Expression and Function of Alpha3 and Beta2 Neuronal Nicotinic Acetylcholine Receptor Subunits in HEK-293 Cells

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EXPRESSION AND FUNCTION OF ALPHA3 AND BETA2 NEURONAL NICOTINIC ACETYLCHOLINE RECEPTOR SUBUNITS IN HEK-293 CELLS

by

Nathan W. Steinhafel

A thesis submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Master of Science

Department of Physiology and Developmental Biology

Brigham Young University

December 2006
GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

Nathan W. Steinhafel

This thesis has been read by each member of the following graduate committee and by majority vote has been found to be satisfactory.

Date ____________________________  Sterling Sudweeks, Chair

Date ____________________________  James Porter

Date ____________________________  Barry Willardson
As chair of the candidate’s graduate committee, I have read the thesis of Nathan W. Steinhafel in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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Chair, Graduate Committee

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Graduate Coordinator

Accepted for the College

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John D. Bell
Associate Dean,
College of Biology and Agriculture
Single-cell real-time quantitative RT-PCR was used to characterize the mRNA expression of rat neuronal nicotinic acetylcholine receptor (nAChR) subunits $\alpha_3$ and $\beta_2$ in CA1 hippocampus stratum radiatum and stratum oriens interneurons. $\alpha_3\beta_2$ co-expression was detected in 43% of interneurons analyzed. The nAChR subtype $\alpha_3\beta_2$ was transiently expressed in cells derived from the human embryonic kidney cell line 293 at mRNA levels found in the CA1. The functional properties of $\alpha_3\beta_2$ in HEK-293 cells were characterized by whole-cell patch clamping using acetylcholine (ACh) as an agonist. The kinetics of $\alpha_3\beta_2$ channels were further analyzed by altering the level of $\alpha_3$ DNA transfected into HEK-293 cells. Varying the $\alpha_3$ concentration by more than 100,000 fold did not
significantly alter the majority of the kinetics; the 10%-90% rise-time was the main characteristic found to be significantly different. A decrease in $\alpha_3$ concentration illustrated a significant increase in rise time. This and future studies will further our understanding of the extensive role neuronal nAChRs play in modulating hippocampal activity and consequently influencing cognition and memory.
ACKNOWLEDGEMENTS

I would like to express my thankfulness to Dr. Sterling Sudweeks the opportunity he afforded me to conduct research in his laboratory. More importantly, I have valued his friendship, thought provoking conversation, and the countless hours of mentored-teaching he offered me as a graduate student.

I would also like to acknowledge the contribution of many students, working in Dr. Sudweek’s lab, for their assistance in isolating plasmids, maintaining and preparing transfected HEK-293 cells, and running real-time PCR with me for these data, particularly, Sean Georgi, Rick Burgon, and John Mizukawa.

I would also like to thank the following individuals for their support, suggestions, and insight throughout the course of my graduate program: Dr. James Porter, Dr. Dixon Woodbury, and Dr. Barry Willardson.

Most importantly, I would like to thank my beautiful wife, Emily Steinhafel, for her encouragement, patience and unyielding support. I have loved our delightful conversation and her tender influence on the HEK-293 cells while patch clamping during the evening hours.
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INTRODUCTION

Ionotropic neuronal nicotinic acetylcholine receptors (nAChRs) are heterogeneous cationic channels that are widely expressed in the central and peripheral nervous system (CNS, PNS), where their opening is controlled by endogenous acetylcholine (ACh) or exogenous agonists such as nicotine. They consist of homopentameric or heteropentameric subtypes that are involved in a variety of physiological processes including neuronal development, learning and memory formation, and reward (Jones and Yakel 1999; Gotti, Zoli et al. 2006). More importantly, nAChR dysfunction throughout the CNS has been shown to be key in the cognitive impairments of neurodegenerative diseases including Alzheimer’s (Nordberg, Alafuzoff et al. 1986; Perry 1995; Dani 2001), schizophrenia (Freedman, Coon et al. 1997; Breese, Lee et al. 2000), and even aging (Jones and Yakel 1999). However, many of the cellular and molecular mechanisms of these diverse conditions and their involvement with nAChRs are currently unknown. Different types of cognitive pathologies seem to involve dysfunction of specific combinations of α and β nicotinic receptor subtypes (Martin-Ruiz, Court et al. 1999; Durany, Zochling et al. 2000; Leonard, Breese et al. 2000). Accordingly, drugs could be designed to interact selectively at a nAChR subtype and therefore provide different therapeutic opportunities for treating cognitive related diseases. Better understanding of the role nicotinic receptor subtypes play in the CNS and PNS may provide development of safe and effective therapeutic agonists for treating a variety of cognitive dysfunctions. Therefore, to enhance our understanding of the pharmacological and physiological
properties of neuronal nAChRs, we must investigate how their subunits assemble to form functional channels.

Neuronal nAChRs are members of a large, structurally related ligand-gated ion channel superfamily, together with GABA<sub>A</sub>, glutamate, glycine, and 5-HT<sub>3</sub> receptors (Ortels et al., 1995). Neuronal nAChRs are located in a variety of CNS locations (i.e. basal ganglia, thalamus, cerebellum, striatum, and hippocampus) (Bourin, Ripoll et al. 2003; Gotti, Zoli et al. 2006), and can be found in both pre-synaptic and post-synaptic locations. ACh-mediated innervation acting through nAChRs regulates crucial processes such as transmitter release, cell excitability, and neuronal integration (Gotti, Fornasari et al. 1997; Newhouse, Potter et al. 1997; Wonnacott 1997; Jones and Yakel 1999). Like every member of the ligand-gated ion channel family, the nAChR is a transmembrane receptor protein complex consisting of five polypeptide subunits arranged around a central pore that is permeable to cations (Bourin, Ripoll et al. 2003) (Figure 1). Thus far separate genes for α2-10 and β2-4 nAChR subunits have been identified. These receptors are known to be expressed in a variety of two- and three-way subunit combinations (Dani 2001; Gotti, Zoli et al. 2006) (Figure 2). Although many subunit combinations have been identified in the CNS, the most commonly discovered mammalian neuronal nAChRs with a high affinity for agonists contain α7 or α4/β2 subunits which form homomeric or heteromeric complexes, respectively (Changeux, Bertrand et al. 1998). In addition, α3/β4, a highly expressed nAChR in the autonomic nervous system, has also been found to be a combination involved in cognitive
Figure 1. Pentameric assembly of a nACHR. Shown: a dimer nACHR subtype, α4β2 (Illustration by: Georgi 2004).
Figure 2. Regional distribution and subunit organization of the main nicotinic acetylcholine receptor subtypes in the rodent CNS. The subtypes present in the cortex, cerebellum, hippocampus, interpeduncular nucleus, medial habenula and pineal gland have been identified by binding, immunoprecipitation and/or immunopurification assays in tissue from rat and/or wild-type and/or receptor subunit knockout mice. The subtypes present in the amygdala, hypothalamus, locus coeruleus, olfactory bulb, raphe nuclei, spinal cord, substantia nigra–ventral tegmental area and thalamus have been deduced from in situ hybridization, single-cell PCR and binding studies of tissues obtained from rat and/or wild-type and/or knockout mice [Illustration and reviewed by: (Gotti, Zoli et al. 2006)].
function (Alkondon, Rocha et al. 1996; Albuquerque, Alkondon et al. 1997; Gotti, Zoli et al. 2006). The CNS location as well as the pentameric co-assembly of different neuronal nAChR subunits largely determines the physiological action and biophysical and pharmacological properties of neuronal nAChRs (Wada, Wada et al. 1989; Alkondon, Rocha et al. 1996; Changeux, Bessis et al. 1996; Gotti, Fornasari et al. 1997).

The hippocampus is highly heterogeneous in neuronal nAChR expression (Albuquerque, Pereira et al. 1997; Frazier, Rollins et al. 1998; McQuiston and Madison 1999; Sudweeks and Yakel 2000; Gotti, Zoli et al. 2006). The hippocampus has direct connections to the entorhinal cortex (via the subiculum) and the amygdala. Outputs from these structures can then have an effect on many other areas of the central nervous system. For instance the entorhinal cortex projects to the cingulate cortex, which has a connection to the temporal lobe cortex, orbital cortex, and olfactory bulb. Thus, all of these areas can be influenced by hippocampal output, primarily from the output of the CA1 region. Nicotinic acetylcholine receptors within hippocampal interneurons are involved in fast synaptic transmission in the hippocampal CA1 stratum oriens and stratum radiatum subfields. There is cholinergic innervation to the hippocampus from the medial-septum diagonal band complex from the basal forebrain (Frotscher and Leranth 1985) and there are cholinergic synapses between normal hippocampal interneurons (Matthews, Salvaterra et al. 1987). Also, previous research has shown that hippocampal interneurons contain GABAergic projections onto the more frequent CA1
pyramidal neurons, regulating the excitability of the pyramidal cells (Jones and Yakel 1999).

Furthermore, it has previously been demonstrated that rat hippocampal interneurons express neuronal nAChRs in a manner homologous to humans (Jones and Yakel 1997). Of the 11 different nAChR subunits known; eight of these (α2-α5, α7, and β2-β4) are expressed in the rat hippocampus (Sudweeks and Yakel 2000). The nAChRs in hippocampal interneurons are in a key location to be involved in modulating cognitive functions because interneurons play a major role in coordinating hippocampal activity (Freund and Buzsaki 1996; Jones and Yakel 1999). Altered synaptic plasticity has been shown to change the dynamic interactions among hippocampal networks causing age-related cognitive impairments (NIA/NIH 1999; Pauly 1999; Bourin, Ripoll et al. 2003; Grassi, Palma et al. 2003; Lamb, Melton et al. 2005). Consequently, nAChRs are assumed to be involved in cognitive diseases including Alzheimer’s disease (AD). For example, revealed by behavioral and electrophysiological studies, evidence implicates the α7 nAChR subtype as being linked to AD (Gotti, Fornasari et al. 1997). AD is characterized by accumulation of senile plaques, mainly composed of beta-amyloid peptide (Aβ). Even though the exact causes of AD are unknown, different pathogenesis hypotheses implicating nAChRs made up of α7 subunits have been proposed, with the receptors exerting a direct or indirect action on the mechanism of Aβ toxicity. The application of Aβ has recently been shown to impede nAChR function in rat hippocampal neurons (Liu, Kawai et al. 2001; Pettit, Shao et al. 2001).

Many studies suggest that interneurons within the hippocampus express primarily homomeric and heteromeric combinations of α7 and α4/β2, respectively.
However, various studies involving pharmacology (Xiao and Kellar 2004), electrophysiology (Khiroug, Harkness et al. 2002), knock-out mice and RT-PCR (Sudweeks and Yakel 2000) have revealed a variety of other heteromeric nAChR subunit combinations. For example, previous pharmacological studies have suggested that α7-containing nAChRs are functionally expressed in most hippocampal interneurons, but the incomplete block by α7-specific antagonists suggest that there is a class of non-α7-containing receptors (Alkondon, Pereira et al. 1998; Frazier, Rollins et al. 1998; McQuiston and Madison 1999). Furthermore, comparing the properties of heterologously expressed channels with those of native nAChRs from hippocampal electrophysiological recordings, it is clear that composition of the native receptors is more complicated than just α7 and α4β2. For example in the CA1 stratum oriens the slow nAChR-mediated response usually expected from an α4-containing nAChR (Albuquerque, Pereira et al. 1995) was not as sensitive to the broad spectrum non-α7 nAChR antagonist, dihydro-β-erythroidine (DHβE); (McQuiston and Madison 1999), suggesting the involvement of an alpha subunit such as α2 or α3. For instance, this response was not blocked by α-CTX MII (which does block α3 containing receptors), suggesting that it may be partly mediated by an α2-containing receptor (McQuiston and Madison 1999; Khiroug, Khiroug et al. 2004). Accordingly, the α2 as well as the α3 subunit might be a key component in forming functional two-way and even three-way nAChRs.

The relatively small number of subtype-specific pharmacological agents available has limited progress in characterizing the roles of many subunit combinations. In order to more accurately understand the function of nAChRs in areas with highly
heterogeneous expression, such as the hippocampus, we need to conduct studies with tools other than just pharmacology. Quantitative information on the relative expression levels of individual neuronal nAChR subunit mRNA may provide an avenue for effectively defining and categorizing neuronal nAChR subtypes. Single-cell RT-PCR studies along with in situ hybridization have revealed significant levels of α2-α5, α7, and β2-β4 nAChR subunit mRNA expression in interneurons within the hippocampus CA1 stratum oriens and stratum radiatum and other regions of the CNS (Wada, Wada et al. 1989; Sudweeks and Yakel 2000). In addition, it has been demonstrated that many of these subunits are significantly co-expressed together in individual neurons (Sudweeks and Yakel 2000).

Expressing recombinant ion channels in heterologous expression systems has been traditionally used to characterize their functional properties (e.g., kinetics, conductance, ion selectivity, agonist activity, pharmacology, etc.). Successful expression of two-subunit neuronal nAChR combinations has been achieved using human Xenopus laevis oocytes (Elliott, Ellis et al. 1996; Chavez-Noriega, Crona et al. 1997; Fenster, Rains et al. 1997; Nelson and Lindstrom 1999; Figl and Cohen 2000; Khiroug, Harkness et al. 2002) and less commonly human embryonic kidney- (HEK-) 293 cells (Figure 3) (Wang, Nelson et al. 1998; Nelson, Wang et al. 2001; Khiroug, Harkness et al. 2002; Xiao and Kellar 2004). To date, the characterization of the rat neuronal α3β2 nAChR in HEK-293 cells has been rarely studied (Xiao and Kellar 2004). However more frequent studies have been done using Xenopus laevis oocytes.
Figure 3. Confocal microscopy of HEK-293 cells with α3β2 nAChRs. Green reflects labeling of α3 subunits by Alexa 488-labeled mAb 210. Red reflects secondary labeling by Alexa 594-labeled GART antibody against mAb 295 which bound β2 subunits. Yellow is a result of overlap in the labeling of colocalized subunits [photo and research by: (Nelson, Wang et al. 2001)].
(Luetje and Patrick 1991; Chavez-Noriega, Crona et al. 1997; Fenster, Rains et al. 1997; Figl and Cohen 2000). Moreover, very few, if any, studies have yet been carried out using three-way rat neuronal nAChR subunit combinations in HEK-293 cells. In this study, we use single-cell real-time quantitative RT-PCR to characterize the mRNA expression of rat neuronal nAChR α3 and β2 nAChR subunits mRNA in CA1 hippocampus stratum radiatum and stratum oriens interneurons. This provides insight into how often these subunits are expressed together, and what the actual mRNA ratios are in individual interneurons when the subunits are co-expressed.

Our study constitutes one of the first studies, to date, to identify native rat neuronal nAChR subunit mRNA co-expression ratios, reconstitute them in HEK-293 at the same ratios to form functional channels, and analyze their functional properties in HEK-293 cells. This and future studies will further our understanding of the substantial role these receptors play in modulating hippocampal activity and thereby influencing cognition and memory.
MATERIALS AND METHODS

Slice Preparation and Cytoplasm Aspiration

All CA1 hippocampal slice and interneuron aspiration experiments were prepared and carried out as described previously (Sudweeks and Yakel 2000). In brief, coronal brain slices (either 300 or 350 µm thick) were made from Wistar rats (8-23 days old) using a Vibratome 1000-Plus (Pelco, Redding, CA). Cut in ice-cold oxygenated (95% O₂, 5% CO₂) artificial cerebro-spinal fluid (ACSF in mM: 124 NaCl, 2 KCl, 1 NaH₂PO₄, 26 NaHCO₃, 11 Glucose, 2 CaCl₂, 1 MgSO₄), the slices were placed in room-temperature oxygenated ACSF for at least 30 minutes prior to placing in microscope recording chamber.

Individual hippocampal interneurons from the CA1 stratum oriens and stratum radiatum were visually identified using an upright microscope with infrared lighting (Figure 4) and aspirated into a standard whole-cell patch-clamp pipette (Figure 5) (Borosilicate capillaries, Harvard Apparatus, Kent, England) containing 5 µL intracellular fluid (ICF in mM: 10 MgCl₂, 0.1 CaCl₂, 1 EGTA, 10 HEPES, 135 K-Gluconate, Na-2 ATP).

Electrophysiology in CA1 Hippocampal Interneurons

A whole-cell patch clamp of the interneuron was obtained in voltage-clamp mode prior to cytoplasm aspiration. Interneuron membrane potentials were held at negative 70mV.
Figure 4. Rat hippocampal slice indicating regions where interneurons were aspirated. Inset shows a stratum radiatum interneuron (Photo by: Sudweeks 2002)
Figure 5. Screenshot of a whole-cell patch-clamp pipette on an interneuron in the CA1 stratum oriens prior to aspiration (Photo by: Burgon 2005).
**Primers and Probes**

Primers and probes were purchased from Invitrogen. The primers and probes were designed using either Vector NTI version 7.0 (Invitrogen) or Primer Express version 2.0 (ABI Prism) software (Burgon 2005).

**RT Reaction**

A cDNA library representing each CA1 hippocampal interneuron was made by running a reverse transcription reaction using BIORAD iScript cDNA Synthesis Kit with a final volume of 10 µl (Burgon 2005).

**Multiplex Reaction and Real-time Quantitative PCR**

A multiplex PCR reaction was run (15 cycles) for each aspirated interneuron using all neuronal nAChR primers as well as primers for 18s rRNA with a final volume of 75 µl. The multiplex reaction was run using reagents by Invitrogen including Platinum® Taq DNA Polymerase and PCR nucleotides (10mM). A second round of PCR was run (60 cycles) for each specific target (18s, α2, α3, and β2) using an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) utilizing BIORAD iTaq Supermix with ROX (Invitrogen). Cycle threshold values for each target were compared to the reference gene 18s for analysis (more in *Real-Time Analysis*).

Standard curves (efficiency of amplification tests) for each cDNA target were developed by running 60-cycle real-time quantitative PCR assays on positive controls (rat whole-brain homogenate) for six known concentrations (100, 33.3, 10, 3.33, 1, 0.333 ng cDNA/µL). Upstream (primer +) and downstream (primer –) primer concentrations were adjusted to optimize amplification as reported previously. The
efficiency of the amplification reaction is calculated using the slope of the log (concentration) vs. CT plot. The formula for PCR efficiency (Bustin 2004):

\[ 10^{(-1/slope)} - 1 \]

Reaction efficiencies were run in triplicate and the amplification efficiencies were compared using an ANOVA to determine if there were significant differences between any of the primer/probe sets (18s, α2,α3, and β2) (Burgon 2005).

**Real-Time Analysis**

After running the real-time quantitative PCR on hippocampal interneurons, raw fluorescence (Delta Rn) values across 60 cycles were curve-fit using a Boltzmann Sigmoidal function with an output of either 2000 or 4000 data points in the new curve using GraphPad 4.0 (Graphpad, Prism) software. The second derivative graph for the curve-fit data was then determined, also using Graphpad. The cycle threshold (CT) value used for quantitative analysis was determined by finding the cycle number (along the x-axis) corresponding to the maximum Delta Rn value (along the y-axis), as described previously (Bustin 2004).

**Primer Efficiencies Analysis**

Triplicate reactions of each cDNA target were averaged and a linear regression equation was calculated (SLOPE function, Microsoft Excel) of the CT values corresponding to the six known concentrations (100, 33.3, 10, 3.33, 1, 0.333 ng cDNA/µL) in the standard curve primer efficiency tests. The PCR efficiency was then determined by incorporating the slope of the linear equation using the formula described above (see *Multiplex Reaction and Real-time Quantitative PCR*) (Burgon 2005).
**mRNA Expression Analysis**

For comparison between cDNA targets, fold expression values from the triplicate CT averages were calculated as reported previously, but compared to the CT value corresponding to the lowest level of cDNA detection (Livak and Schmittgen 2001). Significance between relative levels of mRNA expression was calculated by comparing mean fold expression values using a Mann-Whitney test (calculated using InStat ver. 3.05, Graphpad software) (Burgon 2005).

**Plasmid DNA Preparation**

The rat α3 and β2 genes were put in the pcDNA 3.1 plasmid (Invitrogen) using a digestion and ligation protocol from New England Biolabs and Bioline, respectively. Green florescence protein plasmid was kindly provided by Dr. Michael Stark (Brigham Young University, Provo, UT). All plasmids were grown-up using a transformation protocol by Yeastern Biotech and carried out in accordance with its guidelines. A plasmid isolation and purification protocol was followed by HiSpeed®, a plasmid purification kit by QIAGEN Inc. (USA).

**Tissue Culture**

Human embryonic kidney (HEK) -293 cells were kindly provided by Dr. Barry Willardson (Brigham Young University, Provo, UT). HEK-293 cells were grown and maintained on 60 mm surface petri dishes coated with nunclon (Nunc) containing 1.6 ml of complete medium: Dulbecco’s Modified Eagle medium (DME) (Nova-Tech, Inc. Grand Island, NE) supplemented with 10% fetal bovine serum (FBS) and 1.25% Penicillin. HEK-293 cells were stored in an incubator humidified to 37 degrees Celsius containing 5% CO₂. HEK-293 cells were grown up three to four days until a suitable
density of cells was achieved (75-85% confluence). This was visually approximated using a light microscope.

Approximately 24 hours preceding transfection, 1ml of HEK-293 cells at 75-85% confluence were triturated and plated onto 30 mm diameter petri dishes coated with nunclon (Nunc) containing 1ml of warmed complete medium in order to obtain 50-70% confluence the following day (Figure 6). This was followed by incubation (37 degrees Celsius, 5% CO₂) overnight.

Expression in HEK-293 Cells

Transfection into HEK-293 cells was accomplished using a protocol by Mirus. TransIT-293 Reagent (3-4 µl per 1 µg plasmid DNA) was placed directly into 200 µl of serum-free DME and incubated at room temperature for 15-20 minutes. DNA (α2 or β2, and GFP) (Table 1) was added to the TranIT-293 Reagent and mixed by gentle pipetting. This reagent/DNA complex was allowed to incubate at room temperature for 15-30 minutes. The reagent/DNA complex was placed drop wise onto HEK-293 cells. This was followed by incubation (37 degrees Celsius, 5% CO₂) overnight. Two control cells were used: Non-transfected HEK-293 cells and GFP-only transfected HEK-293 cells.

Twenty-four hours preceding electrophysiological recordings, three 10.5 mm diameter cover slips treated with thermanox (Nunc) were positioned in a 30 mm petri dish containing 1 ml warmed complete medium. Subsequently, 500 µl triturated transfected HEK-293 cells were placed drop wise overlaying the cover slips. The cells were then returned to incubate (37 degrees Celsius, 5% CO₂) for approximately 12-15 hours.
Figure 6. Light microscopy of HEK-293 cells grown on a 30 mm petri dish treated with nunclon (Nunc) 12 hours prior to transfection (Screen shot by: Steinhafel 2003)
<table>
<thead>
<tr>
<th>Rat neuronal nAChR combination</th>
<th>[α3 in pcDNA 3.1]</th>
<th>[β2 in pcDNA 3.1]</th>
<th>[GFP-pcL plasmid]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0 µg</td>
<td>0 µg</td>
<td>1 µg</td>
</tr>
<tr>
<td>α3β2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1/(2&lt;sup&gt;17&lt;/sup&gt;) µg</td>
<td>1 µg</td>
<td>1 µg</td>
</tr>
<tr>
<td>α3β2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 µg</td>
<td>1 µg</td>
<td>1 µg</td>
</tr>
</tbody>
</table>

Table 1. Relative nAChR subunit concentration added to transfection reagent complex. α3β2<sup>a</sup> contains ~100,000 fold less α3 compared to α3β2<sup>b</sup>.
Electrophysiological Recordings on HEK-293 Cells

Transfected HEK-293 Cells were visually identified using fluorescence on an inverted microscope. Whole-cell configuration patches were formed on transfected HEK-293 cells growing on the cover slips. Recordings were performed in an extracellular like solution (ECF) containing (in mM) NaCl (126), KCl (6), CaCl$_2$·2H$_2$O (1.2), MgCl (2.5), HEPES (10), Glucose (11) (pH 7.4). Current responses were obtained by voltage-clamp recording at a holding potential of ~70 mV using a MultiClamp 700A and pCLAMP 9.2 software (Axon Instruments) (Figure 7). Traces were filtered at 1 kHz and sampled at 10 kHz. Electrodes containing an intracellular like solution [ICF in mM: 5 NaCl, 147 KCl, 1.2 CaCl$_2$·2H$_2$O, 20HEPES, 11 Glucose, 5 ATP (pH 7.2)] were formed from borosilicate glass capillaries (Harvard Apparatus, Kent, England) and had resistances of typically 3-6 MΩ when filled with ICF. ACh was freshly prepared in ECF solution, placed into a borosilicate glass electrode, and applied using the Picospritzer® III (Parker Hannifin Instrumentation). All compounds were obtained from Fischer Scientific and Sigma-Aldrich.

Recording Acquisition and Analysis

Peak currents, 10-90 rise times, 90-10 decay times, curve-fitting and desensitization were measured and using Clampfit 9.2.0.11 (Axon Instruments) and analyzed with Microsoft Excel and GraphPad 4.0 (Graphpad, Prism). A standard two-tailed T Test was used to test differences between the two α3β2 groups based on these individual variables. A Mann-Whitney Test, a common post-hoc test, was used to verify significant differences in comparisons not amenable to using parametric statistics. On all tests, P values less than 0.05 were considered significant. GraphPad together with
Microsoft Excel was used to identify descriptive statistics including means and standard deviations.
Figure 7. Illustration of a screen shot simulating one-electrode voltage-clamp in whole cell mode (Photo by: NeuroSearch, Denmark).
RESULTS

Neuronal nAChR subunit expression from rat CA1 hippocampal interneurons

Ninety-Three interneurons were aspirated from acutely prepared rat hippocampal slices from the CA1 stratum oriens and stratum radiatum were individually analyzed for their mRNA expression of the neuronal nAChR α2–10, and β2-β4 subunits, along with 18s rRNA. Out of 93 hippocampal interneurons analyzed 10.8% had no detectable nAChR subunits, 45.2% had a unique combination, 25.8% had a combination that was observed twice, 9.7% had a combination that was observed 3 times, and 8.6% had a combination that was observed 4 times (Figure 8). The most common single cell combinations were α3α4α5α7β2 and α3α5β2β3β4, both of which contain α3 and β2. A z-test of two proportions did not demonstrate any significant differences between the proportion of cells expressing the α3 or β2 subunits when comparing the stratum radiatum and the stratum oriens cells in this sample, so the data from the two cell layers was pooled into a single set. α3β2 was detected in 43% (n=36) of interneurons containing nicotinic receptors (n=83).

Heterologous expression of rat α3β2 nAChR channels

Various combinations of the plasmids containing the α3 and β2 neuronal nAChR genes were successfully transfected into HEK-293 cells along with a plasmid containing green fluorescent protein (GFP). The GFP allowed for the visual detection of those cells which had been successfully transfected (Figure 9). A 500 ms application (unless stated otherwise) of ACh [10mM] was used to elicit nAChR current responses in whole-cell voltage-clamped HEK-293 cells expressing the rat α3β2 neuronal nAChR subunit combination at a holding potential of -70 mV (Figure 10) but
did not excite the non-transfected HEK-293 cells (n=10) or the GFP-only transfected HEK-293 cells (n=14). The existence and function of α3β2 nAChRs after a significant reduction in α3 transfection validates the capability of HEK-293 cells to assemble and/or process the mature protein properly. Dissimilar characteristics of these two receptors suggest different possible mechanisms of assembly and therefore two different possible stoichiometries.

10%-90% Rise Times

The 10%-90% peak rise times from ACh elicited currents were measured using Clampfit and analyzed by GraphPad (see Materials and Methods). The HEK-293 cells transfected with α3β2 (n=9, unless stated otherwise) at a DNA ratio of approximately 1/(2^17):1 revealed a faster average rise time (10-90%) of 378.7 ms ± 188.7. Those cells transfected with α3β2 (n=23, unless stated otherwise) at DNA ratios 1:1 were observed having a slower average rise time of 724.3 ms ± 244.0 (Figure 11). A two-tailed T Test (P value = 0.006) together with a Mann-Whitney Test (P value = 0.0022) revealed a significant difference in rise times between these two groups of receptors (Table 2).

90%-10% Decay Times

HEK-293 cells transfected with α3β2 1/(2^17):1 revealed channels having an average decay time (90-10%) of 2502 ms ± 888.5. Those cells transfected with α3β2 1:1 were observed containing channels having an average decay time of 3051 ms ± 1792 (Figure 12). A two-tailed T Test together with a revealed no significant differences in decay times between these two groups of receptors (Table 3).
PCR Data: # of times a unique nACHR combination was observed

Figure 8. PCR Data on CA1 rat hippocampal interneurons. The number of times a specific group of nAChR subunits were found in an interneuron. n=93. α3α4α5α7β2 and α3α5β2β3β4 were seen in 4 cells each., interestingly, α3 and β2 were found in both of these groups.
Figure 9. Microscopy of transient transfected HEK-293 cells expressing α3β2 nACHRs and green-florescent protein (GFP) when exposed to blue UV-light. Cells which do not fluoresce were not successfully transfected with either α3β2 or GFP (data not shown) (Screen shot by: Steinhafel 2003).
Figure 10. Altering the $\alpha_3$ concentration of $\alpha_3\beta_2$ did not significantly alter the kinetics when expressed in HEK-293 cells. The application of ACh (10mM; indicated by the 500 ms horizontal bar) on $\alpha_3\beta_2$ nAChR transfected cells induced a rapid inward current at a -70 mV holding potential that rapidly desensitized.
Desensitization

When subjected to prolonged exposure to nicotinic agonists, nicotinic acetylcholine receptors undergo desensitization, resulting in an inactive receptor that does not allow for the passage of ions. Rates of recovery from desensitization are dependent on the receptor subunit composition, length of agonist exposure, and on the agonist used to induce desensitization. Furthermore, nicotinic receptor desensitization may contribute to behavioral changes in humans or animals subjected to prolonged nicotine exposure pharmacologically or through the use of tobacco products.

We measured desensitization by applying another 500 ms pulse of ACh (10mM) 3 seconds following the initial stimulus. All HEK-293 cells transfected with α3β2 1/(2^17):1 (n=7) showed very little desensitization when peak2/peak1 was compared (Mean=0.984 pA ± 0.1857). When the α3 subunit concentration was increased to equal the concentration of β2 (n=15), little desensitization was observed (Mean=0.9690 pA ± 0.2223) (Figure 13). Again, a two-tailed T Test confirmed that there was no significance in desensitization between the two nAChR combinations (Table 5).

Decay Tau

The logarithmic decay (τ) characteristics for both nAChR subunit groups were very similar and a two-tailed T Test showed no significant differences among them (Table 6). HEK-293 cells containing α3β2 1/(2^17):1 (n=8) and α3β2 1:1 (n=20) had mean decay τ values of 1440 ± 605 and 1766 ± 1054, respectively (Figure 14). Again, a two-tailed T Test confirmed that there was no significance in desensitization between the two nAChR combinations (Table 5).
Peak Current

HEK-293 cells expressing α3β2 1/\(2^{17}\):1 revealed the largest mean peak value of 231.7 pA ± 248.9 whereas α3β2 1:1 showed a mean peak value of 70.46 pA ± 45.90 (Figure 15). A two-tailed T Test (P=0.010) together with a Mann-Whitney Test (P=0.0183) showed significant differences between these two α3β2 nAChR groups (Table 6).
Figure 11. 10%-90% Rise times. n=29.
### Unpaired t test

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| **Table 2.** 10%-90% Rise time statistical analysis. A two-tailed T-test (P value = 0.0006) revealed together with a Mann-Whitney test (P=0.0022) revealed a significant difference in rise times between these two groups of α3β2 receptors. |
Figure 12. 90%–10% Decay times for both nAChR groups. n=29.
Table 3. 90%-10% Decay time statistical analysis. A two-tailed T Test revealed no significant differences between the two \(\alpha3\beta2\) groups.
Figure 13. $\alpha_3\beta_2$ nAChR Desensitization in HEK-293 cells. $n=29$. 
**Unpaired t test**

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*Table 4. Desensitization statistical analysis.*
Figure 14. Decay tau (τ) for α3β2 in HEK-293 cells. n=29
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Table 5. Decay tau (τ) statistical analysis.
Figure 15. Peak current for both α3β2 nAChR groups. n=29.
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Table 6. Peak current statistical analysis. A two tailed T-test (P value = 0.001) together with a Mann-Whitney Test (P value = 0.0183) revealed significant differences in peak current amplitude between these two groups of α3β2 receptors.
DISCUSSION

Neuronal nAChR subunits are widely expressed in the hippocampus, and are thought to participate in a diversity of physiological and pathological processes associated with cognition and memory (Scoville and Milner 1957; Levin 1992; Newhouse, Potter et al. 1997; Liu, Kawai et al. 2001). However, the specific cellular roles of these ion channels in these processes remain largely undetermined (Jones, Sudweeks et al. 1999). While the major subtype of nAChRs in the hippocampus is thought to be composed of $\alpha_7$ and $\alpha_4\beta_2$ channels, other subunits ($\alpha_{2-5}$, $\alpha_7$, and $\beta_{2-4}$) have been shown to be significantly expressed in the CA1 stratum radiatum and stratum oriens and may be contributing to the formation of functional channels (McQuiston and Madison 1999; Sudweeks and Yakel 2000). Our single-cell real-time quantitative RT-PCR data has shown that 8.6% of 93 CA1 hippocampal interneurons had $\alpha_3\alpha_4\alpha_5\alpha_7\beta_2$ and $\alpha_3\alpha_5\beta_2\beta_3\beta_4$, two combinations that were seen in four cells each. These two unique combinations were the two most common-single cell combinations found and $\alpha_3\beta_2$ was a subcomponent among them. $\alpha_3\beta_2$ is the simplest combination that can make functional nAChR channels from both of these two most commonly observed expression profiles. Therefore, $\alpha_3$ could be one of the major components that could contribute to the physiological and pathological processes occurring in the hippocampus. Our aim is to further understand the selective function of $\alpha_3\beta_2$ nAChRs in the CA1 region of the hippocampus. The objective in performing this study was to recreate and characterize functional rat $\alpha_3\beta_2$ neuronal nAChR channels in HEK-293 cells at expression ratios found with single-cell real-time quantitative RT-PCR. In addition, as part of my thesis I also looked at the changes in
α3β2 receptor kinetics when the α3 subunit concentration was altered. To-date, a small amount of studies have looked at rat α3β2 neuronal nAChRs in HEK-293 cells despite the number successful studies using *Xenopus* oocytes (Luetje and Patrick 1991; Elliott, Ellis et al. 1996; Chavez-Noriega, Crona et al. 1997; Fenster, Rains et al. 1997; Nelson and Lindstrom 1999; Figl and Cohen 2000). For this reason, our studies are carried out using HEK-293.

The ion channel properties of recombinant α3-containing neuronal nAChR may be strongly dependent on the host cell type (Lewis, Harkness et al. 1997). Previous studies show have shown that α3β2 illustrates a longer decay time and a smaller peak current when expressed in *Xenopus* oocytes (Elliott, Ellis et al. 1996). Comparatively, other studies using HEK-293 cells (Nelson, Wang et al. 2001) reveal that α3β2 did not produce as long of a decay time which is analogous to our findings. These findings suggest the possibility of differing α3β2 stoichiometries based on host cell type.

Although α3 and β2 have been shown to express at mRNA ratios (i.e. 1:1) found within CA1 interneurons, they are capable of functioning at altered ratios in HEK-293 cells. This suggests that there may be a variety of stoichiometries for α3β2 present within hippocampal interneurons. Other nAChRs such as α4β2 have been proposed to be expressed within oocytes as (α4)2(β2)3 (Anand, Conroy et al. 1991; Cooper, Couturier et al. 1991). However, various functional studies have suggested that there is more than one stoichiometry for the α4β2 nAChR subtype (Zwart and Vijverberg 1998). For instance, patch clamp recordings from *Xenopus* oocytes expressing α4β2 nAChR have demonstrated that single channel conductance depends on the α:β ratio of mRNA injected into the oocyte (Papke, Boulter et al. 1989). When the relative levels of
expression of the α4 and β2 were varied by nuclear injection of three α:β ratios into Xenopus oocytes, different sensitivities to ACh and d-tubucurarine were obtained using voltage clamp recording (Zwart and Vijverberg 1998). Recently reported, using metabolic labeling with [35S]methionine to measure subunit stoichiometry, the majority of subunit stoichiometry of α4β2 nAChRs expressed in HEK cells was (α4)3(β2)2, yet long-term (overnight) nicotine exposure increased the proportion of nAChRs with a (α4)2(β2)3 stoichiometry (Nelson, Kuryatov et al. 2003). The (α4)2(β2)3 stoichiometry was found to have a higher sensitivity to ACh activation compared to (α4)3(β2)2. Interestingly, Nelson et al. also demonstrated that transient transfection with additional β2 subunits increased upregulation of the nAChRs with high sensitivity to activation. Thus, there is speculation that at least two α4β2 stoichiometries may exist in the mammalian brain, making low sensitive and high sensitive α4β2 nAChRs (Nelson, Kuryatov et al. 2003; Moroni, Zwart et al. 2006). The shift in assembly toward the (α4)2(β2)3 form caused by long-term nicotine exposure could be important in understanding nicotine addiction.

Other studies have studied the effect of acute and chronic nicotine exposure on the desensitization and upregulation of different α4β2 subunit ratios [i.e. 1α4:4β2, 2α4:3β2, and 4α4:1β2 (Lopez-Hernandez, Sanchez-Padilla et al. 2004) or 1α4:10β2, 10α4:1β2 (Nelson, Kuryatov et al. 2003; Moroni, Zwart et al. 2006)] expressed in Xenopus oocytes. Using immunoblotting, Lopez-Hernandez et al. showed that the presence of the α4 subunit in the oocyte plasmatic membrane increased linearly with the amount of α4 mRNA injected. Contradictory to our study, the 1α:4β subunit ratio exhibited the lowest ACh- and nicotine-induced macroscopic current, whereas 4α:1β
presented the largest currents at all agonist concentrations tested. Lopez-Hernandez et al. found that chronic nicotine displayed a reduced state of activation in all three $\alpha 4\beta 2$ subunit ratios, and interestingly only the $2\alpha 4:3\beta 2$ was up-regulated. Both Moroni et al. and Lopez-Hernandez et al. showed that an increase in $\alpha 4$ to $\beta 2$ ratio elicits a larger peak amplitude current, opposite to our findings. Therefore, it is possible that the subunit type and ratio determines the functional state of activation and up-regulation. Furthermore, independent structural sites may regulate receptor activation. This provides evidence that nAChR subtype kinetics is largely based on subunit composition. Moreover, it is likely that the relative subunit ratios and the independent structural sites of $\alpha 3$ and $\beta 2$ regulate $\alpha 3\beta 2$ kinetics based on different host cell types.

Our knowledge on the pharmacological profile of many two-way combinations have illustrated differences in nAChR binding and very few pharmacological studies have been carried out using three-way combinations (Luetje and Patrick 1991; Xiao and Kellar 2004). In Luetje’s study, an $\alpha 2\beta 2$-expressing oocyte elicited a smaller current than $\alpha 3\beta 2$-expressing oocytes by an equal concentration of ACh. In sharp contrast, the current elicited in an $\alpha 2\beta 2$-expressing oocyte by nicotine was much larger current than an $\alpha 3\beta 2$-expressing oocyte. It’s clear that the $\alpha 2\beta 3$ and $\alpha 3\beta 2$ nAChRs alter their responses to varying agonists in oocytes. Few studies have characterized the pharmacological profile of nAChRs using agonists in HEK293 cells despite successful studies using oocytes. Xiao et. al. have determined the binding affinities for many agonists (including ACh and nicotine) at heteromeric nAChRs composed of rat $\alpha 2$–4 subunits in combination with $\beta 2$ or $\beta 4$ in HEK-293 cells. $\alpha 3\beta 2$ demonstrated $\alpha$ 4-fold greater affinity for ACh than $\alpha 2\beta 2$. In addition, $\alpha 3\beta 2$ demonstrated a much higher
affinity for nicotine compared to $\alpha 2\beta 2$. Accordingly, further studies are needed using pharmacology on two- and three way nAChR combinations in HEK-293 to further characterize the pharmacological profile of these channels found in native hippocampal tissues (Luetje and Patrick 1991; Alkondon and Albuquerque 1993; Chavez-Noriega, Crona et al. 1997; Xiao and Kellar 2004).

In summary we have demonstrated that rat $\alpha 3\beta 2$ nAChRs are capable of functional expression in HEK-293 cells at DNA ratios found using single-cell real-time quantitative RT-PCR. Furthermore, we have demonstrated that $\alpha 3\beta 2$ 1/(2^17):1 and $\alpha 3\beta 2$ 1:1 are significantly different in 10%-90% rise time and peak current amplitude. However, these two groups are not significantly different among the majority of their functional properties. These findings may help elucidate the functional characteristics of function nAChRs in native hippocampal CA1 interneurons. This information would increase our understanding of how nAChRs may be involved in such pathological conditions of Alzheimer’s disease, schizophrenia, and how nAChRs may be specifically modulated to treat these conditions with therapeutic drugs. Further studies involving $\alpha 2\beta 2$, $\alpha 3\beta 2$, and $\alpha 2\alpha 3\beta 2$ could further elucidate the properties of nAChRs and the role they play in synaptic transmission in the hippocampus.
FUTURE DIRECTIONS

The objective of this work was to heterologously express, in HEK-293 cells, rat neuronal α3β2 nAChRs to analyze the kinetics of these protein channels. To more accurately examine the functional properties of this nAChR receptor subtype further experimentation including quantitative single-cell RT-PCR, electrophysiology, pharmacological, and must be performed.

The techniques currently to identify nAChR receptor subtypes in the hippocampal interneurons include cellular localization of subunit mRNA (by in situ hybridization or single-cell real-time quantitative RT-PCR) or proteins (by immunoprecipitation or immunocytochemistry), and the assessment of subtype composition and pharmacology (by binding in tissue homogenates or immunopurification). Many of these techniques could similarly be used to fully characterize the α3β2 nAChR subtype when transiently expressed in HEK-293 cells.

Single-cell real-time quantitative RT-PCR is highly sensitive and specific and could be used to determine whether HEK-293 cells are expressing α3β2 at the same ratios that they were transfected. Interpreting the data obtained using this technique requires some general caveats: namely, there may not always be a correlation between mRNA levels and the concentration of surface expressed receptors. The total amount of subunit proteins must assemble properly to make functional channels.

To further validate the expression α3β2 in HEK-293 cells, surface labeling using immunohistochemistry along with radioimmuno assays could be employed to detect and even quantify surface expression of α3β2 nAChRs. However, the use of α3- and β2-specific antibodies are not precisely accurate, and evidence exists that they may
cross-react with other subunits as demonstrated by the similar staining patterns in tissues obtained from wild-type and subunit knockout mice (Jones and Wonnacott 2005). Also, not all nAChR subunits have antibodies that are accessible for use yet.

Pharmacological studies include a pharmacological comparison using alternative nicotinic agonists (i.e. nicotine, cytisine, choline, epibatidine, and 1, 1-dimethyl-4-phenylpiperazinium) will provide an agonist profile for $\alpha 3\beta 2$. Rapid application of different nicotinic agonists at varying concentrations will make available a concentration/response family of currents. The dose concentration/response relationships for each nicotinic agonist could therefore be plotted and the EC$_{50}$ values for each agonist could then be determined. In addition, the maximum responses as well as the efficacy of each nicotinic agonist could be compared to the efficacy of ACh.

The recent discovery of Conus peptides in the venom of cone snails has made it possible to discriminate subtypes in both binding and functional tests. One typical example is $\alpha$Conotoxin MII ($\alpha$CntxMII), which can block the response of ACh to heterologously expressed $\alpha 6\beta 2$ and $\alpha 3\beta 2$ subtypes. The specificity of $\alpha$CntxMII for native subtypes has been demonstrated in previous binding studies and it’s use has helped to define different interfaces in the same receptor in vivo (McIntosh, Azam et al. 2004; Gotti, Zoli et al. 2006). The block of $\alpha 3\beta 2$ in HEK-293 cells with $\alpha$CntxMII in the presence of ACh would further confirm the capability of $\alpha 3\beta 2$ expression in HEK-293 cells.

These and other similar studies would help to further elucidate the role of various neuronal nAChR subtypes and help explain why there is so much functional diversity in this neurotransmitter system.
REFERENCES


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CURRICULUM VITAE

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EDUCATION

2005 – Present  Southern California College of Optometry, Fullerton, California.  
Doctor of Optometry (expected date of completion: May 2009).

2003 – 2006  Brigham Young University, Provo, Utah.  
Master’s of Science, Department of Physiology and Developmental Biology.

Bachelor of Science, Major: Zoology.

PROFESSIONAL RESEARCH AND CLINICAL WORK EXPERIENCE

Sep 2005 – May 2006  Southern California College of Optometry Eye Care Center, Fullerton, California.  
Department: Medical Records. Supervisor: Luis Ospina.  
Department: Optometry Library. Supervisor: Denise Hess.

Sep 2003 – Aug 2005  Brigham Young University, Provo, Utah.  
Jun 2006 – Aug 2006  Graduate Student. Department: Physiology & Developmental Biology. Supervisor: Dr. Sterling Sudweeks, PhD.

Laboratory Technician. Department: Kinesiology. Supervisor: Dr. Li Li Ji, PhD.

Laboratory Technician. Department: Botany. Supervisor: Dr. Edgar Spalding, PhD.

(Jan 2001- June 2001) Assisted Kevin M. Folta, Ph.D. – Assistant Professor of Molecular Biology.


Pharmaceutical Technician. Supervisor: Dr. Paul Osteiner, PharmD.
### PROFESSIONAL TEACHING EXPERIENCE

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<td>Sep 2006 – Present</td>
<td>Southern California College of Optometry, Fullerton, California. Graduate Teaching Assistant: Human Physiology I and Ocular Anatomy. Supervisor: Dr. James LaMotte, O.D. Ph.D.</td>
<td></td>
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<tr>
<td>Jan 2004 – May 2004</td>
<td>Brigham Young University, Provo, Utah. Graduate Teaching Assistant: Human Physiology. Department: Physiology &amp; Developmental Biology. Supervisor: Dr. William Winder, PhD.</td>
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### PUBLICATIONS


### RESEARCH GRANT FUNDING

Sep 2003 – August 2005 Graduate Research Assistantship, Brigham Young University, Provo, Utah. and NIH/NIEHS TIP award funding through S. Sudweeks.

### PROFESSIONAL CONFERENCES AND MEETINGS ATTENDED

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PROFESSIONAL NATIONAL MEMBERSHIPS AND LOCAL ORGANIZATIONS

2005 – Present  Member, American Optometric Student Association (AOSA)
2005 – Present  Member, National Optometric Student Association (NOSA)
2005 – Present  Member, California Optometric Association (COA)
2005 – Present  Member, Wisconsin Optometric Association (WOA)
2005 – Present  Spanish Optometric Student Association (SOSA), Southern California College of Optometry, Fullerton, California.

2004 – 2005  Pre-Optometry Club, Brigham Young University, Provo, Utah.

HONORS AND AWARDS

Apr 2004  Dean’s List, Brigham Young University, Provo, Utah.
June 2003  Graduate Scholarship, Brigham Young University, Provo, Utah.
May 1999  Track & Field Coach’s Pick Award. University of Wisconsin at Oshkosh, Oshkosh, Wisconsin.