\beta 4$: PNS, habenulo-interpeduncular system</p> <p data-bbox="1057 867 1321 894">$\alpha 4\beta 2$: Thalamus, cortex</p> <p data-bbox="1057 926 1382 980">$\alpha 6\beta 3$: Monoamine-containing nuclei</p> <p data-bbox="1057 1001 1393 1029">$\alpha 2$: Interpeduncular nucleus</p>

Figure 1.4: Neuronal nAChR subtypes. Schematic representations of homomeric and heteromeric receptor subtypes depicted as groups of colored circles with ligand-binding sites depicted as yellow diamonds and the pore region depicted as an unfilled pentagon. Biophysical characteristics of homomeric and heteromeric nAChR subtypes are provided in the center column with primary regions of expression for specific subtypes listed in the right most column (Leonard and Bertrand, 2001; Wang and Sun, 2005).

The function of neuronal nAChRs is determined, in part, by the sub-cellular localization of the receptor. nAChRs are located at the soma, where activation directly contributes to excitability through the influx of sodium and calcium (Pidoplichko et al., 1997), at presynaptic terminals, where they can modulate neurotransmitter release (McGehee et al., 1995), and also at postsynaptic terminals, where they receive signals from cholinergic neurons (Albuquerque et al., 2009). The targeting of nAChRs is regulated by the interaction of cellular trafficking proteins with residues in the intracellular loop between the M3 and M4 transmembrane spanning domains of individual receptor subunit proteins (Dani and Bertrand, 2007b).

Characterization of the biophysical and pharmacological properties of nAChRs was made possible by the use of heterologous expression systems (McGehee and Role, 1995). By injecting nAChR mRNA or cRNA into *Xenopus* oocytes, nAChR subunits can be expressed either independently or in combination with other subunits. These experiments demonstrated that the $\alpha 7$, $\alpha 8$, and $\alpha 9$ subunits are able to form functional homomeric receptors, while other α subunits require the presence of an additional β subunit to form functional receptors (Couturier et al., 1990b; Gerzanich et al., 1997). Functional heteromeric nAChRs result from co-expression of CHRNA2 – CHRNA6 with the CHRNB2 – CHRNB4 nAChR subunit genes (Boulter et al., 1987; Wada et al., 1988). When activated by agonist, nAChRs allow for the flow of monovalent and divalent cations eliciting a rapid inward current that desensitizes (Barnard et al., 1982) and is potentiated by calcium ions (Vernino et al., 1992). Most high affinity nAChRs in the CNS contain the $\alpha 4$ and $\beta 2$ subunits while most low

affinity receptors are presumably homomeric $\alpha 7$ receptors (Albuquerque et al., 2009).

The CHRNA5/A3/B4 gene cluster

Couturier and colleagues were the first to discover the colocalization of the CHRNA5, CHRNA3 and CHRNB4 genes in the avian genome. Shortly after, a remarkably similar CHRNA5/A3/B4 cluster of homologous genes was discovered in the rat genome by Boulter and colleagues (Boulter et al., 1990). It was later elucidated that the clustering of these three nAChR subunit genes is conserved throughout vertebrates, with similar CHRNA5/A3/B4 clusters found in the mouse (Eng et al., 1991) pig (Tammen, 1998) and human genomes (Fig. 1.5) (Raimondi et al., 1992). To date, no clustered subunit genes analogous to CHRNA5, CHRNA3 and CHRNB4 have been identified in the Xenopus, Zebrafish, Drosophila or *C. elegans* genomes (Le Novere and Changeux, 1995; Tsunoyama and Gojobori, 1998). The conservation at the CHRNA5/A3/B4 locus paired with the observation that these genes are co-expressed in a variety of cell types and tissues suggests that these subunit genes are coordinately regulated (Gotti et al., 2006; Improgo et al., 2010b). Functional characterization of the promoter regions of each of the three clustered subunit genes revealed that as expected, several transcription factors act to positively influence expression of each of the clustered genes including, Sp1, Sp3, Sox10, and SCIP/Tst-1/Oct-6 (Improgo et al., 2010b).

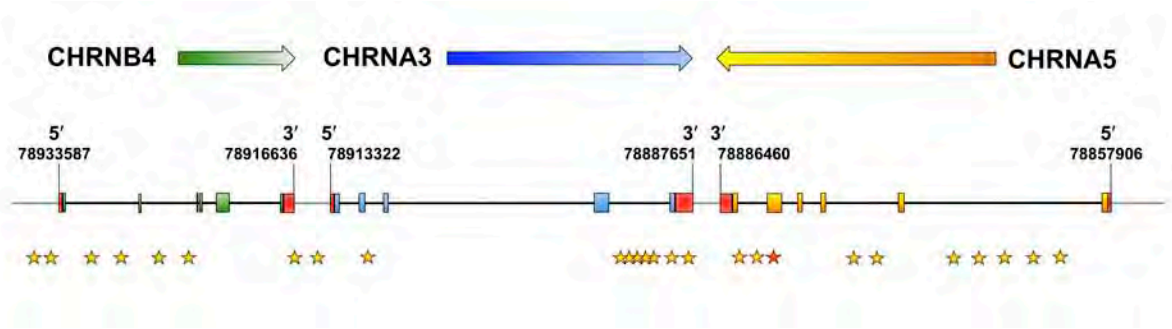


Figure 1.5: The CHRNA5/A3/B4 gene cluster. Schematic representation of the human CHRNA5/A3/B4 gene cluster. Each gene is drawn to scale with colored boxes representing exons and red boxes representing untranslated regions. Horizontal black lines represent introns while horizontal gray lines represent intragenic regions. Colored arrows indicate the direction of transcription. The 25 stars below the CHRNA5/A3/B4 locus indicate the position of SNPs linked to either to an increased risk of nicotine dependence or lung cancer.

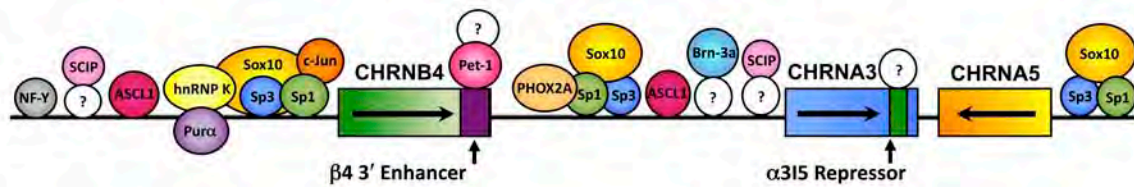


Figure 1.6: Transcriptional regulation of the CHRNA5/A3/B4 locus. Coding regions of the nAChR subunit genes are represented as colored boxes with arrows indicating the direction of transcription. Two transcriptional regulatory elements, the intronic repressor in the fifth intron of CHRNA5 (A315) and the 3' enhancer in the CHRNB4 gene, are shown (green and purple boxes, respectively). Horizontal black lines depict the non-coding regions of DNA. Transcription factors that regulate expression of these genes are depicted as colored circles and are touching each other if they directly interact. Circles labeled with a “?” indicate transcription factors whose identities have yet to be identified. In the case of Sp1, multiple binding sites have been identified in each of the nAChR genes, however, for clarity, the sites are represented as a single green circle at each promoter region.

However, it is important to note that the expression patterns of the clustered subunit genes do not completely overlap, indicating that in addition to the coordinate regulation, unique regulatory mechanisms also act to control expression of the individual subunit genes (Fig. 1.6). Nicotinic receptors containing $\alpha 5$, $\alpha 3$, and $\beta 4$ subunits are the most abundant nAChRs expressed in the peripheral nervous system (PNS) (Conroy and Berg, 1995; Improgo et al., 2010b), where they play a crucial role in mediating fast synaptic transmission in autonomic ganglia (Rust et al., 1994; Conroy and Berg, 1995; Flores et al., 1996). They are also expressed centrally (Leonard and Bertrand, 2001; Gotti et al., 2007), particularly in the habenulo-interpeduncular pathway where they are hypothesized to play a role in reward and motivation (Grady et al., 2009).

In *Xenopus* oocytes, co-expression of rat $\alpha 3$ and $\beta 4$ nicotinic receptor subunit cRNA results in functional receptors that are activated by ACh with an EC_{50} of 79-180 μM and a single channel conductance, γ , of 22 pS (McGehee and Role, 1995; Improgo et al., 2010b). Interestingly, the $\alpha 5$ subunit fails to form functional receptors when co-expressed with either the $\beta 4$ or $\beta 2$ subunits alone (Boulter et al., 1987; Couturier et al., 1990a). However, functional channels are formed if $\alpha 5$ is co-expressed with both the $\alpha 4$ and $\beta 2$ subunits (Ramirez-Latorre et al., 1996) or with both the $\alpha 3$ and $\beta 4$ subunits (Wang et al., 1996; Groot-Kormelink et al., 2001). Surprisingly, inclusion of the $\alpha 5$ subunit in the $\alpha 3\beta 4$ receptor has little impact on the ACh/nicotine concentration-response relationship. However, the $\alpha 5\alpha 3\beta 4$ subtype does have an

accelerated rate of desensitization when compared to the $\alpha 3\beta 4$ receptor (Wang et al., 1996; Wang and Sun, 2005). The $\alpha 3\beta 4$ subtype has an EC_{50} for ACh that is significantly higher than the $\alpha 4\beta 2$ subtype, which has an ACh EC_{50} of 0.8 μM . Cytisine is the most potent agonist at the $\alpha 3\beta 4$ subtype (EC_{50} of 15 μM) whereas nicotine is the most potent agonist at the $\alpha 4\beta 2$ (EC_{50} of 0.13-0.3 μM) subtype (McGehee and Role, 1995; Improgo et al., 2010b).

It has been shown previously that nAChR subtypes in the brain containing the $\alpha 5$, $\alpha 3$ and $\beta 4$ subunits are involved in the pathogenesis of nicotine-induced seizures (Wang et al., 2002; Kedmi et al., 2004; Salas et al., 2004b) as well as nicotine-induced hypolocomotion (Salas et al., 2004b). Specifically, CHRN $\beta 4$ knock out (KO) mice exhibit decreased signs of nicotine withdrawal (Salas et al., 2004a) as well as autonomic cardiac and intestinal dysfunction (Wang et al., 2003). CHRN $\alpha 3$ KO mice exhibit poor growth, decreased survival, absence of bladder contractility and widely dilated pupils (Xu et al., 1999b), whereas CHRN $\alpha 5$ KO mice display altered autonomic function and decreased sensitivity to nicotine induced seizures (Salas et al., 2003b). Recently, $\alpha 3$ - and $\beta 4$ -containing nAChR subtypes in the habenulo-interpeduncular pathway have been shown to mediate ACh release (Grady et al., 2009).

A series of candidate gene analyses and genome-wide association studies (GWAS) has identified single nucleotide polymorphisms (SNPs) in the chromosomal locus encoding the clustered nAChR subunit genes as risk factors for nicotine

dependence, lung cancer, chronic obstructive pulmonary disease, alcoholism and peripheral arterial disease (Saccone, 2007; Amos, 2008; Berrettini, 2008; Bierut, 2008; Hung et al., 2008; Schlaepfer, 2008; Stevens et al., 2008; Thorgeirsson, 2008; Weiss et al., 2008; Caporaso et al., 2009; Freaty et al., 2009; Saccone et al., 2009; Sasaki et al., 2009; Wang et al., 2009b; Wang et al., 2009a). As a result of the correlation of lung cancer susceptibility with variability in the clustered subunit genes, new attention has been brought to the $\alpha 5\alpha 3\beta 4$ subtype shifting the focus from the more commonly studied $\alpha 7$ and $\alpha 4\beta 2$ subtypes (Sciamanna et al., 1997; Picciotto et al., 1998; Wang et al., 2001; Walters et al., 2006; Breitling et al., 2009; Paleari et al., 2009; Sun et al., 2009).

Nicotinic receptors and lung cancer

A variety of evidence exists indicating that nAChRs play a role in lung carcinogenesis (Improgo et al., 2010a). First, nAChRs are expressed in both normal and lung cancer cells with several nAChR subunit genes, including CHRNA5, CHRNA3 and CHRNB4, overexpressed in lung cancer (Schuller, 1989; Maneckjee and Minna, 1990; Maus et al., 1998; Wang et al., 2001; Song et al., 2003; Lam et al., 2007; Sartelet et al., 2008; Improgo et al., 2010d). In addition, the $\alpha 7$, $\alpha 4\beta 2$, $\alpha 3\beta 2$, and $\alpha 3\beta 4$ nAChR subtypes have been shown to mediate cellular processes involved in the pathogenesis of lung cancer (Schuller, 2009). Activation of these receptors potentiates the development and progression of lung cancer through several different mechanisms.

Activation of nAChRs by either nicotine or ACh can enhance cell proliferation. It has been shown previously that ACh activates signaling pathways vital for growth and differentiation of human epithelial cells (Grando, 2008). Nicotine treatment has been shown to initiate a signal cascade that leads to inactivation of cyclins and cyclin-dependent kinases, disassociation of E2F1 from the retinoblastoma protein, activation of proliferative promoters by E2F1 and entry into S-phase (Dasgupta and Chellappan, 2006; Egleton *et al.*, 2008). Nicotine also upregulates a wide variety of growth factors and their receptors (Sher *et al.*, 1998).

Nicotine can also confer resistance to apoptosis in lung cancer cells (Maneckjee and Minna, 1994). Nicotine's ability to protect cells from apoptosis appears to involve several pathways. One such pathway involves activation of the anti-apoptotic protein B cell lymphoma gene 2 by protein kinase C α and phospholipase C (Mai *et al.*, 2003). Consistently, nicotine also inactivates the proapoptotic functions of the Bax and Bad proteins (Jin *et al.*, 2004; Xin and Deng, 2005).

Nicotine also functions as a pro-angiogenic agent, activating angiogenesis through the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways (Heeschen *et al.*, 2001). Nicotine enhances angiogenesis by promoting endothelial cell migration, proliferation, survival, tube formation, and nitric oxide production (Cooke and Ghebremariam, 2008).

β4-containing nicotinic receptors

The β4 nAChR subunit was initially identified in 1989 (Duvoisin et al., 1989) in a screen of a rat cDNA library. Duvoisin and colleagues were able to demonstrate that in *Xenopus* oocytes, the newly discovered β4 subunit could form functional channels with the α2 – α4 subunits. A year later the sequence of the CHRNA4 gene was reported for the first time along with CHRNB4 (Boulter et al., 1990). Subsequent studies on β4-containing receptors like α3β4 and α5α3β4 revealed that these receptors are insensitive to α-Bgtx and display a slower time course of desensitization when compared to other receptor subtypes like the α7 homomeric receptor (Fig. 1.4) (Couturier et al., 1990a; Role, 1992). Much like the CHRNB4 and CHRNA3 subunit genes, the CHRNA4 gene is widely expressed in the PNS with a more restricted pattern of expression in the CNS (Gotti and Clementi, 2004). CHRNA4 expression is relatively high in trigeminal sensory neurons (Flores et al., 1996; Liu et al., 1998) as well as the superior cervical, dorsal root, sphenopalatine and otic ganglia (Mandelzys et al., 1994; Rust et al., 1994; Zoli et al., 1995). CHRNA4 is also expressed in the adrenal medulla (Di Angelantonio et al., 2003) with lower expression in the retina (Moretti et al., 2004). In the CNS, CHRNA4 expression is particularly high in the olfactory bulb, pineal gland, medial habenula and interpeduncular nucleus (Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997; Grady et al., 2009) with lower expression in other thalamic nuclei, the cortex, hippocampus, spinal cord, cerebellum and midbrain (Hellstrom-Lindahl et al., 1998; Quik et al., 2000; Azam et al., 2002; Perry et al., 2002; Keiger et al., 2003; Gahring et al., 2004; Turner and Kellar, 2005).

As discussed above, the transcription factors Sp1, Sp3, Sox10 and SCIP/Tst-1/Oct-6 positively impact transcription of the CHRNA4 gene as well as the CHRNA5 and CHRNA3 genes (Fig. 1.6). In addition to these factors, CHRNA4 expression is also positively regulated by c-Jun (Melnikova and Gardner, 2001). Transactivation by Sp1, Sp3, c-Jun and Sox10 is abolished when the Sp-binding site on the CHRNA4 promoter (referred to as a CA box) is mutated. Conversely, synergistic activation of the CHRNA4 promoter is observed when Sp1 is supplied in concert with Sox10, Sp3 or c-Jun (Melnikova et al., 2000b; Melnikova and Gardner, 2001). Co-immunoprecipitation experiments demonstrated that all of these factors physically interact (Melnikova et al., 2000a). These findings suggest the existence of a positively acting multi-subunit transcriptional regulatory complex that assembles on the CHRNA4 promoter (Fig. 1.6). This result is consistent with the hypothesis that Sp1 is critical for transcription from the CHRNA4 promoter and likely nucleates the regulatory complex that drives expression of CHRNA4.

Two additional transcription factors have been shown to interact with the CHRNA4 promoter, Pur α and heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Du et al., 1997; Du et al., 1998) (Fig. 1.6). These proteins interact with another motif, the CT box, located directly upstream of the CA box. hnRNP K is capable of repressing Sp factor-mediated transactivation of the CHRNA4 promoter (Du et al., 1998) and also physically interacts with Sox10 (Melnikova et al., 2000a). Similar to hnRNP K, Pur α is capable of interacting with Sox10 (Melnikova et al., 2000a). Moreover, Pur α and hnRNP K themselves physically interact (Melnikova et al., 2000a). These

proteins may participate in the multi-subunit complex described above to modulate expression of the CHRNA4 gene in the appropriate cellular context. *In vitro* binding experiments demonstrated that each factor binds preferentially to the opposing single strand elements of the CT box, suggesting that some local DNA helix unwinding may occur (Krecic and Swanson, 1999). Interestingly, Pur α and hnRNP K have been shown to function together to negatively impact transcription of genes in other systems and the same may be occurring at the CHRNA4 promoter (Fig. 1.7) (Da Silva et al., 2002).

While the molecular machinery responsible for the incorporation of a particular subunit into a mature nAChR is poorly understood, regulation at the level of transcription undoubtedly plays an important role. The transcriptional regulatory mechanisms controlling expression of the CHRNA4 gene are not completely understood, however, and therefore served as the focus of my studies.

I have focused on investigating the transcriptional regulation of the CHRNA4 gene both *in vitro* and *in vivo*. Specifically, I demonstrated that a set of transcription factors shown previously to enhance CHRNA4 promoter activity *in vitro* are capable of interacting with the CHRNA4 promoter in the context of native chromatin using both neuronal-like cell lines and rodent brain tissue. In addition, I have developed a transgenic animal model to investigate the importance of the CHRNA4 promoter CA box regulatory element *in vivo*.

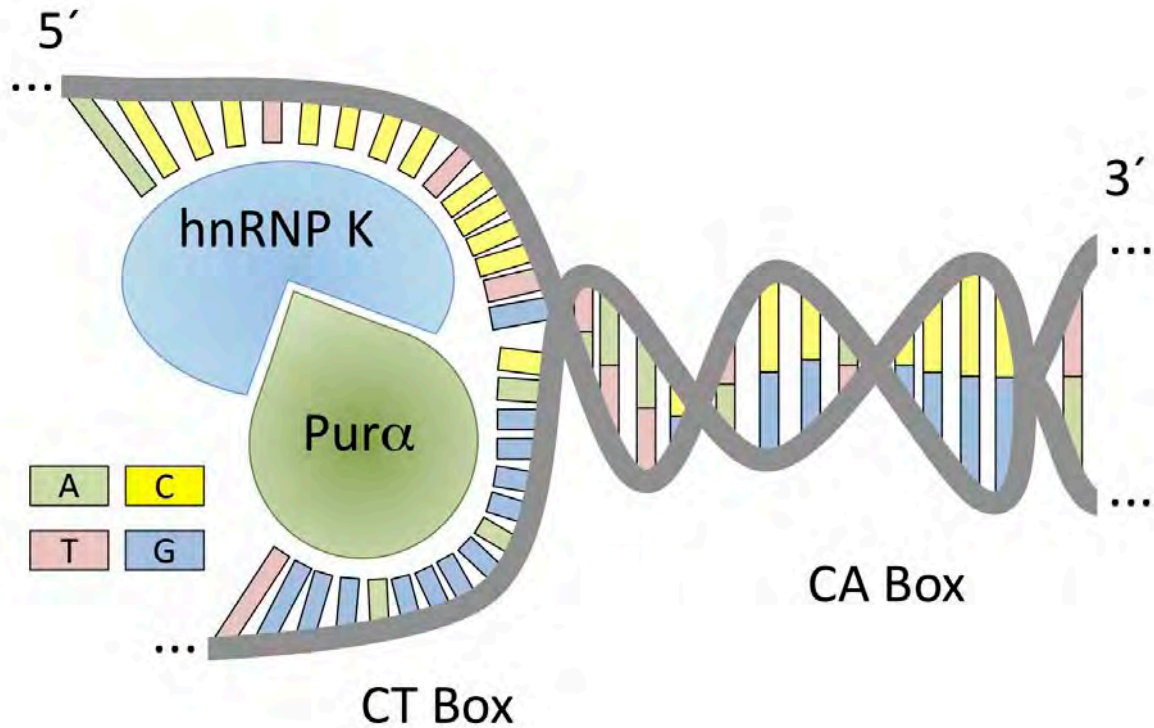


Figure 1.7: Protein-DNA interaction at the CHRNA4 promoter CT box.

Nucleotide sequences of the CT and CA box are represented as colored boxes with each base represented as either green (A), pink (T), yellow (C) or blue (G) boxes. The transcription factors hnRNP K (blue) and Purα (green) are depicted as colored shapes interacting with single stranded regions of the CT box shown. Nucleotide sequences that physically interact with either hnRNP K or Purα *in vitro* have been depicted as colored boxes orientated towards the hnRNP K or Purα proteins. Because hnRNP K and Purα physically interact, they have been depicted here as interlocking shapes.

Using this model system I was able to demonstrate that the CA box regulatory element is critical for CHRNA4 promoter activity *in vivo* and that mutation of this sequence results in decreased interaction of the regulatory factor Sp1 with the CHRNA4 promoter. Finally, I have investigated the role that the transcription factors hnRNP K and Pur α play at the CHRNA4 promoter.

CHAPTER II: MATERIALS AND METHODS

Cell culture and differentiation

PC12 cells (Greene and Tischler, 1976) were maintained in Dulbecco's modified Eagle's medium (Cellgro) containing 10% fetal bovine serum (GIBCO) and 5% heat inactivated horse serum (GIBCO). PC12 cells were differentiated for 72 hours with nerve growth factor (NGF) (Millipore) as described previously (Hu et al., 1994).

OBL21 cells (Ryder et al., 1990) were grown in Dulbecco's modified Eagle's medium (Cellgro) containing 10% fetal bovine serum (GIBCO). Neuro 2A cells (Olmsted et al., 1970) were maintained in minimum essential medium (GIBCO) containing 10% fetal bovine serum (GIBCO). SN17 cells (Hammond et al., 1990) were maintained in Dulbecco's modified Eagle's medium (Cellgro) containing 10% fetal bovine serum (GIBCO).

ChIP

The ChIP experiment was originally designed to map the location of post-translational modified histone proteins (Kuo and Allis, 1999). This technique has since been adapted to investigate the interaction of transcription factor proteins with a locus or DNA region of interest (Collas, 2010). The ChIP assay allows for the investigation of protein-DNA interaction in the native chromatin environment. This technique provides more physiologically relevant data when compared to electrophoretic mobility shift assays where nuclear extracts are incubated with short-labeled DNA oligonucleotides. In electrophoretic mobility shift assays, DNA is not

associated with histone proteins or organized into higher order chromatin structures. This is a considerable advantage of the ChIP technique, as it is well known that chromatin structure can profoundly impact protein-DNA interaction and transcription (Li et al., 2007).

ChIP Protocol: ChIP experiments performed on cells grown in culture were carried out using a modified version of the ChIP protocol provided in the Millipore ChIP assay kit, catalogue #17-295. Cells used for these experiments were grown in 150 cm culture dishes (Corning) to a confluency of ~80%, yielding approximately 10^7 cells. Proteins were cross-linked to DNA by adding formaldehyde directly to culture medium to a final concentration of 1% and incubating for 10 minutes at 37°C. Cross-linking was then stopped by the addition of glycine to a final concentration of 0.125 M, followed by a 10-minute room temperature incubation. Culture media was aspirated off and cells were then washed twice with ice cold PBS + protease inhibitors (1 µg/ml aprotinin, 1 µg/ml pepstatin A and 1 mM phenylmethylsulfonyl fluoride (Sigma)). Cells were scraped into ice-cold PBS containing protease inhibitors and collected by centrifugation for 4 minutes at 2,000 rpm at 4°C. The resulting pellet was then resuspended again in ice cold PBS containing protease inhibitors and placed on ice. Cell number was determined using a hemocytometer in order to ensure that each individual ChIP was performed using approximately 10^6 cells as a source of chromatin. Cells were processed in batches of 10^7 cells, enough for 10 individual ChIP reactions. After quantification and any necessary adjustments, cells were collected again by centrifugation for 4 minutes at 2,000 rpm at 4°C and

supernatant was removed. The resulting pellet was re-suspended in 2 mls SDS lysis buffer (1%SDS, 10 mM EDTA 50 mM Tris-HCL, pH 8.1) with protease inhibitors in a pre-cooled 15 ml conical tube (Fisher). Once in SDS lysis buffer, cells were allowed to incubate on ice for 10 minutes. Cells were then sonicated in the 15 ml conical tube, which was kept in an ice water bath throughout the sonication protocol. The intensity and duration of the sonication was optimized in order to shear chromatin into an average size of 500 base pairs. After sonication, fragmented chromatin was transferred to a pre-cooled microfuge tubes and centrifuged at 13,000 rpm for 10 minutes at 4°C, in order to eliminate cellular debris. The supernatant was carefully removed from the debris pellet and then transferred to a fresh pre-cooled 15 ml conical tube stored on ice. Chromatin was then diluted 10 fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL, pH 8.11, 67 mM NaCl), containing protease inhibitors. At this point, the Input unprecipitated control sample is set aside. Diluted chromatin was pre-cleared by adding 80 µl salmon sperm DNA/protein A-agarose beads (Millipore catalog #16-157C) for each individual ChIP reaction (800 µl was used for a batch of 10 individual ChIPs) and incubating for 30 minutes with rotation at 4°C. After the 30-minute incubation, salmon sperm DNA/protein A-agarose beads were spun down for 2 minutes at 1,500 rpm at 4°C and the cleared supernatant was transferred to a fresh pre-cooled conical tube on ice. Diluted chromatin samples were then aliquoted to pre-cooled siliconized IP tubes (Fisher) kept on ice. Each individual ChIP reaction was performed using 2 ml of diluted chromatin. 5 µg of the desired antibody was then added to each individual ChIP reaction and samples were incubated at 4°C with rotation, overnight.

The following morning, 60 μ l of salmon sperm DNA/protein A-agarose beads were added to each IP. CHIP samples were allowed to incubate with salmon sperm DNA/protein A-agarose beads for 1 hour at 4°C with rotation, in order to allow protein A / antibody / transcription factor / DNA complexes to form. Salmon sperm DNA/protein A-agarose beads were collected by centrifugation at 1000 rpm at 4°C for 1 minute. Following centrifugation the supernatant was carefully removed and discarded. Protein A / antibody / transcription factor / DNA complexes were washed with 1 ml of a low salt buffer (0.1%SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH8.1, 150 mM NaCl) for 5 minutes at 4°C with rotation. Salmon sperm DNA/protein A-agarose beads were the collected by centrifugation at 1000 rpm at 4°C for 1 minute and supernatant was carefully removed and discarded. Additional washes were carried out in the same manner for high salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.1, 500 mM NaCl), lithium chloride (1% IGEPAL – CA 630, 1% Deoxycholic acid, 1 mM EDTA, 10 mM Tris, pH 8, 0.25 M LiCl) and TE (10 mM Tris, pH 8, 1 mM EDTA) wash buffers. After the final wash step, beads were collected by centrifugation at 1,000 rpm at 4°C for 1 minute a final time and supernatant was carefully removed. The salmon sperm DNA/protein A-agarose beads were then eluted by adding 400 μ l of elution buffer (0.1 M NaCHO₃, 1% SDS – must be prepared fresh) to each CHIP sample. After briefly vortexing, samples were incubated at room temperature for 30 minutes, with rotation. Salmon sperm DNA/protein A-agarose beads were then collected by centrifugation at 1,000 rpm for 1 minute at room temperature. The resulting supernatant was transferred to freshly labeled tubes and the eluted beads were discarded. 8 μ l of 5 M NaCl was added to

each eluted ChIP reaction and samples were incubated at 65°C for 4 hours in order to reverse protein-DNA crosslinking. After the 4-hour incubation at 65°C 40 µl of 10X Proteinase K buffer (2%SDS, 1 M Tris-HCL, pH 8.0, 50 mM EDTA, 2 M NaCl) and 2 µl of a 10 mg / ml proteinase K stock (Sigma) was added to each individual ChIP sample. Samples were then incubated at 45°C for 2 hours in order to digest protein. Following proteinase K treatment, DNA was isolated with phenol:chloroform extraction, followed by ethanol precipitation. The resulting ChIP-derived DNA was then used as template for conventional or quantitative real-time PCR (qRT-PCR).

ChIP experiments using brain tissue as a source of chromatin were performed using a modified version of the protocol discussed above. Rodent brain tissue was ground into a powder using a mortar and pestle (Coors) cooled with liquid nitrogen, on dry ice. Approximately 100 mg of brain tissue was used for each individual ChIP. Once ground to a powder, 1 g of brain tissue (enough for one experiment with 10 individual ChIPs) was triturated in 4 mls PBS with 1% formaldehyde. Cells were incubated in PBS + formaldehyde for 10 minutes in a 37°C water bath. Cross-linking was then stopped by the addition of glycine to a final concentration of 0.125 M, followed by a 10-minute room temperature incubation with rotation. After the 10-minute incubation, tissue slurry was transferred to 2 pre-cooled 2ml dounce homogenizers on ice (Kontes Glass Co.). The brain tissue was homogenized with 10-15 pestle strokes using the "tight" pestle. Cells were then transferred to 2, 2 ml pre-cooled tubes and collected by centrifugation at 1,500 rpm for 5 minutes at 4°C. Supernatant was removed and cells and each pellet was washed in 1 ml ice-cold

PBS containing protease inhibitors. Cells were then collected again by centrifugation at 1,500 rpm for 5 minutes at 4°C and were subsequently washed an additional time. The resulting pellets were resuspended in 1 ml of SDS lysis buffer (1%SDS, 10 mM EDTA 50 mM Tris-HCL, pH 8.1) containing protease inhibitors and then combined to reach a final volume of 2 mls. Brain tissue ChIPs were carried out as described above for the remainder of the procedure.

Cell lines used in the ChIP assay

PC12 Cells: The rat pheochromocytoma cell line PC12 is a widely used model system for NGF-induced neuronal differentiation (Greene and Tischler, 1976). Upon NGF treatment these cells extend long neurite-like processes and acquire many of the biochemical and physiological properties associated with autonomic neurons (Rogers et al., 1992). NGF treated PC12 cells exhibit increased expression levels of several nAChR subunit genes including the CHRNA4 gene (Rogers et al., 1992). PC12 cells treated with NGF were used in the ChIP experiments described below due to the fact that these cells will be actively transcribing the CHRNA4 gene. In these cells, it is most likely that the factors that act to control transcription of this gene will be present at the CHRNA4 promoter.

OBL21 Cells: The OBL21 cell line was produced by retroviral vector-mediated transduction of the avian myc oncogene. Mitotic progenitor cells of postnatal mouse olfactory bulb were infected in order to produce a clonal cell line that exhibits a

stable neuronal phenotype (Ryder et al., 1990). OBL21 cells extend long processes and display growth cones. These cells also express the 150 kD neurofilament protein (a common neuronal marker (Zopf et al., 1987)) as well as voltage-gated potassium channels, but do not express voltage-gated sodium channels (Ryder et al., 1990). OBL21 cells were used in ChIP assays because of their neuronal-like morphology and also due to the fact that the CHNRB4 gene is expressed in the olfactory bulb (Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997).

Neuro 2A Cells: The mouse neuroblastoma cell line Neuro 2A was derived from a spontaneously occurring neuroblastoma in an albino mouse strain (Olmsted et al., 1970). These cells produce a microtubular protein, which is believed to play a role in the contractile system giving axoplasmic flow in nerve cells. When cultured these cell rapidly adhere to substrate and extend branching processes, giving them a neuronal-like morphology. Neuro 2A cells were used in ChIP assays because of their neuronal-like morphology and also because they express relatively high levels of several nAChR subunits including the CHRNA5, CHRNA3 and CHRNB4 genes.

Antibodies used in the ChIP assay

Anti-acetyl Histone protein H4 (Millipore – 06-866): rabbit polyclonal antiserum raised against amino acids 2-19 of *Tetrahymena* histone H4. 5 µl of H4 antibody was used in each H4 ChIP positive control reaction.

Normal Rabbit IgG: (Santa Cruz Biotechnology – sc-2027)

Normal Mouse IgG: (Santa Cruz Biotechnology – sc-2025)

Sp1 (PEP 2) X (Santa Cruz Biotechnology – sc-59X): an affinity purified rabbit polyclonal antibody raised against amino acids 528-546 mapping within an internal region of Sp1 of rat origin.

Sp3 (D-20) X Santa Cruz Biotechnology – sc-644X) an affinity purified rabbit polyclonal antibody raised against a peptide within the C-terminus of Sp3 of human origin.

Sox10 (H-140) X (Santa Cruz Biotechnology – sc-48824X) a rabbit polyclonal antibody raised against amino acids 311-450 mapping near the C-terminus of Sox-10 of human origin.

c-Jun (N) X (Santa Cruz Biotechnology – sc-45X): an affinity purified rabbit polyclonal antibody raised against a peptide within the N-terminus of c-Jun of mouse origin.

hnRNP K (H-300) (Santa Cruz Biotechnology – sc-25373): an affinity purified rabbit polyclonal antibody raised against amino acids 1-300 of hnRNP K of human origin.

Pur α (Abcam – Ab79936): an affinity purified rabbit polyclonal antibody raised against a peptide containing residues 1-100 of Pur α of human origin.

Amplification of ChIP-derived DNA

Conventional PCR

ChIPs performed on cells grown in culture: PC12 cell ChIP-derived DNA was used as template in PCRs with the following primers designed to recognize a segment of the rat CHRN4 promoter containing the CT and CA boxes, 5'-

TAAGCTGCCTCGGGTGA ACTAAGA-3', 5'-TGTCTGGGGGAACCTGTGGCTAT-3'. Neuro 2A and OBL21 CHIP-derived DNA was used as template in PCRs with the following primers designed to recognize a segment of the mouse CHRN4 promoter containing the CT and CA boxes 5'- TTGGGTAAGCCAGGCTAAGA-3', 5'- GGTCCCGAGACTTTCTCACA-3'. Both mouse and rat CHIP-derived PCR products were sequenced to confirm the specificity of the PCR reaction. Following amplification, PCR products were electrophoresed through a 2% agarose gel.

Fetal and adult rat brain ChIPs: Rat brain CHIP-derived DNA was used as template in PCRs with the rat primers discussed above, designed to recognize a segment of the rat CHRN4 promoter containing the CT and CA boxes, 5'- TAAGCTGCCTCGGGTGA ACTAAGA-3', 5'-TGTCTGGGGGAACCTGTGGCTAT-3'. Following amplification, PCR products were electrophoresed through a 2% agarose gel.

CA box mutant transgenic brain tissue ChIPs: Each CHIP-derived DNA sample was used in PCR with the rat primers discussed above designed to amplify a segment of the *rat mutant transgenic* CHRN4 promoter, 5'- TAAGCTGCCTCGGGTGA ACTAAGA-3', 5'-TGTCTGGGGGAACCTGTGGCTAT-3'. The same CHIP-derived DNA was also used as template in a separate set of reactions using the mouse primers discussed above, which are designed to amplify a segment of the *endogenous wild type mouse* CHRN4 promoter, 5'- TTGGGTAAGCCAGGCTAAGA-3', 5'- GGTCCCGAGACTTTCTCACA-3'. Following

amplification, PCR products were electrophoresed through a 2% agarose gel. Densitometry values for ChIP-DNA derived PCR products were obtained using the Ultra-Violet Products EpiChemi³ Darkroom imaging system and UVP analysis software (UVP). Densitometry values obtained for each ChIP reaction were normalized to the corresponding value obtained for the Input control sample, in order correct for differences in starting material. Student's t-test was used for statistical analysis.

qRT-PCR

Quantification of CHRN4 promoter fragment enrichment in OBL21, PC12 and Neuro 2A ChIP-derived DNA samples was performed using the absolute quantification method (Chini et al., 2007) using SYBR green supermix with ROX (BioRad). Serial dilutions of a plasmid containing a fragment of either the rat (used for PC12 ChIPs) or mouse (used for the OBL21 and Neuro 2A ChIPs) CHRN4 promoter were used to generate a standard curve for the quantification of the ChIP-derived DNA samples. ChIP-derived DNA from the rat PC12 cell line was amplified using the primers discussed above designed to recognize a segment of the rat CHRN4 promoter containing the CT and CA boxes, 5'-TAAGCTGCCTCGGGTGAAGTAAGA-3', 5'-TGTCTGGGGGAACCTGTGGCTAT-3'. ChIP-derived DNA from the mouse OBL21 and Neuro 2A cells were amplified using the primers discussed above, designed to recognize a segment of the mouse CHRN4 promoter, 5'-TTGGGTAAGCCAGGCTAAGA-3', 5'-GGTCCCAGACTTTCTCACA-3'. Each ChIP-derived DNA sample was run in

triplicate and values obtained for each ChIP were normalized to the value obtained for the Input ChIP positive control (a 2% sample of un-precipitated DNA) in order to control for differences in starting material. Data from these experiments are presented as a percentage of the value obtained for the Input control or % Input.

Construction of the mutant CHRN4 promoter / lacZ transgene

The 2,346-base pair SacI/HindIII fragment of the *rat* CHRN4 gene promoter was excised from the pSGB4SH construct (Bruschweiler-Li et al., 2010) and used as the template for mutagenic PCR. For mutagenesis, the primers were designed to make 3 base pair substitutions in the CA box within the context of the 2.3-kb promoter fragment. After mutagenesis, the resulting fragment was ligated back into the pSGMAR backbone in order to generate the pSGMutB4SH construct. The SacI/HindIII fragment in the resulting construct was sequenced in order to ensure that only the expected mutations were made (Genewiz, CA) (Fig. 4.3).

Determination of transgene copy number

Transgene copy number of the mutant transgenic lines was determined using absolute quantification-based real-time PCR (Yuan et al., 2007). PCR reactions were performed with primers designed to amplify a fragment of the lacZ coding sequence present in the CHRN4 promoter / lacZ transgene. The sequence of the upper strand primer was 5' - GATTTCCATGTTGCCACTCGCTTTA-3' while that of the lower strand primer was 5' - TTCAGCAGCAGCAGACCATTTTCAA-3'. All PCR reactions were set up in triplicate and included 100 ng of genomic DNA as template.

Three individuals from each founder line were used to determine copy number. Two positive control samples of known copy number (a generous gift from Ricardo Medina, University of Massachusetts Medical School) were used in this analysis. These control samples were isolated from transgenic animals that contain a targeted lacZ coding sequence. One of these animals has 1 copy of the lacZ transgene and the other has 2 copies. The value obtained for the single copy positive control sample was set as 1 in each experiment. This value was used to estimate the copy number for all other samples including the control sample with 2 copies. In order to determine copy number for our 6 mutant CHRNA4 / lacZ transgenic lines, 3 DNA samples from each line were run in qRT-PCR experiments. The quantities derived from the standard curve for each set of 3 animals were averaged and then divided by the value for the 1-copy positive control.

Generation of transgenic mice

pSGMutB4SH was digested with NotI to release the mutated CHRNA4 / lacZ transgene. Following agarose gel electrophoresis, the transgene fragment was excised and the DNA was extracted from the gel using a QIAquick Gel Extraction Kit (QIAGEN). The purified DNA was injected into pronuclei followed by implantation into pseudopregnant females. The C57BL/6 x SJL F2 hybrid mouse strain was used for all transgenic experiments. Transgenic founders were identified by PCR. Founders were mated with C57BL/6 x SJL F2 hybrid mice to establish transgenic lines. Adequate measures were taken to minimize pain and discomfort to the animals. All procedures were conducted in accordance with the rules of the

Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

Histochemical analysis of transgenic mice

Two ages of transgenic mice were studied: ED18.5 and PD30. The embryonic time point was selected due to the fact that it is several days after the initiation of CHRN4 expression (Zoli et al., 1995). The postnatal time point was selected in order to accurately compare the expression patterns of our reporter gene to previous in situ hybridization studies that investigated CHRN4 expression in mice at this time point (Winzer-Serhan and Leslie, 1997). Mice were anesthetized with pentobarbital and perfused transcardially with cold 0.1 M sodium phosphate buffer/2 mM MgCl₂ followed by fixative (cold 4% paraformaldehyde). Tissues were then dissected and post-fixed for 5-6 hours (ED18.5) or 4 hours (PD30). Fixed tissues were transferred to 30% sucrose/2 mM MgCl₂, in 1X PBS and incubated at 4°C overnight. Tissues were embedded in Tissue-Tek (Miles) and quick frozen on dry ice. If not used immediately, the samples were stored at -80°C. Sectioning was done on a Leica CM3050S cryostat at -28°C generating either 14 µm (ED18.5) or 25 µm (PD30) thick sections that were transferred directly onto Superfrost glass slides (Fisher). Slides were air-dried at room temperature, washed with sodium phosphate buffer and then incubated overnight at 37°C with β-gal staining solution (0.1 M NaHPO₄, 0.1 M NaH₂PO₄, 2 mM MgCl₂, 0.1% sodium deoxycholate, 0.02% NP-40, 10 mM K₃(Fe)CN₆, 10 mM K₄(Fe)CN₆, 1 mg/ml X-gal). In order to minimize any variability in the β-Gal staining results, sections from mutant transgenic lines and the

corresponding WT transgenic and non-transgenic lines were stained at the same time and in the same batch of staining solution. Two animals from each transgenic founder line were studied in depth. Following β -gal staining, slides were washed with 1X PBS and incubated in distilled water either for 1 h (ED18.5) or overnight (PD30). Slides were then counter-stained with Neutral Red (1% w/v in 37 mM sodium acetate), dehydrated through a graded series of ethanol solutions (50%, 70%, 90% and 100%) and cleared with xylene. The slides were air-dried overnight at room temperature in a fume hood followed by the application of cover slips. Microscopy was done using a Zeiss Axiovert 200M microscope with a high resolution Retiga 1300R CCD camera and Slidebook image analysis software. Anatomical analysis was done with the aid of the Paxinos and Franklin mouse brain atlas (Franklin and Paxinos, 2001) and the Kaufman atlas of mouse development (Kaufman, 1998).

Northern blot analysis

Expression of Sox10 mRNA in the OBL21 cell line was determined by Northern blot analysis as previously described (Fanger et al., 1995) using a radioactively labeled Sox10 cDNA.

Quantification of gene expression using qRT-PCR

Quantification of hnRNP K, Pur α , CHRNA5, CHRNA3 and CHRNB4 gene expression in OBL21, PC12 and Neuro 2A cells was performed using the $-\Delta$ CT relative quantification method (Fernandez et al., 2003) with an ABI 7500 Real Time

PCR System. In these experiments, expression of a gene of interest is expressed relative to the expression level of the housekeeping gene β 2-microglobulin (β 2M).

Quantification of gene expression in the siRNA experiments was performed using the $2^{-\Delta\Delta CT}$ relative quantification method (Livak and Schmittgen, 2001). In these experiments, β 2M was used as the endogenous control to quantify gene expression, while the negative control siRNA treated cells are used as a calibrator. In each set of experiments, total RNA was isolated from cells using the RNeasy kit (Qiagen). cDNAs were generated using the RETROscript kit (Ambion). Each of the three clustered nAChR subunit genes along with hnRNP K, Pur α , β 2M and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were amplified using TaqMan[®] probes (ABI).

Transient Transfections

Neuro 2A or SN17 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers instructions. Cells were transfected with either the WT or mutant construct and a luciferase expression construct, pGL-Promoter, in which the SV40 promoter drives expression of the firefly luciferase gene. (Promega). These cells were allowed to incubate in transfection medium for 2 days and then harvested and assayed for β -gal (Galacto-Star, Applied Biosystems) and luciferase (Luciferase Assay System, Promega) activities in triplicate using a Lumimark microplate luminometer (Bio-Rad).

To correct for differences in transfection efficiencies between dishes, the β -gal activity in each sample was normalized to the luciferase activity in that same sample. The Student's t-test was used for statistical analysis.

Silencer select® siRNAs specific for hnRNP K, Pur α or a negative control siRNA (ABI) were transfected into Neuro 2A cells at a final concentration of 5 nM. siRNA was delivered to Neuro 2A cells using Lipofectamine 2000 (Invitrogen).

Western blot analysis

Expression of Sox10 protein in OBL21 cells was determined by Western blot analysis as described previously (Scofield et al., 2008). Anti-Sox10 antibody (Santa Cruz Biotechnology - sc-48824) was used at a dilution of 1: 200.

hnRNP K, Pur α , nAChR α 3, α 5, β 4, β -Actin and β 2M protein levels in siRNA treated Neuro 2A cells were also determined by Western blot analysis. Proteins from siRNA treated cells were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) then electrophoretically transferred to nitrocellulose membranes. The resulting membranes were probed with anti-hnRNP K, Pur α , nAChR α 3, α 5, β 4, β -Actin or β 2M antibodies. Signal intensities were measured following chemiluminescent detection. Values for hnRNP K, Pur α , nAChR subunits α 3 α 5 or β 4 were normalized to the corresponding value obtained for either β 2m or β -Actin, in order to correct for differences in the amount of lysate loaded into each well. In siRNA western experiments, negative control siRNA and either hnRNP

K or Pur α siRNA treated cell lysates were run on the same gels in order to more accurately quantify any reduction in protein levels. The anti-nAChR α 3 (Santa Cruz Biotechnology – sc5590), anti-nAChR α 5 (Abcam – ab41173), anti-nAChR β 4 (R&D antibodies – As-5656S), anti-hnRNP K antibody (Santa Cruz Biotechnology - sc-25373) and anti-Pur α antibody (Abcam – Ab79936) were used at a dilution of 1:400, whereas the anti- β -Actin antibody (Sigma – A2228), and anti- β 2m antibody (Santa Cruz Biotechnology – sc-51509) were used at a dilution of 1:5000. Image acquisition and analysis were performed using a BioRad VersaDoc system and software.

CHAPTER III: PROTEIN-DNA INTERACTION AT THE CHRNB4 PROMOTER CA

BOX

Disclaimer: I performed all of the experiments described in this chapter with the exception of the Northern and Western blots shown in Figure 3.3 (OBL21 cells express high levels of Sox10 mRNA and protein), which were performed by Zhongming Mou.

Abstract

nAChRs are involved in a plethora of fundamental biological processes ranging from muscle contraction to the formation of memories. These receptors are pentameric proteins whose subunits are encoded by distinct genes, with the biophysical properties of nAChRs governed by subunit composition. The incorporation of a particular subunit into a mature receptor is governed in part by the cell-type specific transcriptional regulation of each subunit gene. Here, using the ChIP assay, I report the interaction of Sp1, Sp3, Sox10 and c-Jun with the CHRNB4 promoter in neuronal-like cell lines as well as rodent brain tissue. These results are in agreement with previous data from our lab demonstrating that these factors functionally interact with the CHRNB4 promoter *in vitro*. Taken together, these data strongly suggest that Sp1, Sp3, Sox10 and c-Jun regulate expression of the CHRNB4 subunit gene in the mammalian brain.

Introduction

Neuronal nAChRs play an essential role in an array of physiological processes including learning, memory, and attention (Leonard and Bertrand, 2001; Gotti and Clementi, 2004; Albuquerque et al., 2009). The subunit composition of a particular nAChR is governed, at least in part, by the transcriptional regulation of individual subunit genes. Our laboratory has had a long-standing interest in the transcriptional mechanisms underlying expression of the CHRNA/A3/B4 subunit genes. The three nAChR subunits encoded by these genes make up the predominant nicotinic receptor subtype expressed in the peripheral nervous system (PNS) (Leonard and Bertrand, 2001). Recently, GWAS studies have linked variability in the CHRNA5/A3/B4 locus to an increased risk of nicotine dependence and lung cancer (Greenbaum and Lerer, 2009; Improgo et al., 2010c).

As only a small subset of neurons in the CNS express the CHRNB4 gene (Dineley-Miller and Patrick, 1992; Zoli et al., 1995; Gahring et al., 2004) it is likely that receptors including this subunit play an important role in mediating cholinergic signaling in and around these brain regions (Duvoisin et al., 1989; Gahring et al., 2004). Studies done using CHRNB4 KO animals have demonstrated that the $\beta 4$ subunit plays a critical role in the molecular mechanism underlying nicotine induced withdrawal (Salas et al., 2004a). Furthermore, blockade of $\alpha 3\beta 4$ nAChRs results in a reduction of opioid and stimulant self-administration, suggesting that nAChRs that contain the $\beta 4$ subunit may be involved in mediating withdrawal syndromes elicited by other drugs of abuse (Glick et al., 2002).

Gaining a better understanding of the transcriptional regulation of the CHRN4 gene would contribute not only to a more complete understanding of cholinergic signaling in the brain, but also nicotine addiction and withdrawal. Here, I focus on elucidating the interaction of transcription factors with the CHRN4 gene promoter in the chromatin environment.

Previous work from our laboratory has identified a 226-base pair segment of DNA in the 5'-flanking region of the CHRN4 gene capable of driving luciferase expression in a cell type specific manner (Hu et al., 1994; Liu et al., 1999). Deletional analysis led to the identification of a unique regulatory element, referred to as a CA box (Fig. 3.1), located upstream of the CHRN4 initiator methionine. Interestingly, a C – T mutation of a similar CA box regulatory element located in the β globin gene promoter resulted in a 50% reduction in promoter activity (Kulozik et al., 1991). By introducing similar mutations to the CHRN4 promoter CA box, we have previously shown that this regulatory element is critical for luciferase expression from the CHRN4 promoter in neuronal-like cell lines (Hu et al., 1994). Using electrophoretic mobility shift assays we also previously showed that the transcription factors Sp1 (Bigger et al., 1996; Bigger et al., 1997), Sp3 (Bigger et al., 1997), and Sox10 (Liu et al., 1999) bind to the CA box *in vitro*. In addition, each of these factors is capable of transactivating expression of a luciferase reporter gene driven by the CHRN4 promoter, as is the transcription factor c-Jun (Bigger et al., 1997; Liu et al., 1999; Melnikova and Gardner, 2001).

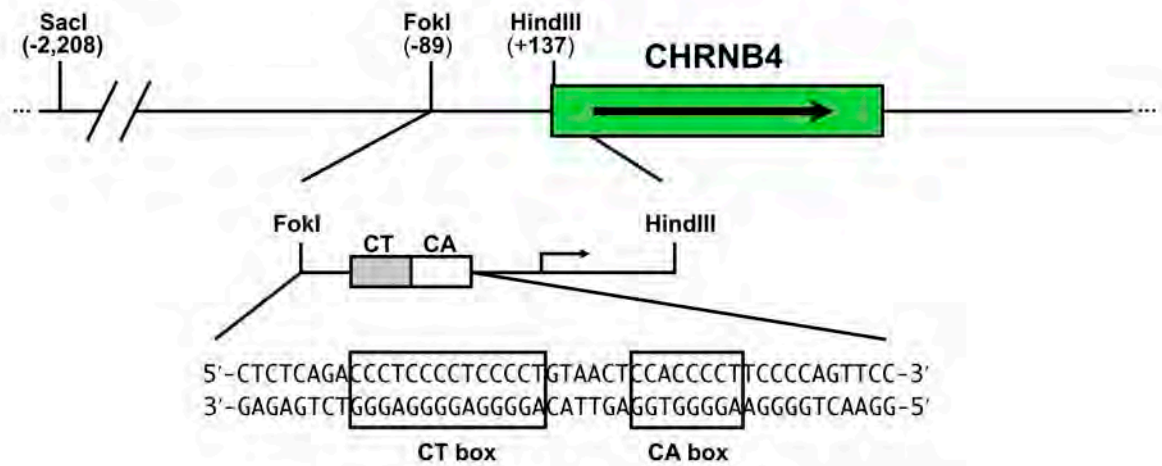


Figure 3.1: The CT and CA box regulatory elements of the CHRNA4 gene promoter. The CHRNA4 gene is depicted in green with the arrow inside denoting the direction of transcription. Nucleotide positions of the SacI, FokI, and HindIII sites are shown relative to the major transcription initiation site of the CHRNA4 gene indicated by the bent arrow. The CT and CA boxes are shown in grey and white, respectively with their nucleotide sequences shown below the schematic representation.

Interestingly, transfection of c-Fos had no impact on CHRNA4 promoter activity and there is no consensus AP-1 (c-Jun and c-Fos heterodimer binding site) site in the CHRNA4 promoter. Co-immunoprecipitation studies have demonstrated that Sp1 and Sp3 (Bigger et al., 1997), as well as Sox10 and the Sp factors (Melnikova et al., 2000b; Melnikova et al., 2000a), physically interact. Furthermore, transient transfections have demonstrated that these factors transactivate the CHRNA4 promoter synergistically when co-transfected into neuronal-like cell lines (Bigger et al., 1997; Melnikova et al., 2000b). Similarly, Sp1 and c-Jun also synergistically transactivate the CHRNA4 promoter in transient transfection experiments (Melnikova and Gardner, 2001).

Therefore, I hypothesize that Sp1, Sp3, Sox10 and c-Jun act to enhance expression of the CHRNA4 gene by interacting with the CHRNA4 promoter CA box, in the chromatin environment. To test whether these four regulatory factors interact with the CHRNA4 promoter, in neuronal-like cells as well as in the mammalian brain, ChIP experiments were performed using the PC12 and OBL21 cell lines as well as chromatin isolated from fetal and adult rat brain tissue. These experiments assay for the interaction of Sp1, Sp3, Sox10 and c-Jun with the CHRNA4 promoter in the chromatin environment. Previous Sp1, Sp3 and Sox10 *in vitro* binding experiments were performed using short DNA oligonucleotides (Bigger et al., 1997; Melnikova et al., 2000b), whereas the ChIP assay is performed using fragmented chromatin where DNA is associated with histone proteins and may be organized into higher order structures.

Results

Sp1, Sp3 and c-Jun interact with the CHRNB4 promoter in differentiated PC12 cells.

To investigate whether Sp1, Sp3 and c-Jun interact with the CHRNB4 promoter on cellular chromatin, ChIP assays were performed using differentiated PC12 cells. PCR reactions using ChIP-derived DNA from Sp1, Sp3 and c-Jun immunoprecipitations resulted in the amplification of a 207-base pair fragment of the rat CHRNB4 promoter containing the CA box (Fig. 3.2). The specificity of antibody-transcription factor complexes was demonstrated by negative results in ChIP assays using either normal mouse IgG or no antibody (Fig. 3.2). An additional negative control was performed by substituting water for ChIP-derived DNA at the final PCR step (Fig. 3.2). Immunoprecipitation with an anti-histone protein H4 antibody was performed as a ChIP positive control, which resulted in the amplification of the expected fragment (Fig. 3.2). In addition, a sample of fragmented chromatin prior to immunoprecipitation was used as a positive control (referred to as "Input"), which also amplified the expected 207-base pair product (Fig. 3.2). Taken together, these data indicate that Sp1, Sp3 and c-Jun are present at the endogenous CHRNB4 promoter in NGF-treated PC12 cells.

Sp1, Sp3, Sox10 and c-Jun interact with the CHRNB4 promoter in the neuronal-like cell line OBL21.

Our laboratory has previously shown that Sox10 binds to and transactivates the CHRNB4 promoter in neuronal-like cell lines, but not in non-neuronal cell lines (Liu et al., 1999). As PC12 cells do not express Sox10 (data not shown),

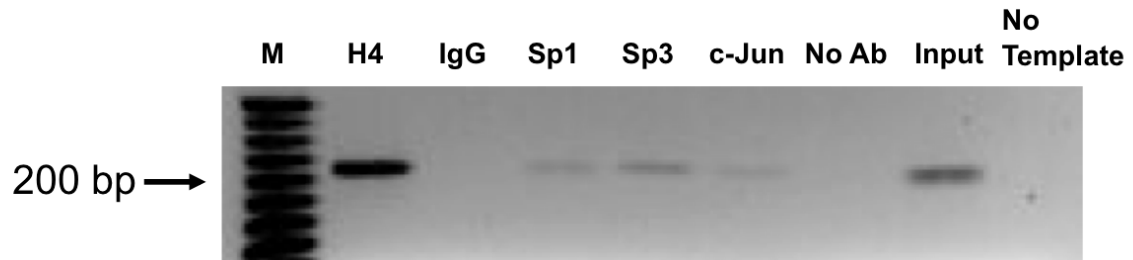


Figure 3.2: Differentiated PC12 chromatin immunoprecipitation. ChIP-derived DNA was used as template for PCR reactions designed to amplify a fragment of the rat CHRN4 promoter containing the CA box. Amplification was observed for both histone H4 (“H4”) and input (“Input”) ChIP positive controls. The expected PCR product was also produced in Sp1, Sp3 and c-Jun reactions (“Sp1”, “Sp3”, “c-Jun”). No amplification was observed when using DNA immunoprecipitated by normal mouse IgG (“IgG”), no antibody (“No Ab”) or in no-template PCR reactions (“No Template”). The arrow indicates the 200-base pair marker in the 100-base pair ladder (“M”). Each ChIP experiment was carried out a minimum of three times.

ChIP analyses were extended to another cell line, the mouse olfactory bulb derived cell line OBL21 (Ryder et al., 1990), which express high levels of Sox10 mRNA and protein (Fig. 3.3 A and B respectively). ChIP experiments with OBL21 cells were carried out as described above for PC12 cells. PCR reactions with ChIP derived DNA from Sp1, Sp3, Sox10 and c-Jun IPs resulted in the amplification of the expected 243-base pair product of the mouse CHRN4 promoter, containing the CA box (Fig. 3.4, lanes 4-7). Once again, the specificity of antibody-transcription factor complexes was confirmed by the failure of ChIP-derived DNA from mouse IgG and mock immunoprecipitations to produce the CHRN4 promoter PCR product (Fig. 3.4, lanes 3 and 8). Finally, H4 and Input ChIP positive controls showed strong bands (Fig. 3.4, lanes 2 and 9) and there was no amplification in the no template PCR control (Fig. 3.4, lane 10). These data indicate that Sp1, Sp3, Sox10 and c-Jun interact with the CHRN4 promoter in OBL21 cells.

Sp1, Sp3, Sox10 and c-Jun interact with the CHRN4 promoter in fetal and adult rat brain tissue.

In order to assay for the interaction of Sp1, Sp3, Sox10 and c-Jun with the CHRN4 promoter in the most physiologically relevant setting, ChIP experiments were performed using chromatin derived from rodent brain tissue. PCR reactions using fetal rat brain ChIP-derived DNA as template resulted in the amplification of the expected rat CHRN4 promoter fragment for Sp1, Sp3, Sox10 and c-Jun ChIPs (Fig. 3.5 A).

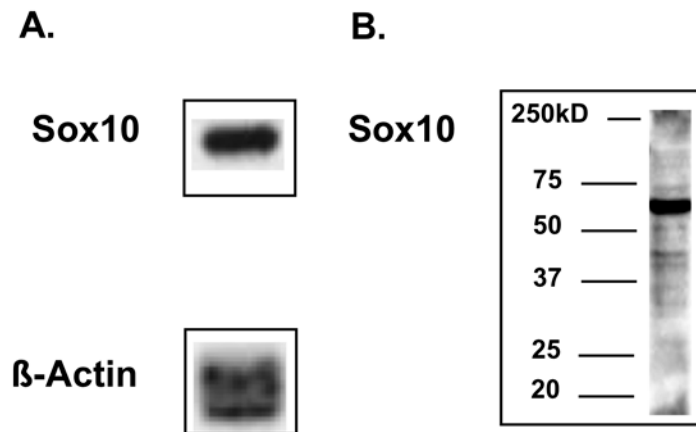


Figure 3.3: OBL21 cells express high levels of Sox10 mRNA and protein. (A) Northern blot analysis of OBL21 cells revealed relatively high levels of Sox10 mRNA. Northern analysis was also carried out for β -actin message as a loading control. (B) Western blot analysis was carried out on OBL21 cell lysates and showed abundant levels of Sox10 protein (MW = 68 kDa). Lines on the left of the blot indicate the positions and weights of protein standards.

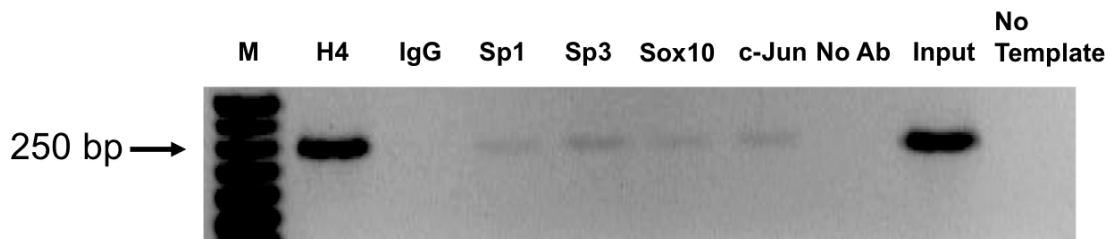


Figure 3.4: OBL21 chromatin immunoprecipitation. ChIP-derived DNA was used as a template for PCR reactions designed to amplify a 243-base pair product of the mouse *CHRNA4* promoter. The expected product was observed in PCR reactions for both histone H4 (“H4”) and input (“Input”) positive controls. Amplification was also observed in Sp1, Sp3, Sox10 and c-Jun immunoprecipitations (“Sp1”, “Sp3”, “Sox10”, “c-Jun”). No amplification was seen in PCR reactions using DNA immunoprecipitated by normal mouse IgG (“IgG”), no antibody (“No Ab”) or in no-template PCR reactions (“No Template”). The arrow indicates the 250-base pair marker in the 100-base pair ladder (“M”). Each ChIP experiment was carried out a minimum of three times.

The specificity of antibody-transcription factor complexes in these ChIP experiments was demonstrated by failure of ChIP-derived DNA from mouse IgG and no antibody immunoprecipitations to produce the expected product (Fig. 3.5 A). These experiments indicate that Sp1, Sp3, c-Jun and Sox10 interact with the CHRN4 promoter, at the CA box, in fetal rat brain.

I hypothesized that Sp1, Sp3, Sox10 and c-Jun would also interact with the CHRN4 promoter in adult rat brain. In order to test this hypothesis, I performed an additional round of ChIP assays using adult rat brain tissue. These experiments were performed using whole brain tissue as a source of chromatin for ChIP assays. Due to the restricted pattern of CHRN4 expression, I would expect there to be regional differences in ChIP results. However, conventional microdissection procedures were not suitable for the removal of the very small brain regions where CHRN4 is highly expressed (e.g., medial habenula and the interpeduncular nucleus). Therefore, whole brain homogenate was used. PCR using ChIP DNA from Sp1, Sp3, Sox10 and c-Jun immunoprecipitations resulted in the amplification of the expected fragment of the rat CHRN4 promoter (Fig. 3.5 B). No amplification occurred in PCR reactions using DNA derived from mouse IgG and mock immunoprecipitations (Fig. 3.5 B), further suggesting that positive ChIP results are specific to the antibodies used in the immunoprecipitations.

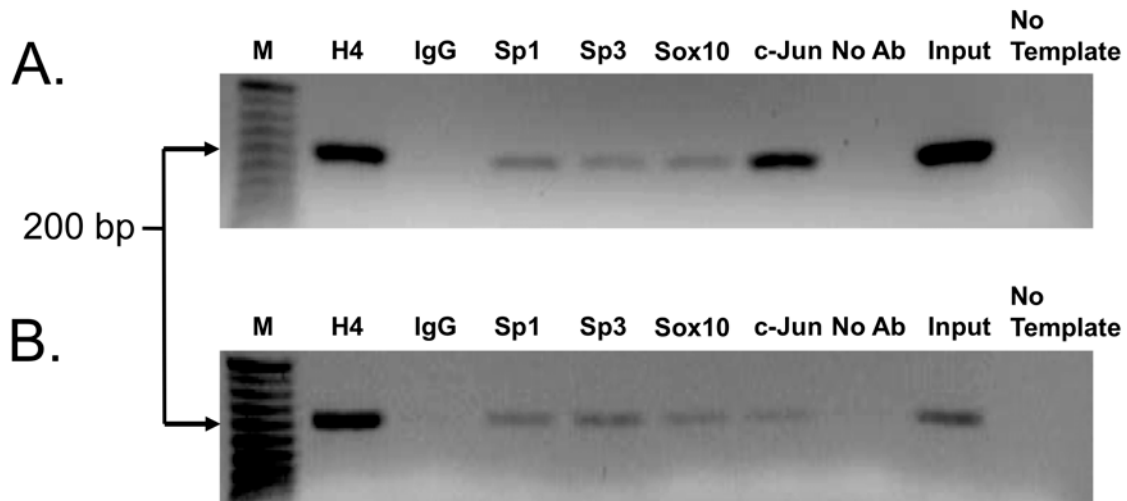


Figure 3.5: Rat brain chromatin immunoprecipitation. (A) Fetal rat brain ChIP-derived DNA was used as template for PCR reactions designed to amplify a segment of the rat CHRN4 promoter. Histone H4 (“H4”) and input (“Input”) positive controls produced the expected product. Amplification was also observed for Sp1, Sp3, Sox10 and c-Jun PCR reactions (“Sp1”, “Sp3”, “Sox10”, “c-Jun”). ChIP negative controls, normal mouse IgG (“IgG”), mock immunoprecipitation (“No Ab”) and no-template PCR control (“No Template”) produced no amplified products. The arrow indicates the 200-base pair marker in the 100-base pair ladder (“M”). (B) Adult rat brain ChIP-derived DNA was used in PCR reactions designed to amplify the rat CHRN4 promoter. Again, both the histone H4 (“H4”) and input (“Input”) positive controls produced the expected product. Amplification was also observed for Sp1, Sp3, Sox10 and c-Jun PCR reactions (“Sp1”, “Sp3”, “Sox10”, “c-Jun”). Finally, ChIP negative controls, normal mouse IgG (“IgG”), mock immunoprecipitation (“No Ab”) and no-template PCR control (“No Template”) produced no amplified products. The arrow indicates the 200-base pair marker in the 100-base pair ladder (“M”). Each ChIP experiment was carried out a minimum of three times.

Discussion

nAChR subunit genes are expressed in a variety of locations in both the PNS and CNS (Gotti et al., 2007; Albuquerque et al., 2009). However, the molecular mechanisms underlying the temporally and spatially specific expression of nAChR subunit genes remain to be completely elucidated. Here I extended our previous *in vitro* work on protein-DNA interactions that occur at the CHRNA4 promoter CA box. In this study, using both cell lines and brain tissue as a chromatin source for CHIP assays, I have shown that Sp1, Sp3, Sox10 and c-Jun interact with the CHRNA4 promoter in the chromatin environment. These data confirm and extend our previous results indicating that Sp1, Sp3, Sox10 and c-Jun interact with the CHRNA4 promoter *in vitro*, as it is well known that chromatin structure can profoundly impact protein-DNA interaction (Li et al., 2007).

The positive CHIP results for the transcription factors Sp1 and Sp3 are in agreement with previous data from our lab indicating an interaction between these proteins and the CHRNA4 promoter *in vitro* (Bigger et al., 1997). In addition to those present in the CHRNA4 promoter, there are several functionally relevant Sp1 binding sites in the CHRNA3 and CHRNA5 subunit gene promoters (Campos-Caro et al., 1999; Deneris et al., 2000; Valor et al., 2002). Due to the fact that these genes are located in a tight cluster in the mammalian genome, they are likely subject to some level of coordinate regulation. (Boulter et al., 1990; Yang et al., 1997). This coordinate regulation is likely responsible for directing the overlapping expression patterns of the clustered subunits in the mammalian nervous system (Winzer-Serhan and

Leslie, 1997; Albuquerque et al., 2009). It is my hypothesis that the coordinate regulation of these clustered subunits takes place at least in part through the action of Sp factors (Campos-Caro et al., 1999; Liu et al., 1999; Deneris et al., 2000; Valor et al., 2002).

I have also shown here that Sox10 interacts with the CHRNB4 promoter in the chromatin environment, this finding is also corroborated by previous results from our laboratory indicating that the Sox10 protein can interact with and transactivate the CHRNB4 promoter *in vitro* (Liu et al., 1999; Melnikova et al., 2000b).

There is no consensus c-Jun binding site in the CHRNB4 promoter and the c-Jun protein does not interact with the CHRNB4 promoter using *in vitro* binding assays. However, c-Jun synergistically transactivates the CHRNB4 promoter when co-transfected with Sp1 (Melnikova and Gardner, 2001). This result is in agreement with the data presented here, demonstrating that c-Jun is associated with the endogenous CHRNB4 promoter in neuronal-like cell lines and in rodent brain tissue. c-Jun is most likely recruited to the CHRNB4 promoter through a protein-protein interaction with Sp1, as mutation of Sp binding sites in the CHRNB4 promoter abrogates transactivation by c-Jun (Melnikova and Gardner, 2001).

By assembling previous data from our lab as well as the ChIP data discussed above, I have generated a model of protein-DNA interaction at the CHRNB4 promoter CA box (Fig. 3.6). Given the high GC content of the CA box, transcription factors in the

Sp family were likely candidates for proteins that may interact with the CHRNA4 promoter. Using electrophoretic mobility shift assays we previously demonstrated that both the Sp1 and Sp3 are capable of interacting with the CHRNA4 promoter CA box *in vitro*. As expected, mutation of the CA box abrogated this interaction (Bigger et al., 1996; Bigger et al., 1997). In addition to their ability to directly interact with the CHRNA4 CA box *in vitro*, both Sp1 and Sp3 are capable of transactivating the CHRNA4 promoter (Bigger et al., 1997). Furthermore, co-immunoprecipitation assays demonstrated that Sp1 and Sp3 directly interact and when these factors are supplied in concert, synergistic transactivation of the CHRNA4 promoter is observed (Bigger et al., 1997). Finally, ChIP assays discussed in this chapter have demonstrated that these factors interact with the CHRNA4 promoter in the chromatin environment, in the mammalian brain (Fig. 3.5). Taken together these data indicate that Sp1 and Sp3 directly interact with the CHRNA4 promoter in order to enhance expression of the CHRNA4 gene. As a result, I have depicted Sp1 and Sp3 contacting the CHRNA4 CA box, as well as each other (Fig. 3.6).

When PC12 cells are differentiated with NGF, CHRNA4 gene expression is increased (Rogers et al., 1992). The signal cascade that results from the recognition of NGF by its receptor causes the phosphorylation and subsequent activation of the c-Jun transcription factor. NGF treatment also causes an increase in c-Jun expression (Greenberg et al., 1986).

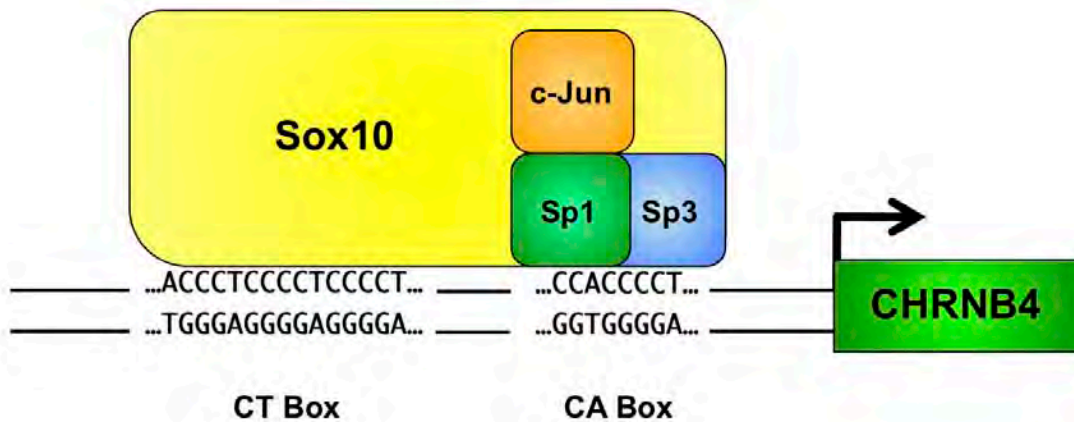


Figure 3.6: Assembly of positive-acting factors at the CHRNB4 gene promoter. The coding region of the CHRNB4 gene is represented as a light green box with arrow above the coding region indicating that CHRNB4 expression is turned on. The double stranded nucleotide sequences of two transcriptional regulatory elements, the CT and CA boxes, are also shown. The transcription factor Sox10 is depicted as a yellow colored oval with Sp1, Sp3, and c-Jun depicted as colored squares above the CA box.

Since both CHRN4 and c-Jun expression are increased during NGF treatment in these cells, c-Jun was an attractive candidate for a possible factor involved in controlling expression of the CHRN4 gene. As discussed above, electrophoretic mobility shift assays revealed no interaction of c-Jun with the CHRN4 promoter. This result was not surprising considering that the CHRN4 promoter contains no c-Jun binding site. Despite the lack of direct interaction with the CHRN4 promoter *in vitro*, transient transfection assays revealed that c-Jun is capable of transactivating the CHRN4 promoter (Melnikova and Gardner, 2001). Using lysates from NGF treated PC12 cells, we have previously shown that Sp1 and c-Jun directly interact (Melnikova and Gardner, 2001). In addition, when the site of Sp factor binding in the CHRN4 promoter (the CA box) is mutated, c-Jun is no longer capable of transactivating the CHRN4 promoter (Melnikova and Gardner, 2001). Furthermore, when Sp1 and c-Jun are supplied in concert, synergistic transactivation of the CHRN4 promoter is observed (Melnikova and Gardner, 2001). These data suggest that c-Jun exerts its positive effect on CHRN4 gene expression by virtue of its interaction with Sp1. The c-Jun ChIP assays discussed above demonstrate that this factor is also associated with the CHRN4 promoter in the chromatin environment. In addition to cross-linking proteins to DNA, formaldehyde is also capable of causing some protein-protein cross-linking as well. This allows for the detection of proteins that are associated with DNA through a protein intermediate. However, formaldehyde cross-linking is not suitable for the detection of large multisubunit protein complexes due to its short 2Å spacer arm length (Orlando, 2000). This phenomenon explains the discrepancy between the positive results for c-Jun in the

ChIP assays discussed above and the negative result in the c-Jun electrophoretic mobility shift assays done previously in our lab (Melnikova and Gardner, 2001). Taken together, these data suggest that c-Jun is present at the CHRN4 promoter by virtue of its interaction with Sp1, and most likely does not directly interact with DNA. As a result, I have depicted c-Jun contacting Sp1, but not directly interacting with DNA at the CHRN4 promoter CA box (Fig. 3.6).

Analysis of the CHRN4 expression patterns during development of the nervous system revealed considerable spatial and temporal overlap with the expression of the Sox10 transcription factor (Zoli et al., 1995; Kuhlbrodt et al., 1998). The co-expression of Sox10 and CHRN4 in a variety of cell types during development lead to the hypothesis that Sox10 may act to regulate CHRN4 expression.

Electrophoretic mobility shift assays, using a segment of the CHRN4 promoter containing the CT and CA boxes, demonstrated that Sox10 interacts with the CHRN4 promoter *in vitro*. However, competition of Sox10 binding using a CA box mutant probe was not complete, suggesting that Sox10 interacts with the CHRN4 promoter in a region that overlaps the CA box (Liu et al., 1999). Transient transfection assays revealed that in addition to directly interacting with the CHRN4 promoter, Sox10 is also capable of transactivating the CHRN4 promoter *in vitro* (Liu et al., 1999). Interestingly, co-immunoprecipitation assays revealed that Sox10 is capable of physically interacting with Sp1 and Sp3, and can also synergistically transactivate the CHRN4 promoter when supplied in concert with either Sp1 or Sp3 (Melnikova et al., 2000b). In the ChIP assays performed on neuronal-like cells as

well as brain tissue I obtained positive results for Sox10, indicating that Sox10 interacts with the CHRN4 promoter in the chromatin environment. Taken together, these data indicate that Sox10 interacts with the CHRN4 promoter, as well as the Sp1 and Sp3 transcription factors, in order to enhance expression of the CHRN4 gene. As a result, I have depicted Sox10 interacting with a region of DNA that encompasses the CA box. Due to the synergy observed with the Sp factors and their direct physical interaction, Sox10 was depicted contacting the Sp1 and Sp3 proteins, as well as the CHRN4 promoter DNA sequence (Fig. 3.6).

These experiments have provided insight into the interaction of regulatory factors with the CHRN4 gene promoter CA box in the chromatin environment, where DNA is associated with histone proteins and may be organized into higher order structures. Using the ChIP assay, I was able to demonstrate these factors are associated with the endogenous CHRN4 promoter, at the CA box, in neuronal-like cell lines and in rodent brain tissue. Taken together, these data suggest that Sp1, Sp3, c-Jun and Sox10 assemble at the CHRN4 promoter CA box to enhance transcription of the CHRN4 gene in the mammalian brain. The majority of the data presented here were published in 2008 (Scofield et al., 2008).

Limitations and Future Perspectives

In order to control for extensive formaldehyde cross-linking in CHRN4 ChIP experiments, additional control PCRs should be performed on ChIP-derived DNAs. In these control reactions, ChIP-derived DNA would be amplified with primers

designed to recognize the segment of DNA where the transcription factors of interest *do not* bind. Negative results in these PCRs would indicate that the association of Sp1, Sp3, Sox10 and c-Jun with the CHRNB4 promoter observed in the experiments discussed above is not a result of extensive cross-linking.

In addition to negative sequence controls, ChIP experiments could also be performed with negative control immunoprecipitations. In this additional ChIP negative control, an antibody that recognizes a transcription factor which *does not* interact with the CHRNB4 promoter is used. For example, in PC12 cells the Sox10 gene is not expressed, thus we would expect negative results in Sox10 PC12 ChIPs. Obtaining negative results in the additional ChIP negative controls would indicate that the positive results obtained in PC12 ChIPs are not due to non-specific antibody-DNA interactions or improper DNA fragmentation.

As an additional layer of control, amplification of ChIP derived DNA could also be performed using positive control PCR primer sets. These primers would be designed to recognize segments of DNA that are known sites of interaction for the transcription factors of interest. Obtaining positive results in these additional ChIP-derived DNA PCRs would further demonstrate that the interaction of Sp1, Sp3, Sox10 and c-Jun with the CHRNB4 promoter is specific and not an artifact of the experimental procedure.

CHAPTER IV: THE CA BOX IS AN ESSENTIAL REGULATORY FEATURE OF THE CHRNB4 PROMOTER

Disclaimer: I performed all experiments described in this chapter with the exception of the generation of the WT transgenic construct and the sectioning and staining of the tissue shown in Figures 4.2 and 4.3 (CHRNB4 promoter activity in ED18.5 transgenic mice and CHRNB4 promoter activity in the CNS of PD30 transgenic mice). Generation of the WT CHRNB4 promoter / lacZ transgenic construct was performed by Lei Bruscheiler Li. The tissues shown in Figures 4.2 and 4.3 were sectioned and stained by Ellen Trang and Sarah Binke, respectively. Finally, injection of DNA and all subsequent steps up to and including the generation of founder animals were performed by the University of Massachusetts Medical School Transgenic Animal Modeling Core for both the WT and mutant transgenic animal lines.

Abstract

GWAS studies have underscored the importance of the clustered neuronal nAChR subunit genes with respect to nicotine dependence as well as lung cancer susceptibility. CHRNB4, which encodes the nAChR β 4 subunit, plays a major role in the molecular mechanisms that govern nicotine withdrawal. Thus, elucidating how expression of the CHRNB4 gene is regulated is critical for understanding the pathophysiology of nicotine addiction. We previously identified a CA box regulatory element, (5'– CCACCCCT –3') critical for CHRNB4 promoter activity *in vitro*. We

further demonstrated that a 2.3-kb fragment of the CHRNA4 promoter region containing the CA box is capable of directing cell-type specific expression of a reporter gene to a myriad of brain regions that endogenously express the CHRNA4 gene (Bruschweiler-Li et al., 2010). To test the hypothesis that the CA box is critical for CHRNA4 promoter activity *in vivo*, transgenic animals expressing a mutant form of the CHRNA4 promoter were generated. Reporter gene expression was not detected in any tissue or cell type at embryonic day 18.5 (ED18.5). Similarly, we observed dramatically reduced reporter gene expression at postnatal day 30 (PD30) when compared to wild type transgenic animals. Finally, we demonstrated that CA box mutation results in decreased interaction of the transcription factor Sp1 with the mutant CHRNA4 promoter. Taken together, these results demonstrate that the CA box is critical for CHRNA4 promoter activity *in vivo*.

Introduction

Cholinergic signaling mediated by nAChRs is involved in an array of physiological functions including learning, memory and attention (Albuquerque et al., 2009). Interestingly, substantial alterations in cholinergic signal transduction are observed in numerous neurological disorders including Alzheimer's disease, schizophrenia, epilepsy and nicotine addiction (Whitehouse et al., 1988; Steinlein et al., 1995; De Fusco et al., 2000; Perry et al., 2001; Isacson et al., 2002; Perl et al., 2003; Teaktong et al., 2003). Thus, further elucidating the molecular mechanisms that act to control expression of the nAChR subunit genes will contribute not only to our

understanding of neuronal development but to a greater understanding of the pathophysiology of several neurological disorders as well.

Neuronal populations in both the CNS and PNS express multiple nAChR subtypes (Goldman et al., 1987; Corriveau and Berg, 1993; Vernallis et al., 1993; Conroy and Berg, 1995; Gotti et al., 1997; Zoli et al., 1998; Genzen et al., 2001). Each distinct nAChR subtype displays unique biophysical and pharmacological properties, which are determined by subunit composition (McGehee and Role, 1995). The specific subunits assembled into a particular receptor subtype is dictated, at least in part, by the cell-type specific transcription of the individual subunit genes (Albuquerque et al., 2009).

Three of the twelve neuronal nAChR subunit genes, those encoding the $\alpha 5$, $\alpha 3$ and $\beta 4$ subunits, are located in a highly conserved genomic cluster (Boulter et al., 1990). These three receptor subunit genes are co-expressed in a variety of cell types and tissues in the nervous system (Gotti et al., 2006). The co-expression of these three genes is thought to result from the coordinate regulation of the CHRNA5/A3/B4 locus (Albuquerque et al., 2009). As a likely consequence of their co-expression, the $\alpha 5\alpha 3\beta 4$ receptor subtype is the predominant nAChR expressed in the PNS where they play a crucial role in mediating fast synaptic transmission in autonomic ganglia (Rust et al., 1994; Conroy and Berg, 1995; Flores et al., 1996). Despite their largely overlapping patterns of expression, there are a small subset of regions where these genes appear to be uniquely regulated (Gotti et al., 2007; Grady et al., 2009). For

example, all three of the clustered nAChR subunit genes are expressed in the hippocampus, habenula and interpeduncular nucleus whereas only CHRNA3 and CHRNA5 are expressed in the ventral tegmental area (Improgo et al., 2010b).

nAChR subunit gene KO mice have proven to be a valuable tool for deciphering the function of specific nAChR subtypes in the nervous system. CHRNB4 KO animals display significantly milder somatic symptoms of nicotine withdrawal (Salas et al., 2004a). These results demonstrate that the β 4 subunit plays a major role in the molecular mechanism underlying nicotine dependence. There is also some indication that blockade of α 3 β 4 nAChRs results in a reduction of opioid and stimulant self-administration, suggesting that nAChRs that contain the β 4 subunit are involved in mediating withdrawal syndromes elicited by other drugs of abuse (Glick et al., 2002). Thus, elucidating the molecular mechanisms underlying expression of the CHRNB4 gene will improve our understanding of nicotine addiction and withdrawal.

The expression pattern of the CHRNB4 gene has been investigated by several groups, (Dineley-Miller and Patrick, 1992; Zoli et al., 1995; Winzer-Serhan and Leslie, 1997) yet the mechanisms that control its expression are not completely understood. We previously identified an 8-base pair transcriptional regulatory element in the CHRNB4 promoter that is critical for promoter activity *in vitro* (Bigger et al., 1996). This regulatory element, deemed the CA box due to its nucleotide

composition, is also the binding site for several transcription factors *in vivo* (Scofield et al., 2008).

We recently demonstrated that a 2.3-kb fragment of the CHRNB4 promoter region containing the CA box is capable of directing cell-type specific and developmentally regulated expression of a reporter gene *in vivo* (Bruschweiler-Li et al., 2010). In these experiments we used a 2.3-kb fragment of the rat CHRNB4 promoter to drive expression of a lacZ reporter gene in transgenic mice. The lacZ reporter gene was placed upstream of a nuclear localization sequence in order to restrict reporter gene expression to the nucleus. This was done in order to more easily compare our data to previous CHRNB4 *in situ* hybridization studies (Fig. 4.1) (Dineley-Miller and Patrick, 1992; Zoli et al., 1995). We also included a matrix attachment region (MAR) in the transgenic construct, which has been shown previously to inhibit position- and copy number-dependent effects on transgene expression (Fig. 4.1) (Phi-Van and Stratling, 1996). In the CHRNB4 promoter / lacZ transgenic animals we observed a striking recapitulation of CHRNB4 expression patterns at ED18.5. For example, we observed β -gal reactivity in the cortex, spinal cord, intestine and tongue (Fig. 4.2 A, A-D), all regions that endogenously express the CHRNB4 gene (Zoli et al., 1995; Winzer-Serhan and Leslie, 1997; Flora et al., 2000b; Glushakov et al., 2004). We also observed a striking recapitulation of CHRNB4 expression in the CNS at PD30. β -galactosidase (β -gal) expression was detected in the layer 5 of the frontal cortex, as well as in the piriform cortex, association cortex, and caudate putamen (Fig. 4.3 A-D) (Bruschweiler-Li et al., 2010).

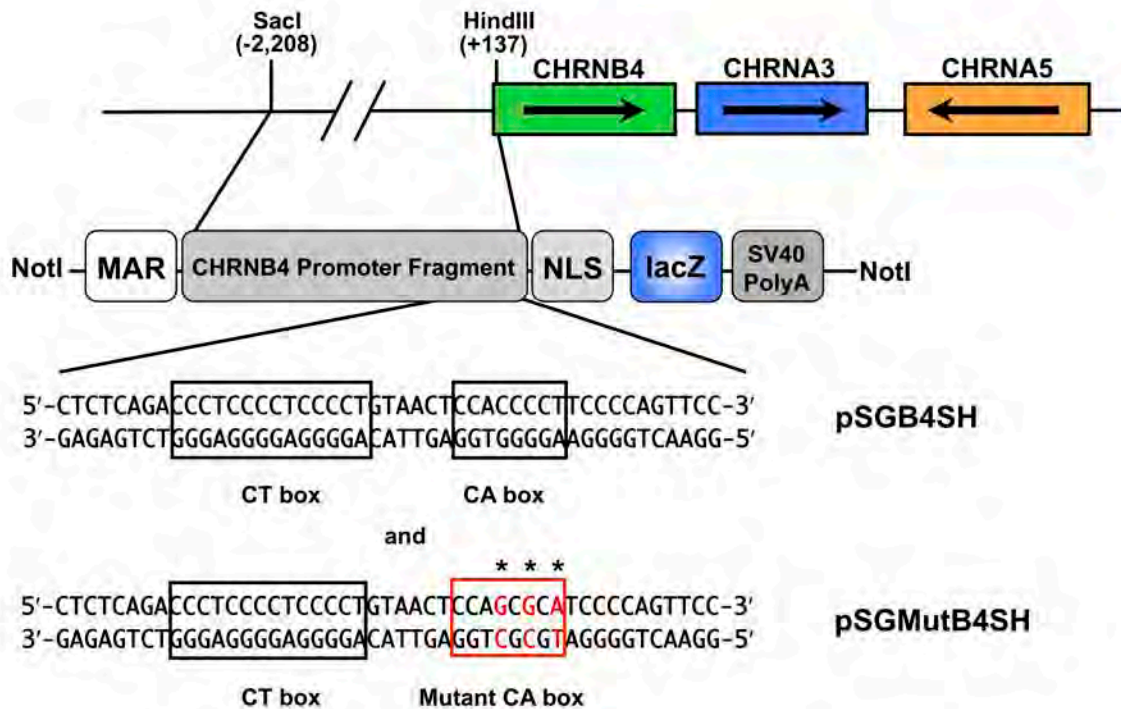


Figure 4.1: WT and mutant CHRNA4 promoter / lacZ transgene architecture.

The CHRNA5/A3/B4 gene cluster is depicted as boxes with arrows depicting the direction of transcription. Below the clustered nAChR subunit genes is a schematic of the linearized construct used to generate the transgenic animals. MAR, matrix attachment region; NLS, nuclear localization sequence. Shown below this schematic are the nucleotide sequences of the WT and mutant CA boxes. Mutations made to the CA box in the mutant transgenic construct are shown in red with asterisks above the mutated nucleotides.

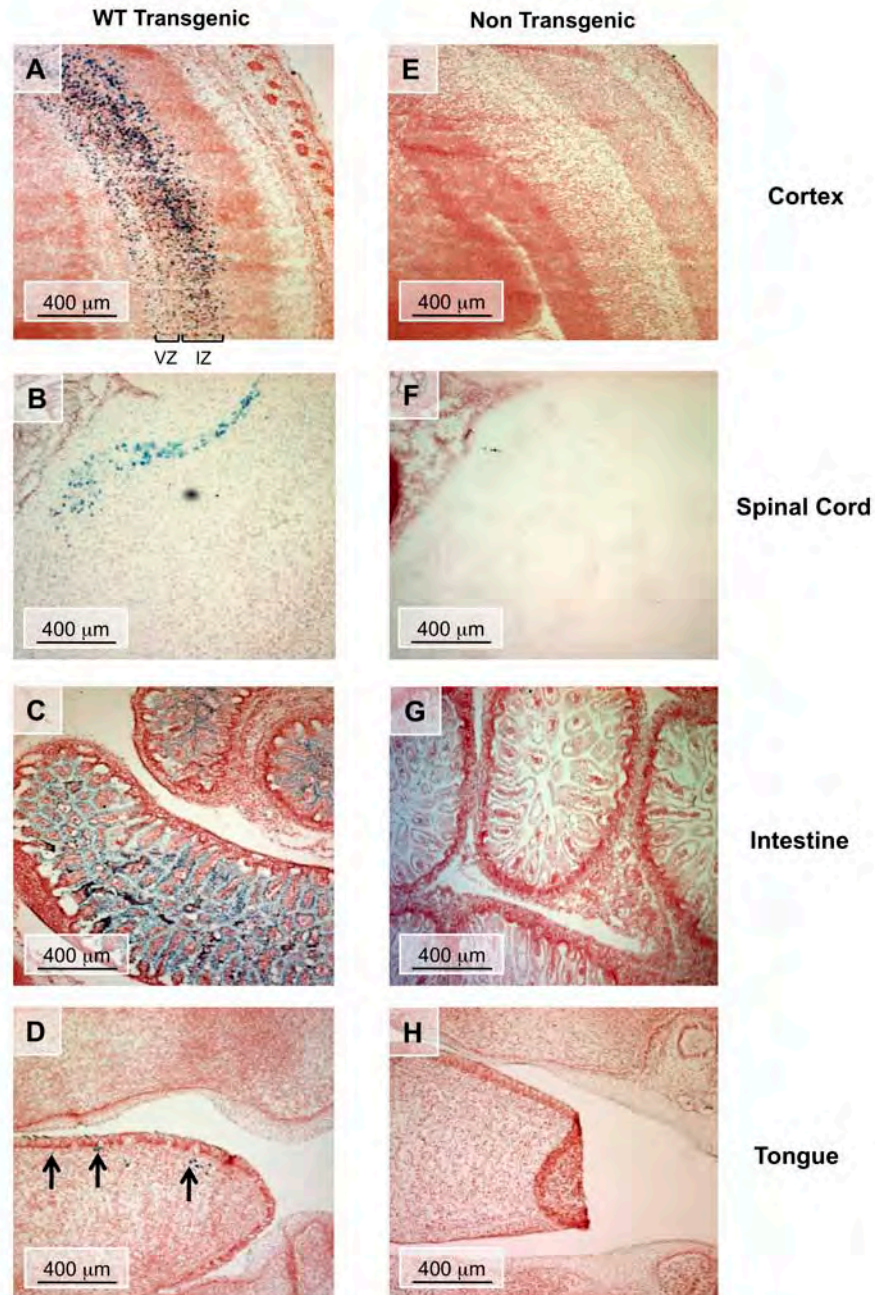


Figure 4.2: CHRN B4 promoter activity in ED18.5 transgenic mice. Sagittal sections of WT transgenic line 54 (A - D) and non transgenic (E - H) ED18.5 mouse embryos are shown. These sections were simultaneously stained for β -Gal activity and then counter-stained with neutral red. (A and E) cortex (IZ, intermediate zone; VZ, ventricular zone); (B and F), upper cervical region of the spinal cord; (C and G), mucosa of the small intestine; (D and H) tongue. β -gal staining of the tongue appears to be restricted to or near the longitudinal intrinsic muscle. Arrows in panel D indicate β -gal-expressing cells.

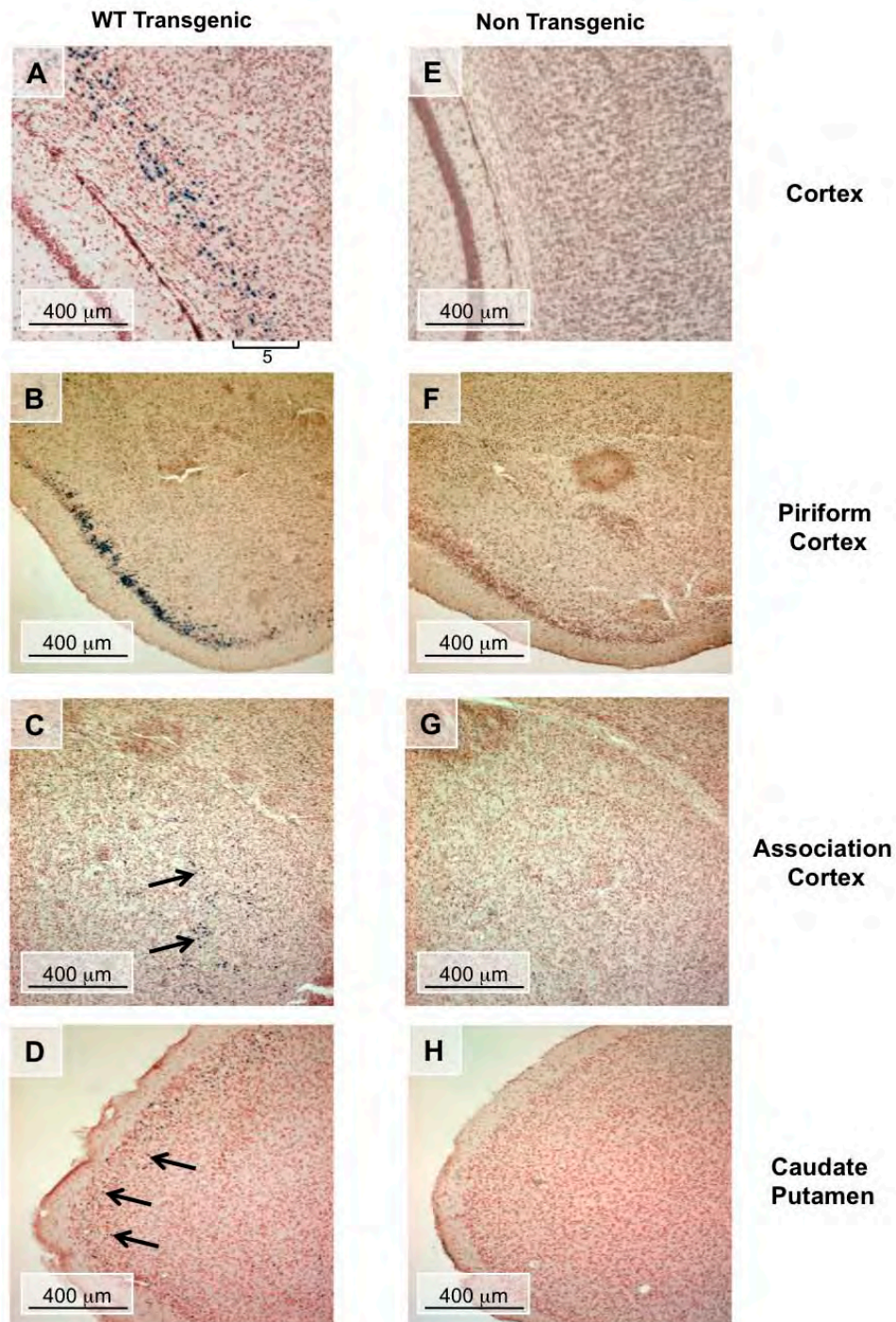


Figure 4.3: CHRNb4 promoter activity in the CNS of PD30 transgenic mice. Coronal sections of WT transgenic line 39 (A - D) and non transgenic (E - H) PD30 mouse brains are shown. These sections were simultaneously stained for β -Gal activity, and then counter-stained with neutral red. (A and E) layer 5 of the cortex ("5"); (B and F) piriform cortex; (C and G) association cortex; (D and H) caudate putamen; Arrows in panels C and D indicate β -Gal-expressing cells.

These brain regions are also areas of endogenous CHRNA4 gene expression (Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997; Gahring et al., 2004). To date, this transgenic model is the only system that accurately recapitulates the endogenous patterns of CHRNA4 during development in the PNS and CNS. Moreover, this transgenic model is also the only system that recapitulates the endogenous expression patterns of CHRNA4 in non-neuronal tissues like the intestine and tongue (Bruschweiler-Li et al., 2010).

In this work, I sought to determine whether the CA box regulatory element within the CHRNA4 promoter region is indeed critical for the temporally and spatially restricted transcriptional activity of the CHRNA4 promoter *in vivo*. In order to test this hypothesis I generated transgenic animals using a transgenic construct with 3 base pair substitutions made to the CA box (Fig. 4.1).

Results

Mutation of the CA box significantly reduces CHRNA4 promoter activity in vitro

Previously, we showed that mutagenesis of the CA box within the CHRNA4 promoter virtually eliminates promoter activity *in vitro* (Bigger et al., 1996). When both the WT and mutant CHRNA4 promoter transgenic constructs were tested *in vitro*, significantly reduced levels of β -Gal activity were observed in the two neuronal-like cell lines Neuro 2A and SN17 (Fig. 4.4).

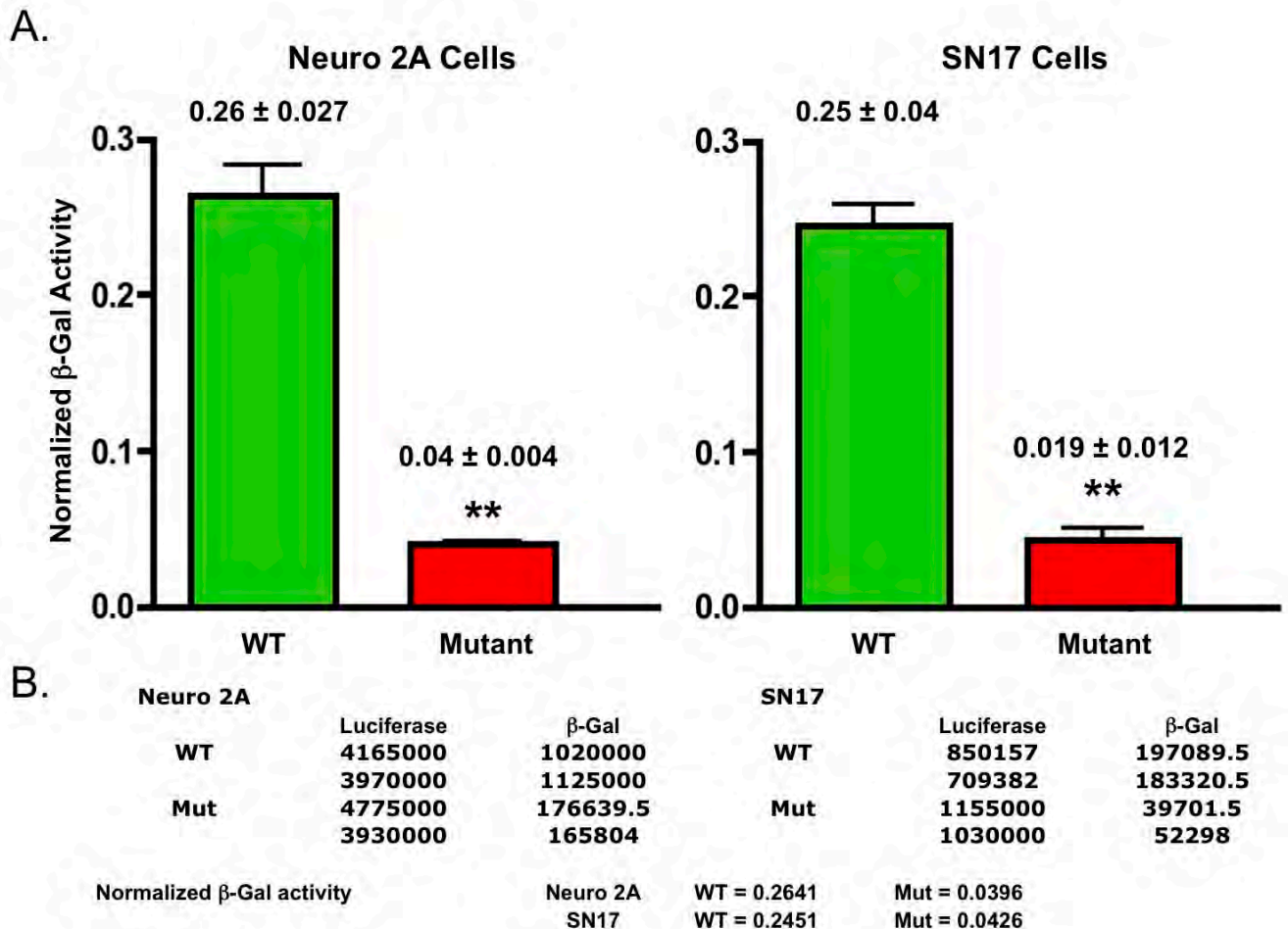


Figure 4.4: WT and mutant CHRN4 promoter activity in vitro. (A) DNA from either the WT (green) or mutant (red) transgenic constructs was transfected into Neuro 2A (left) or SN17 cells (right) along with a luciferase construct in which the SV40 promoter drives expression of the firefly luciferase gene. β -Gal and luciferase activities were measured with a luminometer. β -Gal activity was normalized to luciferase activity in order to correct for differences in transfection efficiencies. The data shown here are an average of 2 independent experiments, error bars represent standard error of the means, $n=2$. Student's t-test indicated that CA box mutation significantly decreased the β -gal activity of the mutant transgenic construct in both cell lines, $p<0.01$ (**). (B) Raw data for the graphs shown above, values reported are relative light units.

These results indicated that the mutant construct was suitable for use in the generation of transgenic animals. A total of 6 CHRNB4 mutant promoter / lacZ founder lines (lines 19, 25, 28, 30, 33, and 83) were generated and analyzed for β -Gal activity at both ED18.5 and PD30. Using a quantitative PCR approach (Bruschweiler-Li et al., 2010), copy number analysis revealed that line 19 has 10 copies, line 25 has 63 copies, line 28 has 69 copies, line 30 has 25 copies, line 33 has 23 copies and line 83 has 37 copies (Fig. 4.5).

CA box mutation eliminates CHRNB4 promoter activity in transgenic animals at ED18.5

In the WT CHRNB4 promoter / lacZ transgenic lines, we observed reporter gene expression in an array of regions of endogenous CHRNB4 gene expression at ED18.5 in both the CNS and PNS, including the spinal cord, intestine, and cortex (Fig. 4.6 A-C) (Zoli et al., 1995; Bruschweiler-Li et al., 2010). In contrast, no reporter gene expression was seen in any of these tissues at ED18.5 in any of the mutant CHRNB4 promoter / lacZ transgenic lines (Fig. 4.6 E-G). CHRNB4 gene expression is observed early during prenatal development in the dorsal root ganglia (DRG) where it co-assembles with the $\alpha 3$ and $\alpha 5$ subunits to form the $\alpha 5\alpha 3\beta 4$ nAChR subtype. This receptor subtype has been shown previously to be the predominant subtype expressed in the PNS (Conroy and Berg, 1995) where it plays a role in nicotinic transmission at the synapses of autonomic ganglia (Vernallis et al., 1993). At ED18.5, we observed β -gal expression in the DRG of the WT transgenic lines

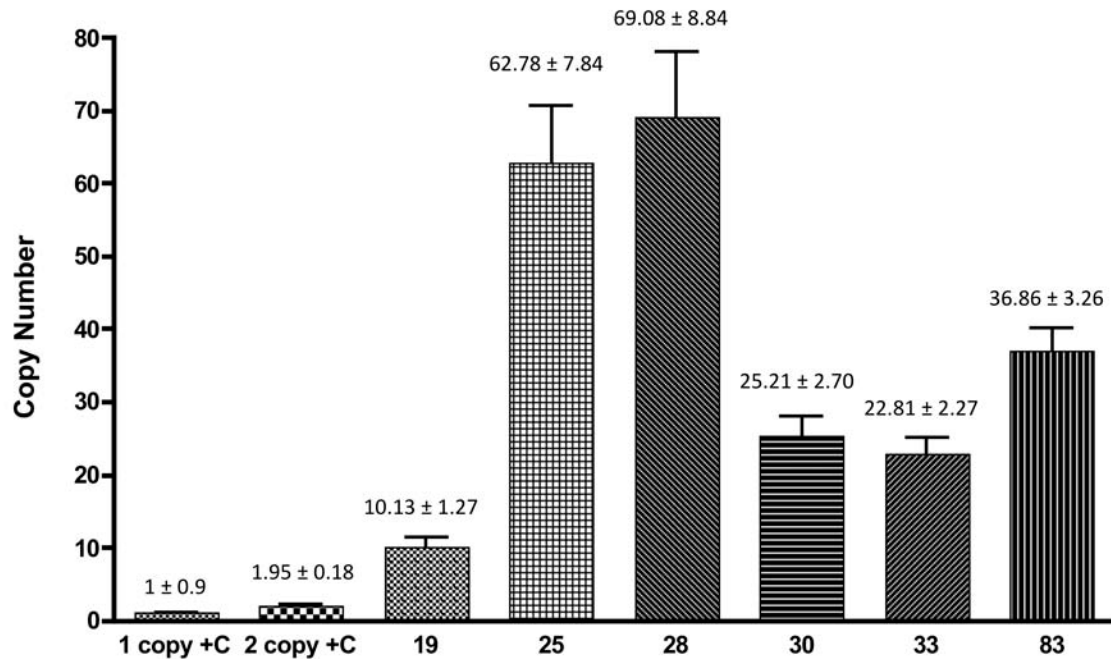


Figure 4.5: Copy number of the mutant CA box transgenic lines. Transgene copy number was determined using absolute quantification-based real-time PCR (Yuan et al., 2007) designed to amplify the lacZ sequence in the transgenic construct. A positive control sample from a mouse containing a single copy of a lacZ gene (1 copy +C) was used to normalize values obtained for CA box mutant transgenic lines 19, 25, 28, 30, 33 and 83. An additional positive control isolated from a mouse that has 2 copies of a lacZ transgene was also analyzed (2 copy +C). The data shown here are an average of values obtained for three individual animals from each transgenic line, error bars represent standard error of the means.

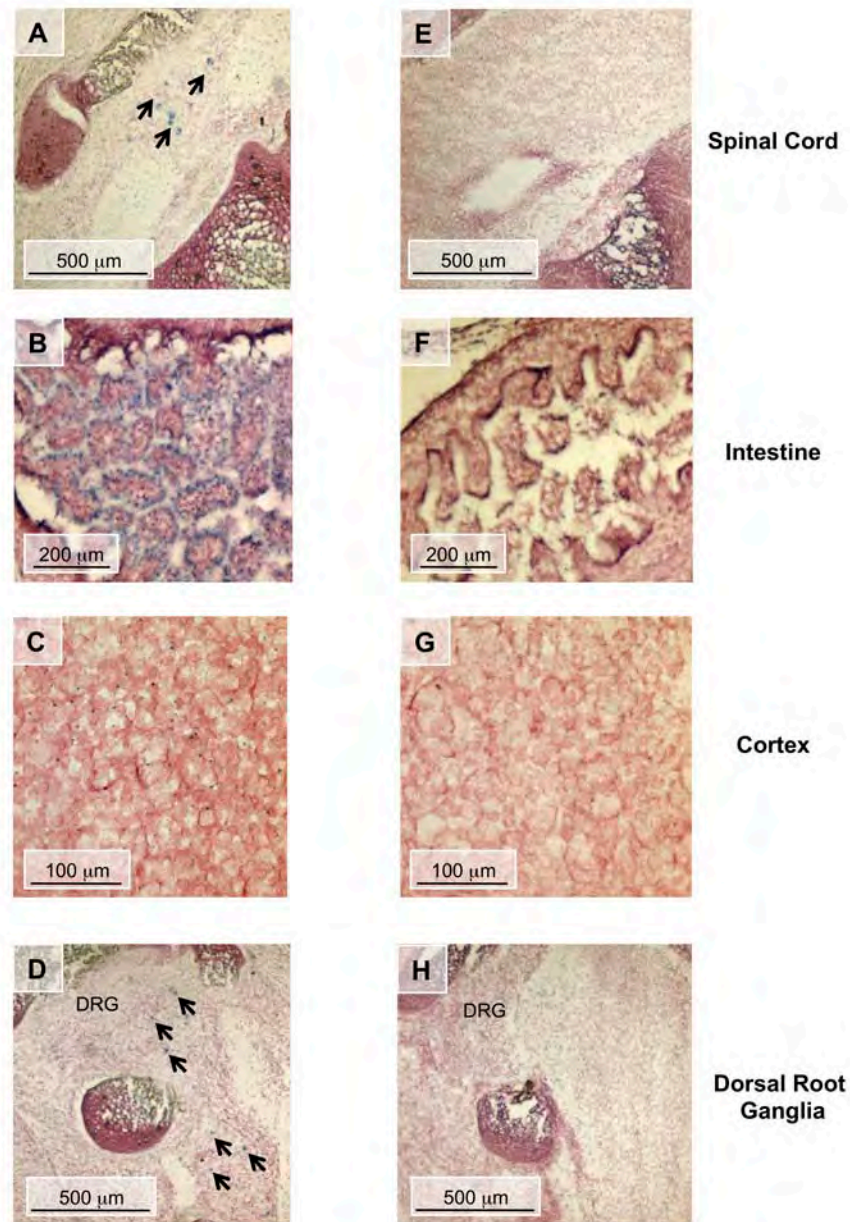


Figure 4.6: CHRN B4 promoter activity in ED18.5 WT and mutant transgenic mice. Sagittal sections of WT transgenic line 54 (A - D) and mutant transgenic line 28 (E - H) ED18.5 mouse embryos are shown. These sections were simultaneously stained for β -Gal activity and then counter-stained with neutral red. (A and E) lower lumbar region of the spinal cord; (B and F), mucosa of the small intestine; (C and G) cortex (β -gal-expressing cells are located in the intermediate zone); (D and H) lower lumbar dorsal root ganglion (DRG). Arrows in panels A, C and D indicate β -gal-expressing cells.

(Fig. 4.6 D) at levels that closely resemble what has been reported in previous CHRN4 in situ experiments done by Changeux and colleagues (Zoli et al., 1995). However, no β -gal expression was observed in the DRG of any of the six mutant transgenic lines (Fig. 4.6 H).

Mutant transgenic animals express very low levels of β -gal at PD30 in the CNS

β -Gal activity was observed in PD30 WT transgenic lines in several areas that endogenously express the CHRN4 gene including the piriform cortex, medial habenula, and the subiculum (Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997; Gahring et al., 2004; Bruschweiler-Li et al., 2010) (Fig. 4.7 A-C). Out of the 6 mutant transgenic founder lines investigated, no β -gal expression was observed in any region of the brain at PD30 in 4 of these lines, lines 25, 30, 33, and 83 (Table 4.1). The remaining 2 lines, mutant lines 19 and 28, expressed extremely low levels of the β -gal in a small subset of brain regions (Table 4.1). Interestingly, no expression of β -gal was observed in any of the mutant transgenic lines in several areas of endogenous CHRN4 expression including the piriform cortex, medial habenula, or the subiculum (Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997; Gahring et al., 2004) (Fig. 4.7 E-G). In addition, mutant transgenic lines 19 and 28 displayed fewer β -gal positive cells when compared to the WT transgenic lines in several areas of endogenous CHRN4 expression. For example, I observed a drastically reduced number of β -gal positive cells in the dentate gyrus of mutant transgenic line 28 when compared to WT transgenic line 39 (Fig. 4.6 D, H) (Table 4.1).

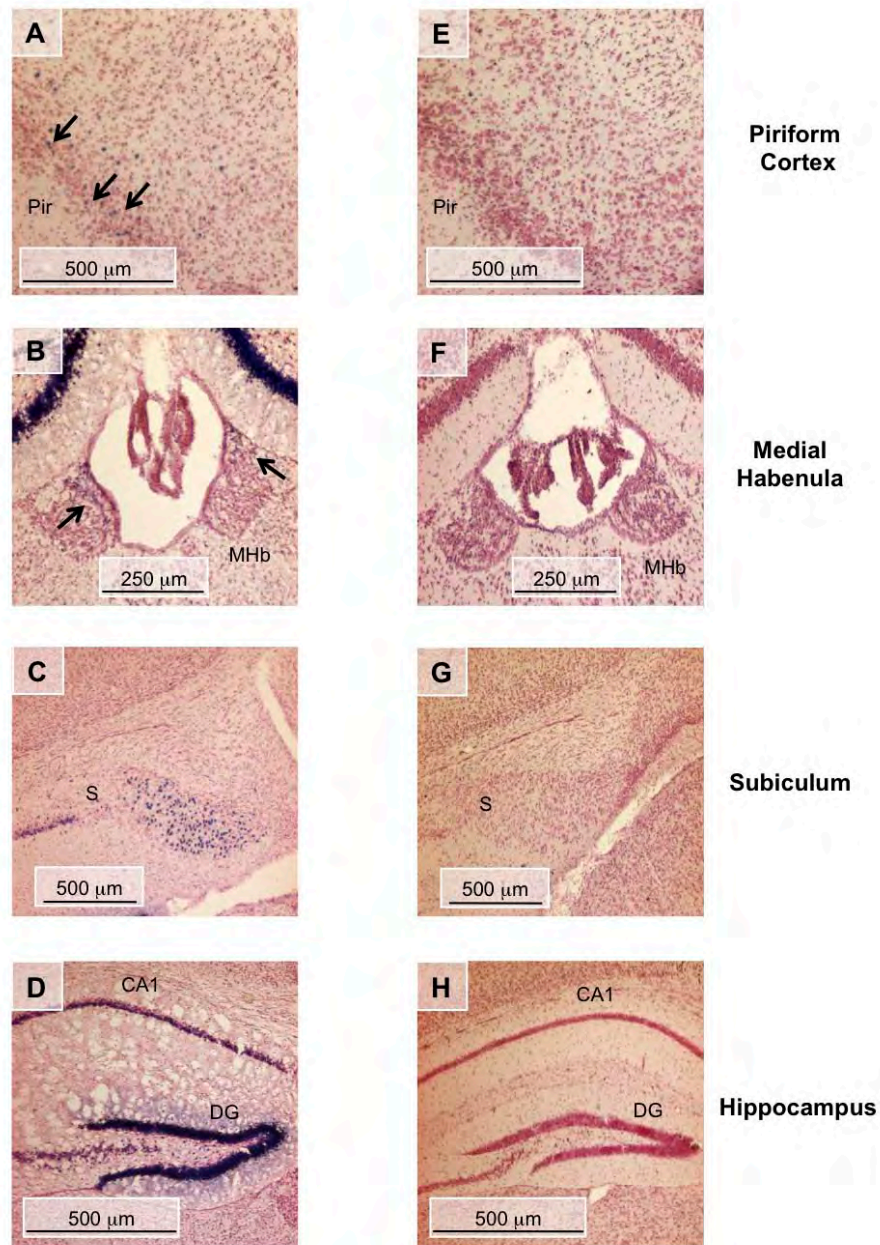


Figure 4.7: CHRNb4 promoter activity in the CNS of PD30 WT and mutant transgenic mice. Coronal sections of WT transgenic line 39 (A - D) and mutant transgenic line 28 (E - H) PD30 mouse brains are shown. These sections were simultaneously stained for β -Gal activity and then counter-stained with neutral red. (A and E) piriform cortex (Pir); (B and F) medial habenula (Mhb); (C and G) subiculum (S); (D and H) hippocampus, cornu ammonis 1 (CA1), dentate gyrus (DG); Arrows in panels A and B indicate β -Gal-expressing cells.

Table 4.1: CHRNA4 promoter activity in the CNS of PD30 WT and mutant transgenic mice.

	Wild type Transgenic				Mutant CA Box Transgenic					
Founder Line:	30	39	54	447	19	25	28	30	33	83
Brain Region										
(Pir) Piriform Cortex	++	+++	++++	+++	-	-	-	-	-	-
(Cpu) Caudate Putamen	+	+	++	++	-	-	-	-	-	-
(MHb) Medial Habenula	-	+++	++	+	-	-	-	-	-	-
(DG) Dentate Gyrus	++	++++	++	++	+	-	+	-	-	-
(S) Subiculum	-	+++	++++	+	-	-	-	-	-	-
(Cb) Cerebellum	-	-	-	-	-	-	-	-	-	-
(SN) Substantia Nigra	-	-	-	-	-	-	-	-	-	-
(IPn) Interpeduncular Nucleus	-	-	-	-	-	-	-	-	-	-
Transgene Copy Number	7	26	12	10	10	62	69	25	23	37

Expression levels were scored as follows: -, no expression; +, low level; ++, intermediate level; +++, high level; +++++, very high level.

Consistent with our previous observations at ED18.5, β -gal expression was detected in the DRG of 4 WT transgenic lines at PD30 while no expression of β -gal was detected in the DRG in any of the 6 mutant transgenic lines (Fig. 4.8). In the WT transgenic animals, there are higher numbers of lacZ positive cells in the DRG of ED18.5 transgenic animals (Fig. 4.6, D) when compared to PD30 WT transgenic animals (Fig. 4.8, A) This result is in agreement with previous in situ hybridization studies demonstrating that DRG expression of CHRN4 is highest early during development (Zoli et al., 1995; Winzer-Serhan and Leslie, 1997). Taken together, these data suggest that the CA box regulatory element plays a critical role in directing CHRN4 promoter activity *in vivo*.

CA box mutation results in decreased levels of Sp1 association with the CHRN4 promoter in vivo

We previously demonstrated that, in addition to virtually eliminating promoter activity, mutation of the CHRN4 promoter CA box substantially reduced binding and transactivation by the transcription factor Sp1 *in vitro* (Bigger et al., 1996; Bigger et al., 1997). In order to test the hypothesis that CA box mutation results in reduced association of the Sp1 transcription factor with the CHRN4 promoter *in vivo*, brain tissue from mutant CHRN4 promoter / lacZ transgenic line 28 was used as a source of chromatin for ChIP assays. Using these animals I can assay for the interaction of Sp1 with the endogenous mouse CHRN4 promoter or the transgenic rat CA box mutant CHRN4 promoter.

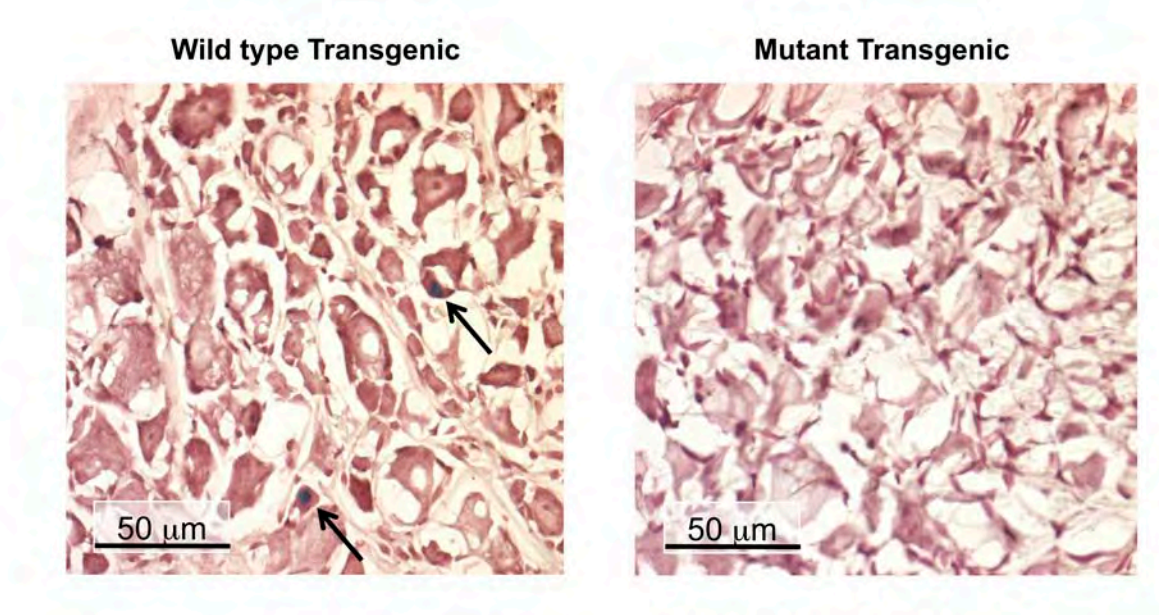


Figure 4.8: CHRN4 promoter activity of in the DRG of PD30 transgenic mice. Sections of WT transgenic line 54 (left) and mutant transgenic line 28 (right) PD30 DRG are shown. These sections were simultaneously stained for β -Gal activity and then counter-stained with neutral red. Arrows in panel A indicate β -Gal-expressing cells.

ChIP-derived DNA from the mutant transgenic brain tissue experiments was used as template in PCR reactions with primers that recognize either the endogenous mouse CHRN4 promoter region containing the CA box, or the corresponding segment of the rat transgenic promoter containing the mutant CA box. Consistent with our previous work (Scofield et al., 2008), PCR using Sp1 ChIP-derived DNA as template resulted in amplification of the endogenous mouse CHRN4 promoter to levels that were approximately 3-fold higher than controls (Fig. 4.9, black bars), indicating an interaction of Sp1 with the endogenous CHRN4 promoter. Conversely, PCR reactions using the same Sp1 ChIP-derived DNA as template *did not* result in amplification of the mutant transgenic CHRN4 promoter above background levels (Fig. 4.9, white bars), indicating that there was no or significantly reduced interaction between Sp1 and the mutant CHRN4 promoter. The H4 ChIP positive control is performed using an antibody directed against acetylated histone protein H4. Acetylation of the H4 protein is highly associated with actively transcribed genes (Hebbes et al., 1988; Jeppesen and Turner, 1993). As the mutant CA box CHRN4 promoter is significantly less active, this may be a possible explanation for the differences in the endogenous (black) and transgenic (white) H4 ChIPs.

Discussion

The biophysical diversity of nAChRs is a result of the large variety of subunit combinations that can assemble into functional receptors (Gotti et al., 2006).

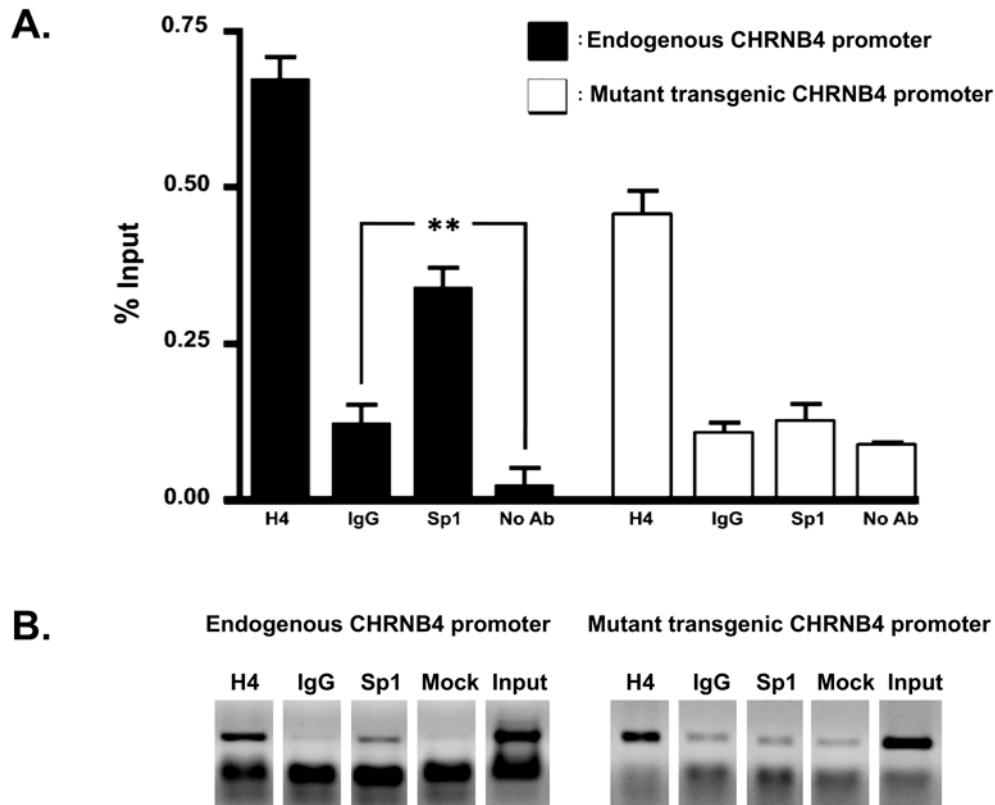


Figure 4.9: CA box mutation results in decreased association of Sp1 with the CHRNb4 promoter. (A) ChIP experiments were performed using brain tissue from mutant CHRNb4 promoter / lacZ transgenic line 28. Transgenic brain ChIP-derived DNA was used as template for PCR designed to amplify either the WT endogenous mouse CHRNb4 promoter (black bars), or the CA box mutant transgenic rat CHRNb4 promoter (white bars). (H4) anti-acetylated histone protein H4; (IgG) normal rabbit IgG; (Sp1) Transcription factor Sp1; (No Ab) Mock IP. The data shown here are an average of 4 independent experiments, expressed as percent Input. Student's t-test statistical analysis indicated a significant difference between the Sp1 ChIPs and the WT IgG and mock ChIP controls, $p < 0.01$ (**). Student's t-test statistical analysis was also performed on the transgenic mutant CHRNb4 promoter values (white bars) and indicated no significant difference between Sp1 ChIPs and the IgG and mock ChIP controls at the mutant CHRNb4 promoter. Error bars represent standard error of the means, $n=4$. (B) Representative agarose gels for either the endogenous wild type CHRNb4 promoter PCR (left) or the mutant transgenic CHRNb4 promoter PCR (right).

While the exact molecular mechanisms controlling the incorporation of a particular subunit into a mature receptor are not completely understood, regulation at the level of transcription plays a critical role. Consistent with this hypothesis, the CHRNA5, CHRNA3, and CHRNB4 genes are co-expressed in a variety of cell types in the PNS where the $\alpha 5\alpha 3\beta 4$ receptor subtype is the predominant nicotinic receptor subtype expressed (Rust et al., 1994; Conroy and Berg, 1995). The co-expression of these subunits is likely the result of coordinate regulation of the CHRNA5/A3/B4 locus by shared transcriptional regulatory features, an example of which is the positive regulation of these three genes by the transcription factor Sp1 (Yang et al., 1995; Bigger et al., 1997; Campos-Caro et al., 1999; Improgo et al., 2010b). Data from our lab as well as from others indicate that Sp1 likely acts in concert with cell-type specific regulatory factors (e.g., Nuclear transcription factor Y and Paired mesoderm homeobox protein 2A) in order to direct expression of the clustered subunit genes to the appropriate cell types (Campos-Caro et al., 1999; Melnikova et al., 2000b; Valor et al., 2002; Benfante et al., 2007; Scofield et al., 2008).

Several groups, including our own, have intensively studied the transcriptional regulatory mechanisms that act to control expression of these three subunit genes. These studies have uncovered several regulatory elements (discussed below) that are involved in directing cell-type specific expression of the clustered nAChR subunit genes. In these studies, I have used mutant CHRNB4 promoter transgenic mice to demonstrate that the CA box is a critical mediator of CHRNB4 promoter activity *in vivo*. Mutation of the CA box resulted in absent or substantially decreased

expression of the β -gal in an array of brain regions where β -gal expression is observed in the WT CHRN4 promoter / lacZ transgenic lines (Bruschweiler-Li et al., 2010) and CHRN4 is endogenously expressed (Winzer-Serhan and Leslie, 1997; Gahring et al., 2004). Taken together, these results demonstrate that the CA box is crucial for CHRN4 promoter activity *in vivo*.

Previous studies done by Deneris and colleagues have uncovered additional transcriptional regulatory elements that influence expression of the CHRN4 gene. One such element, deemed the conserved non-coding region #4 (CNR4), is located approximately 30-kb upstream of the CHRN4 gene (Fig. 4.10, A) (Xu et al., 2006). CNR4 appears to contain regulatory information needed to direct expression of the CHRN4 gene to the pineal gland and interpeduncular nucleus (Xu et al., 1999a), two areas of endogenous CHRN4 gene expression where we do not see β -gal expression in the WT (Bruschweiler-Li et al., 2010) or mutant CHRN4 promoter / lacZ transgenic lines. Additional experiments done by Deneris and colleagues demonstrated that CNR4 is critical for the coordinate expression of the CHRN4 and CHRNA3 genes in the pineal gland as well as in the superior cervical ganglion. Based on its high degree of conservation and its distal location from the genes it acts to regulate, CNR4 is thought to function as a locus control region-like regulatory domain (Xu et al., 2006). In addition to CNR4, Deneris and colleagues have also characterized a transcriptional enhancer element located in the CHRN4 3'-untranslated region (called the β 43' enhancer) (Fig. 4.10, A).

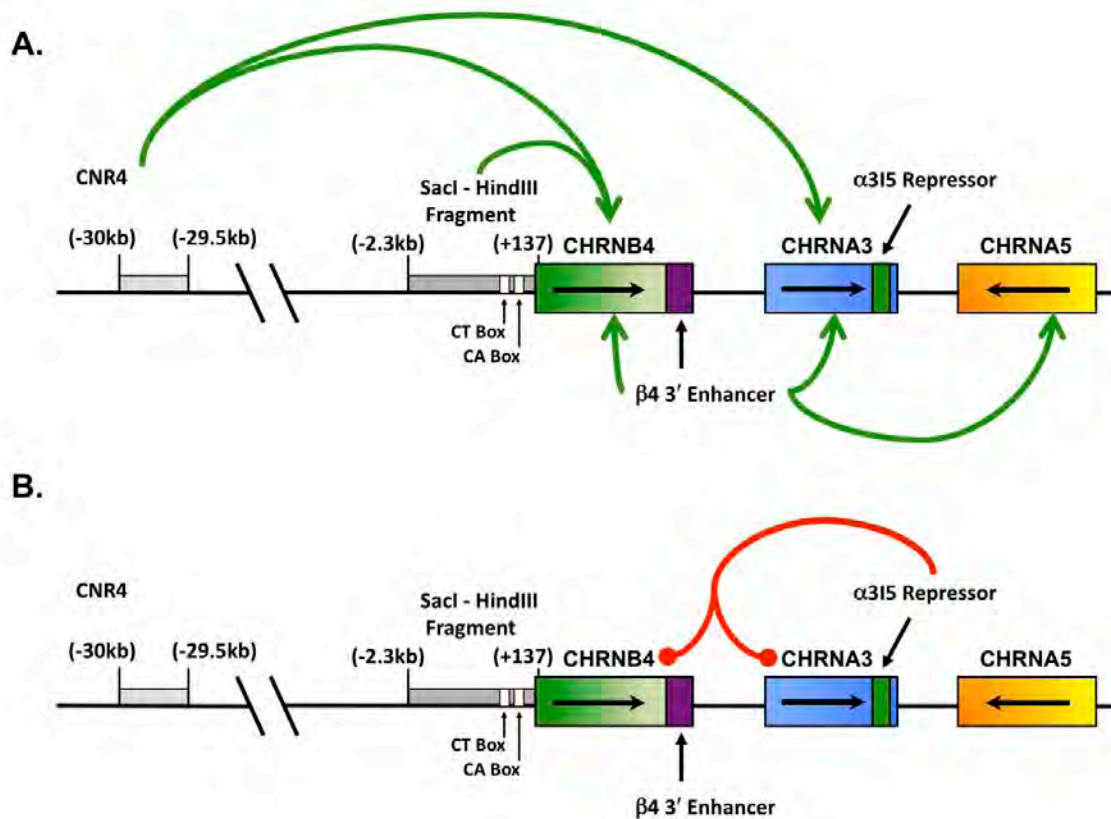


Figure 4.10: Positive and negative regulation of the CHRNB4 locus. (A and B) Coding regions of the clustered subunits are represented as colored boxes with arrows that indicate the direction of transcription. Four transcriptional regulatory elements are depicted in this figure: $\alpha 315$ (green box), the $\beta 4$ 3' enhancer (purple), the Sacl – HindIII fragment of the *CHRNB4* promoter region (dark grey box), and the distal CNR4 regulatory region (white box). The boundaries of the latter two regions are labeled relative to the transcriptional start site of the *CHRNB4* gene. Green arrows in A denote positive regulatory effects in a neuronal context whereas red lines in B indicate negative transcriptional regulation occurring in non-neuronal cells.

Interestingly, mutation of several E26 transformation-specific sequence (ETS) factor binding sites in the enhancer can severely diminish expression of the CHRNA5, CHRNA3 and CHRNB4 genes in the adrenal gland (Xu et al., 2006) (Fig. 4.10, A). Furthermore, mutation of ETS binding sites within the $\beta 43'$ enhancer also diminished expression of these genes in the superior cervical ganglion (Xu et al., 2006). Apart from the positive regulatory elements described above, we have previously shown that there is a transcriptional repressor element present in the fifth intron of the CHRNA5 gene (A3I5) (Fuentes Medel and Gardner, 2007) (Fig. 4.10, B). The A3I5 repressor is capable of inhibiting transcription from CHRNA3 and CHRNB4 gene promoters in an orientation- and position-independent manner. Interestingly, repression of transcription by A3I5 was cell-type specific, only occurring in cells with little or no expression of the clustered nAChR subunit genes (Fuentes Medel and Gardner, 2007). It is clear from these data that several transcriptional regulatory elements located around the clustered subunit genes act in concert to provide the necessary regulatory information to drive expression of the required subunits in the appropriate cell type (Yang et al., 1997; Francis and Deneris, 2002; Xu et al., 2006; Fuentes Medel and Gardner, 2007; Bruschweiler-Li et al., 2010).

ChIP experiments done on mutant transgenic brain tissue demonstrated a reduced interaction of Sp1 with the mutant CA box CHRNB4 promoter (Fig. 4.9). To date, Sp1 is the only factor that has been investigated using CA box mutant transgenic brain tissue. These data are in agreement with previous studies from our lab indicating that CA box mutation abrogates Sp factor binding and transactivation of

the CHRNA4 promoter *in vitro* (Bigger et al., 1996; Bigger et al., 1997). Because the CHRNA4 promoter lacks a TATA box, Sp1 could play a crucial role in tethering the basal transcription machinery to the CHRNA4 promoter, as it does at other TATA-less promoters (Pugh and Tjian, 1991). The lack of Sp1 interaction with the mutant CA promoter provides a plausible molecular mechanism for the severely reduced CHRNA4 promoter activity observed in the CA box mutant CHRNA4 promoter / lacZ transgenic lines. CA box mutation may also impede the transactivation ability of additional transcriptional regulatory factors that associate with the CHRNA4 promoter by virtue of protein-protein interactions with Sp1 (Melnikova et al., 2000a; Scofield et al., 2008). This is likely the case for transcription factor c-Jun, which physically interacts with Sp1 and is capable of activating transcription from the CHRNA4 promoter *in vitro*, despite the fact that the CHRNA4 promoter lacks a c-Jun binding site. At the WT CHRNA4 promoter, Sp1 and c-Jun synergistically activate transcription, however, when the CA box is mutated, the CHRNA4 promoter is no longer responsive to either Sp1 or c-Jun (Melnikova and Gardner, 2001). In addition, the transcription factor Sox10 also synergistically transactivates the CHRNA4 promoter when supplied in concert with Sp1 (Melnikova et al., 2000b). Similar to what is observed with c-Jun, CA box mutation also dramatically reduces Sox10's ability to activate transcription from the CHRNA4 promoter (Liu et al., 1999). From these data it appears that Sp1 may act to nucleate a complex of positive-acting transcription factors needed for cell-type specific expression for the CHRNA4 gene *in vivo*. However, additional experimentation is required to test the hypothesis that

Sp1 binding is upstream of the binding of the other factors shown previously to interact with the CHRN4 promoter CA box *in vivo*.

In conclusion, these results demonstrate that the CA box is a key determinant of CHRN4 promoter activity at ED18.5 and PD30 in the PNS and CNS. Furthermore, these data also indicate that Sp1 plays a critical role in directing the positive transcriptional regulatory effect of the CA box *in vivo*. Positive regulation of the CHRN4 promoter by Sp1 at the CA box is a crucial aspect of the intricate regulatory cascade that ensures accurate expression of the CHRN4 gene, allowing nAChRs containing the $\beta 4$ subunit to participate in both normal physiological processes (Dani and Bertrand, 2007a; Albuquerque et al., 2009), as well as tobacco-related pathological conditions (Salas et al., 2004a; Salas et al., 2009).

These studies provide insight into the protein-DNA interactions that govern expression of the CHRN4 gene in the mammalian brain. Furthermore, these data indicate that the CA box is an essential regulatory element in the CHRN4 promoter, and that mutation of this element reduces promoter activity as well as the interaction of the transcription factor Sp1 with the CHRN4 promoter *in vivo*.

Limitations and Future Perspectives

Although there was no CHRN4 promoter activity in the mutant transgenic animals at ED18.5, it remains unclear if the CA box is involved in directing basal promoter activity or if it governs the developmentally and temporally regulated expression of

the CHRNA4 gene. Due to the dramatically reduced promoter activity observed in the mutant transgenic animals, it is most likely that the CA box and its interaction with Sp1 is critical for basal activity of the CHRNA4 promoter. This hypothesis is consistent with findings from other groups demonstrating that regulation by Sp1 is essential for the basal activity of other TATA-less promoters (Botella et al., 2001; Kim et al., 2010; Yu et al., 2010). Future studies should focus on testing this hypothesis by investigating the association of RNA polymerase II and the general transcription factors with the wild type and mutant CA box CHRNA4 promoter. These experiments would provide insight into the molecular mechanism behind the reduction of transcriptional activity observed at the mutant CA box CHRNA4 promoter.

In order to further elucidate how CA box mutation impacts transcription factor occupancy at the CHRNA4 promoter, additional Sp3, Sox10 and c-Jun ChIP assays should be performed using mutant transgenic animal brain tissue. These experiments could test the hypothesis that CA box mutation impacts the interaction of Sp3, Sox10 and c-Jun with the CHRNA4 promoter, in the mammalian brain. I would expect the additional ChIP assays to demonstrate little to no interaction of Sp3, Sox10 and c-Jun with the mutant promoter as CA box mutation abrogates transactivation of the CHRNA4 promoter by these factors *in vitro* (Bigger et al., 1997; Melnikova et al., 2000b; Melnikova and Gardner, 2001).

In the work discussed above we used a conventional transgenic approach to investigate WT and mutant CHRNA4 promoter activity *in vivo*. The integration site of the CHRNA4 promoter reporter construct and the number of transgene integration events are not controlled. As discussed above, we included a MAR element in our transgenic constructs to control for integration position-dependent effects as well as variability in levels of transgene expression (Phi-Van and Stratling, 1996). Due to the fact that we observed very similar results in mutant transgenic lines 19 and 28, which had the largest difference in copy number (Fig. 4.5), I was not concerned that the copy number was impacting the results.

The hypothesis that the CA box is critical for activity of the CHRNA4 promoter *in vivo* could also be investigated with CHRNA4 promoter CA box mutant knock in transgenic mice. Through the use of homologous recombination techniques, mice could be generated with the mutations discussed above made to the endogenous CHRNA4 promoter CA box. Transgenic mice generated with this technique would not be subject to the complications of transgene integration site or number of transgene integrations. Using these animals, future experiments would be able to focus on the impact that CA box mutation has on endogenous CHRNA4 gene expression. I would expect that, as observed *in vitro* and in the transgenic animals discussed above, CHRNA4 promoter activity would be severely diminished in the mutant CA box knock in animals. As a result, these mice would likely express the CHRNA4 gene at very low levels, if at all. When using mutant CA box knock in animals in behavioral assays it is most likely that these animals would display

characteristics similar to the CHRN4 KO animals. Thusly, I would expect that the mutant CA box knock in animals would have an altered anxiety related-response (Salas et al., 2003a), be less sensitive to nicotine induced seizures (Salas et al., 2004b), and display an overall reduction in the somatic symptoms of nicotine withdrawal (Salas et al., 2004a). These experiments would be able to test the hypothesis that CA box mutation causes a functionally relevant decline in CHRN4 gene expression. If I were running the laboratory I would make the CA box knock in mouse as it would be the most elegant way to determine the relevance of CA box mutation in vivo, and would allow me to test the hypothesis that CA box mutation results in a decline in CHRN4 expression that ultimately affects behavior.

Additional gene expression analysis assays could be performed using the knock in mice to test the hypothesis that the reduced expression of CHRN4 results in an augmented nAChR subtype expression profile. Because mutant CA box knock in mice would likely have extremely low levels of CHRN4 gene expression, they may also have reduced expression of $\alpha 3$ subunit mRNA, much like CHRN4 KO mice (Salas et al., 2004b). I could test this hypothesis by comparing the ability of an $\alpha 3\beta 4$ receptor specific antagonist to block nicotine-induced whole cell currents in WT, CHRN4 KO and mutant CA box knock in animals. In these experiments, the efficacy of the $\alpha 3\beta 4$ receptor selective conotoxin AU1B to block nicotine induced whole cell currents in neurons of WT animals would be compared to what is observed in CHRN4 KO and mutant CA box knock in animals (Azam and McIntosh, 2009). Neurons in the habenulo-interpeduncular pathway would be

selected for recording as they express high levels of the CHRNA3 and CHRNB4 genes (Salas et al., 2009). As a result of diminished CHRNB4 and subsequently CHRNA3 gene expression, there would be fewer $\alpha 3\beta 4$ receptors and subsequently a less potent inhibition of nicotine induced currents by AU1B in the CHRNB4 KO and mutant CA box knock in animals. These results would demonstrate that CA box mutation causes a functionally relevant decline in CHRNB4 gene expression, resulting in fewer functional $\beta 4$ -containing receptors.

CHAPTER V: DISCUSSION

Signal transmission through nAChRs is essential for the function of the nervous system, with cholinergic signaling involved in the molecular mechanisms underlying learning, memory formation and attention (Albuquerque et al., 2009). In order to perform specific biological functions, physiologically diverse neuronal nAChR subtypes are expressed in unique patterns throughout the mammalian brain (Gotti et al., 2007; Albuquerque et al., 2009). By precisely controlling the expression of the individual nAChR subunit genes, highly specialized neurons can produce the required nAChR subtypes needed in order to perform specific biological functions. Thus, elucidating the underpinnings of the transcriptional regulation of the nAChR subunit genes is essential for gaining a thorough understanding of neuronal cholinergic signaling.

Three of the twelve genes that encode neuronal nicotinic receptor subunits, CHRNA5, CHRNA3, and CHRNB4, are located in a tight genomic cluster, suggesting that they are subject to some level of coordinate regulation (Boulter et al., 1990; Improgo et al., 2010b). The $\alpha 5\alpha 3\beta 4$ subtype is the most prevalent nAChR expressed in the PNS (Conroy and Berg, 1995). In the CNS, receptors that contain these subunits play a central role in mediating ACh release in the habenulo-interpeduncular pathway (Quick et al., 1999; Grady et al., 2009). Recently, independent studies from several laboratories have shown that genetic variability at the CHRNA5/A3/B4 locus can be linked not only to an increased risk of nicotine

dependence, but also to an increased risk of lung cancer (Greenbaum and Lerer, 2009). Taken together, these findings highlight the importance of the $\alpha 5\alpha 3\beta 4$ nAChR subtype with respect to susceptibility to tobacco addiction and tobacco related disease. Hence, the $\alpha 5\alpha 3\beta 4$ nAChR may play an important role in the molecular mechanisms that govern tobacco addiction, adding an additional component to the more commonly studied $\alpha 4\beta 2$ and $\alpha 7$ receptor subtypes implicated in the pathophysiology of tobacco related disease (Picciotto et al., 1998; Schuller et al., 2000; Wang et al., 2001; Tapper et al., 2004; Kuryatov et al., 2005; Walters et al., 2006). In this work, I have focused on the transcriptional regulation of the CHRNB4 gene, which has been shown previously to be critical for nicotine withdrawal symptoms in mice (Salas et al., 2004a).

Transcription factor interaction with the CHRNB4 promoter CA box

The data presented in Chapter III confirm and extend previous data from our lab indicating that Sp1, Sp3, and Sox10 interact with the CHRNB4 promoter at the CA box region *in vitro* by demonstrating that these proteins also interact with the CHRNB4 promoter in the chromatin environment (Bigger et al., 1997; Melnikova et al., 2000b). I have also shown that c-Jun interacts with the CHRNB4 promoter in the chromatin environment (Scofield et al., 2008), which is consistent with previous data from our lab demonstrating that c-Jun is capable of transactivating the CHRNB4 promoter *in vitro* (Melnikova and Gardner, 2001). In combination, these data strongly suggest that Sp1, Sp3, Sox10 and c-Jun functionally interact with the CHRNB4

promoter, at the CA box, to promote transcription of the CHRNA4 gene in the mammalian brain.

My data on protein-DNA interaction at the CHRNA4 promoter contribute to a more thorough understanding of the molecular mechanisms that control expression of the CHRNA4 gene. These data can be compiled with additional results from our lab as well as the Deneris, Criado and Fornasari groups, to present a detailed description of the transcriptional regulatory mechanisms that control expression of the CHRNA5, CHRNA3 and CHRNA4 genes (Fig. 4.9). From the data presented in both Chapters III and IV, it is most likely that the association of Sp1 with the CHRNA4 promoter CA box aids in the recruitment of additional positive-acting co-factors, including Sp3, Sox10 and c-Jun, which ultimately enhance transcription of the CHRNA4 gene. Similar to the CHRNA4 gene promoter, the CHRNA5 and CHRNA3 promoters also lack TATA boxes (Boulter et al., 1990) and contain several functional Sp1 binding sites (Yang et al., 1995; Campos-Caro et al., 1999; Flora et al., 2000a; Terzano et al., 2000; Campos-Caro et al., 2001), indicating that Sp1 may play a crucial role in regulating expression of the CHRNA5 and CHRNA3 genes as well.

Transgenic Animal Models

Transgenic animals were first used to study the transcriptional regulatory mechanisms of nAChR subunit genes by Changeux and colleagues. The Changeux group used a 1.163-kb segment of the $\beta 2$ promoter to drive expression of lacZ in transgenic mice (Bessis et al., 1995). These experiments demonstrated that the $\beta 2$

promoter confers neuron-specific expression of β -gal in a pattern that closely resembles endogenous β 2 gene expression. In addition, Changeux and colleagues demonstrated that the 1.163-kb β 2 promoter fragment used in the transgenic construct contains both positive and negative regulatory elements, a hallmark of neuronal gene promoters (Bessis et al., 1995).

Several groups have used transgenic model systems to investigate the function of regulatory elements the control expression of the CHRNA5/A3/B4 genes. For example, the intragenic region between the CHRNA4 and CHRNA3 genes, containing the β 43' enhancer and the CHRNA3 promoter region, was used to drive expression of lacZ in transgenic mice by the Deneris group (Yang et al., 1997). In these animals, reporter gene expression resembled expression of the CHRNA3 gene, with β -gal reactivity restricted to the olfactory bulb, medial habenula, locus coeruleus and medial geniculate. However, no reporter gene expression was observed in the PNS, indicating that the regulatory information that directs expression of the CHRNA4 and CHRNA3 genes to PNS tissues is not contained in this segment of DNA. However, additional analysis of the β 43' enhancer region indicated that this regulatory element is capable of directing neuron-specific expression.

In these experiments, Deneris and colleagues used a construct where the β 43' enhancer was placed upstream of the SV40 viral promoter and a luciferase reporter gene. The resulting construct was then electroporated in a variety of cell lines,

having the highest activity in neuronal-like PC12 cells (McDonough and Deneris, 1997). In an extension of this analysis Deneris and colleagues transfected the $\beta 43'$ enhancer / reporter construct into dissociated rat sympathetic ganglia cultures, which contain nAChR-positive neurons as well as nAChR-negative non-neuronal cells. These experiments demonstrated that the $\beta 43'$ enhancer confers neuron-specific activity, as 90% of neurons transfected with the enhancer construct were positive for the luciferase and cells transfected with a control construct lacking the $\beta 43'$ enhancer displayed a significant reduction in neuron-specific expression (McDonough et al., 2000). One possible explanation for the lack of PNS expression of the reporter gene in the transgenic animals discussed above could be the differences between the experimental methods used to investigate the activity of the $\beta 43'$ enhancer. It is possible copy number- and the integration position-dependent factors may have impacted the ability of the transgenic construct to direct expression to neurons in the PNS. Moreover, neuron-specific expression *in vivo* may require additional regulatory elements not present in the transgenic construct used by the Deneris group.

Using a P1-derived artificial chromosome (PAC) containing 132 kb of DNA, including the entire rat CHRNA5/A3/B4 locus, the Deneris group generated an additional set of transgenic mice that express the rat CHRNA5, CHRNA3 and CHRNB4 genes under direction of transcriptional regulatory information included in the 132-kb PAC construct. These animals express the CHRNA5, CHRNA3 and CHRNB4 transgenes in a variety of regions where they are endogenously expressed including the SCG,

pineal gland, adrenal gland and trigeminal ganglia (Xu et al., 2006). In this model system, mutation of several ETS factor binding sites in the $\beta 43'$ enhancer eliminated expression of all three rat nAChR transgenes in the adrenal gland. These data indicate that the ETS sites in the $\beta 43'$ enhancer are required for expression of the CHRNA5, CHRNA3 and CHRNB4 genes in the adrenal gland *in vivo* (Xu et al., 2006).

The Deneris group has also investigated several regions of non-coding DNA upstream of the CHRNB4 gene that are phylogenetically conserved, with 70% identity across the human, mouse and rat sequences (Xu et al., 2006). As discussed in Chapter IV, when the CNR4 region is deleted from the PAC construct, the resulting transgenic animals do not express the rat CHRNA3 or CHRNB4 transgenes in the pineal gland, indicating that CNR4 is required for expression of the CHRNA3 and CHRNB4 genes in the pineal gland *in vivo* (Xu et al., 2006). As an extension of this analysis, an additional set of transgenic animals was generated with a transgenic construct with CNR4 placed upstream of a luciferase reporter gene. This CNR4 / reporter transgenic model demonstrated that the 599-bp CNR4 segment of DNA is capable of directing expression of β -gal to the medial habenula, interpeduncular nucleus, inferior colliculus, adrenal gland, pineal gland, and the SCG (Xu et al., 2006), all regions of endogenous CHRNB4 gene expression (Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997; Gahring et al., 2004).

We have shown previously that a 2.3-kb segment of the CHRN4 promoter, containing the CA box, is capable of directing expression of β -gal to a variety of tissues and cell types that endogenously express the CHRN4 gene at ED18.5 and PD30, in the CNS and PNS (Bruschweiler-Li et al., 2010). At ED18.5, we observed β -gal expression in WT transgenic animals in the cerebral cortex, piriform cortex, and medial habenula. We also observed β -gal expression in the cervical, thoracic and lumbar root ganglia, as well as the spinal cord (Bruschweiler-Li et al., 2010). This pattern of β -gal expression closely resembles the endogenous patterns of CHRN4 gene expression in the PNS and CNS (Dineley-Miller and Patrick, 1992; Zoli et al., 1995; Winzer-Serhan and Leslie, 1997; Gahring et al., 2004). As discussed in Chapter IV, we did not observe β -gal expression in the pineal gland, this is likely due to the fact that our transgenic construct does not contain the CNR4 regulatory element which has been shown to be required for CHRN4 promoter activity in this region (Xu et al., 2006).

At PD30, we observed β -gal expression in WT transgenic animals in the piriform cortex, dentate gyrus, medial habenula, subiculum and inferior colliculus (Scofield et al., 2008), all regions of endogenous CHRN4 gene expression (Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997; Gahring et al., 2004). As indicated in Chapter IV, we did not observe expression of β -gal in the interpeduncular nucleus in our transgenic mice (Bruschweiler-Li et al., 2010), a region where the CHRN4 gene is known to be highly expressed (Dineley-Miller and Patrick, 1992; Winzer-Serhan and Leslie, 1997; Gahring et al., 2004). This is likely due to the fact that that our

transgenic construct did not include CNR4, which has been shown by the Deneris group to be required for reporter gene expression in the interpeduncular nucleus (Xu et al., 2006).

It is interesting to note that the CHRNB4-CHRNA3 intragenic region, the CNR4 region and the 2.3-kb region of the CHRNB4 promoter used by our lab are all capable of directing expression of a reporter gene to the medial habenula. The amount of redundant regulatory information responsible for gene expression in this region suggests that receptors containing the $\alpha 5$, $\alpha 3$ and $\beta 4$ subunits play an important role in mediating cholinergic signaling in this region. This hypothesis is supported by recent findings indicating that nAChRs containing the $\alpha 3$ and $\beta 4$ subunits are responsible for ACh release in the habenulo-interpeduncular pathway (Grady et al., 2009), and also that nAChRs in this region are necessary for nicotine withdrawal in mice (Salas et al., 2009).

While a significant amount of redundant regulatory sequences exist at the CHRNA5/A3/B4 locus, unique regulatory elements responsible for directing gene expression to very specific regions also exist. As discussed above, the Deneris group has shown that mutation of the $\beta 4 3'$ enhancer abrogates expression of the CHRNA5, CHRNA3 and CHRNB4 transgenes in the adrenal gland, while deletion of CNR4 abrogates expression of CHRNA3 and CHRNB4 subunit genes in the pineal gland (Xu et al., 2006). In WT transgenic animals, we do not observe expression of β -gal in either the pineal or adrenal gland. This is most likely due to the fact that the

transgenic construct used does not contain the $\beta 43'$ enhancer or CNR4. Moreover, the data presented by the Deneris group indicate that the $\beta 43'$ enhancer and the CNR4 regions are required for directing expression to the adrenal and pineal gland, respectively.

Interestingly, our CHRNA4 promoter / reporter transgenic construct is the first to direct reporter gene expression to the subiculum and piriform cortex at ED18.5 and PD30 (Bruschweiler-Li et al., 2010), both regions where the CHRNA4 gene is endogenously expressed (Dineley-Miller and Patrick, 1992; Zoli et al., 1995; Winzer-Serhan and Leslie, 1997; Gahring et al., 2004). Additional studies would be required to determine if the regulatory information contained in the SacI – HindIII fragment of the CHRNA4 promoter is necessary for gene expression in the subiculum and piriform cortex, or if other regulatory elements can also direct expression to these brain regions.

Another advantage of our CHRNA4 promoter / reporter transgenic model is the recapitulation the expression pattern of the CHRNA4 gene in the PNS during development. Not only do we observe reporter gene expression in PNS ganglia where CHRNA4 is known to be highly expressed (Rust et al., 1994), we also observe promoter activity in non-neuronal tissues, like the intestine and tongue (Fig. 4.2 C and D), (Bruschweiler-Li et al., 2010). These results are consistent with previous in situ experiments demonstrating that CHRNA4 message is present in these regions (Zoli et al., 1995; Winzer-Serhan and Leslie, 1997; Flora et al., 2000b;

Glushakov et al., 2004). Our lab is also the first to use transgenic animal models to investigate CHRNA4 promoter activity during development; the studies done by the Deneris and colleagues were performed on adult animals (Yang et al., 1997; Xu et al., 2006).

In order to directly assess the importance of the CA box regulatory element in the CHRNA4 promoter, I generated an additional set of CHRNA4 promoter / reporter transgenic animals with expression of the lacZ gene driven by a mutant CA box CHRNA4 promoter. These experiments demonstrated that mutation of the CA box results in absent or severely reduced reporter gene expression in nearly all areas where expression is observed in the WT transgenic animals *and* where the CHRNA4 gene is endogenously expressed (Fig. 4.5, 4.6 and Table 4.1). This drastic reduction of CHRNA4 promoter activity was observed in CA box mutant transgenic animals at ED18.5 and at PD30, in the CNS and PNS. These data strongly suggest that the CA box is an essential regulatory feature of the CHRNA4 promoter and is required for CHRNA4 promoter activity *in vivo*, which is consistent with previous data from our lab demonstrating that CA box mutation drastically reduces CHRNA4 promoter activity *in vitro* (Bigger et al., 1996).

I have also demonstrated that mutation of the CHRNA4 promoter CA box results in a significant decrease in the interaction of Sp1 with the CHRNA4 promoter in brain tissue (Fig. 4.9). These results are in agreement with previous data from our lab demonstrating that mutation of the CA box eliminates transactivation of the CHRNA4

promoter by Sp1 *in vitro* (Bigger et al., 1997). Taken together, these data strongly suggest that the loss of promoter activity that results from CA box mutation is a consequence of decreased interaction of Sp1 with the mutant CHRN4 promoter. These findings advance the current understanding of the transcriptional regulation of the CHRN4 gene by identifying a critical regulatory element in the CHRN4 promoter and also by demonstrating that regulation of the CHRN4 promoter by Sp1 is necessary for promoter activity *in vivo*.

By assembling multiple regulatory elements into one construct expression one could design a targeting construct that would more accurately mirror expression of the endogenous CHRN4 gene. Using this more comprehensive transgenic construct, one could selectively express any gene of interest in CHRN4 expressing cells. For example, it would be interesting to use a construct like this to drive expression of a modified diphtheria toxin, in order to selectively eliminate CHRN4 expressing neurons and the neuronal circuits that they are located in (Miyoshi and Fishell, 2006; Wang et al., 2006). Using this transgenic model one could test the hypothesis that CHRN4-expressing neurons are critical for nicotine withdrawal or withdrawal syndromes elicited by other drugs of abuse. Given that antagonism of $\alpha 3\beta 4$ -containing receptors in the habenulo-interpeduncular pathway has been shown to reduce opioid and stimulant self administration in rats (Glick et al., 2002), I would expect that animals with selective death of CHRN4-expressing neurons would display an alteration in self administration or reinstatement of drug use following a drug-paired cue during abstinence, when compared to wild type littermates. It has

been shown previously that chronic use of several drugs of abuse, including methamphetamine, cocaine and nicotine, results in atrophy of neurons in the habenulo-interpeduncular circuit, suggesting that the death of these neurons may contribute to addiction-associated behaviors (Ellison, 2002).

Another possibility would be to use the combined regulatory elements to drive expression of the CRE recombinase gene. These mice could be crossed with genetically engineered mice with loxP sites flanking a gene of interest. Using this experimental design, one could eliminate expression of a particular gene, selectively in CHRNA4-expressing cells. This system could be used to eliminate tyrosine hydroxylase expression, and subsequently dopamine neurotransmission, in neurons that express the CHRNA4 gene. These mice could be used to determine if dopamine signaling, in specific neurons, is involved in nicotine addiction and withdrawal. In this manner, one could selectively inactivate modes of neurotransmission in CHRNA4-expressing neurons by targeting key enzymes in their biosynthetic pathways. Similar results could be achieved by using the comprehensive construct to direct expression of shRNA designed to recognize a gene of interest. This method may be more efficient as it would not require crossing CRE transgenic mice to loxP mice, and would still effectively eliminate expression of the target gene in CHRNA4-expressing cells.

Summary

Overall, the work presented here provides insight into the molecular mechanisms that control expression of the CHRNA4 gene. These findings contribute to a growing body of work describing the underpinnings of the transcriptional regulation of the clustered neuronal nAChR subunit genes CHRNA5, CHRNA3 and CHRNA4. Here I have shown that a multicomponent regulatory complex assembles at the CHRNA4 promoter CA box, in the context of native chromatin, to drive expression of the CHRNA4 gene in the mammalian brain. Moreover, this CA box regulatory element is critical for CHRNA4 promoter activity *in vivo*. Mutation of this region results in a severe decrease in promoter activity as well as a decreased association of the transcription factor Sp1 with the CHRNA4 promoter in the mammalian brain. Taken together these results indicate that Sp1, Sp3, Sox10 and c-Jun interact with the CA box to enhance expression of the CHRNA4 in the mammalian brain.

APPENDIX I: PROTEIN-DNA INTERACTION AT THE CHRNB4 PROMOTER CT BOX

Abstract

Our laboratory has had a long-standing interest in the transcriptional mechanisms underlying expression of the CHRNA5, CHRNA3, and CHRNB4 genes, which are located in a tight genomic cluster. Two proteins, heterogeneous nuclear ribonucleoprotein K (hnRNP K) and Pur α were purified by virtue of their ability to interact with the CHRNB4 promoter CT box *in vitro*. Here I analyze the ability of Pur α and hnRNP K to interact with the CHRNB4 promoter in the context of native chromatin using a ChIP approach. These experiments demonstrated that hnRNP K physically interacts with the CHRNB4 promoter in OBL21 cells but not in Neuro 2A or PC12 cells. In contrast, I obtained negative Pur α ChIP results in OBL21, Neuro 2A and PC12 cells. Using the Neuro 2A cell line, I knocked down expression of either Pur α or hnRNP K and measured CHRNA5, CHRNA3 and CHRNB4 gene expression with quantitative RT-PCR. I found that in Neuro 2A cells, hnRNP K knockdown had no impact on expression levels of the CHRNA5, CHRNA3 and CHRNB4 genes despite a significant reduction in both hnRNP K message and protein. Conversely, Pur α knockdown resulted in decreased levels of Pur α , CHRNA5, CHRNA3 and CHRNB4 gene expression. Subsequent analysis of protein levels in siRNA treated cells revealed that Pur α knockdown results in a substantial reduction of Pur α , α 3, and α 5 protein levels, while an increase in β 4 protein levels was detected.

Introduction

Regulation of gene expression at the level of transcription is crucial for both the control of normal cellular processes and for neuronal differentiation. By regulating which genes are transcribed, individual cells can produce specific proteins required to perform unique cellular functions, allowing for the existence of a vast array of highly specialized cells. The production of mRNA is carried out by RNA polymerase II and a set of general transcription factors (Fuda et al., 2009). These factors act to regulate RNA polymerase II's binding, enzymatic activity, and eventual disassociation from the DNA template (Fuda et al., 2009; Selth et al., 2010; Weake and Workman, 2010). An additional set of transcription factors act to enhance or repress transcription by influencing the recruitment of RNA polymerase II to specific genes. These transcription factors are sequence-specific DNA-binding proteins that recognize stretches of DNA called transcriptional regulatory elements (Pan et al., 2010). Regulatory elements are usually located in non-coding stretches of DNA and can be positioned either in close proximity or far distally from the genes they act to regulate (Bulger and Groudine, 2010). Thus, gene expression can be precisely controlled through the interaction of transcription factors with transcriptional regulatory elements. By expressing a particular program of transcription factors, individual cell types can precisely control gene expression, allowing these cells to perform distinct functions.

We previously identified a 19-base pair transcriptional regulatory element in the CHRNA4 promoter referred to as the CT box, because of its nucleotide composition

(Hu et al., 1995). We have also shown previously that mutation of this element results in a substantial reduction of CHRN4 promoter activity in the neuronal-like cell line SN17 (Hu et al., 1995). In addition, we demonstrated, using *in vitro* binding assays, that the CT box specifically interacts with DNA-binding proteins from rat brain nuclear extracts (Hu et al., 1995). Using affinity chromatography, two proteins were isolated by virtue of their affinities for the CHRN4 promoter CT box. Subsequent peptide sequencing revealed the identity of the two CT box-interacting proteins to be Purine rich element binding protein A (Pur α) (Du et al., 1997) and heterogeneous nuclear ribonucleoprotein K (hnRNP K) (Du et al., 1998).

The Pur α protein is a single polypeptide of approximately 40 kDa that interacts specifically with purine-rich motifs (Bergemann and Johnson, 1992). Pur α is one of four Pur proteins in the Pur gene family, which consists of Pur α , Pur β and two isoforms of Pur γ (Gallia et al., 2000). Pur α can positively or negatively impact transcription of target genes and is also capable of interacting with both single-stranded DNA and RNA (Gallia et al., 2000). In addition, Pur α possesses DNA helicase activity (Wortman et al., 2005) and plays a role in several physiological processes including transcription, translation, and cell growth (Gallia et al., 2000). At the CHRN4 promoter, Pur α interacts preferentially with the single-stranded 3' – GGGAGGGGAGGGGA – 5' sequence located on the minus strand of DNA at the CT box (Fig. 1.7).

hnRNP K is a 65kDa member of the ribonucleoprotein complex of heterogeneous nuclear RNA in mammalian cells (Bomsztyk et al., 1997). hnRNP K binds tenaciously to pyrimidine rich sequences (Matunis et al., 1992) and can also either activate or inhibit transcription of target genes (Bomsztyk et al., 2004). Like Pur α , hnRNP K is capable of interacting with both single-stranded DNA and RNA, yet it has higher affinity for single-stranded DNA (Tomonaga and Levens, 1995). hnRNP K plays a role in chromatin remodeling, transcription, RNA editing, and repression of translation (Bomsztyk et al., 2004). At the CHRNA4 promoter, hnRNP K interacts with the single-stranded 5' – CCCTCCCCTCCCCT – 3' sequence located on the plus strand of the CT box. We have shown previously that hnRNP K can inhibit Sp factor binding to the CHRNA4 promoter *in vitro* as well as Sp factor-mediated transactivation of the CHRNA4 promoter drosophila SL2 cells (Du et al., 1998). We have also shown by *in vitro* co-immunoprecipitation that Pur α and hnRNP K physically interact (Melnikova et al., 2000a).

Given the negative transcriptional role that hnRNP K plays at the CHRNA4 promoter and the cooperative down-regulation of transcription in other systems by Pur α and hnRNP K (Da Silva et al., 2002; Wang et al., 2008), I hypothesize that hnRNP K and Pur α act to repress expression of the CHRNA4 gene. Based on the previous data from our lab discussed above, I have constructed the following model of hnRNP K and Pur α mediated repression of CHRNA4 gene expression (Fig. A1.1).

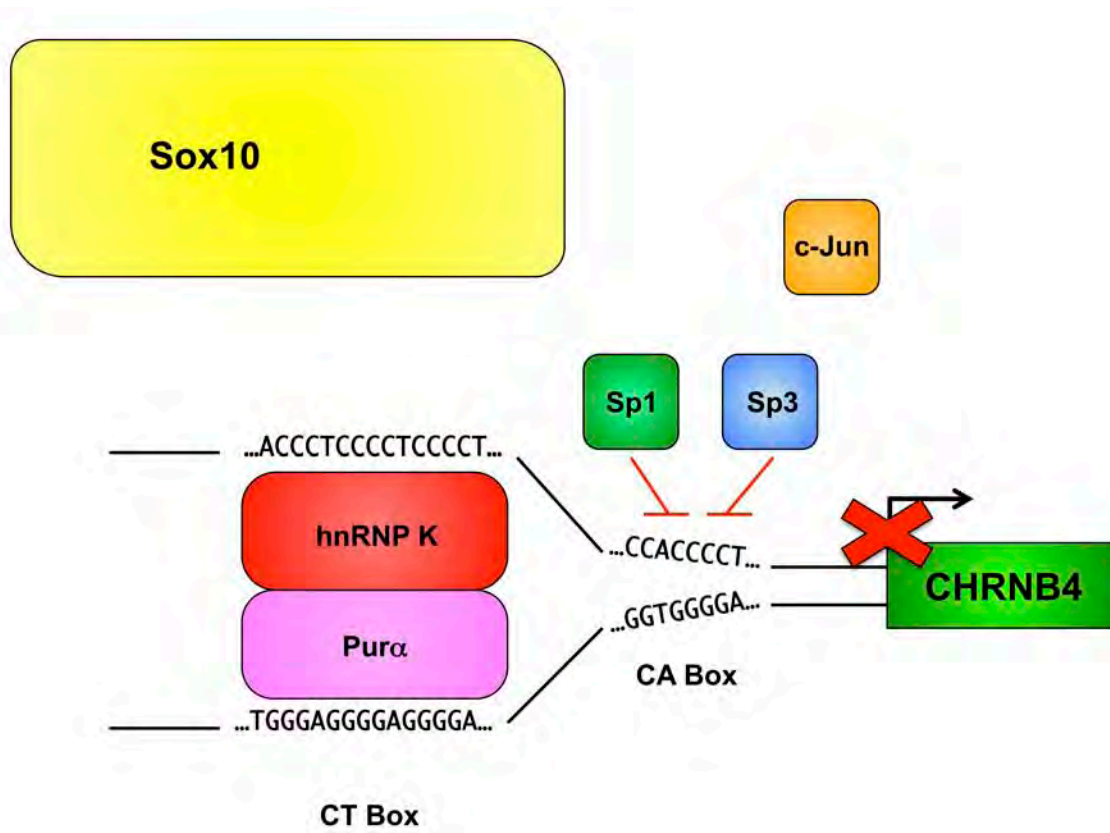


Figure A1.1: Model of hnRNP K and Pur α -mediated repression of CHRNB4 gene expression The coding region of the CHRNB4 gene is represented as a light green box with the red X indicating that CHRNB4 expression is turned off. The double stranded nucleotide sequences of two transcriptional regulatory elements, the CT and CA boxes, are also shown. Transcription factors hnRNP K and Pur α are depicted as red and pink colored ovals, contacting each other and separating the DNA strands at the CT box. Sp1 and Sp3 are depicted as colored squares above the CA box and cannot associate with the CHRNB4 promoter (depicted as red lines) as a result of strand separation by hnRNP K and Pur α . Sox10 and c-Jun are depicted as a yellow oval and an orange square respectively and also do not interact with the CHRNB4 promoter.

Results

OBL21 cells express high levels of hnRNP K and Pur α mRNA and low levels of CHRNA5, CHRNA3 and CHRNB4 mRNA.

Several observations led to development of the hypothesis that hnRNP K and Pur α act to negatively impact transcription from the CHRNB4 promoter: first, hnRNP K and Pur α have higher affinity for the single stranded elements of the CT box (Du et al., 1997; Du et al., 1998); second, both hnRNP K and Pur α have the ability to unwind the DNA duplex (Bomsztyk et al., 2004; Wortman et al., 2005); third, hnRNP K was previously shown to inhibit Sp factor binding and transactivation of the CHRNB4 promoter (Du et al., 1998); finally, these factors have been shown to cooperatively inhibit expression of other target genes (Da Silva et al., 2002; Wang et al., 2008). I began my analysis by investigating the expression levels of the Pur α , hnRNP K, CHRNA5, CHRNA3 and CHRNB4 in the mouse olfactory bulb derived cell line OBL21. qRT-PCR analysis of OBL21 cells revealed that the clustered nAChR subunit gene mRNAs are expressed at low levels while hnRNP K and Pur α mRNA are expressed at high levels. The correlation of high levels of hnRNP K and Pur α expression with low levels of CHRNB4 expression is consistent with the hypothesis that hnRNP K and Pur α act in concert to repress transcription of the CHRNB4 gene (Fig. A1.2). Similar to what was observed for CHRNB4, CHRNA5 and CHRNA3 mRNA was also minimally expressed in OBL21 cells.

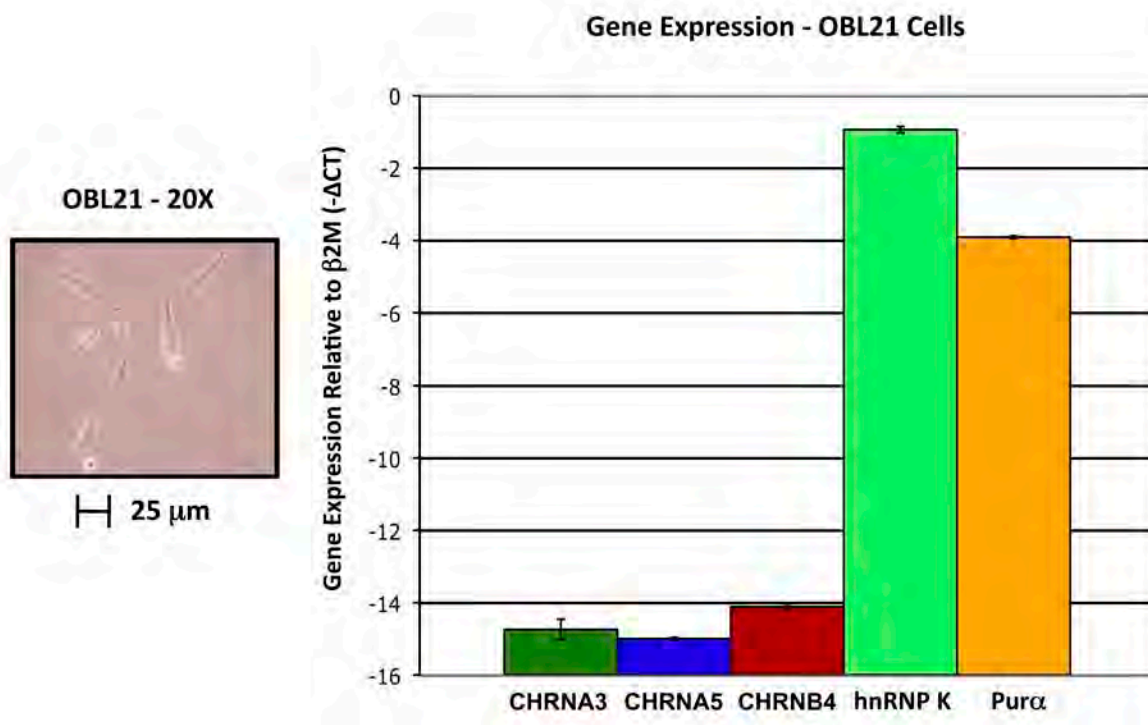


Figure A1.2: Expression levels of hnRNP K, Pur α , CHRNA5, CHRNA3 and CHRNB4 in OBL21 cells. The mouse olfactory bulb derived cell line OBL21 is shown in the bright field image on the left. mRNA levels of CHRNA3 (green), CHRNA5 (blue), CHRNB4 (red), hnRNP K (light green) and Pur α (orange) are expressed relative to the housekeeping gene $\beta 2M$. Error bars represent standard error of the means, $n=3$.

hnRNP K interacts with the CHRNB4 promoter in OBL21 cells

In order to determine if hnRNP K and Pur α interact with the CHRNB4 promoter in cells that express low levels of CHRNB4 mRNA, I performed ChIP assays using the OBL21 cell line (Fig. A1.3). Sox10 ChIP experiments were performed in this cell line as a positive control due to the fact that the interaction of Sox10 with the CHRNB4 promoter has been well characterized both *in vitro* (Liu et al., 1999) and in the context of native chromatin (Scofield et al., 2008). In these experiments, enrichment of the CHRNB4 promoter fragment in the Sox10 and hnRNP K ChIPs was significantly higher than that observed in the IgG and No Ab ChIP negative controls, indicating an interaction of Sox10 and hnRNP K with the CHRNB4 promoter in these cells. Enrichment of the CHRNB4 promoter fragment in the Pur α ChIPs was no higher than what was observed in the IgG and No Ab ChIP negative controls, indicating a negative ChIP result for Pur α at the CHRNB4 promoter in these cells. These data suggest that hnRNP K is present at the CHRNB4 promoter in OBL21 cells, however additional experimentation is required to determine if Pur α is not. Further experimentation would be able to determine if the negative Pur α ChIP result from the use of an antibody not suited for the ChIP assay. Additionally, using the same ChIP-derived DNA, a set of separate positive control PCRs could be performed with primers designed to amplify a segment of DNA shown previously to be a site of Pur α interaction. Data obtained from these experiments would aid in deciphering if Pur α truly does not interact with the CHRNB4 promoter in OBL21 cells, or if the negative results obtained were an artifact of the experimental procedure.

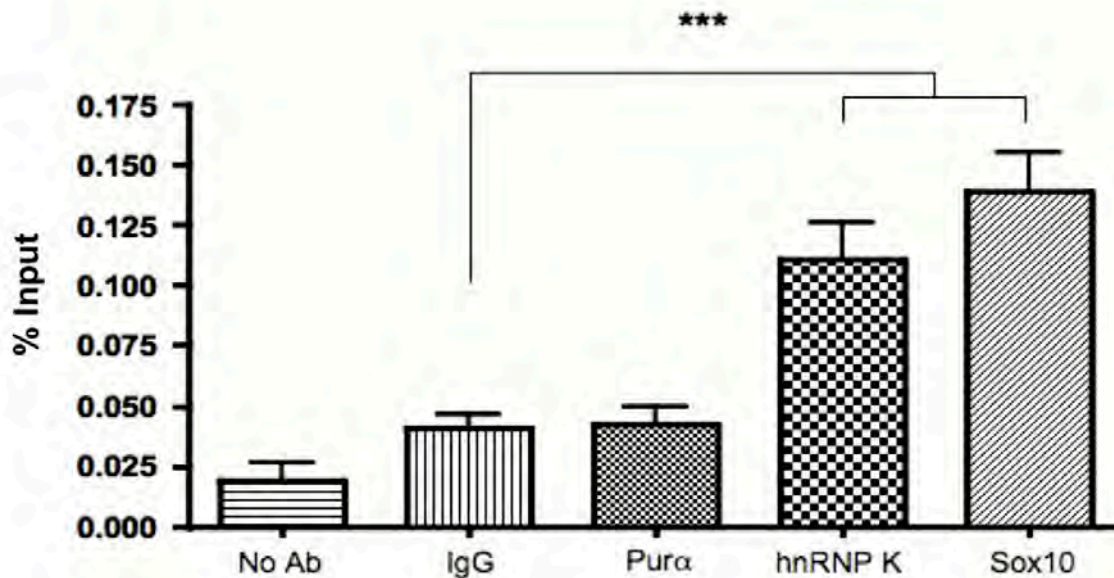


Figure A1.3: hnRNP K and Sox10 interact with the CHRN4 promoter in OBL21 cells. OBL21 cells were used in ChIP experiments to investigate hnRNP K and Pur α interactions with the CHRN4 promoter in the context of native chromatin. Sox10 ChIPs were performed as a positive control. OBL21 ChIP-derived DNA was quantified using absolute quantification-based real time PCR. Data from three individual ChIP experiments were pooled and analyzed for statistical significance using the Student's t-test. Both hnRNP K and Sox10 values differed significantly when compared to the IgG negative control, whereas Pur α did not (***, p value <0.001). Error bars represent standard error of the means, n=3.

Both differentiated and undifferentiated PC12 cells express high levels of hnRNP K and intermediate levels of Pur α

The treatment of the neuronal-like rat pheochromocytoma cell line PC12 with NGF is a well-established model of neuronal differentiation *in vitro* (Greene and Tischler, 1976; Henderson et al., 1994; Hu et al., 1994; Leppa et al., 1998). Following the application of NGF, PC12 cells extend neurite-like processes, change their program of gene expression and become electrically excitable (Greene and Tischler, 1976). Interestingly, CHRN4 is among the genes upregulated in response to NGF (Henderson et al., 1994). Thus, PC12 cells provide a system where expression of the CHRN4 gene can be enhanced *in vitro*. I first measured expression levels of hnRNP K and Pur α in both differentiated and undifferentiated PC12 cells to determine whether these genes are expressed in PC12 cells. qRT-PCR analysis revealed that both hnRNP K and Pur α are expressed in undifferentiated and differentiated PC12 cells (Fig. A1.4). hnRNP K expression is approximately 4-fold higher than Pur α in both cases. No difference in expression levels of hnRNP K or Pur α mRNA were observed when comparing undifferentiated and differentiated PC12 cells (Fig. A1.4).

Neither hnRNP K nor Pur α interacts with the CHRN4 promoter in undifferentiated or differentiated PC12 cells

I expected that if hnRNP K or Pur α were involved in transcriptionally repressing CHRN4 gene expression in PC12 cells, I would observe a reduction in hnRNP K or Pur α association with the CHRN4 promoter following NGF treatment.

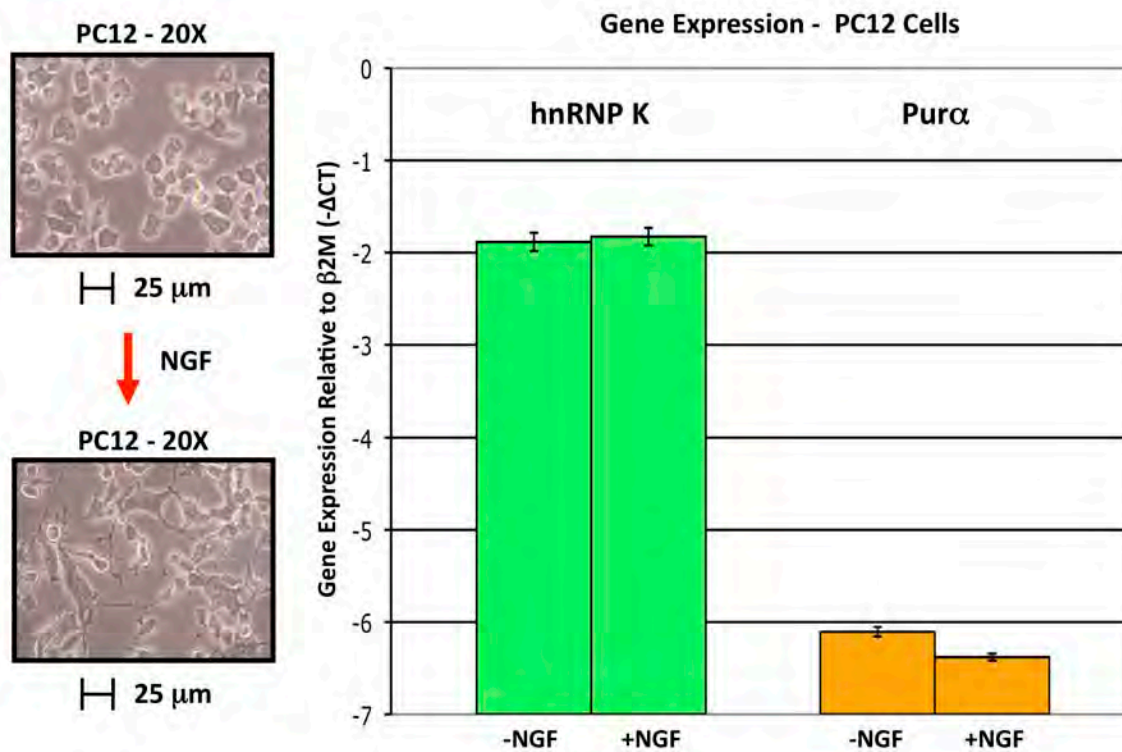


Figure A1.4: hnRNP K and Purα mRNA expression levels in undifferentiated and differentiated PC12 cells. Bright field images of the rat pheochromocytoma cell line PC12 +/- NGF are shown on the left. hnRNP K (light green) and Purα (orange) gene expression levels are depicted in the right panel relative to the house keeping gene β2M. Error bars represent standard error of the means, n=3.

In order to test this hypothesis, I performed hnRNP K and Pur α ChIP assays in undifferentiated and differentiated PC12 cells. In these experiments, enrichment of the CHRN4 promoter fragment in the hnRNP K and Pur α ChIPs was no higher than that of the IgG and No Ab ChIP negative controls, in both undifferentiated and differentiated PC12 cells (Fig. A1.5). These data suggest that hnRNP K and Pur α do not interact with the CHRN4 promoter in PC12 cells. However, due to the large amount of variability and the low n, additional PC12 ChIP experiments need to be performed in order to confirm these results. As discussed above, additional positive control PCRs of ChIP-derived DNA would also aid in deciphering if hnRNP K and Pur α truly do not interact with the CHRN4 promoter in differentiated and undifferentiated PC12 cells.

Neuro 2A cells express high levels of hnRNP K, Pur α , CHRNA3 and CHRN4 mRNAs, while CHRNA5 mRNA is expressed at an intermediate level.

In order to extend our analysis into an additional neuronal-like cell line, I investigated the expression levels of the Pur α , hnRNP K, CHRNA5, CHRNA3 and CHRN4 genes in the mouse neuronal-like cell line Neuro 2A (Olmsted et al., 1970). In Neuro 2A cells, expression of hnRNP K is approximately double what is observed for Pur α (Fig. A1.6). In addition, the CHRNA3 and CHRN4 genes are also highly expressed, with CHRNA5 mRNA expressed at an intermediate level (Fig. A1.6). According to the current model I am testing (Fig. A1.1), in Neuro 2A cells I would expect that hnRNP K and Pur α do not interact with the CHRN4 promoter, given that the CHRN4 gene is highly expressed.

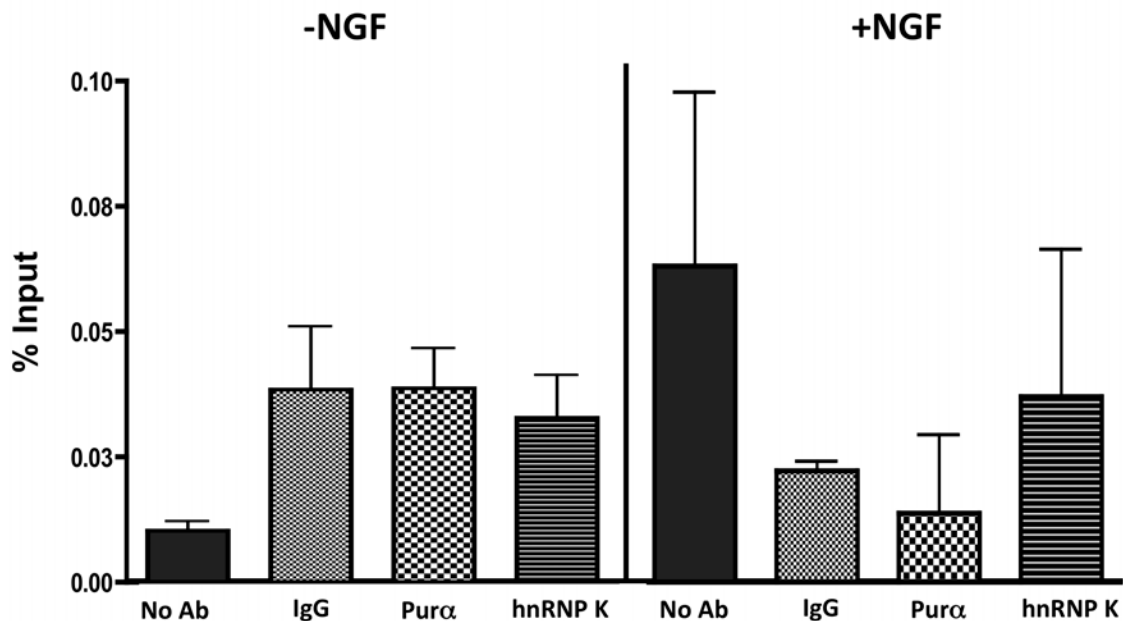


Figure A1.5: hnRNP K and Pur α do not interact with the CHRNA4 promoter in PC12 cells. PC12 cells were used in ChIP experiments to investigate hnRNP K and Pur α interactions with the CHRNA4 promoter in undifferentiated and differentiated PC12 cells. PC12 ChIP-derived DNA was quantified using absolute quantification-based real time PCR. Data from two individual ChIP experiments for either undifferentiated or differentiated PC12 cells were pooled and analyzed for statistical significance using the Student's t-test. Statistical analysis of both undifferentiated and differentiated PC12 cell ChIP experiments revealed no significant differences between ChIP controls and hnRNP K or Pur α . Error bars represent standard error of the means, n=2.

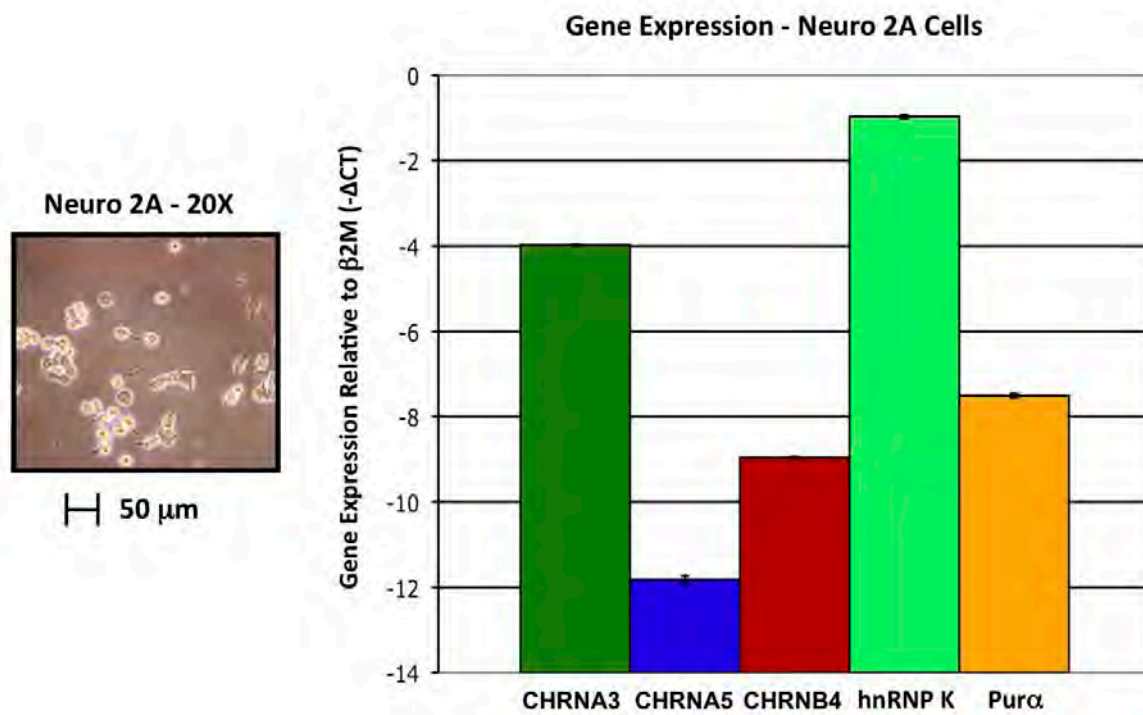


Figure A1.6: expression levels of hnRNP K, Purα, CHRNA5, CHRNA3 and CHRNB4 in Neuro 2A cells. The mouse neuroblastoma derived cell line Neuro 2A is shown in the bright field image on the left. mRNA levels of CHRNA3 (green), CHRNA5 (blue), and CHRNB4 (red) subunits, as well as hnRNP K (light green) and Purα (orange) are expressed relative to the housekeeping gene β2M. Error bars represent standard error of the means, n=3.

hnRNP K and Pur α do not interact with the CHRNB4 promoter in Neuro 2A cells

In order to test the hypothesis that hnRNP K and Pur α do not interact with the CHRNB4 promoter in cells that express high levels of the CHRNB4 gene, ChIP assays were performed using Neuro 2A cells. Enrichment of the CHRNB4 promoter fragment in the hnRNP K and Pur α ChIPs was no higher than background levels observed in the IgG and No Ab ChIP negative controls. These results suggest that hnRNP K and Pur α do not interact with the CHRNB4 promoter in Neuro 2A cells (Fig. A1.7). As the results for both hnRNP K and Pur α were negative in Neuro 2A ChIP experiments, additional positive control PCRs of Neuro 2A ChIP-derived DNA could also be performed in order to confirm that hnRNP K and Pur α do not interact with the CHRNB4 promoter in these cells.

Knockdown of hnRNP K in Neuro 2A cells has no effect on expression of the CHRNA5, CHRNA3 and CHRNB4 genes

In order to assess the possible transcriptional regulatory role that hnRNP K plays at the CHRNA5/A3/B4 locus, siRNA experiments were performed in Neuro 2A cells. In these experiments, expression of hnRNP K, GAPDH, CHRNA5, CHRNA3 and CHRNB4 genes were measured in negative siRNA and hnRNP K siRNA treated cells. In Neuro 2A cells, transfection of hnRNP K specific siRNA resulted in a decrease in hnRNP K message levels of $69.5\% \pm 1.7\%$ (Fig. A1.8). When comparing negative siRNA treated and hnRNP K siRNA treated cells using the Student's t-test, no significant changes in CHRNB4 gene expression were observed, $p = 0.93$ (Fig. A1.8).

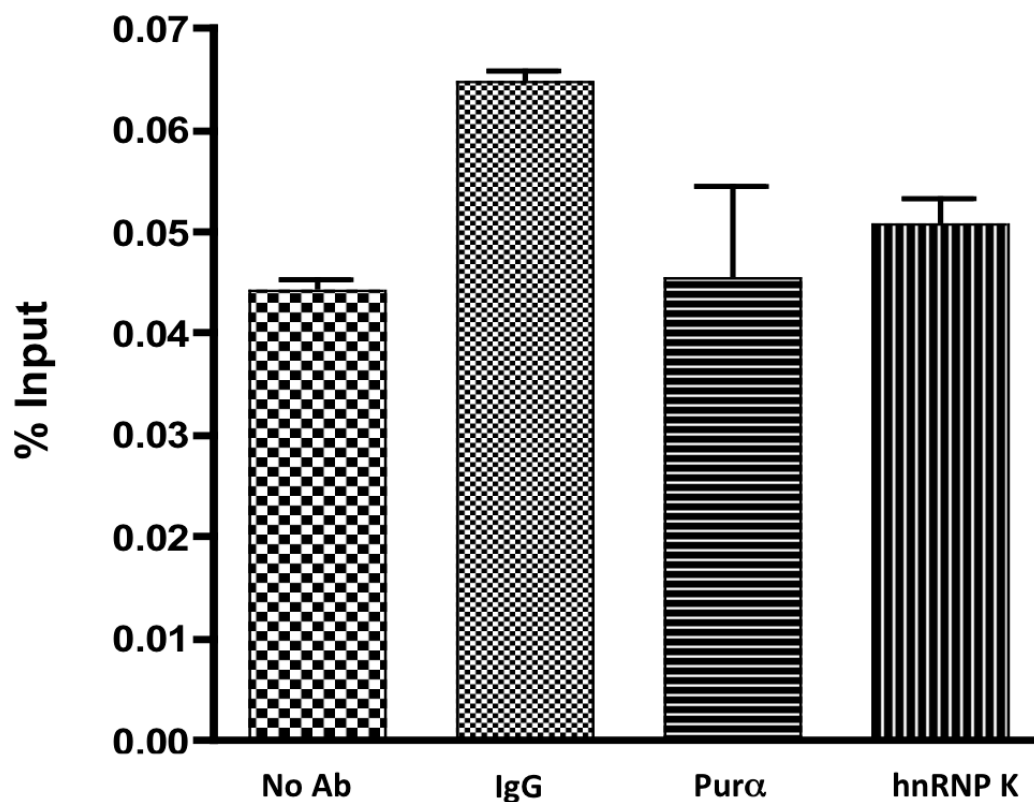


Figure A1.7: hnRNP K and Pur α do not interact with the CHRN4 promoter in Neuro 2A cells. Neuro 2A cells were used in ChIP experiments to investigate hnRNP K and Pur α interactions with the CHRN4 promoter in the chromatin environment. Neuro 2A ChIP-derived DNA was quantified using absolute quantification-based real-time PCR. Data from two individual ChIP experiments were pooled and analyzed for statistical significance using the Student's t-test. Statistical analysis revealed that neither hnRNP K nor Pur α values differed significantly when compared to the IgG or mock IP ChIP negative controls. Error bars represent standard error of the means, n=2.

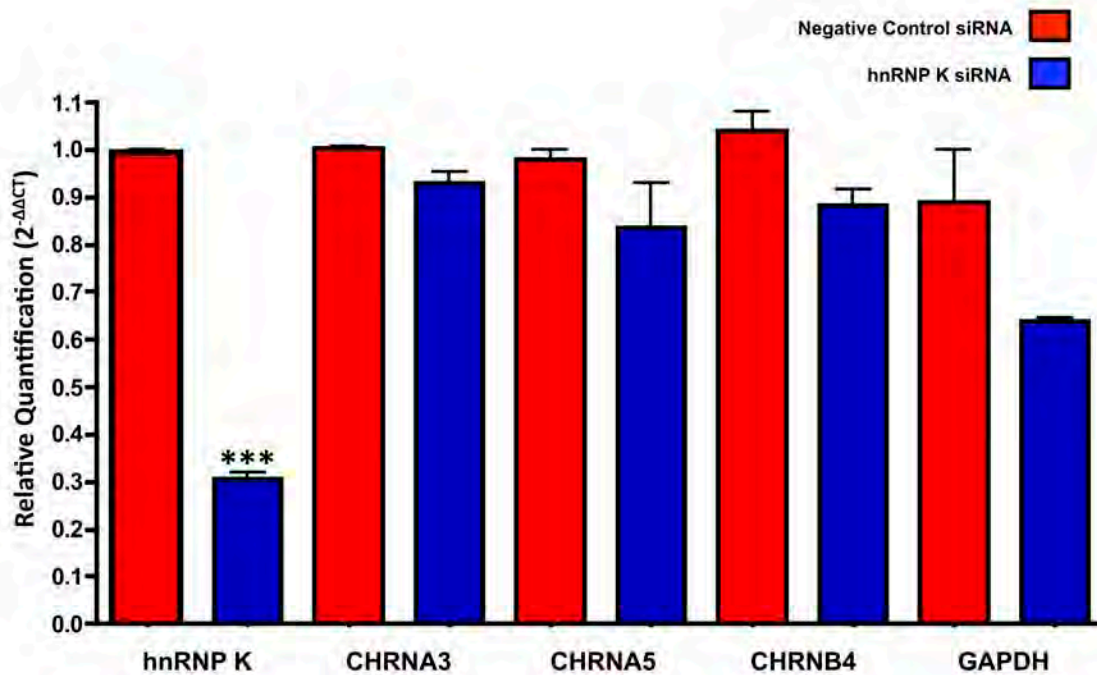


Figure A1.8: hnRNP K knockdown has no impact on CHRNA3, CHRNA5 and CHRNB4 gene expression in Neuro 2A cells. Gene expression data for hnRNP K, CHRNA3, CHRNA5, CHRNB4 and GAPDH in cells treated with negative control siRNA (red), or hnRNP K siRNA (blue). Data from two individual siRNA experiments were pooled and analyzed for statistical significance using the Student's t-test. hnRNP K expression in knockdown cells differed significantly when compared to the negative control siRNA treated cells. ***, p value <0.001. GAPDH expression in knockdown cells did not differ significantly when compared to the negative control siRNA treated cells, p value >0.05. Error bars represent standard error of the means, n=2.

In addition, there were no significant changes in mRNA levels of the other clustered nAChR subunit genes *CHRNA5* and *CHRNA3*, $p = 0.27$ and 0.089 respectively. The expression of a housekeeping gene *GAPDH* was measured as a negative control. When comparing negative siRNA treated and hnRNP K siRNA treated cells, there was also no significant change in *GAPDH* gene expression, $p = 0.1499$.

In order to demonstrate that hnRNP K siRNA treatment of Neuro 2A cells resulted in reduction of both hnRNP K message and protein levels, Western blot analysis of siRNA treated cells was performed. As expected, these results demonstrate that a $69.5\% \pm 1.7\%$ reduction of hnRNP K message results in a significant decrease in hnRNP K protein levels (Fig. A1.9). These data indicate that hnRNP K knockdown was successful and that reduction of hnRNP K message and protein levels has no impact on the expression levels of the *CHRNA5*, *CHRNA3* and *CHRNA4* genes. Taken together, these data suggest that hnRNP K does not regulate expression of the *CHRNA5*, *CHRNA3* and *CHRNA4* genes in Neuro 2A cells. As there was a significant decrease in both hnRNP K message and protein levels, I would expect that a hnRNP K function is reduced in siRNA treated cells. I could further test this hypothesis by performing additional positive controls in future hnRNP K siRNA experiments. By measuring expression of a gene shown previously to be regulated by hnRNP K in negative and hnRNP K siRNA treated cells, I would be able to further test the hypothesis that a 70% reduction in hnRNP K message results in a loss of hnRNP K function.

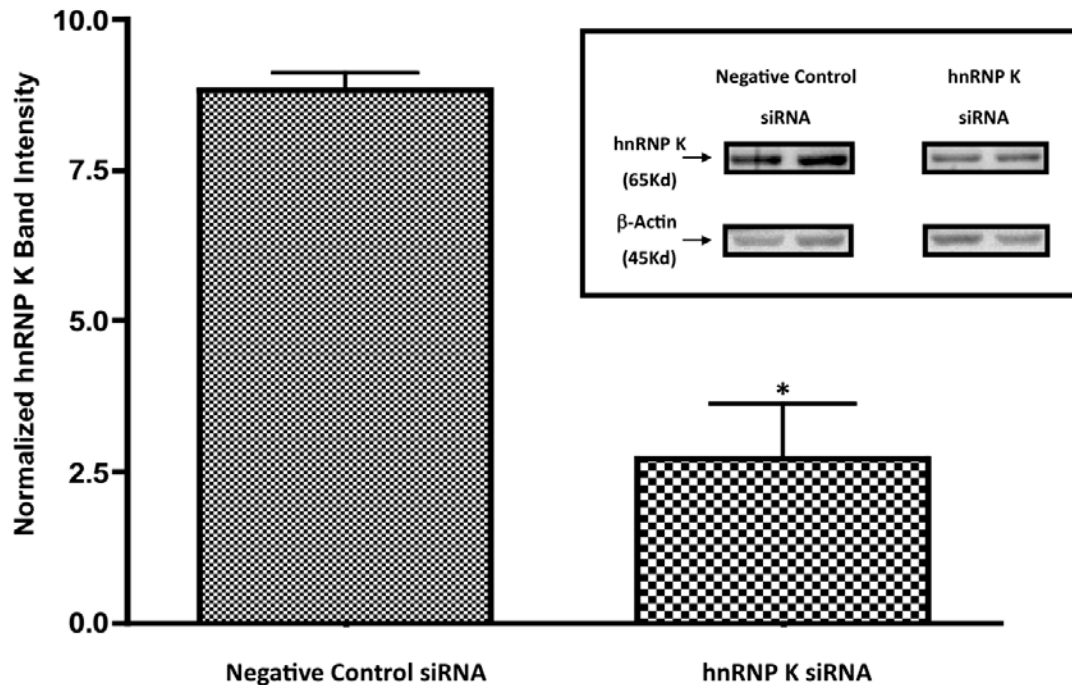


Figure A1.9: hnRNP K knockdown results in a significant decrease in hnRNP K protein levels in Neuro 2A cells. Lysates from hnRNP K and negative control siRNA treated Neuro 2A cells were separated using SDS-PAGE in duplicate. Following electrophoresis, proteins were transferred to a nitrocellulose membrane and probed with anti-hnRNP K or anti-β-Actin antibodies. hnRNP K and β-Actin band intensities were quantified using the VersaDoc gel imaging system and software. hnRNP K band intensity in hnRNP K and negative siRNA treated cells are expressed normalized to the corresponding β-Actin band intensities. Data were analyzed for statistical analysis using the Student's t-test, * indicates a p value <0.05. Error bars represent standard error of the means, n=2.

Knockdown of Pur α in Neuro 2A cells results in decreased expression of CHRNA5, CHRNA3 and CHRNB4 mRNA

In order to investigate the functional role that Pur α plays at the CHRNB4 promoter, siRNA experiments were performed in Neuro 2A cells. Expression of Pur α , CHRNA5, CHRNA3, CHRNB4 and GAPDH were measured in both negative siRNA and Pur α siRNA treated cells. Transfection of Pur α siRNA resulted in a decrease in Pur α message levels of $68\% \pm 12\%$ (Fig. A1.10). Surprisingly, when comparing negative siRNA treated and Pur α siRNA treated cells, significant reductions in CHRNA5, CHRNA3 and CHRNB4 mRNA levels were observed. CHRNA3 gene expression was reduced by $51.5\% \pm 10.7\%$, CHRNA5 gene expression was reduced by $36.54\% \pm 7.7\%$ and CHRNB4 gene expression was reduced by $26.8\% \pm 9.7\%$ (Fig. A1.10). As described above, expression of a housekeeping gene GAPDH was also measured as a negative control. When comparing negative siRNA treated and Pur α siRNA treated cells, no significant change in GAPDH expression was observed. These data indicate that knockdown of Pur α does not result in a non-specific reduction in gene expression. It appears that in Neuro 2A cells, Pur α positively regulates expression of the CHRNA5, CHRNA3 and CHRNB4 genes.

In order to verify that Pur α knock down results in a reduction in Pur α protein levels, Western blot analysis was performed. In these experiments, Pur α siRNA treated cells had significantly lower levels of Pur α protein when compared to negative control siRNA treated cells (Fig. A1.11).

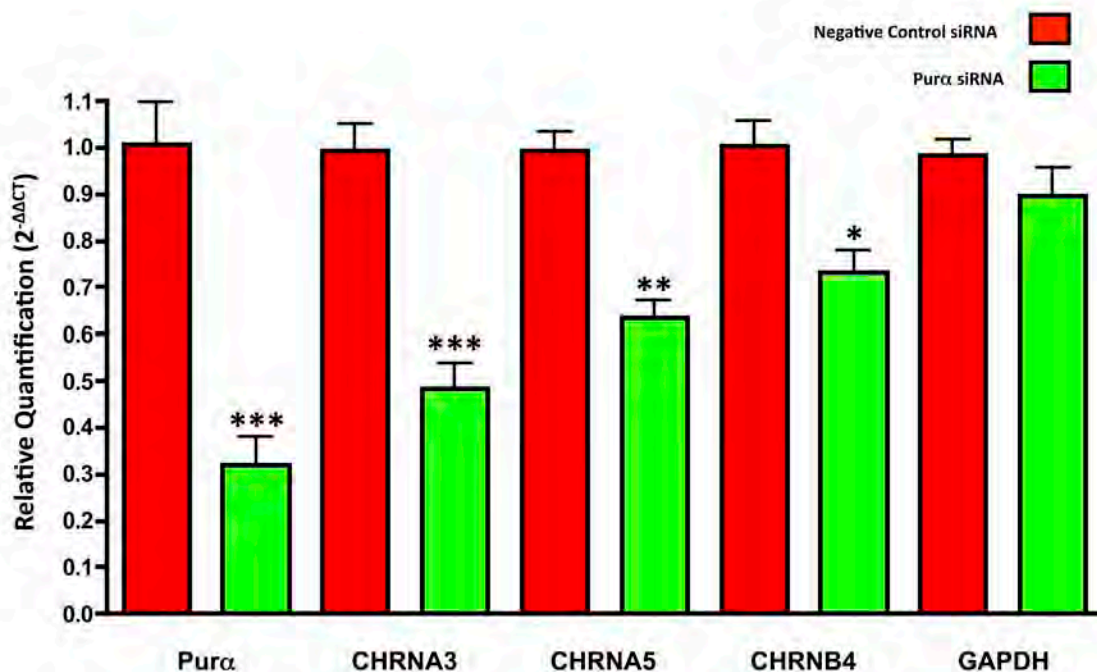


Figure A1.10: Purα knockdown results in significant decreases in CHRNA3, CHRNA5 and CHRNB4 gene expression in Neuro 2A cells. Gene expression data for Purα, CHRNA3, CHRNA5, CHRNB4 and GAPDH in cells treated with negative control siRNA (red), or Purα siRNA (green). Data from four individual siRNA experiments were pooled and analyzed for statistical significance using the Student's t-test. Purα expression in knockdown cells differed significantly when compared to the negative control siRNA treated cells, as did expression of the CHRNA3, CHRNA5 and CHRNB4 genes. ***, p value <0.001; **, p value <0.01; *, p value <0.05. Error bars represent standard error of the means, n=4.

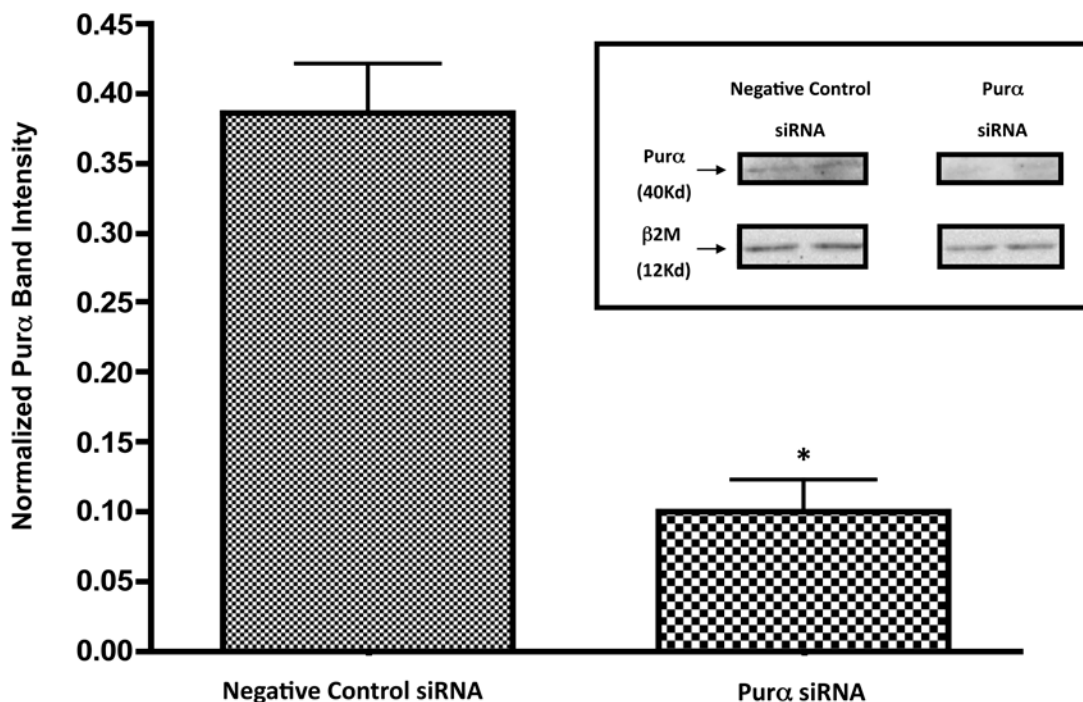


Figure A1.11: Pur α knockdown results in a significant decrease in Pur α protein levels in Neuro 2A cells Lysates from Pur α and negative control siRNA treated Neuro 2A cells were electrophoresed through a 10% SDS polyacrylamide gel in duplicate. Separated proteins were transferred to a nitrocellulose membrane and probed with anti-Pur α or anti- β 2M antibodies. Pur α and β 2M band intensities were quantified using the VersaDoc gel imaging system and software. Pur α band intensity in Pur α and negative siRNA treated cells is expressed normalized to the corresponding β 2M band intensities. Data were analyzed for statistical analysis using the Student's t-test, * indicates a p value <0.05. Error bars represent standard error of the means, n=2.

These data suggest that reduction of Pur α message, and subsequently Pur α protein levels, results in decreased expression of the CHRNA5, CHRNA3 and CHRNB4 genes. Despite the observed reduction in Pur α protein levels following siRNA treatment, detection of Pur α protein should be optimized further in order more robustly quantify the reduction of Pur α protein levels following siRNA treatment. Additionally, Pur α Western blot experiments of siRNA treated cells could also be performed in parallel using a mouse monoclonal anti-Pur α antibody generated by Johnson and colleagues, shown to be suitable for Western blot analysis (Liu et al., 2005).

In order to test the hypothesis that reduction of CHRNA5, CHRNA3 and CHRNB4 mRNA results in a corresponding reduction at the protein level, additional Western blot experiments were performed using siRNA treated cells. In these experiments, Neuro 2A cells were treated with either negative control or Pur α siRNA then harvested and split into two aliquots. The first set of cells was used for gene expression analysis (Fig. A1.12, A) while the second set of cells was used for Western blot analysis (Fig. A1.12, B). qRT-PCR analysis of Pur α siRNA treated cells indicated a $78.2\% \pm 10.2\%$ reduction of Pur α gene expression, a $60.3\% \pm 2\%$ reduction of CHRNA3 gene expression, a $37.9\% \pm 10\%$ reduction of CHRNA5 gene expression and a $34.9\% \pm 10\%$ reduction in CHRNB4 gene expression, consistent with the experiment presented in Fig. A1.10. Importantly, Pur α siRNA treatment did not impact levels of GAPDH gene expression (Fig. A1.12 A).

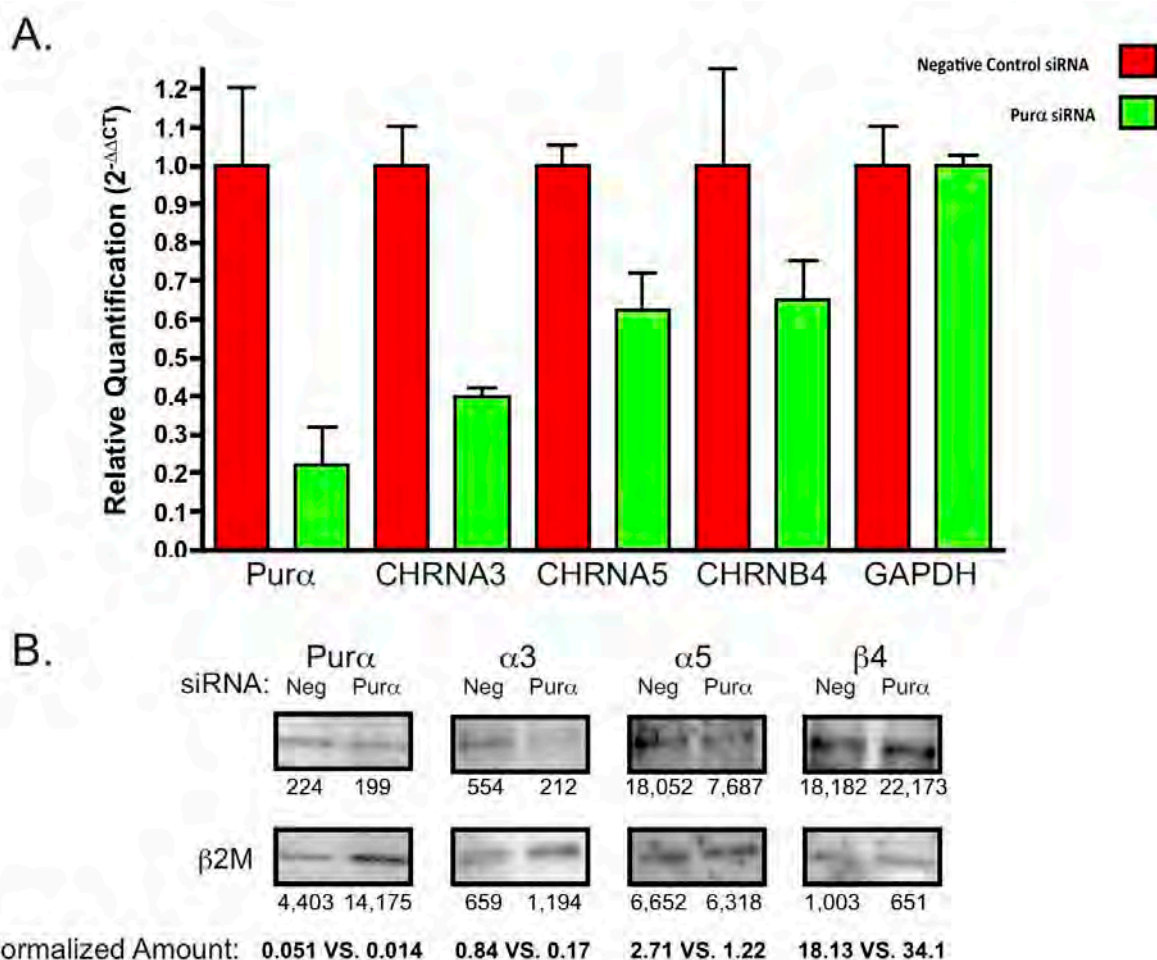


Figure A1.12: Purα knockdown results in reduced nAChR subunit α3, and α5 protein levels in Neuro 2A cells. (A) Gene expression data for Purα, CHRNA3, CHRNA5, CHRNB4 and GAPDH in cells treated with negative control siRNA (red), or Purα siRNA (green). Error bars represent standard error of the means obtained from triplicate measurements of a single experiment, n=1. (B) Lysates from Purα and negative control siRNA treated Neuro 2A cells were analyzed as described for Fig. A1.11. Purα, nAChR α3, α5 and β4 subunits, and β2M band intensities were quantified using the VersaDoc gel imaging system and software.

Western blot analysis of Pur α siRNA treated Neuro 2A cells indicated reduced levels of the Pur α , nAChR α 3 and α 5 proteins while an increase in nAChR β 4 subunit protein was observed (Fig. A1.12 B). This result is difficult to interpret given that a reduction in α 5 protein was observed, yet the decrease in CHRNA5 message is almost identical to what was observed for CHRNB4. It is possible, however, that the detection of nAChR α 5, α 3, and β 4 protein levels was not accurate due to limitations of the commercially available anti-nAChR antibodies. These concerns were brought to light in a paper published around the time I was performing these experiments. In this study, immunoreactivity observed during Western blot analysis of nAChR protein was identical when using lysates from either wild type mice, or the corresponding nAChR subunit KO mice (Moser et al., 2007). Among the antibodies shown to be non-specific were the anti-nAChR α 3 and β 4 subunit antibodies used in the experiments discussed above (Fig. A1.12). These data suggest that the antibodies used may not be suitable for Western blot analysis (Moser et al., 2007). The specificity of the anti-nAChR α 5 subunit antibody used in Figure A1.12 has yet to be tested using α 5 KO animals.

Other groups have had some success measuring nAChR subunit protein levels using custom made antibodies (Lindstrom, 2000a; Kuryatov et al., 2005; Tumkosit et al., 2006). Future nAChR β 4 Western blot analysis could be performed using antibodies developed by other groups, such as the monoclonal anti- β 4 antibody MAb 337 (Liu et al., 2009a) or the polyclonal anti- β 4 antibody 4886 (Zhou et al., 2001).

Discussion

In order to test the hypothesis that hnRNP K and Pur α negatively regulate CHRNA4 gene expression, I first examined expression levels of the CHRNA5, CHRNA3 and CHRNA4 genes, as well as hnRNP K and Pur α , in several cell lines. This analysis revealed high expression of hnRNP K and Pur α in all cell lines tested including OBL21, PC12 +/- NGF and Neuro 2A cells. Expression of CHRNA5, CHRNA3 and CHRNA4 genes was low in OBL21 cells and higher in PC12 +/- NGF and Neuro 2A cells. In OBL21 cells, where expression of the CHRNA4 gene is low, I observed an interaction of hnRNP K with the CHRNA4 promoter (Fig. A1.2). In undifferentiated and differentiated PC12 cells (Fig. A1.4) as well as Neuro 2A cells (Fig. A1.6), where expression of the CHRNA4 gene is higher, I see no interaction of hnRNP K with the CHRNA4 promoter. These data are in agreement with the hypothesis that hnRNP K negatively regulates expression of the CHRNA4 gene. siRNA mediated knockdown of hnRNP K had no impact on expression levels of the CHRNA4 mRNA or the other clustered nAChR subunits CHRNA5 and CHRNA3 in Neuro 2A cells (Fig. A1.8). As these genes are expressed at high levels in Neuro 2A cells (Fig. A1.6), this finding is also consistent with our previous hypothesis. From these data it appears that, at least in Neuro 2A cells, hnRNP K does not regulate expression of the CHRNA5, CHRNA3 and CHRNA4 genes.

Due to its ability to directly interact with hnRNP K and its ability to interact with the opposing strand of the CT box *in vitro*, we previously hypothesized that Pur α acts in concert with hnRNP K to negatively regulate expression of the CHRNA4 gene (Fig.

A1.1) (Du et al., 1997; Du et al., 1998). Surprisingly, I obtained negative results in Pur α ChIP assays in each of the cell lines investigated. However, further experimentation is required to determine if the negative Pur α ChIP data (Figures A1.3, A1.5 and A1.7) are the result of a lack of interaction between Pur α and the CHRN4 promoter in the chromatin environment (where DNA is associated with histone proteins and may be organized into higher order chromatin structures) or the result of the use of an anti-Pur α antibody that is not suitable for the ChIP assay. This issue could be addressed by using additional anti-Pur α antibodies, and also by performing additional ChIP positive control PCRs.

Interestingly, Pur α knockdown experiments in Neuro 2A cells revealed that a 68% \pm 12% reduction of Pur α message resulted in decreased CHRNA5, CHRNA3 and CHRN4 mRNA levels (Fig. A1.12). Furthermore, the reduction in clustered nAChR subunit gene expression was not a result of a general dampening of transcription, as GAPDH mRNA levels were unchanged during Pur α knockdown. In an attempt to extend the analysis of Pur α 's regulation of the clustered subunit genes in Neuro 2A cells, Western blot analysis was performed on negative and Pur α siRNA treated cells. These data suggest that the reduction of nAChR CHRNA3 and CHRNA5 message observed during Pur α knockdown results in reduced levels of the α 3 and α 5 proteins. Conversely, I detected an increase in β 4 protein levels despite the reduction in message observed following Pur α siRNA treatment (Fig. A1.12). Further experimentation is required in order to determine to what extent, if any, Pur α

knockdown impacts $\alpha 5$, $\alpha 3$ and $\beta 4$ nAChR subunit protein levels. As discussed above, these experiments should be performed using antibodies whose specificity has been verified using the corresponding nAChR KO animals.

Contrary to our previous hypothesis these data indicate that, at least in Neuro 2A cells, Pur α acts to promote transcription of the CHRNA4 gene. Positive regulation of CHRNA4 gene expression by Pur α may be achieved through an interaction with Sp1. A similar interaction between Pur α and Sp1 has been shown to occur at the myelin basic protein (MBP) gene promoter (Tretiakova et al., 1999). In this system, both Sp1 and Pur α interact with the MBP promoter at overlapping regions of DNA, similar to what is observed at the CHRNA4 promoter CT / CA region (Bigger et al., 1997; Du et al., 1997). At the MBP promoter, binding of Sp1 results in increased recruitment of Pur α . Furthermore, overexpression of both factors results in synergistic activation of the MBP gene promoter (Tretiakova et al., 1999). If we consider that Pur α is not a repressor and acts to positively influence expression of the CHRNA4 gene, the reduction of CHRNA4 gene expression following Pur α knockdown could be explained by the dampening of the cooperative effects of Pur α and Sp1 at the CHRNA4 gene promoter. Due to the fact that Sp1 also positively regulates expression of the CHRNA5 and CHRNA3 subunit genes (Campos-Caro et al., 1999; Benfante et al., 2007), inhibition of Pur α - Sp1 interactions could also explain the reduction in CHRNA5 and CHRNA3 mRNA observed in the Neuro 2A Pur α siRNA experiments (Fig. A1.10).

We have also shown previously that Pur α directly interacts with the transcription factor Sox10 (Melnikova et al., 2000a). This finding is of particular significance in light of the fact that Sox10 positively regulates expression of each of the three clustered nAChR subunit genes (Melnikova et al., 2000b; Improgo et al., 2010b) and interacts with the CHRNA4 promoter both *in vitro* (Liu et al., 1999) and in the context of native chromatin (Scofield et al., 2008). Despite the direct physical interaction, to date no functional interaction between Sox10 and Pur α has been reported.

These findings advance the current understanding of the transcriptional regulation of the CHRNA4 gene by detailing the interaction of hnRNP K and Pur α with the CHRNA4 promoter in several neuronal-like cell lines with varying levels of CHRNA3/A5/B4 gene expression. These studies demonstrate that in Neuro 2A cells, Pur α acts to enhance transcription of the CHRNA5, CHRNA3 and CHRNA4 genes, providing an alternate hypothesis for the role that Pur α plays at the CHRNA4 promoter, and providing the first evidence for positive regulation of nAChR subunit genes by Pur α . Given its interaction with factors that positively influence CHRNA4 promoter activity and its positive regulation of CHRNA4 gene expression in Neuro 2A cells, I hypothesize that Pur α participates in a multi-subunit transcriptional regulatory complex that acts to *positively* influence transcription of the CHRNA4 gene (Fig. A1.13). This hypothesis could be tested further with experimentation aimed at discovering protein-protein interactions between Pur α and additional transcription factors that act to regulate expression of the CHRNA4 gene.

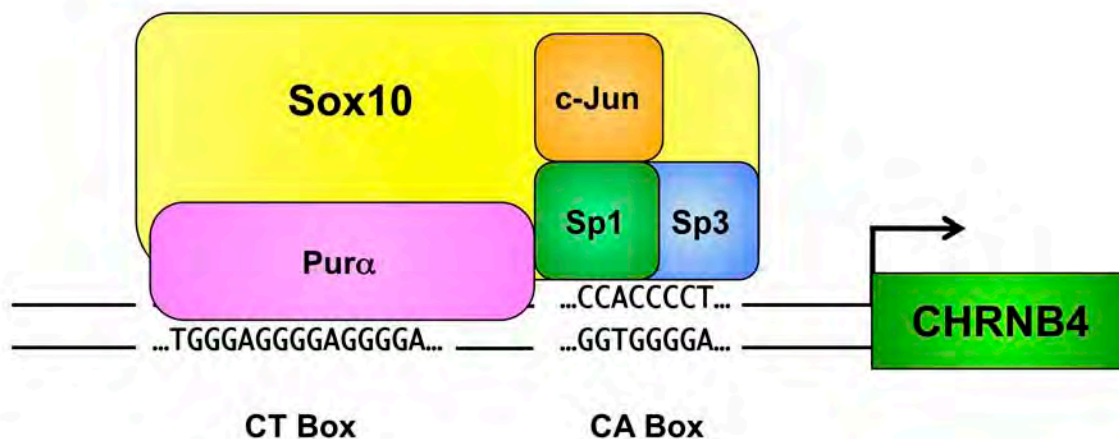


Figure A1.13: Revised model of positive-acting factors at the CHRNA4 gene promoter. The coding region of the CHRNA4 gene is represented as a light green box with arrow above the coding region indicating that CHRNA4 expression is turned on. The double stranded nucleotide sequences of two transcriptional regulatory elements, the CT and CA boxes, are also shown. Transcription factors Sox10 and Purα are depicted as yellow and pink colored ovals with Sp1, Sp3, and c-Jun depicted as colored squares above the CA box. Factors shown previously to physically interact are depicted touching each other.

Limitations and Future Perspectives

These studies provide insight into the functional role that the two transcription factors, hnRNP K and Pur α , play at the CHRNA4 promoter. As hnRNP K was found to interact with the CHRNA4 promoter in OBL21 cells siRNA experiments should be extended to OBL21 cells as well as other cell lines with low levels of CHRNA4 gene expression. These experiments may yield evidence that will contribute to a better understanding of the role that hnRNP K plays at the CHRNA4 promoter. siRNA experiments were not performed in OBL21 and PC12 cells due to technical difficulties in the optimization of the transfection procedure. However, the Neuro 2A cell line was easily transfected and cells remained healthy during the siRNA treatment. In Neuro 2A cells, knockdown of Pur α was found to negatively impact transcription of the CHRNA5, CHRNA3 and CHRNA4 genes, while in the ChIP experiments no interaction of Pur α with the CHRNA4 promoter was observed. In addition to the positive control PCRs discussed above, as well as the use of different anti-Pur α antibodies, future Pur α ChIP experiments could be performed in a set of cell lines or tissue types with varying levels of CHRNA4 gene expression.

As discussed above, Pur α ChIP derived DNA should also be used as template in PCR with primers designed to amplify a promoter fragment of a gene that has been shown previously to be regulated by Pur α . One possible candidate for a Pur α ChIP positive control is the neuron-specific FE65 gene. Much like the CHRNA4 gene, FE65 is transcribed from a TATA-less promoter, is positively regulated by Sp1 and is expressed primarily in neurons (Zambrano et al., 1997; Yu et al., 2010). ChIP-

derived DNA could be used as template in PCR with primers designed to amplify the region of Pur α interaction with the FE65 gene promoter. In these experiments, I would expect enrichment of the FE65 promoter fragment in Pur α ChIPs to be several fold higher than IgG and Mock IP negative controls.

In addition to ChIP assays, the Pur α siRNA knockdown experiments should also be extended to cells that have low levels of CHRN4 gene expression. This course of experimentation is particularly important when considering that Pur α has been shown to function as either a repressor or activator of transcription based on its interaction with other co-factors (Gallia et al., 2000). Thus, the expression of cell-type specific cofactors may influence whether Pur α plays a positive or negative transcriptional regulatory role at the CHRN4 promoter. An examination of Pur α knockdown under conditions where CHRN4 is expressed at low levels may provide insight into the possible regulatory role that Pur α plays in cells that express low levels of the CHRN4 gene.

In order to test the hypothesis that fewer functional β 4-containing receptors are expressed in Neuro 2A cells following Pur α siRNA treatment, additional functional assays should be performed. For example, I would expect that when performing whole-cell patch clamp recordings from Pur α siRNA treated cells you would observe a less potent reduction of nicotine-induced currents by the α 3 β 4 subtype selective nicotinic antagonist AuIB (Azam and McIntosh, 2009) when compared to negative siRNA treated cells. These data would indicate that fewer functional α 3 β 4 receptors

are expressed in Pur α siRNA treated cells as a result of reduced nAChR mRNA production.

These studies have investigated the roles that hnRNP K and Pur α play with respect to the transcriptional regulation of the CHRNA5, CHRNA3 and CHRNB4 genes. Using the ChIP assay, I was able to focus my investigation on the interaction of these proteins with the CHRNB4 promoter in the chromatin environment. These results demonstrated that hnRNP K interacts with the CHRNB4 promoter in OBL21 cells but not in PC12 cells +/- NGF or in Neuro 2A cells. Despite negative results in Pur α ChIP assays, additional experimentation is required to determine if Pur α does not interact with the CHRNB4 promoter in the cell lines tested. In addition, siRNA experiments demonstrated that, in Neuro 2A cells, Pur α positively regulates expression of the CHRNA5, CHRNA3 and CHRNB4 genes, while hnRNP K neither positively nor negatively regulates expression of these genes. These results have provided the first evidence suggesting that Pur α positively regulates expression of the CHRNA5, CHRNA3 and CHRNB4 genes and have lead to the modification of our current model of transcriptional control of the CHRNB4 gene (Fig. A1.13).

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