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Expression of nicotinic acetylcholine receptor (nAChR) in peripheral tissues and the effects of nicotine on insulin signaling

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Biology

by

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ABSTRACT OF THE THESIS

Expression of nicotinic acetylcholine receptor (nAChR) in peripheral tissues and the effects of nicotine on insulin signaling

by

Paul Michael Wadensweiler

Master of Science in Biology

University of California, San Diego, 2012

Professor Sushil Mahata, Chair

Background: It was previously observed that nicotine induced insulin resistance in mice. Nicotine activates nicotinic-acetylcholine-receptors (nAChR). Inhibition of nAChR or nitric oxide synthase (NOS) prevented nicotine-induced hyperglycemia. Functional nAChR pentamers are composed of combinations of α1-10, β1-4, γ, δ, and ε subunits. It has been reported that specific activation of α7nAchR prevents diabetes. Therefore, determination of the subunit expression profile in liver and muscle and

analysis of the effects of nicotine on insulin signaling would help elucidate the pathway of nicotine action in liver and muscle. ***Hypothesis:*** Prolonged exposure to nicotine may inhibit insulin-stimulated PI-3 -kinase/Akt signaling in liver and muscle tissues which may not express α7nAChR subunit for protection. ***Methods:*** (i) Analysis of mRNA expressions of the nAChR subunits in liver and muscle by qRT- PCR. (ii) Analysis of the effects of nicotine on insulin-stimulated phosphorylation of Akt and ERK-1/2 in hepatocytes, muscle and chromaffin cells by immunoblotting. ***Results:*** The predominant nAChR subunits are, β4α3α7α10 in adrenal glands, β4α10α3 in liver and β4α10α1β1 in muscle. Liver and muscle do not express α7nAChR. Nicotine treatment (16 hours) inhibited insulin-stimulated Akt phosphorylation in hepatocytes and muscle cells but not in PC-12 cells. This inhibition was reversed by pretreatment with nAChR and NOS-inhibitors. Nicotine treatment stimulated ERK phosphorylation and enhanced insulin effects in hepatocytes but not in muscle cells. ***Conclusions:*** The lack of α7 and differential expression of nAChR subunits in liver and muscle may be responsible for the inhibition of insulin-stimulated Akt signaling by nicotine leading to insulin resistance.

CHAPTER 1: INTRODUCTION

1.1. Acetylcholine Receptor Overview

Acetylcholine receptors (AChRs) are a class of transmembrane ligand-activated receptors, which respond to the neurotransmitter acetylcholine (Witzemann et al., 1990). These receptors are classified within two main categories: the metabotropic muscarinic receptors and the ionotropic nicotinic receptors, based off of their affinity for specific ligands and their mechanism of action (Albuquerque et al., 2009). Both muscarinic and nicotinic acetylcholine receptors share certain similarities. For instance, the endogenous neurotransmitter acetylcholine activates both of these transmembrane receptors throughout the body, and both receptor subtypes show expression in neuronal as well as nonneuronal tissues (Eglen, 2005; Gotti and Clementi, 2004). However, the function and mechanism of action of these two receptor subtypes are very distinct, leading to different roles within the body. The metabotropic muscarinic acetylcholine receptors fall within the class of G protein-coupled seven-transmembrane receptors that work through relatively slow-acting second messenger cascades to activate membrane ion channels and initiate intracellular functions (Eglen, 2005). Their name originates from their significant binding sensitivity to muscarine, a toxin from the mushroom *Amanita muscaria* (Eglen, 2005)**.**

The other subtype of acetylcholine receptor is the nicotinic acetylcholine receptor, which was named due to its high affinity for nicotine. Nicotine is a chemical derived from the tobacco plant, which uses this toxin to deter insect predators (Eastham et al., 1998). It is also widely known as being the active and highly addictive ingredient in smoking and chewing tobacco products. By contrast to the muscarinic counterparts,

nicotinic acetylcholine receptors (nAChR) do not act through the use of second messengers, but instead are fast-acting ligand-gated ion channels (Albuquerque et al., 2009). Physical interaction with an endogenous ligand, such as acetylcholine or nicotine, causes physical transformation in receptor conformation that will initiate changes in channel structure and resultant ion conductance across the cell membrane. Ion flow across a cell membrane alters the electrochemical properties of the cell and induces a specific cellular response dependent upon cell type and location (Albuquerque et al., 2009).

Section 1.2. nAChR Structure

The nAChR shares a structural organization necessary for the functioning of all ligand-gated ion channels. The first structural property essential to function is that the proteins comprising the receptor span the entire thickness of the membrane, creating a channel that is selectively permeable to specific ions. The receptor is therefore exposed to three separate electrochemical environments including the extracellular, the intracellular and the transmembrane spaces, which influence structure and function of the receptor protein (Albuquerque et al., 2009). The next fundamental property of the nAChR is its propensity to open and to close upon ligand binding and unbinding, allowing control over ion passage. The quaternary structure of the nAChR protein contains sites that are sensitive to interaction with external molecules including agonists, which activate the receptor to open the ion channel, antagonists, which inhibit the activation of the receptors, as well as other molecules that influence the action of both agonists and antagonists (Albuquerque et al., 2009).

One of the primary functions of the nAChR is within the neuromuscular junction

to initiate muscular contraction upon binding to acetylcholine released from motor neurons synapsing onto the target muscle. The structure of this muscle nAChR subtype has been shown to exist as a heteropentamer comprised of a single β, δ, γ, and two α subunits, arranged in a circular organization around a central channel used for ion passage. These muscle-type nAChR subunits served as the springboard into the elucidation of a diverse collection of many additional nicotinic acetylcholine receptor subunits by screening of brain cDNA libraries (Albuquerque et al., 2009). Studies have identified 9 distinct α subunits including α1, α2, α3, α4, α5, α6, α7, α9, and α10 (α8 was determined to not be present in mammals), four distinct β subunits (β1, β2, β3, and β4) in addition to three other subunits named γ, δ, and ε (Dani and Bertrand, 2007; Gotti and Clementi, 2004).

While each nAChR subunit known to date is distinct from the others, certain structural components stand out as collective similarities to all. The first of these commonalities is a large N-terminal domain of the receptor subunit protein involved in agonist binding on the exterior of the cell membrane. This is true only for the alpha-type subunits, contrary to the beta-type subunits that offer a structural role and are not involved in ligand binding (Sala et al., 2008). In addition, four connected transmembrane sections span the width of the cell membrane to localize each receptor subunit correctly, with the second transmembrane segment forming part of the ion channel. Finally, a brief C-terminal domain remains similar to all of the nAChR subunits (Sala et al., 2008). Aside from these resembling motifs, there still exists a degree of distinctiveness across the spectrum of nAChR subunits, which influences subunit-subunit interaction as well as subunit function (Sala et al., 2008).

Section 1.3. Nicotinic Acetylcholine Receptor Specialization

The diversity in nAChR subunits yields a vast variety of specialized functions and properties of complete nicotinic acetcyholine receptors within the mammalian system. The nAChR subunits interact in specific combinations to produce a spectrum of nAChRs that influence channel characteristics and convey an extraordinary collection of functions depending on the cell type (Wenger et al., 1997). Nonneuronal cells including those in muscle, pancreas, and liver in additional to central and peripheral neuronal cells, such as those in the hippocampus or adrenal gland, preferentially express different subunit arrangements based on the function to be carried out by the cell type. The unique structural properties of distinct nAChR subtypes are specifically associated with the physiological process performed by the receptor, which range from modulating synaptic transmission, initiating muscular contraction, controlling inflammatory processes, initiating sympathetic responses, and regulating cell apoptosis (Albuquerque et al., 2009). Therefore, the unique combinations and stoichiometries of nicotinic acetylcholine receptor subunits add to the potential receptor diversity and corresponding specialization afforded by the complete nAChR (Nelson et al., 2003).

Section 1.4. Regulating nAChR Expression

In order to control the cellular effects caused by agonist binding to the nicotinic acetylcholine receptor, the expression of the receptor subunits may be adjusted by various mechanisms at various stages of the formation of the gene product. The primary level in which subunit expression can be controlled is during transcription of the subunit-encoding genes into mRNA, which can be influenced by various forces including developmental pressures and agonist/antagonist exposure. Cultured muscle cell lines and

bovine PC-12 chromaffin cells have demonstrated cell-specific regulation of nAChR subunit during different stages of in vitro development that have correlated closely with observed changes in the functioning of these proteins (Deneris et al., 1989; Rogers et al., 1992). Analogous results have been obtained from tissues during in vivo development (Kues et al., 1995). Chronic exposure to nicotine, a potent agonist for the nAChR, has been shown to increase, or "upregulate" the expression of these receptors in certain tissues, including the brain. In the brains of chronic smokers, which experience high levels of ongoing nicotine exposure, nAChR upregulation was connected to heightened sensitivity to nicotine through significant increase in the total number of nAChRs. The mechanisms involved are still not fully understood (Perry et al., 1999). However, upregulation is not the universal response of all nAChRs to nicotine, and some subunits even downregulate from chronic nicotine exposure, causing decreased sensitivity to the nicotine agonist (Lai et al., 2005).

The actual number of nAChR subunit compositions does not come close to the sheer number of potential receptor subunit combinations that could be formulated on the basis of the number of subunits available (Wenger et al., 1997). Formation of operative nAChRs requires not only the appropriate number and type of subunits, but also demands that these subunits interact and come together in the accurate orientation (Albuquerque, Physiol. Rev. 2009). Therefore, there seems to be precise cell-specific control and regulation over expression and assembly of subunits into functional receptors, which limits the receptor subtypes produced in specific tissue types. Regulation of the nAChR also occurs at the post-translational phase, including the proper transport and placement of the receptor proteins within the cell membrane (Albuquerque et al., 2009).

Section 1.5. nAChR Functions and Relation to Structure

Homomeric and heteromeric pentamer combinations of α and β nAChR subunits present discrete presynaptic and postsynaptic electrochemical functions within the neural system (Sugano et al., 2006). In the central nervous system, the neuronal nAChR is often composed of diverse arrangements of α and β subunits in a 2:3 ratio respectively, including the presynaptic α4 β2 nAChR. Additionally, the homomeric α7 nAChR is also prevalent in the central nervous system (Sala et al., 2008). Compared to other nAChRs, the homomeric α7 nAChR becomes desensitized more quickly and leads to a larger Ca^{2+} conductance across the cell membrane upon receptor activation due to greater permeability to this cation. Therefore, the activation of the α7 nAChR, has profound impacts on signal transduction pathways, exocytosis of vesicles, and other Ca^{2+}-dependent mechanisms within the cell (Broide and Leslie, 1999; Suzuki et al., 2006).

Within the peripheral nervous system the α3, α5, and β4 subunits appear to be the principal subunits that combine to form mature nAChRs (Sala et al., 2008). At the neuromuscular junction, the muscle nAChR is composed of β1, δ, γ and two α1 subunits and serves to respond to acetylcholine released from motor neurons to initiate muscle contraction (Sala et al., 2008). The α10 subunit has been shown to only form functional nAChRs when combined with the α9 subunit. The α9 α10 nAChRs are found in the pituitary gland, olfactory epithelium, and cochlea where they show unusual characteristics correlated to both nicotinic and muscarinic receptor types (Sala et al., 2008). Currently there is little understanding of the subunit compositions comprising the nAChR in muscle and no knowledge of those found in liver. In addition, there is still much to learn about the role that these receptors play in these peripheral tissues.

Section 1.6. Role of nAChR in Cholinergic Anti-Inflammatory Signaling Pathway, Insulin Action, and Glucose Homeostasis

Within the immune system, cytokines are the messenger molecules that cause cellular responses such as tissue remodeling and inflammation (Gallowitsch-Puerta and Tracey, 2005). While cytokines are crucial in the normal function of the immune system, excess or insufficient levels of cytokines lead to impaired organ function and tissue injury. Thus, cytokine response must be tightly controlled (Gallowitsch-Puerta and Tracey, 2005). Contrary to the slow-acting humoral systems previously known to protect against cytokine toxicity, the newly discovered cholinergic anti-inflammatory pathway is a fast acting, neuronal-based mechanism that acts locally to protect against cytokine over-production during inflammatory response (Gallowitsch-Puerta and Tracey, 2005).

Activation of the nAChR shows a vital role in inflammation via the cholinergic anti-inflammatory pathway. Through either acetylcholine or nicotine interaction, the nAChR causes macrophages to release reduced levels of proinflammatory cytokines, therefore preventing inflammatory tissue damage (Wang et al., 2003). Specifically, the α7 nAChR isoform has been identified as the receptor subtype responsible for this effect because an α7-selective nAChR antagonist has been shown to reverse the nicotine-mediated inhibition of macrophage production of cytokines (Cheng et al., 2007). Therefore, the α7 nAChR has become the target for inflammatory disease therapy (Bencherif et al., 2010). Furthermore, stimulation of the vagus nerve and treatment with cholinergic agonists inhibit systemic inflammation by activating the noradrenergic splenic nerve via the α7 nAChR. These findings suggest the role of the α7 nAChR in

linking the parasympathetic and sympathetic system in the control of inflammation (Vida et al., 2011).

By reducing the inflammatory response, nicotine could potentially alleviate metabolic disorders tied to low-grade inflammation, such as obesity and diabetes, through activation of the nAChR (Baker et al., 2011; Gregor and Hotamisligil, 2010; Olefsky and Glass, 2010). In fact, nicotine treatment reduces the occurrence of Type 1 Diabetes in mice (Mabley et al., 2002) and protects insulin sensitivity in obese rats (Liu et al., 2001; Liu et al., 2003). Moreover, chronic nicotine treatment has recently shown to improve glucose homeostasis in obese, diabetic *db/db* mice (Wang et al., 2011). These studies have also determined that α7-nAChR-knockouts (α7-KO) exhibit heightened infiltration of M1 macrophages in adipose tissue and increased levels of cytokines compared to WT mice. Compared to WT mice that showed dampened pro-inflammatory cytokine and free fatty acid levels with nicotine treatment, α7-KO mice lacked this response to nicotine (Wang et al., 2011). The α7-nAChR agonist, TC-7020, has been shown by other reports to impede weight gain and improve metabolic disorder in *db/db* mice (Marrero et al., 2010). The α7-nAChR shows predominant expression in the hippocampus. Therefore, these findings pointed towards the role of α7-nAChR in mediating JAK2/Stat3 signaling in the central nervous system. This direction was supported because the JAK2 inhibitor, AG-490, blocked the aforementioned TC-7020 effects (Marrero et al., 2010).

While there is well-documented evidence of the advantageous effects of specific nicotine agonists on glucose regulation in insulin resistant obese and diabetic models in vivo, the effects of these nicotine agonists on normal (insulin sensitive) models are still unclear. Cigarette smoking has been reported to promote glucose intolerance and insulin

resistance, placing importance on understanding the effects of chronic nicotine exposure through tobacco smoking on glucose metabolism in people sensitive to insulin (Attvall et al., 1993; Frati et al., 1996; Persson et al., 2000). Further work is necessary to define the effects of nicotine activation of nAChR on insulin signaling and glucose metabolism in peripheral tissues such as in the liver. Activation of the homomeric α7-nAChR may improve insulin sensitivity by reducing the production of pro-inflammatory cytokines in obese and diabetic models of these inflammatory conditions (Liu et al., 2001; Liu et al., 2003; Marrero et al., 2010; Wang et al., 2011). However, since liver shows low expression of the α7-nAChR, the nicotine-mediated activation of other heteromeric nAChR subunits lacking α7 (α3β2, α3β4, α4β2, α1β1 *etc.*) may induce insulin resistance in previously insulin-sensitive cases. For instance, β2-knockout mice lacking the nicotine-induced catecholamine release (Scholze et al., 2007), may also show nicotine resistance in regard to regulation of glucose metabolism.

Our group (Dr. Sushil Mahata's laboratory) previously demonstrated that activation of the nAChR causes two distinct effects on glucose metabolism. In insulin-sensitive mice, activation of the nAChR with nicotine enhances nitric oxide (NO) - and catecholamine-dependent glycogenolysis, upregualtes expression of gluconeogenic genes, and increases glucose production after prolonged exposure. The nAChR activation in insulin sensitive mice fed normal chow diet results in hyperglycemia, despite the fact that nicotine induces glucose-stimulated insulin release and increases blood insulin levels. Therefore, nicotine treatment instigates insulin resistance typical in type II diabetes. This effect is distinct from high-inflammation, insulin-resistant models, in

which the improved insulin sensitivity connected to α7-nAChR activation may be caused by the anti-inflammatory pathway and diminished glycogen breakdown.

Direct nicotine-activation of the nAChR in liver, induces primarily an NO-linked pathway, but unlike in neuroendocrine cells (PC-12 cells), nicotine does not directly evoke cAMP/protein kinase A pathway in HepG2 hepatocytes. Nevertheless, when looking *in vivo*, nicotine activated a sympathetic response stimulating catecholamine release from the adrenal medulla, which bind to adrenergic receptors in liver. Activation of the α- and β-adrenergic pathways in liver leads to indirect activation of cAMP/protein kinase A pathway in liver by nicotine. The effect of nicotine treatment on promoting hyperglycemia is abolished with administration of chlorisondamine (a broad, non-specific nAChR antagonist) and nitric oxide synthase inhibitors. Therefore, the conclusions that can be generated from the literature and the previous work performed in the Mahata lab include (i) Nicotine improves insulin sensitivity in insulin-resistant mice acting through the anti-inflammatory pathway linked to the α7-nAChR, (ii) Nicotine causes hyperglycemic and hyperinsulinemic conditions in insulin-sensitive mice mediated by nAChR induced catecholamine and nitric oxide (NO) production, (iii) Nicotine increases glycogen breakdown and glucose production in insulin-sensitive mice, and (iv) Nicotine may interfere with the insulin signaling causing insulin resistance in previously insulin-sensitive mice. The key question that needs to be answered now is: How does nicotine treatment interfere with the insulin action at the level of insulin signaling that can explain the nicotine-induced insulin resistance observed in previously insulin-sensitive mice feed normal chow diet?

Section 1.7. Objectives of this Work

With that background, the objective of my work was twofold: (1) To determine the mRNA expression profile of nAChR subunits in liver and muscle and compare that with the expression in adrenal gland, a neuroendocrine tissue and (2) to determine the effects of activation of nAChR by nicotine on insulin signaling in liver and muscle cells in culture. To achieve these goals, I used quantitative real time PCR technique to determine the mRNA expressions of nAChR subunits in liver, muscle and adrenal gland, and western blotting technique to determine the insulin stimulated phosphorylations of Akt and ERK in cultured hepatocytes, muscle, and chromaffin cells exposed to insulin, nicotine, chlorisondamine (a broad inhibitor of nAChR) and inhibitors of nitric oxide synthase. The basic approach is depicted schematically below.

CHAPTER 2: MATERIAL AND METHODS

Section 2.1. L6 myotubes culture

L6 myoblast cells were obtained from Amira Klip's Lab (Toronto, Canada). L6 myoblasts were cultured in alpha-MEM with 10% FBS and antibiotics. Cells grew as patches of islands, not as individual cells scattered uniformly throughout the culture plates. When the cultures reached 70-75% confluency, the medium was switched to 2% FBS in MEM to induce differentiation**.** When L6 cultures showed long fused fibers looking similar to crisscrossing highways or areas looking like miniature palm of your hand with fingers representing fibers (usually 7-9 days after switching to 2% FBS in alpha-MEM), cultures were ready for experimentation. For long-term nicotine treatment, nicotine (10 uM) or vehicle (water) was added to the culture in a serum-free MEM medium incubated for 16 hours. For acute treatment, cultures in serum-free medium were exposed to nicotine (10 uM) for 15 minutes with or without insulin (100 nM). Insulin (100 nM) treatment was for 15 minutes. For long term nicotine treated cultures, insulin was added for the last 15 minutes before termination.

Section 2.2. Propagation of ATCC-PC12 cells

100-mm cell culture dishes were coated by adding sufficient 100 µg/ml poly-L-lysine solution to cover the surface and incubated for 60 minutes at 37◦ C. After removing the poly-L-lysine solution, sufficient 100 µg/ml rat tail collagen Type I solution was added to cover the surface of the plate and was incubated for 120 minutes at room temperature. Coated dishes were washed two times, each time with 5 ml sterile water. Cells were grown in coated 100-mm culture dishes containing 10 ml of complete F12K culture medium containing 15% horse serum, 2.5% FBS and antibiotics, incubated in a

humidified incubator with 6% CO2 and set to 37°C. At 70 % confluency, cells were differentiated to form neurite growths, with the help of NGF (100ng/ml). Cells were passaged by splitting 1:5 after scraping off from the master plates. Treatment protocols were the same as in L6 muscle cells.

Section 2.3. Hepatocyte cultures

HepG2 was cultured under the instructions provided by the vendor (ATCC). Primary hepatocytes were prepared by infusion of a collagenase solution (5-6 ml/minute, 40 µg/ml, Liberase, Roche Inc.) through IVC over a period of 10-12 minutes. Hepatocytes were cultured on collagen-coated plates in Williams E medium. This medium was fortified, containing non-essential amino acids, glutamine, antibiotics, and 10% FBS. Six hours after attachment, the long-term nicotine treatment group of cultures were sustained for 16 hours in the same Williams E medium, however serum-free and supplemented with nicotine (20 µM) as indicated. The acute nicotine treatment group of cultures were treated with nicotine (10 µM, for 15 minutes), insulin (10 nM, for 10 minutes), or both (insulin was added 5 minutes after nicotine). Insulin (10 nM) or vehicle was added 10 min prior to harvesting of cultures in which nicotine treatment lasted 16 hours (long-term).

The following compounds were used for treatment of cell cultures: the non-specific nAChR inhibitor chlorisondamine (50 µM Tocris, Ellisville, MO), the NOS inhibitors L-NIL [L-N6- (1-iminoethyl) lysine, 10 µM in culture and 15 µg/g BW *in vivo* by IP, Calbiochem, San Diego, CA) and L-NNA (N^{w}-Nitro-L-Arginine, 10 µM in culture and 15 µg/g BW by IP, Calbiochem), NOSI (a mixture of L-NIL and L-NNA, 10 µM each), and the nAChR agonist nicotine bitartrate (100 µM, Calbiochem).

Section 2.4. Immunoblotting of cell lysates

At the end of incubation, cultures were lysed in a buffer containing 1% NP-40, as well as protease and phosphatase inhibitors as described (Bandyopadhya et al) Homogenates were immunoblotted for eNOS signaling molecules and phospho- and total Akt using antisera from Cell Signaling (Danver, MA). L6 myotubes were similarly treated and subjected to the same immunoblot analyses.

Isolated protein samples were measured for protein concentration and were loaded in pre-cast western blot gels in equal amounts. Gels were run and blotted on nitrocellulose paper for immunocytochemical analysis. After 1 hour of blocking of non-specific binding with 5% milk or 5% BSA depending on the primary antibody to be added, the blots were incubated in primary antibody (1:1000) overnight. The membranes were then washed with TBST (Tris buffered saline, pH 7.4 containing 0.05% Tween-20) to remove excess primary antibody and exposed to secondary antibody (1:5000) for 1 hour. Secondary antibody was always prepared in 5% milk. After 1-hour incubation, the secondary antibody was removed and the membranes were washed with TBST to remove excess secondary. In order to visualize the protein bands of interest, the membranes were then exposed to PICO or FEMTO ECL reagents (from Pierce) for varying lengths of times depending on the strength of the signals in the protein band of interest. The luminescence produced was captured in X-Ray films, which were later used for scanning and densitometric analysis.

Section 2.5. Real-Time PCR

Extraction of liver RNA was carried out using a kit and following the specifications of the manufacturer (RNeasy Plus, Quiagen, Valencia, CA). Following

RNA quantitation and DNase digestion, 100 ng of RNA was transcribed into cDNA in a 20-µl reaction using a High Capacity cDNA Archive kit, analyzed, and amplified. PCR (25-µl reactions) contained 5 µl of cDNA (5x diluted), 2× SYBR Green PCR Master Mix, and 400 nM of each primer (sequences listed in Table 1). Differences in the threshold cycle number (ΔCt) between target and the housekeeping gene *GAPDH* were used to calculate differences in expression.

Section 2.6. Standard Curve Generation

RNA was extracted from muscle tissue harvested from saline treated NCD mice using trizol and an RNA isolation protocol. After RNA quantitation, 200 ng total RNA was transcribed into a pool of cDNA representing endogenous RNA population. Next, each individual subunit primer set (forward and reverse) was used to generate the corresponding cDNA from the cDNA pool by PCR. Thus, all 16 cDNA were generated by using 16 individual subunit primer sets. Each cDNA sample was separated by running on an agarose gel. Each cDNA band in the gel was extracted using a gel extraction kit from Qiagen. The purified cDNA concentration was determined and various dilutions were created. Several different dilutions cDNA were analyzed via RTQPCR against the corresponding primers to generate a standard curve of cDNA concentration vs. Ct value. When the Log of concentrations were plotted against Ct values, a linear curve was generated. These linear standard curves would be used to analyze the RNA concentration within experimental samples by comparing the Ct values obtained during RTQPCR to the corresponding concentrations afforded by the standard curve. This method was an alternative to the method previous described in Section 2.5, which determined expression

values based on comparison to the Ct values generated for the GAPDH housekeeping gene

Section 2.7. Statistics

All data are expressed as the mean ± SEM. Statistical comparisons were determined in the Excel Statistical Program using the two-tailed t-test, and in the commercially available GraphPad Package using paired t test, Mann-Whitney test, one-way ANOVA. Statistical significance was concluded at $p < 0.05$.

CHAPTER 3: RESULTS

Section 3.1. Expression of nicotinic acetylcholine receptor (nAChR) subunits in liver, muscle and adrenal gland determined by quantitative real time PCR (qRTPCR) using glyceraldehydes-3 phosphate dehydrogenase (GAPDH) as the housekeeping gene.

In this method, the difference in Ct (Critical Threshold, defined as the number of cycles required for the fluorescent signal to cross the background level) values between the target gene and GAPDH (ΔCt) is used to determine the relative expression level of the target genes in tissues or cells. A comparison of the expression levels of various nAChR subunits in adrenal gland, liver and muscle is given in Fig.1 as composite for all subunits and the effects of nicotine treatment is shown in Fig.2. Results are summarized in Table 2. While the predominant expression pattern in liver seems to be α2β2, muscle and adrenal glands appear to express varieties of subunits including those (α1 and β1 in muscle and α3 and β4 in adrenal glands) reported in literature to be predominantly expressed (Kues et al., 1995; Sala et al., 2008; Wenger et al., 1997; Witzemann et al., 1990). Chronic nicotine treatment seems to have a suppressing effect only on the α3 expression in adrenal gland. Otherwise, nicotine treatment increases expression of α1 and α5 in liver, α2 in muscle and β2 in adrenal gland. It is to be noted that this probably would be the first published work on the expression pattern of nAChR subunits in liver.

Section 3.2. Expression of nAChR subunits determined by qRTPCR using a standard curve generated by plotting Ct versus Log [Concentration of cDNA] for each primer set used for producing the target cDNA.

The major concern about using the GAPDH method was variability of expression of GAPDH itself in different tissues under various physiological and pathological

conditions. This issue has been well documented in literature (Alexander et al., 1988; Barber et al., 2005; Glare et al., 2002; Higashimura et al., 2011). Other housekeeping genes also suffer from this problem as reported by Brattelid T. et al. (Brattelid et al., 2010). We have noticed a similar problem where Ct values for GAPDH varied between tissues we examined. This led to the concern that expressions determined by the ΔCt method using GAPDH may not correctly represent the true expression levels in the three tissues we examined. Therefore, we sought to re-examine the expression levels by a different method that does not rely on the expression of housekeeping genes.

The standard curve method does not use GAPDH or any housekeeping gene as reference. The concentration of the specific mRNA was calculated from the corresponding standard curve generated by plotting Ct values against different concentrations of cDNA produced by the specific primers set. Figure 3 demonstrates the linear standard curves and the corresponding equations for each subunit. These equations have been used to calculate the concentrations of a subunit in the population of cDNA produced by the reverse transcriptase-reaction with the total RNA extracted from each tissue.

In Figure 4, expression of each subunit in adrenal glands, liver and muscle have been compared. These results can be contrasted with those obtained by GAPDH method (Figure 1 and Table 2). The overall predominant profile of expression in adrenal gland, liver and muscle, as determined by this standard curve method, has been shown in the Figure 5. When compared with the summary in Table 2, Figure 5 shows a different profile. The expression profile for nAChR subunits in adrenal gland shown in Figure 5 corroborates with the most of the published literature (Kues et al., 1995; Rogers et al.,

1992; Sala et al., 2008; Wenger et al., 1997; Witzemann et al., 1990), except for the presence of α10. However, some reports demonstrated the presence of α10 (Elgoyhen et al., 2001) in inner hair cells. Therefore, the predominant profile for subunit expression in adrenal gland, liver and muscle, as shown in Figure 6, is considered reliable and more representative.

The results obtained by using the standard curve method revealed that the expression of α10β4 is the most common feature in these three peripheral tissues. However, tissues differ in the abundance of the α and β subunits. The β4 subunit shows the highest expression in the three tissues. Next to β4, the expression of α3 and α7 is significant in adrenal glands and α10 in liver and muscle. α3 is undetectable in muscle and α1 is undetectable in adrenal gland and liver.

We have yet to verify these expression profiles at the protein level. If protein expression reflects the mRNA expression, then it can be predicted that the signaling response due to the activation of nAChR by agonists would be different for these tissues because of the differences in the subunit composition.

Section 3.3. Effects of stimulation and inhibition of nAChR functions on insulin signaling: Regulation of insulin-stimulated Akt phosphorylation by nicotine, chlorisondamine and NOS inhibitors in hepatocytes, muscle and chromaffin cells.

As discussed in the introduction section, nicotine has profound effect on glucose and insulin metabolism. Therefore, it is important to determine the effects of nicotine on the insulin signaling pathways. We chose cell culture systems to examine the effects of nicotine. Accordingly, we cultured hepatocytes, L6 myotubes, and PC-12 chromaffin cells. Hepatocyte and muscle cultures are the most relevant system to examine the effects

on insulin signaling because insulin-regulated glucose metabolism is primarily carried out in these tissues.

As markers for insulin signaling we choose to focus on insulin stimulated phosphorylation of Akt and ERK because Akt and ERK mediate major actions of insulin such as glucose metabolism, DNA and protein synthesis, and apoptosis. Cells are treated with nicotine for 15 minutes (acute treatment) or for 16 hours (long-term treatment). Insulin or vehicle was added for last 10 minutes (acute treatment) of the incubation.

The strategy for analyzing crosstalk between nAChR and insulin receptor was to manipulate the nAChR functions upstream at the receptor level and also at the downstream signaling level. While nicotine activates nAChR through both α and β subunits, chlorisondamine (CSM), a nicotinic antagonist with broad subunit specificity prevents nicotine-mediated activation of nAChR. NOS inhibitors (NOSi) prevent nAChR downstream signaling without affecting the receptors.

As shown in Figure 7, insulin-stimulated pAkt signaling in muscle cells were not affected by the acute treatment with nicotine but long-term treatment (for 16 hours) led to the inhibition of insulin action on Akt phosphorylation. Similarly, pAkt signaling was not perturbed in hepatocytes by acute nicotine treatment. But 16-hour exposure to nicotine inhibited insulin-stimulated pAkt signals in hepatocytes (Figure 8). These results raised the possibility that inhibition of nAChR functions at the receptor level by CSM or by preventing nicotine-induced NO production by NOSi may prevent the inhibitory effects of chronic nicotine treatment. In fact, treatment with CSM and NOSi for one hour prevented inhibition of insulin-stimulated Akt phosphorylation by chronic nicotine treatment in hepatocytes (Figure 9). In contrast to the results in muscle and hepatocytes,

nicotine treatment, acute or chronic, did not inhibit insulin-stimulated Akt phosphorylation in PC-12 chromaffin cells (Figure 10).

Like Akt phosphorylation, insulin-stimulated phosphorylation of ERK-1/2 in muscle cells was also inhibited by long-term nicotine treatment (16 hours) but not by acute treatment (Figure 11). Interestingly, unlike pAkt signals, insulin-stimulated phosphorylation of ERK-1/2 in hepatocytes was not inhibited at all. In fact, nicotine by itself stimulated ERK phosphorylation and further enhanced insulin stimulated phosphorylation in hepatocytes (Figure 12) suggesting diverse regulation of Akt versus ERK phosphorylation in hepatocytes.

Taken together, these results suggested that nAChR activation may lead to diverse effects on insulin signaling on different tissues, possibly, due to different subunit composition.

CHAPTER 4: DISCUSSION

Our analysis of nAChR expression in peripheral tissues revealed an unusual feature: the presence of α10 mRNA along with β4 mRNA in all three tissues, liver, muscle and adrenal gland. While the β4 subunit was predominant in all three tissues, α10 subunit was quite significant in liver and muscle but was lowest in concentration in adrenal gland. The α10 subunit is generally found in inner ear hair cells (Baker et al., 2004; Elgoyhen et al., 2001) and it has a very close similarity in structure with α9 subunit. Expression studies in oocytes using α10 in combination with α2-α6 or β2-β4 did not generate functional receptors. Although α10 does not form a homomer like α9 does, a heteromeric receptor with α9α10 was shown to be functional (Baker et al., 2004; Elgoyhen et al., 2001). The presence of β4 subunit in bovine chromaffin cells has been previously demonstrated (Wenger, BW, 997). It has been shown by expression studies in oocytes that a combination of α2 or α3 or α4 with β2 or β4 cannot form functional heteromeric nAChR whereas α7, α8 and α9 can form functional homomeric receptors (Boulter et al., 1987; Elgoyhen et al., 1994; Gerzanich et al., 1994; Luetje and Patrick, 1991). From these discussions it appears that α10 subunit may not form any functional receptor in these peripheral tissues examined because of the absence of expression of the α9 subunit. Although we have not yet evaluated the subunit composition in these tissues at the protein level, based on our results on mRNA expression, it may be predicted that receptor compositions could be β4α3α7 in adrenal gland, β4α3 in liver and β4β1α1 in muscle. It may also be predicted that because of these possible differences in subunit composition, there could be some characteristic differences in how these receptors may crosstalk with insulin receptors in these three tissues.

Activation of nAChR by chronic nicotine treatment inhibits insulin-stimulated Akt phosphorylation in hepatocytes and muscle cells but not in PC-12 chromaffin cells. Chronic nicotine exposure also inhibits insulin-stimulated phosphorylation of ERK-1/2 in muscle cells but not hepatocytes. Both CSM nad NOSi can reverese the inhibitory effect of nicotine. In contrast to inhibition of Akt signaling in hepatocytes, nicotine directly stimulates ERK-phosphorylation and enhances insulin-stimulated ERK phosphorylation. These differences in crosstalk between nAChR and insulin receptor could be a reflection of the differences in subunit composition.

The protective effect of NOS inhibition against nicotine-induced inhibition of insulin action points to a mechanism through which muscle and hepatocytes may differ in insulin signaling. As shown in the schematic diagram (Scheme 2), persistent exposure to NO may inhibit IRS-1/2 functions through nitrosylation (Pilon et al., 2011) leading to inhibition of Akt signaling in both hepatocyte and muscle cells. It possible that in hepatocytes ERK phosphorylation takes place in a IRS-independent manner via Shc-GRB2 (SOS)-Ras mediated pathway (Sasaoka and Kobayashi, 2000) whereas in muscle, ERK phosphorylation is IRS-1/PI-3Kinase dependent. As a result, nicotine could inhibit ERK phosphorylation in muscle cells but not in hepatocytes. It is also possible that nicotine-stimulated nAChR may directly activate ERK via activation of RAS. Nicotine treatment did not inhibit Akt signaling in PC-12 chromaffin cells because nicotine evokes additional signaling pathway in chromaffin cells that can activate PI-3 kinase activity independent of IRS-1/2. For example, nicotine can activate PI-3Kinase/Akt and ERK pathways through EGF receptor and CaMKII signaling without involving IRS-1/2 (Nakayama et al., 2002).

In the literature, nicotine-induced activation of ERK created some controversies with regard to which nAChR subunit is actually responsible for phosphorylation of ERK-1/2. Is it α7 homomer (Gubbins et al., 2010; Nakayama et al., 2001) or α3β4 heteromeric receptor (Nakayama et al., 2006)? There is also reports, which demonstrated that the nicotine effect on ERK-1/2 phosphorylation is nAChR independent and mediated by glutamatergic signaling through receptor CaMKII in cortical neurons (Steiner et al., 2007). Therefore, we are not sure whether the direct stimulation of ERK-1/2 phosphorylation in hepatocytes is nAChR dependent or not, but we are sure that nicotine enhances insulin-stimulated ERK phosphorylation in hepatocytes and inhibits the same in muscle cells.

APPENDIX

Table.1. Quantitative RT-PCR primers (5'→ 3') for mouse nAChR isoforms and GAPDH.

α1	Forward	AGGGCGTGAAGTACATTGCAGAG
	Reverse	TCAGCCTCTGCTCATCCTTGTTGA
α2	Forward	AACCTGCTCTGAACTGTCCTGTGT
	Reverse	AATGTTCTGAAAGGCCCAATCCGC
α3	Forward	CATCGTGCTTTACAACAACGCCGA
	Reverse	TTAAAGATGGCCGGAGGGATCCAA
α4	Forward	GGCATGCAAGAAATCACCCTGTGT
	Reverse	ATCCAAACAGGGACCTCAGCTTCA
α5	Forward	ATTGTCATGGCAGAAGCATGGCAG
	Reverse	AAGCTCAAGTCATGTCCAGTGGGT
α6	Forward	TTCCACAGCCCTCCGTGTAGAAAT
	Reverse	ACAGACATAAACCGGAGTGCAGGT
α7	Forward	ACCTGCAGATGCAAGAGGCAGATA
	Reverse	AGGAATGAGCAGGTTGAGGCCATA
α9	Forward	TGACAGCTACTGTGCACGCTATGA
	Reverse	AGAAACGGTCTATGACCTTGGCGA
α10	Forward	AGAGCGTTCCACTCATCGGAAAGT
	Reverse	TGGATGTGCATTAGGGCCACAGTA
β1	Forward	ACCAACTCCCTGAACCACATCACT
	Reverse	TCACTTGGAGGCTTCCGGATGAAA
β2	Forward	ACTTCATCATTCGTCGCAAACCGC
	Reverse	TCTTGGAGATGAGCAGCAGGAACA
β3	Forward	TGCACGCGATGACTAGTGTCTTCA
	Reverse	AGGAGTACAGGCCCAACAATGACA
β4	Forward	TAGTCACCAGCACCTTGCCCATTA
	Reverse	TTGAAGGAGAGCAGGAGCCATTCA
δ	Forward	AGCCCACCTCTCAAATCGTGTCTT
	Reverse	TCTCATCTGTTTGCTCCGGCTTCT
ε	Forward	TGTGGATGCTGTGAACTTTGTGGC
	Reverse	TAGAACCAACGCTGAAGAGCACCA
γ	Forward	TCTGCAGTGGTTGGTGATGGGTAT
	Reverse	AGGCAGAGCTCTTAGGGCTTCAAA
GAPDH	Forward	TATGTCGTGGAGTCTACTGGTGT
	Reverse	GTCATCATACTTGGCAGGTTTCT

Table 2. Expression using GAPDH as a housekeeping gene. The expression results using the GAPDH-comparison method are summarized below, detailing the relative expression levels of each nAChR subunit within each tissue type (adrenal gland, liver, muscle) and the statistically significant effects of chronic nicotine treatment on certain nAChR subunit expression at the mRNA level within the three tissue types.

nAChR subunit Expression in liver		nAChR subunit Expression in muscle		nAChR subunit Expression in adrenal gland	
High (> 0.4%)	Low	High (> 0.4%)	Low	High (> 0.4%)	Low
α2	α1(undetectable)	α1	α3 (undetectable)	α1	α6
β2	α3	α2	α7	α2	α7
	α4	α4	α9	α3	α10
	α5	α5	α10	α4	β1
	α6	α6	β1	α5	
	α7	β2		α9	
	α9	β3		β2	
	α10	β4		β3	
	β1			β4	
	β3				
	β4				
Nicotine effect	Nicotine effect	Nicotine effect	Nicotine effect	Nicotine effect	Nicotine effect
Increase	Decrease	Increase	Decrease	Increase	Decrease
α1		α2		β2	α3
α5					

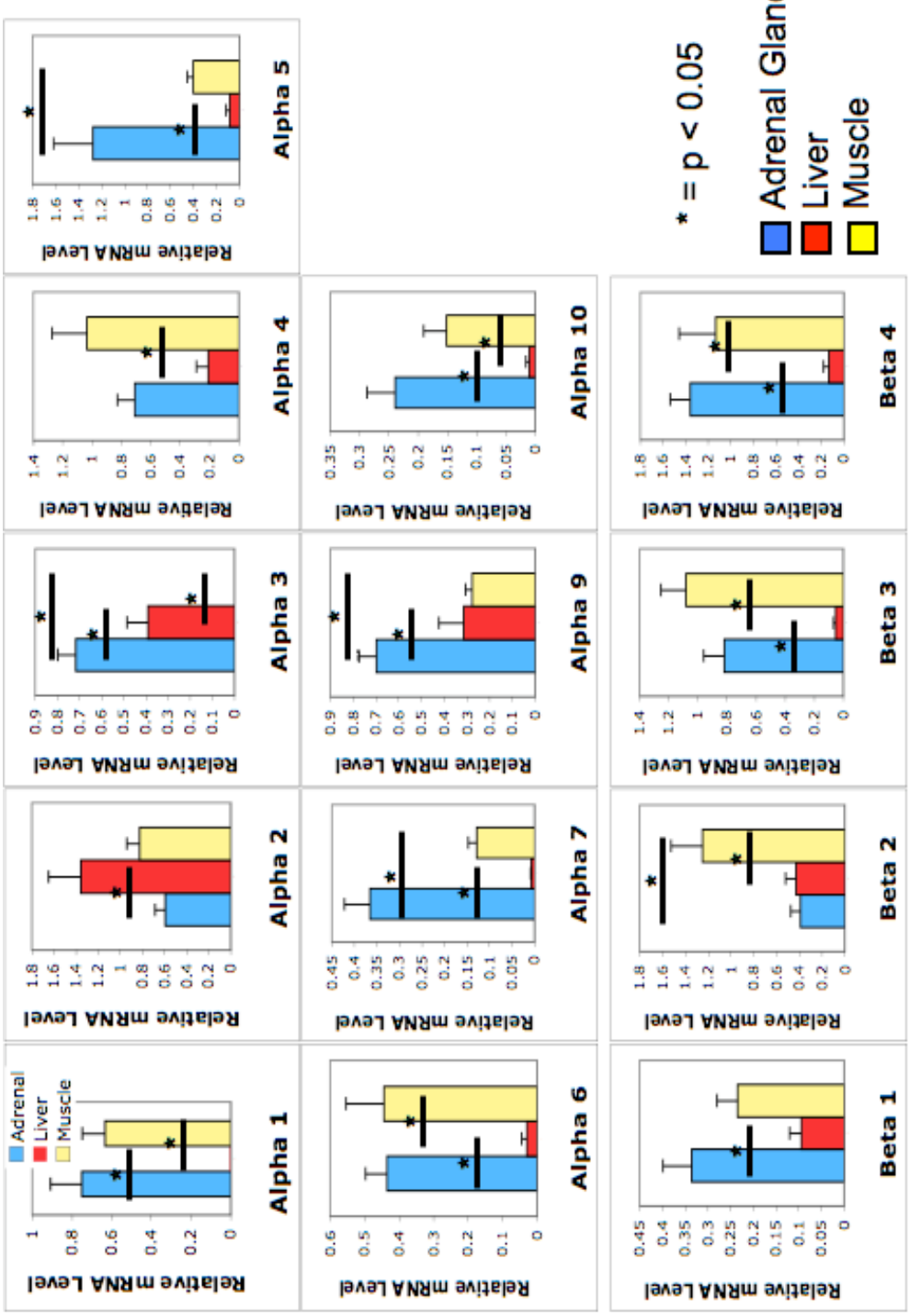


Fig.1. mRNA expression of nAChR a and b-subunits by ΔCt method using GAPDH as reference. Expression at the mRNA level was determined through real time PCR techniques. Expression levels are reported as the percentage of expression compared to the expression of the GAPDH housekeeping gene within the tissue of concern. For each subunit the following tissues from saline treated mice were analyzed for mRNA expression: adrenal gland (blue), liver (red), and muscle (yellow). Significant differences ($p < 0.05$, $n=6$) in expression level between the tissues are indicated by (*)

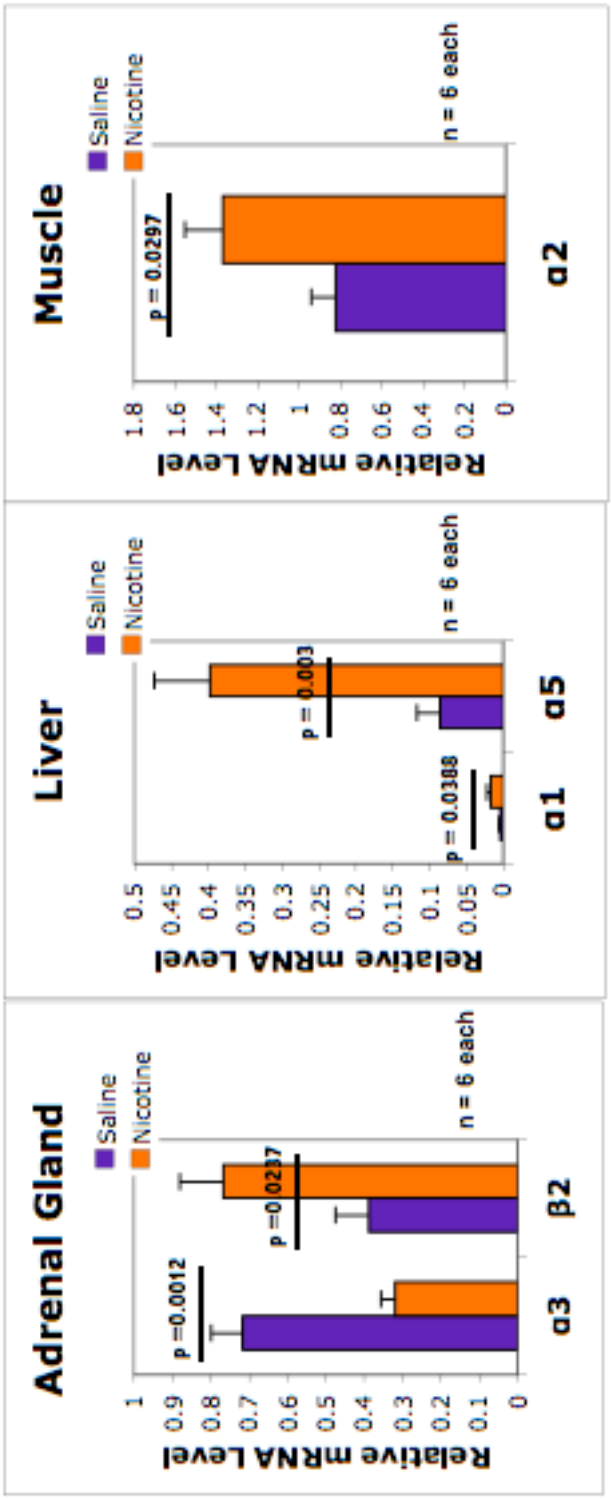


Fig.2. Effects of nicotine treatment on mRNA expression of nAChR subunits. The effect of chronic in vivo nicotine treatment on the nAChR subunit expression at the mRNA level was determined for the three tissue types (adrenal gland, liver, and muscle). Expression levels were determined using the GAPDH-comparison method. The subunits showing statistically significant ($p < 0.05$) changes in expression with nicotine treatment are depicted. n = 6 for each treatment group within each tissue type.

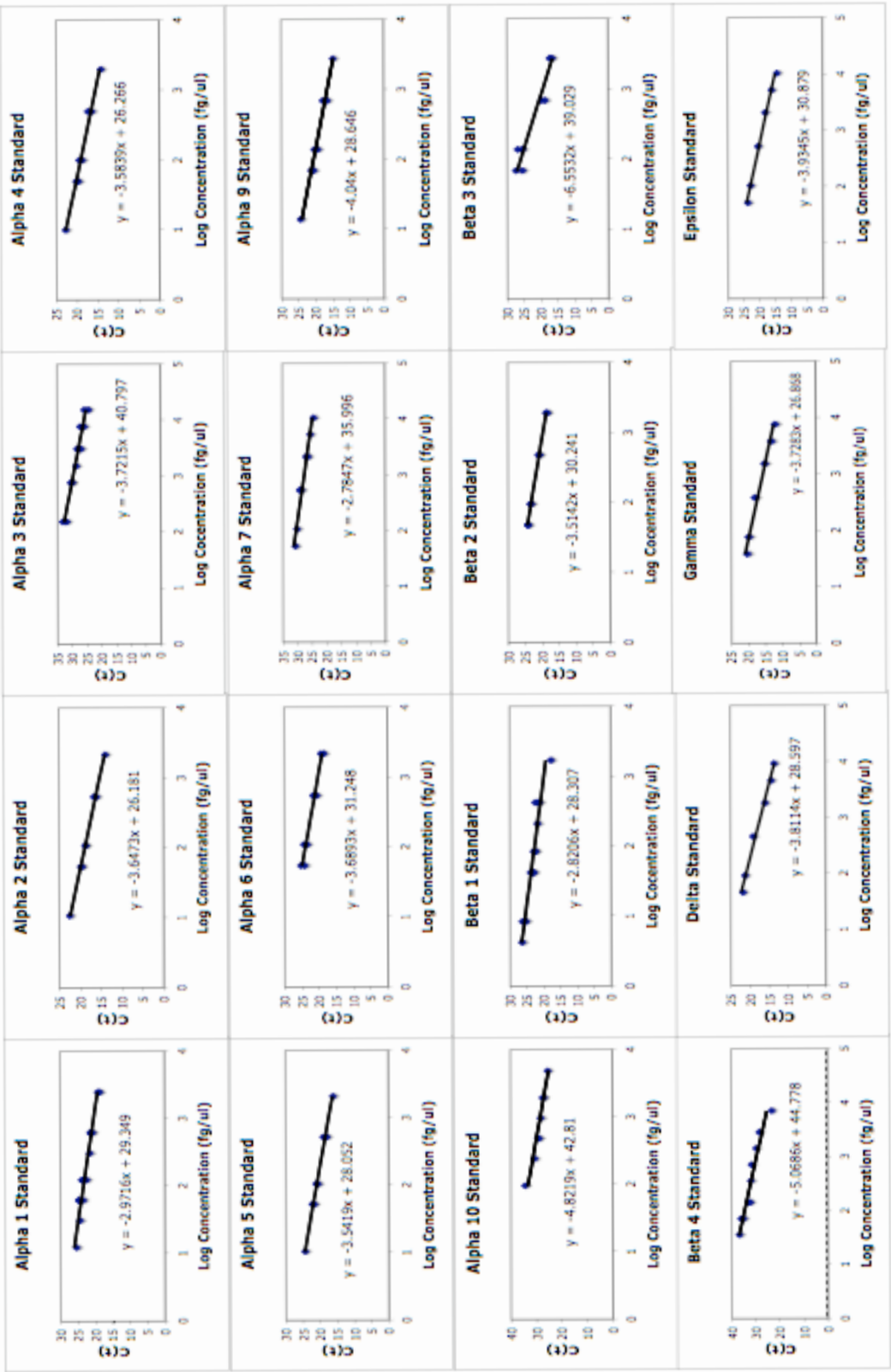


Fig.3. Subunit-specific standard curves for calibration of nAChR mRNA expression by qRTPCR. The standard curves for each nAChR subunit were generated from purified samples of subunit-specific cDNA isolated from saline treated muscle tissue. Graphs are plotted as C(t) vs. Log Concentration (fg/ul) in order to obtain linear regressions used to determine the mRNA expression levels in samples of interest.

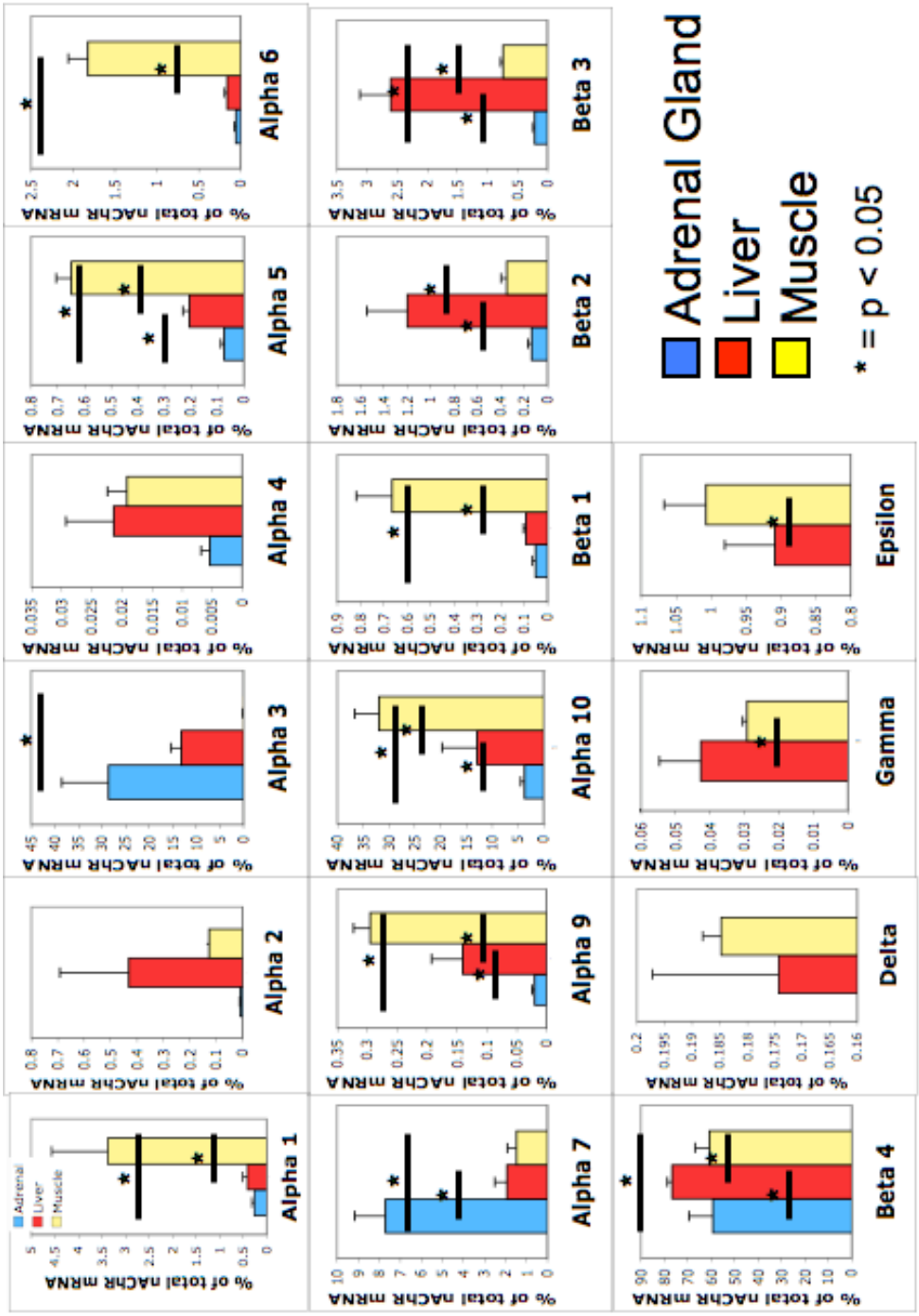


Fig.4. mRNA expression of nAChR subunits by standard curve method. Expression at the mRNA level was determined through real time PCR techniques. Expression levels are reported as the percent expression of the specific subunit compared to the total expression of all of the nAChR subunits for that tissue type. The standard curves were used to calculate the mRNA concentrations for each sample based off of the C(t) values obtained through RTQPCR. For each subunit the following tissues from saline treated mice were analyzed for mRNA expression: adrenal gland (blue), liver (red), and muscle (yellow). Significant differences ($p < 0.05$, $n=6$) in expression level between adrenal and muscle are indicated by (*), between adrenal and liver are indicated by (#), and between liver and muscle are indicated by ($).

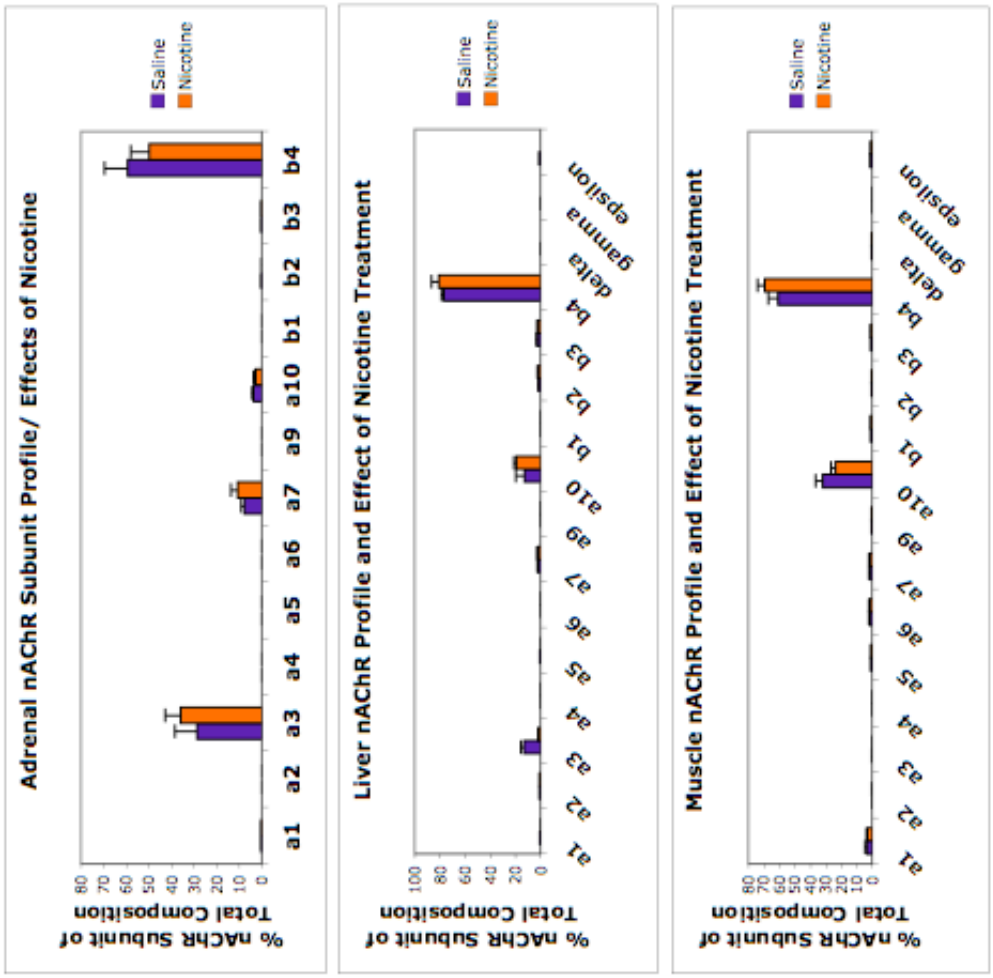


Fig.5. Effects of nicotine treatment on mRNA expression of nAChR subunits. The effect of chronic in vivo nicotine treatment on the nAChR subunit expression at the mRNA level was determined for the three tissue types (adrenal gland, liver, and muscle). Expression levels were determined RTQPCR and the standard curve method. None of the subunits for any tissue type show statistically significant ($p < 0.05$) changes in expression with nicotine treatment. The entire subunit expression distribution is shown for each tissue to gain understanding of the most highly expressed subunits within adrenal gland, liver, and muscle.

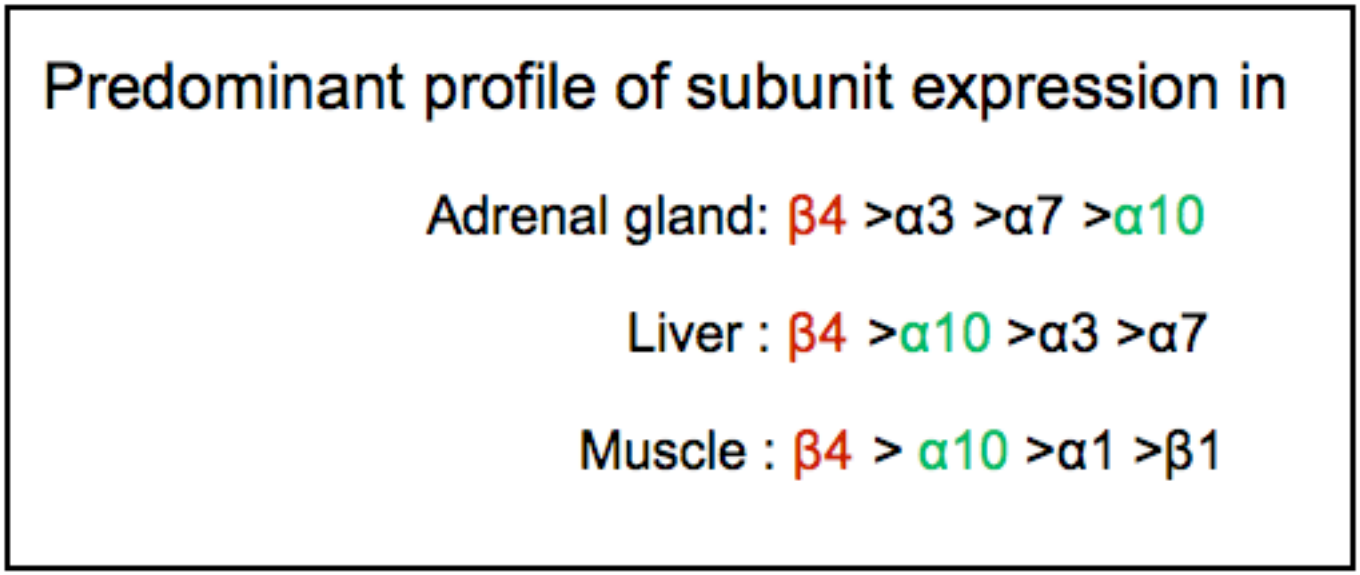
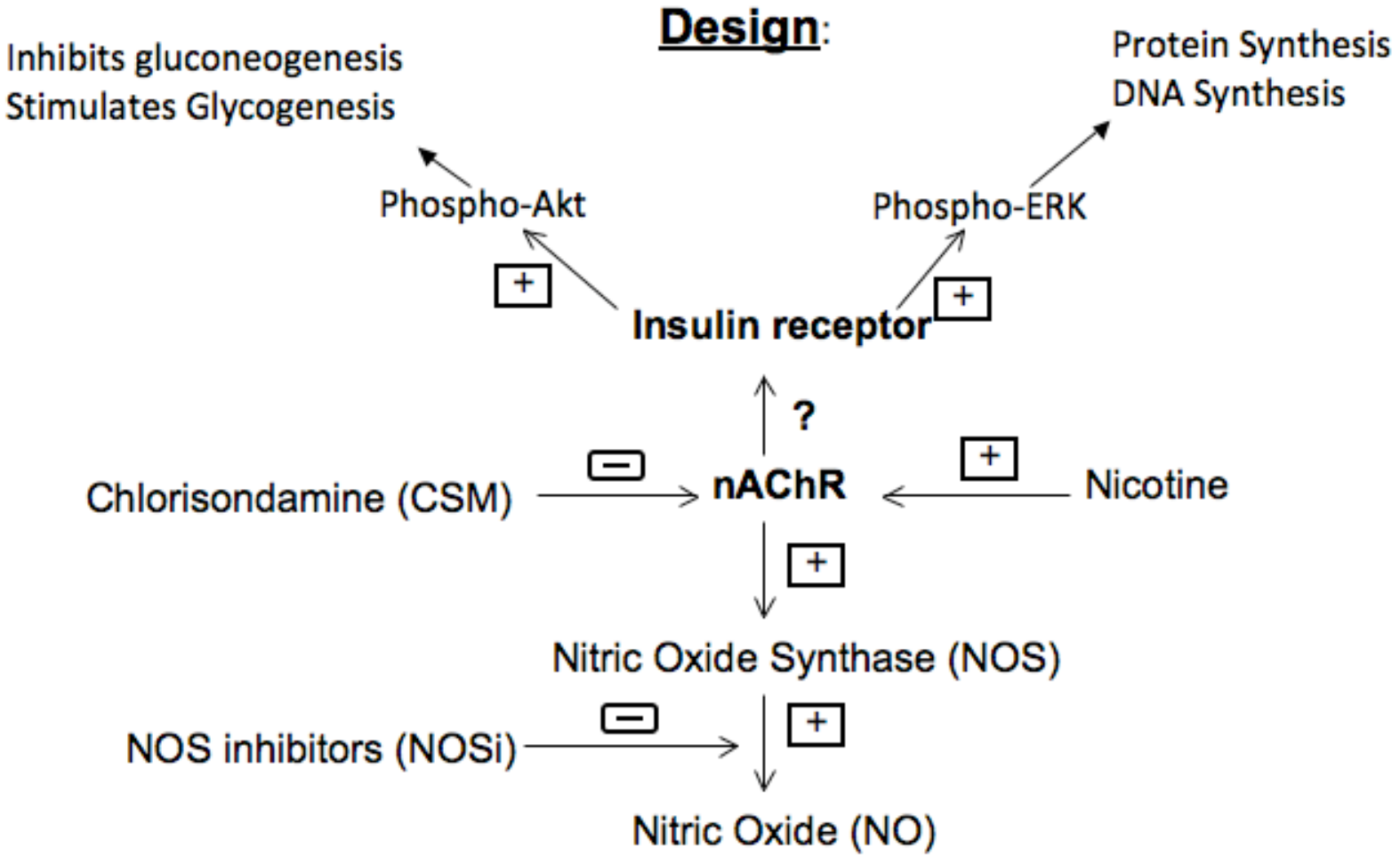


Fig.6. Predominant profile of subunit expression in adrenal gland, liver, and muscle. This figure summarizes the expression results from the standard curve method, highlighting (in color) the similarities in subunit composition within the three tissue types.



Scheme.1. Effects of stimulation and inhibition of nAChR signaling on insulin action. This schematic diagram shows the design for determination of the regulation of insulin-stimulated phosphorylation of Akt and ERK by nicotine. The effects of the inhibitors CSM and NOSi on nicotine modulation of insulin signaling are also depicted.

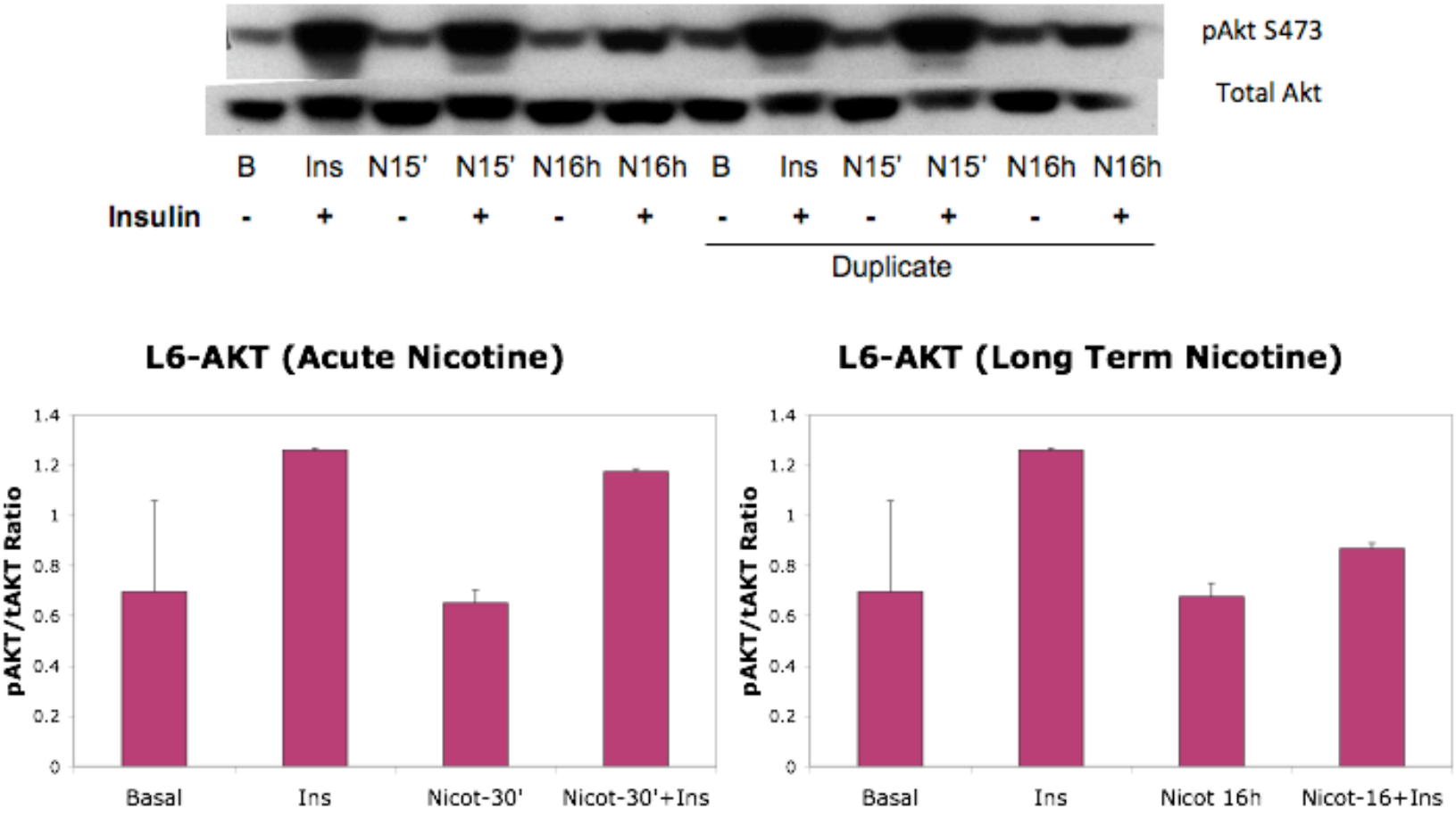
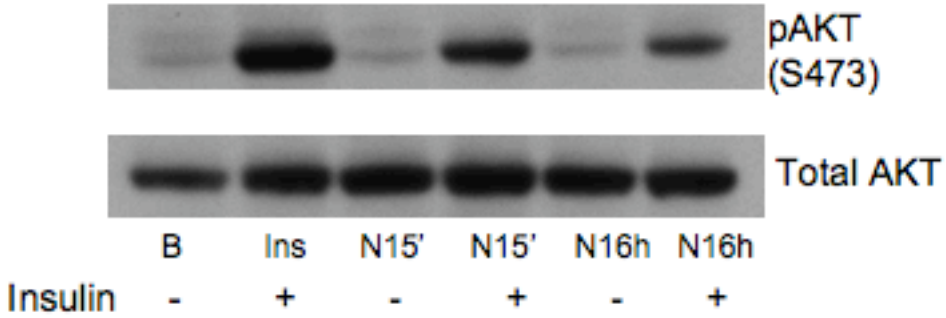


Fig.7. Acute (15 min) nicotine treatment has no effect but 16 hour exposure to nicotine inhibits insulin-stimulated Akt phosphorylation in muscle cells. Graphs of protein levels are obtained from the western blot images above in which a darker band indicates heightened protein levels. The ratio of phosphorylated Akt to total Akt was compared between the treatment groups as a representation of insulin signaling. Treated samples were run on gels and analyzed as averages of duplicates (n=2). Therefore, the changes in Akt phosphorylation could not be tested for statistical significance.



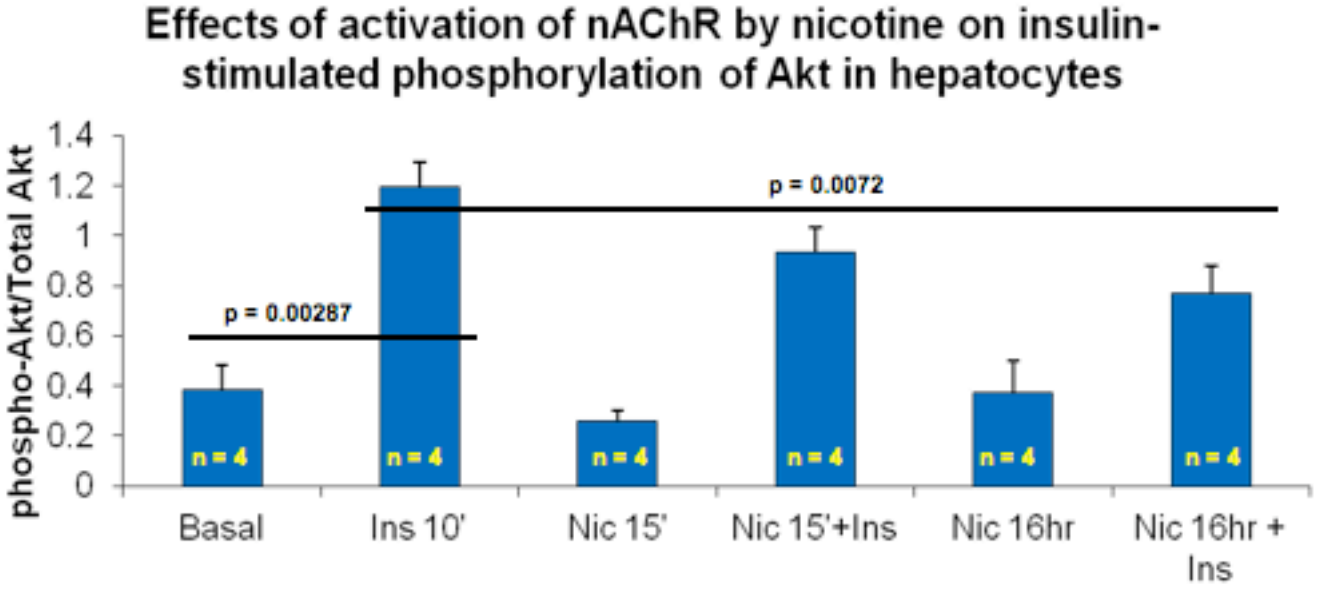
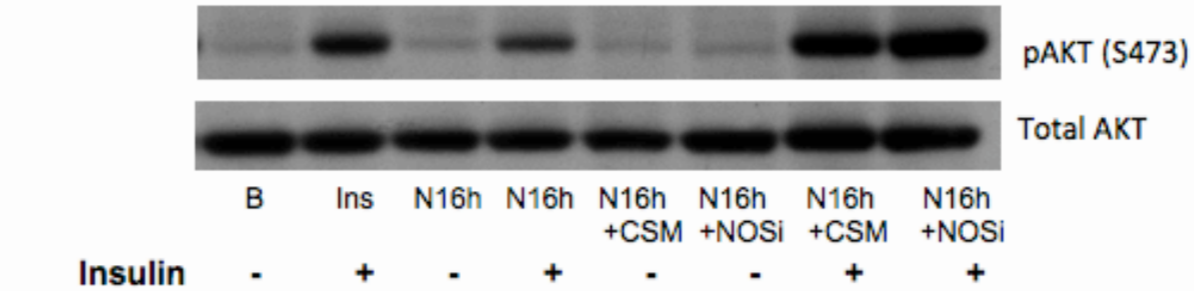


Fig.8. Inhibition of insulin-stimulated phosphorylation of Akt in hepatocytes after nicotine exposure for 16 hours. Graphs of protein levels were obtained from the western blot images above in which a darker band indicates heightened protein levels. The ratio of phosphorylated Akt to total Akt was compared between the treatment groups as a representation of insulin signaling. The number of samples analyzed for each treatment group was n = 4. Statistically significant changes ($p < 0.05$) are indicated on the graph.



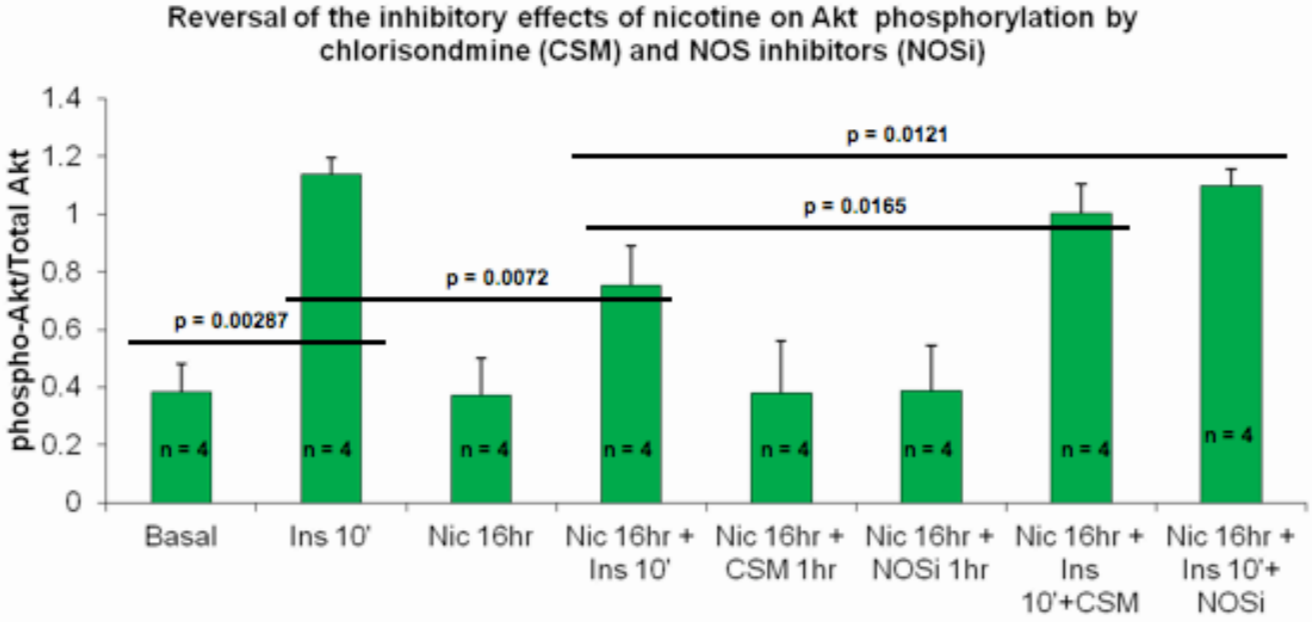


Fig.9. Suppression of nAChR signaling by CSM and NOSi protects against the inhibitory effects of nicotine on insulin-stimulated Akt phosphorylation in hepatocytes. Graphs of protein levels were obtained from the western blot images above in which a darker band indicates heightened protein levels. The ratio of phosphorylated Akt to total Akt was compared between the treatment groups as a representation of insulin signaling. The number of samples analyzed for each treatment group was n = 4. Statistically significant changes ($p < 0.05$) are indicated on the graph.

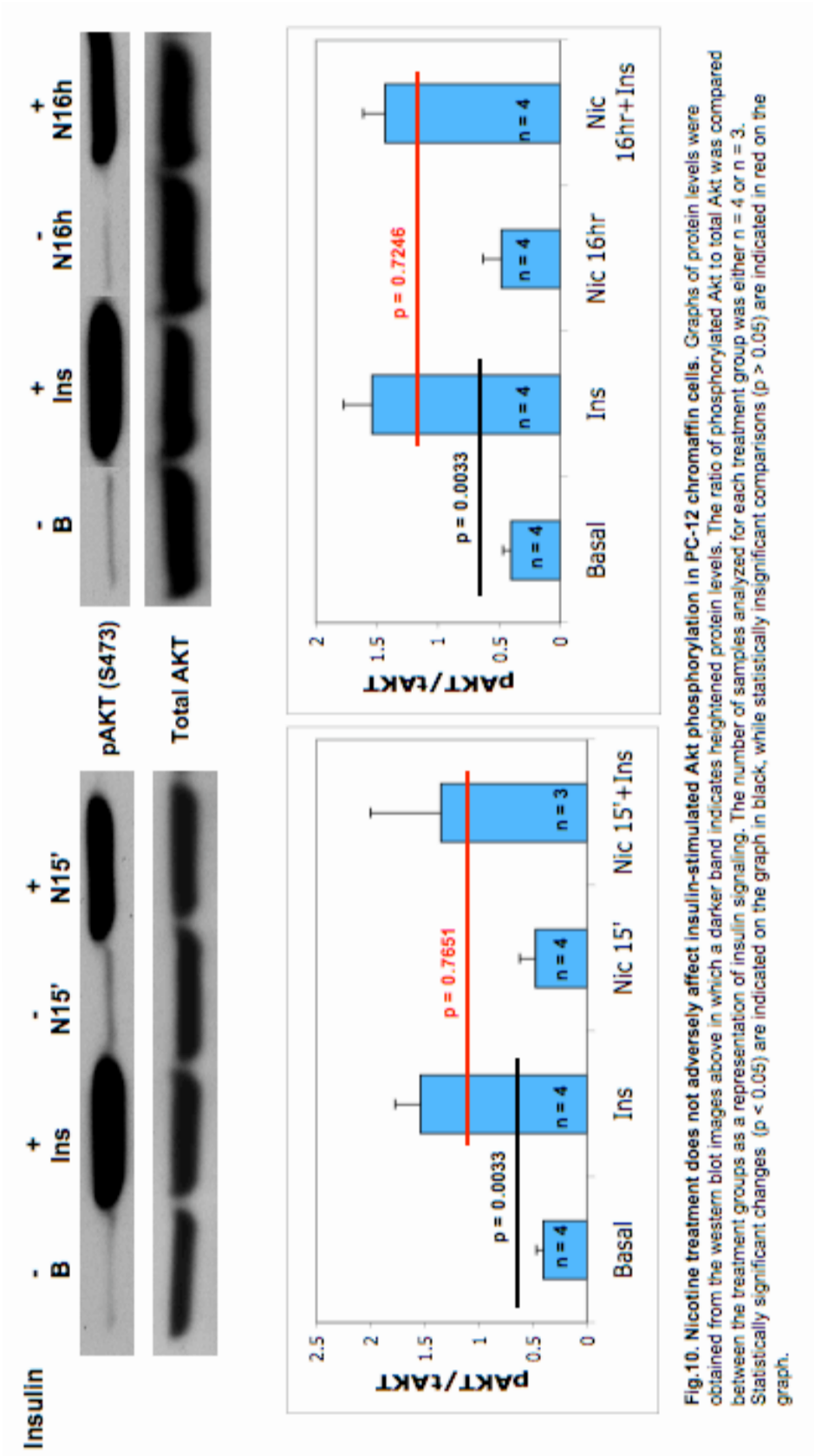


Fig.10. Nicotine treatment does not adversely affect insulin-stimulated Akt phosphorylation in PC-12 chromaffin cells. Graphs of protein levels were obtained from the western blot images above in which a darker band indicates heightened protein levels. The ratio of phosphorylated Akt to total Akt was compared between the treatment groups as a representation of insulin signaling. The number of samples analyzed for each treatment group was either n = 4 or n = 3. Statistically significant changes ($p < 0.05$) are indicated on the graph in black, while statistically insignificant comparisons ($p > 0.05$) are indicated in red on the graph.

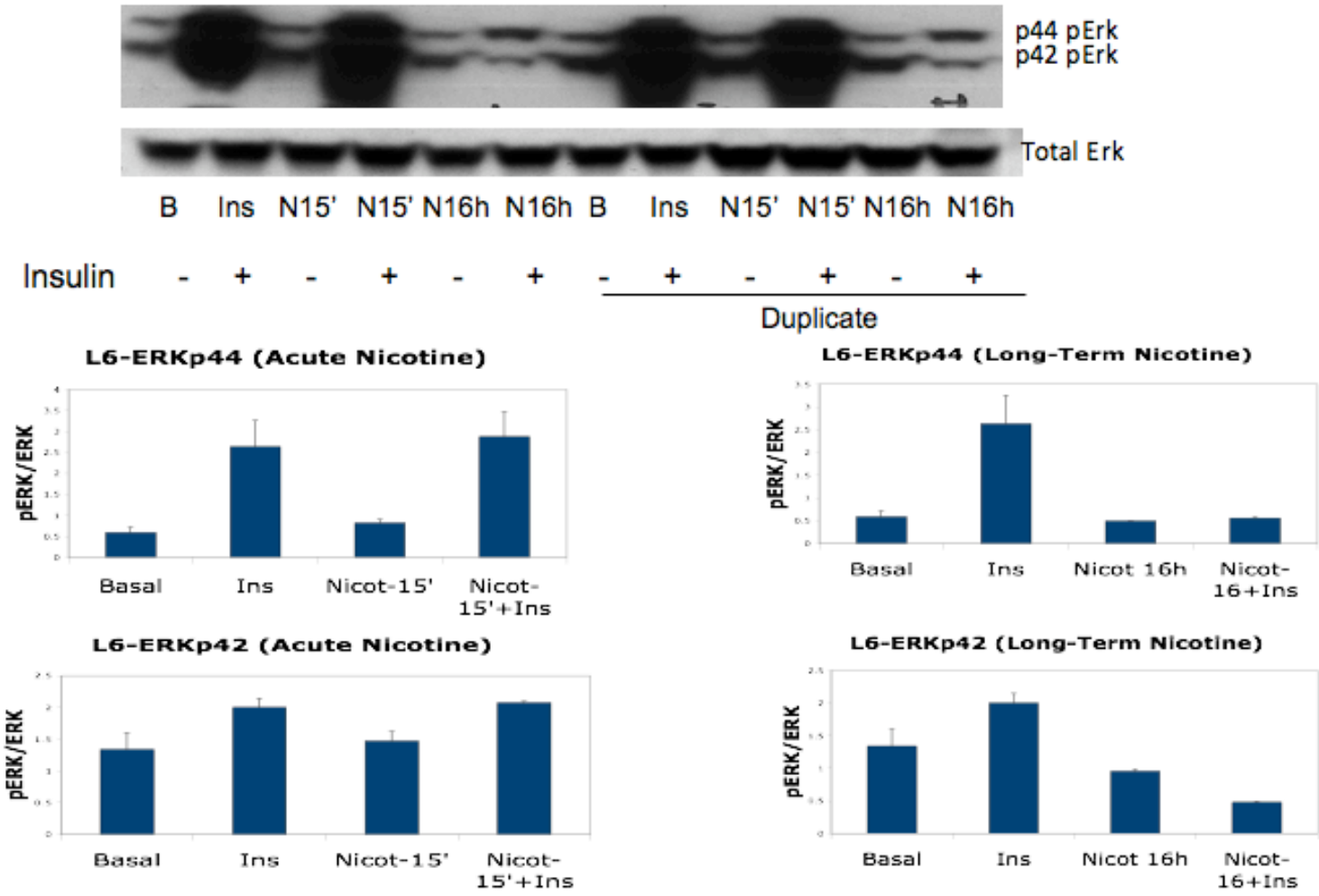


Fig.11. Effects of nicotine on insulin-stimulated ERK phosphorylation in L6 muscle cells: No effect of acute (15 min) treatment but 16 hour exposure inhibits ERK phosphorylation. Graphs of protein levels are obtained from the western blot images above in which a darker band indicates heightened protein levels. The ratio of phosphorylated Erk to total Erk was compared between the treatment groups as a representation of insulin signaling. Treated samples were run on gels and analyzed as averages of duplicates (n=2). Therefore, the changes in Erk phosphorylation could not be tested for statistical significance.

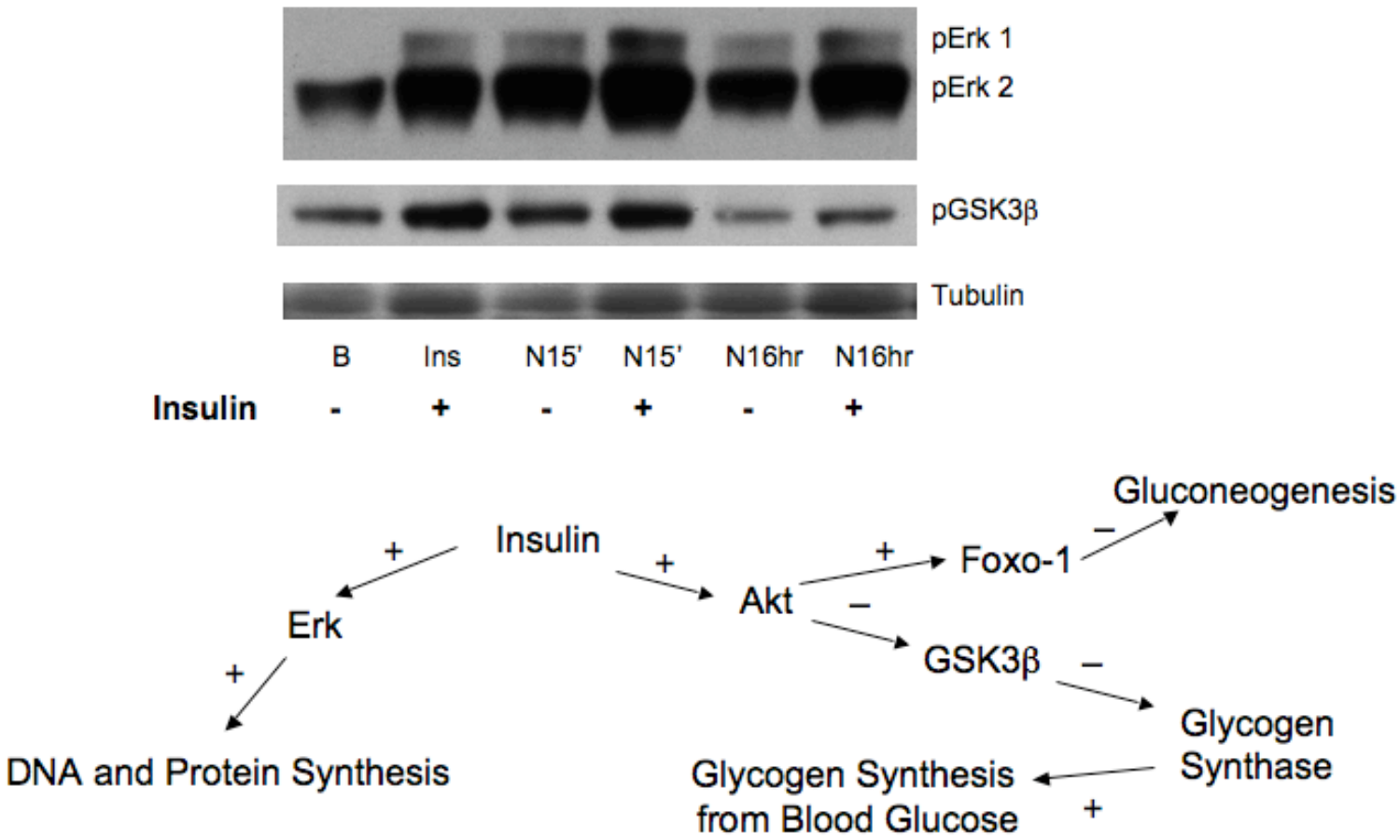
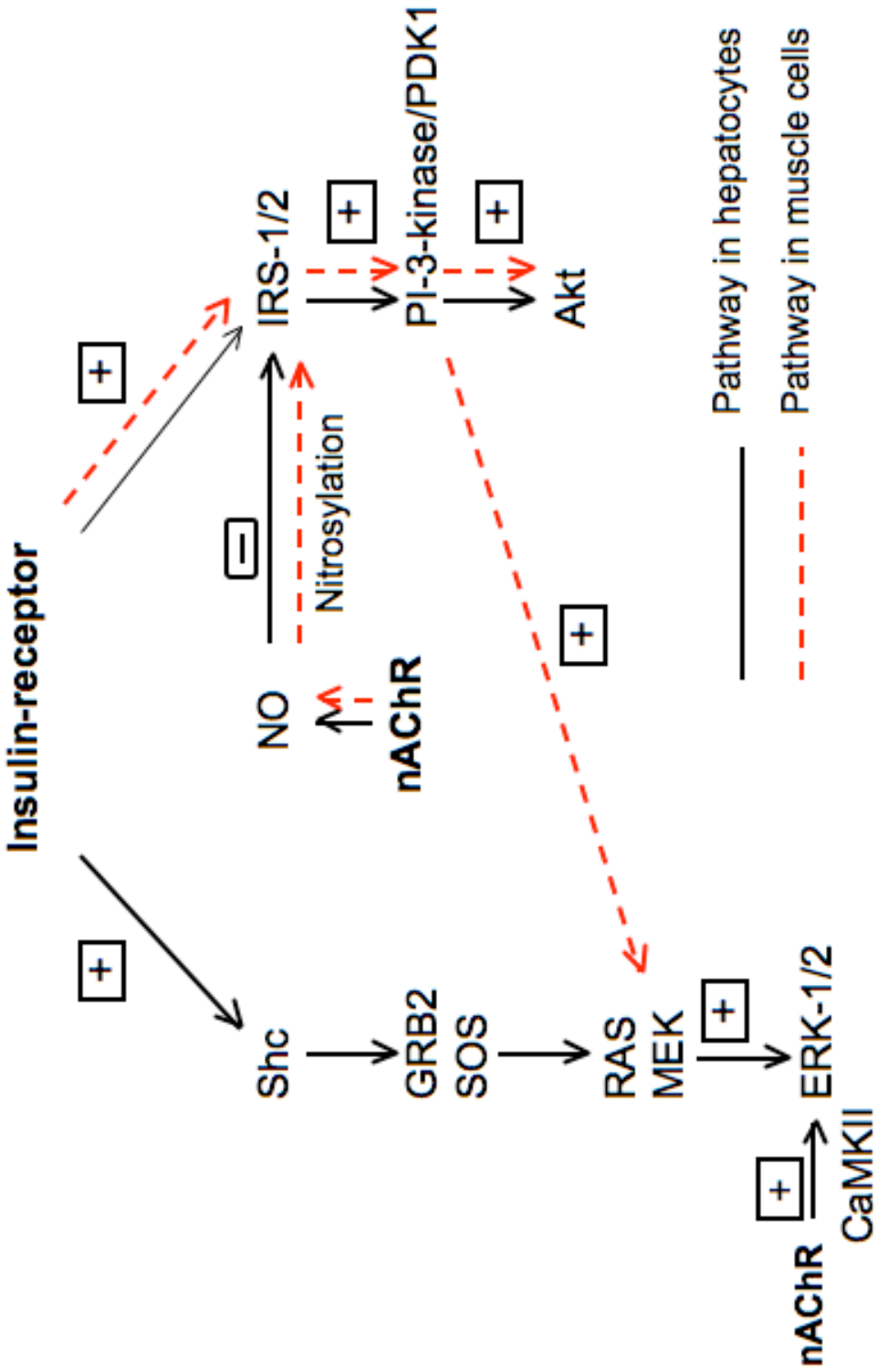


Fig.12. Activation of nAChR by nicotine stimulates Erk phosphorylation in hepatocytes and enhances insulin effect on pERK-1/2 but suppresses phosphorylation of GSK3b. While activation of Akt mediates insulin-stimulated glucose metabolism, the activation of Erk, on the other hand, mediates insulin stimulated DNA and protein synthesis. GSK3B phosphorylates glycogen synthase and inactivates it. However, Akt phosphorylates and inactivates GSK3B, and as a result, keeps glycogen synthase activated. Furthermore, phosphrylated Akt activates Foxo-1 by phosphorylation, which acts to inhibit gluconeogenesis. Western blots show protein amounts in which darker bands indicate higher protein levels. Tubulin is offered as a control for analyzing the levels of phosphorylated ERK and GSK3b. Each western blot shown is a representation of a set of four total blots, with the remaining three not pictured.



Scheme.2. Current understanding of nicotine-mediated insulin signaling. The pathway schematic shows the current understanding of the interaction of signaling molecules involved in nicotine-mediated insulin signaling. Pathways are based upon current literature as well as the results obtained from my experiments. Black arrows indicate pathways present in hepatocytes, while red arrows indicate pathways present in muscle cells.

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