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EXPRESSION OF NICOTINIC ACETYLCHOLINE RECEPTOR mRNA  
AS A FUNCTION OF AGE IN WHOLE HIPPOCAMPUS PREPARATIONS  
FROM WISTAR RATS

by

Kasey C. Welch

A thesis submitted to the faculty of  
Brigham Young University  
as requisite for completion for the degree of  
Master of Science in Neuroscience

Department of Physiology and Developmental Biology  
Brigham Young University

August 2008

BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a thesis submitted by

Kasey Welch

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

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Date

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Date

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Michael R. Stark

BRIGHAM YOUNG UNIVERSITY

As chair of the candidate's graduate committee, I have read the thesis of Kasey Welch 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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Biology

Accepted for the College

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College of Life Sciences

## ABSTRACT

### EXPRESSION OF NICOTINIC ACETYLCHOLINE RECEPTORS AS A FUNCTION OF TIME IN WHOLE HIPPOCAMPUS PREPARATIONS FROM WISTAR RATS

Kasey Welch

Physiology and Developmental Biology

Master of Science

Whole hippocampus preparations, isolated bilaterally, from untreated Wistar rats at various ages (10-90 days old) were analyzed for the mRNA expression of the  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 7$ ,  $\beta 2$ ,  $\beta 3$  and  $\beta 4$  neuronal nicotinic acetylcholine receptor subunits. To do so, RNA was isolated from acutely isolated hippocampal samples, converted to cDNA by means of a reverse transcription reaction, then analyzed with quantitative real-time PCR to determine the relative levels of the mRNAs the cells were expressing at the age when the samples were obtained. The relative expression of the levels of RNA were then compared across age groups by subunits and across subunits by ages. The results suggest that all eight subunits are expressed throughout the life of the rat and that the subunit expression for the Hippocampus varies only slightly as a rat develops.

## ACKNOWLEDGMENTS

I'd like to thank Dr. Scott Steffensen, Dr. Michael Stark, and Dr. Sterling Sudweeks for all their help as my committee. I'd like to thank Dr. Sterling Sudweeks for his superhuman patience and intellect. I would like to especially thank my wife for her encouragement, love, and support.

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## Introduction

Nicotinic Acetylcholine receptors (nAChRs) are part of a large family of ligand-gated ion channels that share similarities in structure. Other members of the family include the GABA<sub>A</sub>, glycine and the 5-HT<sub>3</sub> receptors (Dani 2007). There are two major forms of the nicotinic receptor, those expressed by skeletal muscles at the neuromuscular junction, and those expressed by neurons. The neuronal nAChRs are expressed throughout both central (CNS) and peripheral (PNS) divisions of the nervous system. The channels allow the passage of cations across the membrane and open in response to the endogenous neurotransmitter acetylcholine and exogenous ligands such as nicotine. A functional receptor/ion channel is composed of five subunits (see figure 1).

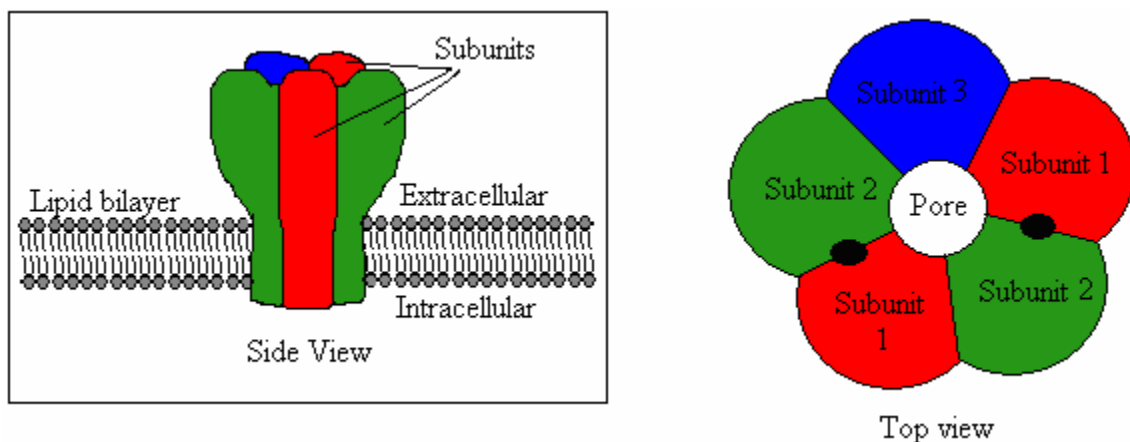


Figure 1 - The look of a hypothetical channel composed of 3 different subunits. The three different subunits are designated by the three different colors; subunit 1 is red, subunit 2 is green, and subunit 3 is blue. The binding sites are designated as black circles along the borders of subunits 1 and 2. The pore forms the channel that when opened allows the passage of ions.

To date many subunits have been cloned that are expressed in neuronal tissue, they have been named  $\alpha$ 2-10 and  $\beta$ 2-4. The subunits are similar but small changes in the individual subunit sequences give rise to changes in the functional and pharmacological properties of the ion channel subtypes (i.e., changes occur in the kinetics or pharmacological profiles of how the channels perform (Dani 2007)). To demonstrate how channels would function differently we can look at the actual channel that is made up of only  $\alpha$ 7s. The kinetics of the channel is unique because the channel opens, closes, and desensitizes quickly in comparison to other channels occurring in vivo. The differing subunits have also been found to have different affinities for various substances, the  $\alpha$ 7 subunit is also more sensitive to choline which is a by-product of the breakdown of the neurotransmitter acetylcholine (Albuquerque 1998). Changes in subunit composition also affect conductivity; for example, the  $\alpha$ 7 homomeric channel conducts calcium while many other subtypes do not (Castro 1995). The varied subunit expression changes the electrical activity of the neurons expressing the channels and has implications for how the neurons develop and function over their lifetimes. A loss or change of function in these receptors can cause changes in cognition and learning and have been implicated in various disease states like schizophrenia (Freedman, Coon et al. 1997; Breese, Lee et al 2000), Alzheimer's disease (Nordbeg, Alafuzoff et al. 1986; Perry 1995; Dani 2007), and epilepsy (Dani 2007).

The connection between the nAChR and Alzheimer's disease was first established in the 1970's when studies showed that individuals with Alzheimer's exhibited lower levels of acetylcholine (Davies 1976; Perry 1977). Another study was done that also linked the low levels of acetylcholine to impaired cognition around the same time

(Bowen 1976). Since that time research has continued to investigate the changes to cholinergic pathways that occur in the brain. We have also continued to learn other details of the disease. We now know that hyperphosphorylated tau and beta amyloid plaques also are present with Alzheimer's disease. Studies have looked at nAChRs in the disease and the relationship between nAChRs and other factors or indicators (Woodruff-Pak 2002, Klein 2004)

Schizophrenia has been treated with nicotinic like substances since the middle of the 1900's. Research on the effects of nicotinic like substances on schizophrenia have occurred since the 1950's (Hoffer 1957). The connection between the hippocampus and schizophrenia is more recent with data appearing in the 1990's (Freedman 1995, Freedman 1994). Research has continued to narrow in on the nAChRs with research being conducted on individual subunits (Bitner 2007, Freedman 2000).

Epilepsy was linked to the hippocampus and the nicotinic system located there in 1985 (Milner 1985). Research has continued with the specific drugs administered to the hippocampus and the effects on specific kinds of epilepsy (Martin-Garcia 2005).

The hippocampus is known to be critically involved in the processes of learning and long-term memory formation. First indications of the importance of the region of hippocampus arose when an individual suffered brain damage to a specific region of the brain. The damage was localized to the region including the hippocampus of both hemispheres. The gentleman, now known as H.M., suffered from the inability to learn anything new since his trauma. H.M.'s memories of before the accident remained intact and he was able to recall many things that he had learned before the trauma occurred, even into his later years in life. Since that time we have learned that the

hippocampus is the specific region of the brain that aids in new memory formation.

The hippocampus receives multiple cholinergic inputs that synapse onto pyramidal cells, granular cells, interneurons, and neurons of the Hilus. The neuronal nAChRs can be expressed in a variety of locations on an individual neuron. Neuronal nAChRs have been shown to appear on presynaptic, postsynaptic, and even extrasynaptic sites. Postsynaptic nicotinic transmission occurs rarely in the mammalian brain, but has been shown to occur in the GABAergic hippocampal interneurons in the rat (Sudweeks 2000). The neuronal nAChRs subunit mRNAs expressed by hippocampal interneurons are the focal point of this project. Hippocampal interneurons express a wide variety of receptor subunits and serve to modulate the signals that are passed through the hippocampus. Preliminary results from the Sudweeks lab suggest that interneurons are an incredibly diverse population and that over 100 different subtypes of neuronal nAChRs could exist in these cells. Single-cell RT-PCR data from our lab demonstrate that the  $\alpha 2$ ,  $\alpha 3$ ,  $\alpha 4$ ,  $\alpha 5$ ,  $\alpha 7$  and  $\beta 2$ ,  $\beta 3$ ,  $\beta 4$  subunit mRNAs are all commonly expressed by hippocampal interneurons in young rats (ages 10-28 days old)(unpublished). Other studies have looked at the expression of certain subunits over time but have not looked at all the subunits (Zhang X 1998, Adams 2006). The timing of expression for many neuronal nAChR subunits is still unknown during the continued development as the rats age. As we seek to establish the kinetics of specific combinations of subunits, it is important to know the timing of receptor subunit co-expression, and what implications that has for the expression of various subtypes of the neuronal nAChR. These findings could have future implications about how exposure to substances such as alcohol, nicotine, and other substances affect the expression profile

and could lead to altered mental states. There are studies that look at how exposure to substances like ethanol and nicotine affect the Nicotinic subunits (Robles 2008, Alkondon 2007, de Fiebre 2003). By establishing a normal expression profile in untreated rats it may facilitate the study of exogenous substances in the future.

**The initial hypothesis for this study was that rats would express different subtypes of the neuronal nAChR in hippocampal interneurons as they age, starting with a very complex expression profile in young rats and leading to a less complicated expression profile in older rats. By establishing that some subunit expression is transient and that certain channel subtypes become physiologically unimportant as the rats age, the study of functional channels in the hippocampus would be greatly simplified. However, as presented in detail below, our results demonstrate that this simplified picture is not the case, and that hippocampal interneurons continue to express a very rich variety of neuronal nAChR subunits as the rats age.**

## **Objectives**

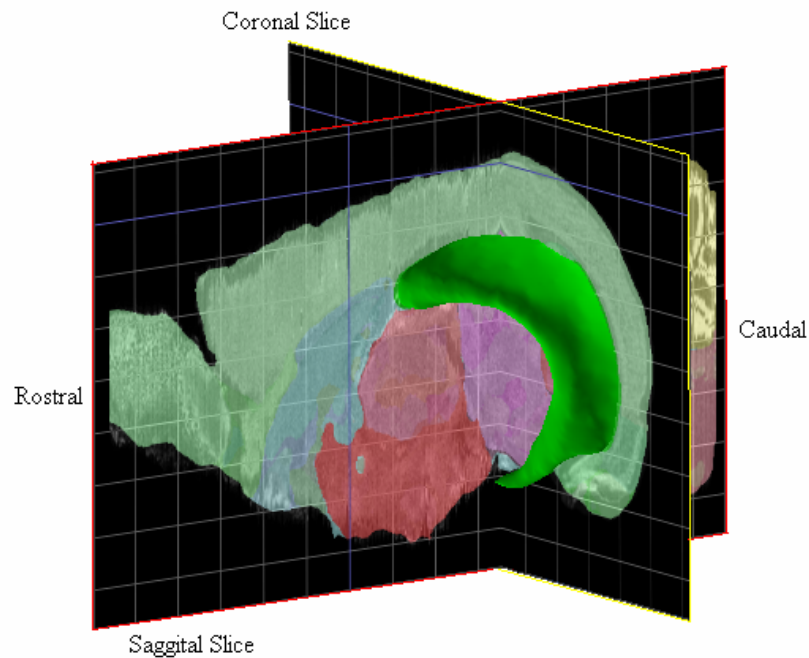
We collected samples of the hippocampus from rat pups ranging in age from 10 days old to 90 days old. Most electrophysiological studies conducted in regards to neuronal nAChRs have used animals of the ages between 14 and 28 days old. We have studied the single-cell expression of the interneurons during this time (<28 days old) and according to our findings there are over 100 combinations of neuronal nAChR subunits for this age

range. Having such a high possibility of different receptor combinations greatly complicates the further study of the expressed neuronal nAChR channels and makes the functional characterization of all of the possible combinations very difficult.

## **Methods**

Wistar rats were generously donated from a breeding colony maintained by Dr. Scott Steffensen's lab (BYU Department of Psychology). Rats maintained a 12h light/dark cycle and were fed/watered ad libitum in accordance with prescribed techniques to maintain general well being. Rats that had undergone no treatment were sacrificed on specified days to allow for a sampling during postnatal day 10 to postnatal day 90. The rats were sacrificed so that there were replicate samples for each day studied. Samples were collected every 2-4 days from day 10 to day 30, from day 31-60 samples were collected every 5 days and from day 61-90 samples were collected every 10-11 days. The number of rats sampled per age was between 2 or 3 rats which produced up to 6 samples per age. The rats were sacrificed by first being anesthetized by halothane in a chamber. Exposure to the halothane lasted only as long as required to produce an areflexic state (roughly one minute). Upon removal from the chamber the effectiveness of anesthesia was tested by the "toe pinch" method. Rats were then be sacrificed by decapitation. The skull was exposed and cut open to allow removal of the intact brain. Upon removal the brain was washed with artificial cerebral spinal fluid, split down the middle to divide it into two hemispheres and allow access to the hippocampal formation (see figure 2).

This is a 3D view of the hippocampus (in darker green). The Saggital and coronal slices are provided to help orient the hippocampus in the brain.



Courtesy of  
Brain Explorer  
Allen Brain Institute For Brain Science  
Version 1.4.1  
November 15, 2007

Figure 2- This is a 3-D rendering of the hippocampus of the left lobe. The dark green portion is the hippocampus. The other colored areas are automatically generated by the Brain Explorer (Allen Brain Institute For Brain Science, Seattle, WA). The hippocampus can be removed intact by gently pulling back the cortex and severing the connections at the two tips of the crescent moon shape.

The hippocampus from each lobe was excised and immediately placed in a BioMasher (ISC BioExpress, Kaysville, UT) setup with pores of 20 $\mu$ m-60 $\mu$ m and placed in centrifuge. The sample was then spun through the BioMasher into 250 $\mu$ L Trizol (Invitrogen, Carlsbad, CA) solution. The Trizol solution arrested the degradation of the RNA contained in the sample and allowed for the subsequent isolation of the RNA. The RNA from the whole hippocampus was isolated using the standard procedure from

manufacturer. RNA was then quantified by 1  $\mu$ l sample spectrophotometry on a Nanodrop 1000 (Nanodrop, Wilmington, DE). The RNA was then used to create cDNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA). As per directions from the iScript, the amount of RNA to be used for template for the reverse transcriptase reaction was standardized to close to the maximum of 1  $\mu$ g per reaction. This also served to standardize the amount of RNA contained in each sample, in an attempt to avoid false high or low readings because of higher or lower amounts of hippocampus isolated. The cDNA was used to run real time RT-PCR. Ribosomal 18s was used as a standard to allow comparison of the results from multiple PCR runs to be compared to each other, as well to other subunits. The ABI prism 7000 sequence detection system was used (Applied Biosystems, Foster City, CA). The cycling protocol was: 1 cycle at 50° for 2 minutes, 1 cycle at 95° for 3 minutes, then 60 cycles with three steps; step one was 15 seconds at 95°, step two was 20 seconds at 55° and step three was 30 seconds at 72°. Mixtures of PCR reaction were constructed from purified deionized water, iTaq Supermix with ROX (BioRad, Hercules, CA), primers and probes designed on ABI Primers Express 2.0 (Applied Biosystems, Foster City, CA) and template obtained as described previously. The relative expression of the subunits can be quantified using the data generated by quantitative PCR and the  $\Delta\Delta C_t$  method (Livak 2001). The  $\Delta\Delta C_t$  method first establishes the difference between the Cycle threshold (initial rise of fluorescence) of the target gene (nAChR subunit) minus the  $C_t$  of the standard gene (18s), this sets the rise of the 18s gene as zero and allows for accurate comparisons between samples of different sizes. In order to more accurately compare real-time RT-PCR data from reactions run at different times, the raw fluorescence data generated from the ABI



7000 machine was curve fitted using Prism 4 software (Graphpad, San Diego, CA). Microsoft Excel (Microsoft, Redmond, WA) was used to sort and arrange data in a manner that graphpad could use, graphpad was then used to fit the data to a Boltzman-Sigmoidal curve and to generate a greater number of data points to allow more specific analysis of the data. From the curve fit, the cycle number for the initial rise in the fluorescence signal (as determined by the maximum of the second derivative of the curve) was used to calculate the cycle threshold (Ct). 18s rRNA from each sample acted as an internal control, and was set as the origin point for all the reactions generated from each sample. For example, if one sample has more tissue and therefore more RNA, than another sample, then the amount of 18s rRNA will be higher, and will be used to offset the apparent increase in the subunit expression. Because every cell expresses 18s rRNA, the amount of 18s cDNA was always much higher than nAChR cDNA. In order to move the initial rise of the 18s rRNA signal out of the range when the ABI system establishes a baseline, the cDNA samples used as a template for 18s rRNA PCR was diluted 1:1000 to move the initial rise to the 20-30 cycle range. The Ct of the triplicate 18s reactions for each sample was averaged to set a consistent value to use for the zero point. The averaged 18s value was then subtracted from the value of each reaction. The difference would give a number of cycles different from zero to when the subunit appeared. The Ct values from all reactions were then averaged to give a reference value of subunit expression (i.e., "1 fold"). Then each individual run was subtracted from the average to give a value of number of cycles different from the average. The cycle difference was then raised to  $2^n$  to give the relative fold expression. The value for each individual reaction was then grouped with values obtained from samples of the same age and tested

for normality of the grouping. An ANOVA test (using InStat software version 3.05, Graphpad, San Diego, CA.) was run to test for differences in the expression from one age to another. To normalize the “fold expression” distribution, the log was taken for each value. The log-normalized values for each age were then averaged to produce a point on a graph that would allow for linear regression to be done. The  $R^2$  value was calculated in Excel for the linear regression line that was fit to the graph.

## **Results**

All 8 subunits tested for in this study were found in the majority, if not all, of samples in each of the age categories from day 10 through day 90. The average fold expression by day was tested by ANOVA (InStat software version 3.05, Graphpad, San Diego, CA.) to see if there were significant differences in expression between the days. The resulting data only found significant difference between a few days in each sample, when the extreme high points were compared to the extreme low points of the profiles. The fold expression of the subunits did not show a statistically significant change from beginning to end. The failure to see significant changes could have been caused by having too small a sample size. I did power calculations (StatMate software version 2.00, Graphpad, San Diego, CA.) by subunit. I grouped the data into two groups, the samples that were above the median in one group and the samples that were below the median in another. I averaged the standard deviation for the groups to run the t- test. The results of the power calculations are contained in the individual subunit paragraphs below. The result of this study shows that the neuronal nAChR subunit mRNA expression profile for the rat

hippocampus does not decrease in complexity over time. Since the subunit expression remained constant there is the possibility that receptors can be constructed from any of the eight subunits throughout the ages examined.

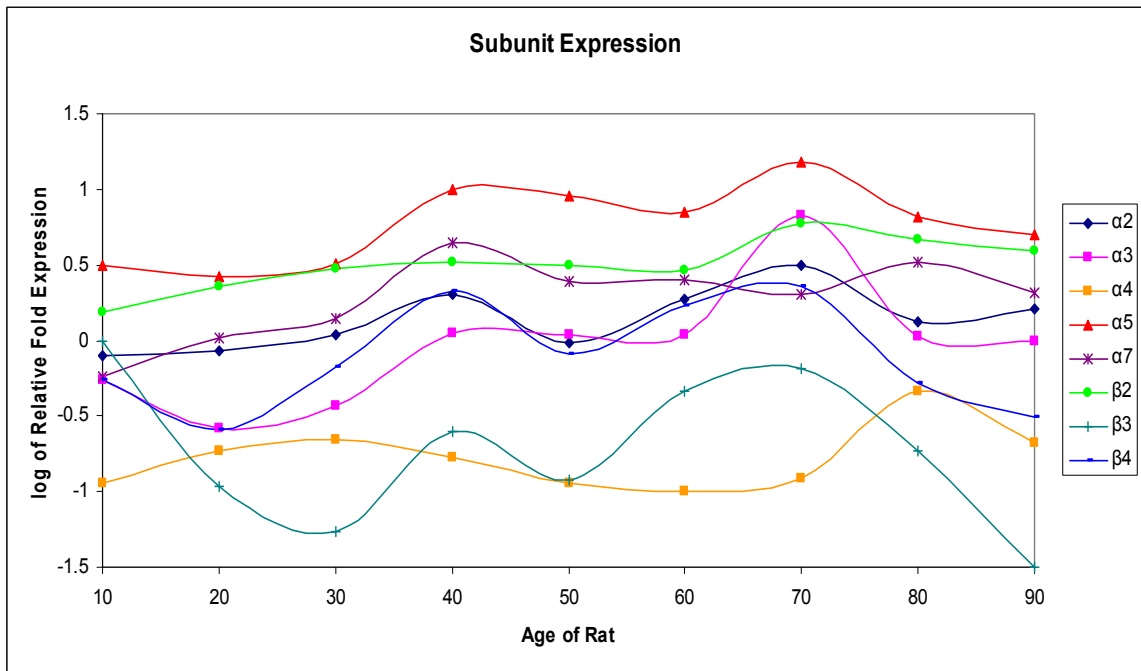


Figure 3- The relative expression of all tested subunits. The y-axis is the relative expression compared to the average expression of all the subunits from all ages. The x-axis is the age of the rat. The data was binned into day ranges. The day 10 bin is all data gathered from day 10-19 for each subunit, the day 20 bin was all data generated from day 20-29, and so on.

The analysis also helped to reveal that although the expression does not change much as the rat ages, the levels of expression between the subunits were significantly different. To compare the subunit expression to each other I simplified the graph by placing the average relative fold expression from each day into bins of 10 day lengths (see figure 3). The resulting graph shows the relative expression levels of each subunit. Overall, the  $\alpha 5$  nAChR subunit had the highest overall expression, while  $\alpha 4$  and  $\beta 3$  had the lowest

expression levels. In this figure, the lowest expressing subunit ( $\alpha 4$ ) is set as the reference for fold expression (i.e., expression of 1 fold) and all of the subunits that had levels of expression that were significantly different from it are marked with asterisks.

To simplify the discussion and significance of our results, I will present each subunit individually in the following paragraphs.

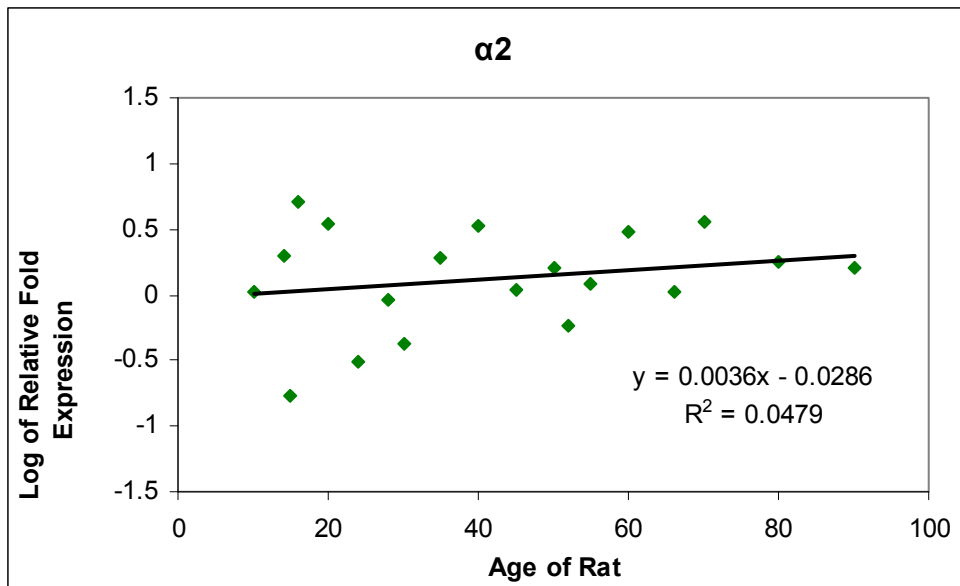


Figure 4- Graph of the values generated from samples tested for  $\alpha 2$ . The Green dot is the log of the values for the average expression for that day compared to the average expression of all subunits from every day. Linear regression and  $R^2$  Value are to help visualize possible trends.

$\alpha 2$ - (Figure 4) Out of 106 samples tested, only two samples did not test positive for  $\alpha 2$  mRNA. The negative samples came from different, non-consecutive ages. The graph produced by calculating a linear regression line to the fold expression data shows only a slightly positive slope, suggesting that as a rat ages the  $\alpha 2$  subunit expression remains

fairly constant. The overall change of the expression level is very slight (slope of fit line is 0.0036). The values of the average fold expression for the subunit varies more during the earlier days and seems to vary less widely as the rat ages. This may be a result of the varying neuronal environment that occurs early on in post-natal rat brain development. As the brain continues to age, it makes sense that less variability occurs during the later time points. Two general trends seem to appear for  $\alpha_2$ ; a sustained expression of the subunit over time and a decrease in the variability from day to day. Calculations showed that statistical power was in the 85%-90% range (Statmate 2.0, Graphpad software, San Diego, CA.).

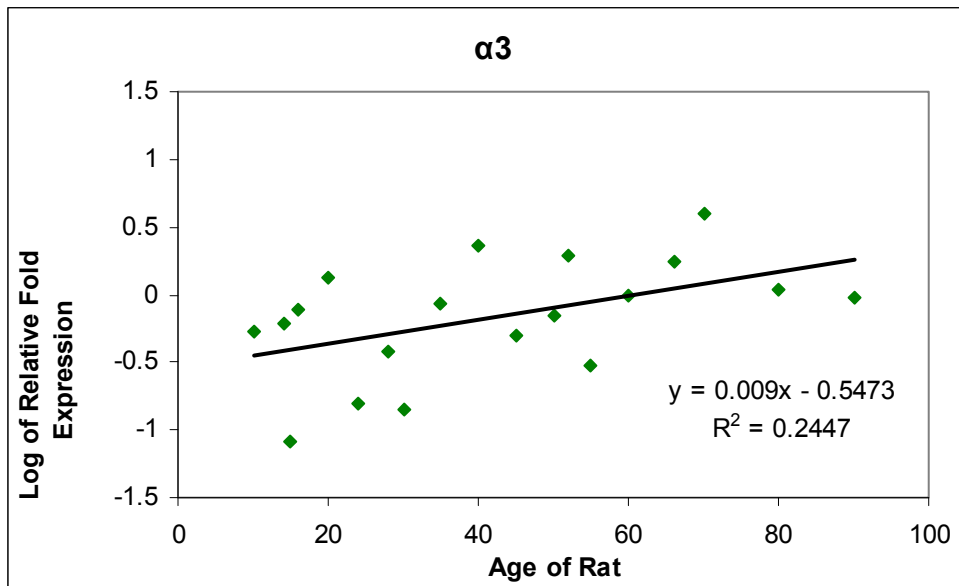


Figure 5- Graph of the values generated from samples tested for  $\alpha_3$ . The Green dot is the log of the values for the average expression for that day compared to the average expression of all subunits from every day. Linear regression and  $R^2$  Value are to help visualize possible trends.

$\alpha_3$ -(figure 5) The  $\alpha_3$  subunit appeared in 99 of 106 or in 93% of the samples, showing

that  $\alpha 3$  is prevalent in the interneurons of the hippocampus throughout life.  $\alpha 3$  showed the clearest example of a modest increase in expression as the rat aged (the slope of the linear regression line was highest at 0.009 ). The  $R^2$  value, at 0.24, was also higher than any of the other subunits as well. Calculations showed that statistical power was in the 70%-75% range (Statmate 2.0, Graphpad software, San Diego, CA.).

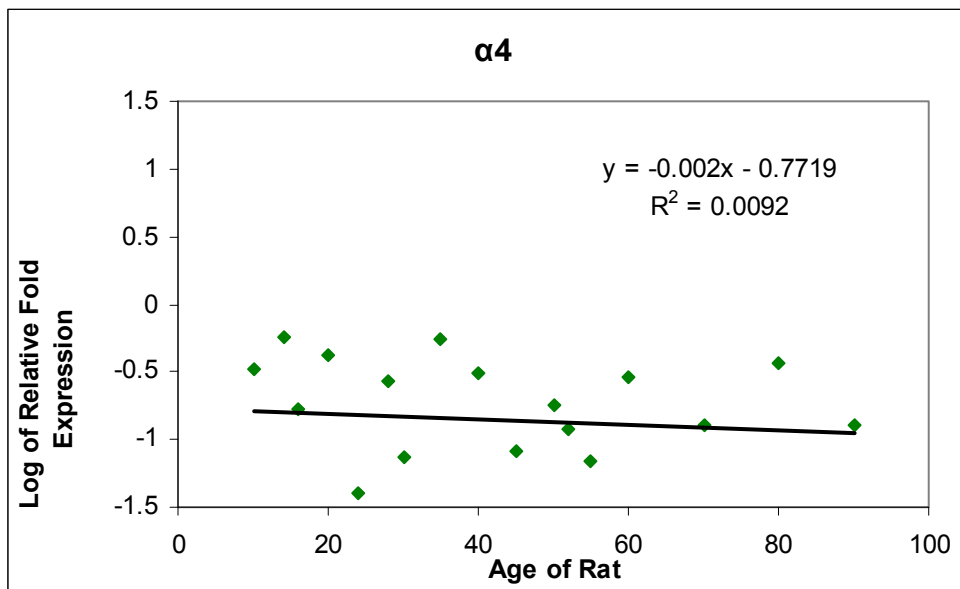


Figure 6- Graph of the values generated from samples tested for  $\alpha 4$ . The Green dot is the log of the values for the average expression for that day compared to the average expression of all subunits from every day. Linear regression and  $R^2$  Value are to help visualize possible trends.

$\alpha 4$ -(figure 6) The  $\alpha 4$  subunit was detected in 96 of the samples for an expression in over 90%. Similar to the  $\alpha 2$  subunit, it also showed a decrease in the variability as the rats were sampled at later dates. The linear regression line showed a slightly negative slope, but overall the slope of -0.002 is small and ANOVA testing showed that the subunit was not different from day 10 to day 90. Calculations showed that statistical

power was in the 90%-95% range (Statmate 2.0, Graphpad software, San Diego, CA.).

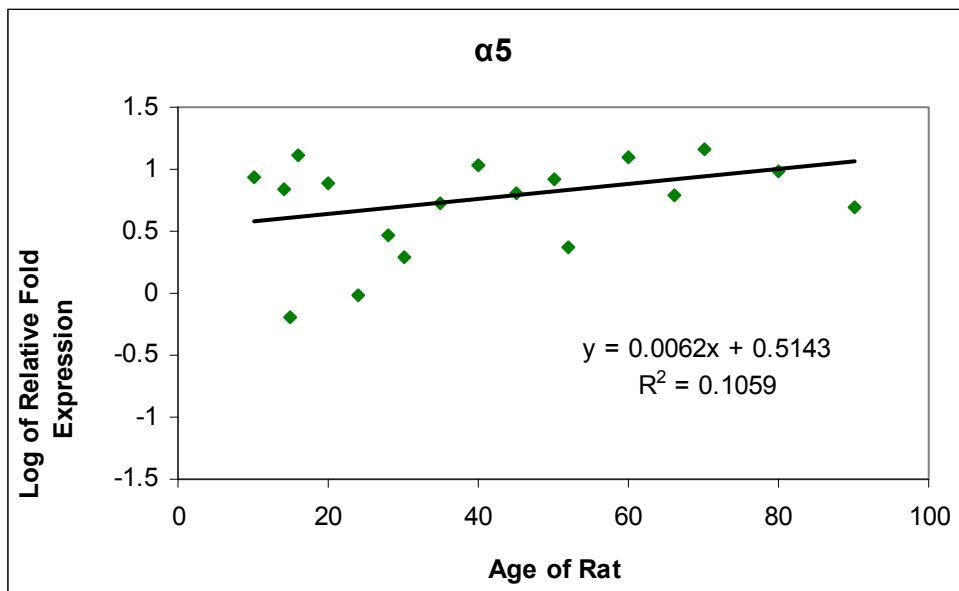


Figure 7- Graph of the values generated from samples tested for  $\alpha 5$ . The Green dot is the log of the values for the average expression for that day compared to the average expression of all subunits from every day. Linear regression and  $R^2$  Value are to help visualize possible trends.

$\alpha 5$ - (figure 7)  $\alpha 5$  had the highest relative fold expression of all the subunits. It appeared in 94%, or 100 out of 106 samples. Like many of the other subunits it showed high variability early in the life cycle of the rat which decreased with time. The slope is again very slight of only 0.006, and an  $R^2$  value of 0.11. Calculations showed that statistical power was in the 75%-80% range (Statmate 2.0, Graphpad software, San Diego, CA.), indicating a slightly increased risk of having a type II error.

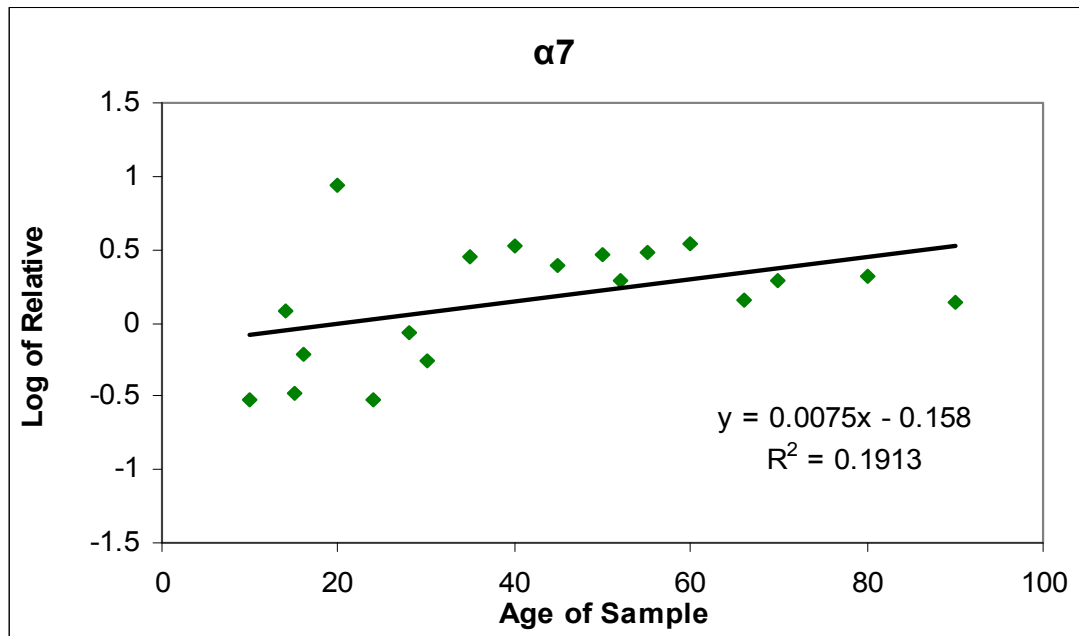


Figure 8- Graph of the values generated from samples tested for  $\alpha 7$ . The Green dot is the log of the values for the average expression for that day compared to the average expression of all subunits from every day. Linear regression and  $R^2$  Value are to help visualize possible trends.

$\alpha 7$ - (figure 8) Expressed in 99 of 106 samples,  $\alpha 7$  was also identified as being prevalent throughout the life cycle investigated in this study. This subunit seemed to lose the high variability quickly, reaching a point of relative stability in expression at around 30 days postnatal. This contributes to the low slope of 0.0075, but the relatively high  $R^2$  value of 0.19. Calculations showed that statistical power was in the 85%-90% range (Statmate 2.0, Graphpad software, San Diego, CA.).



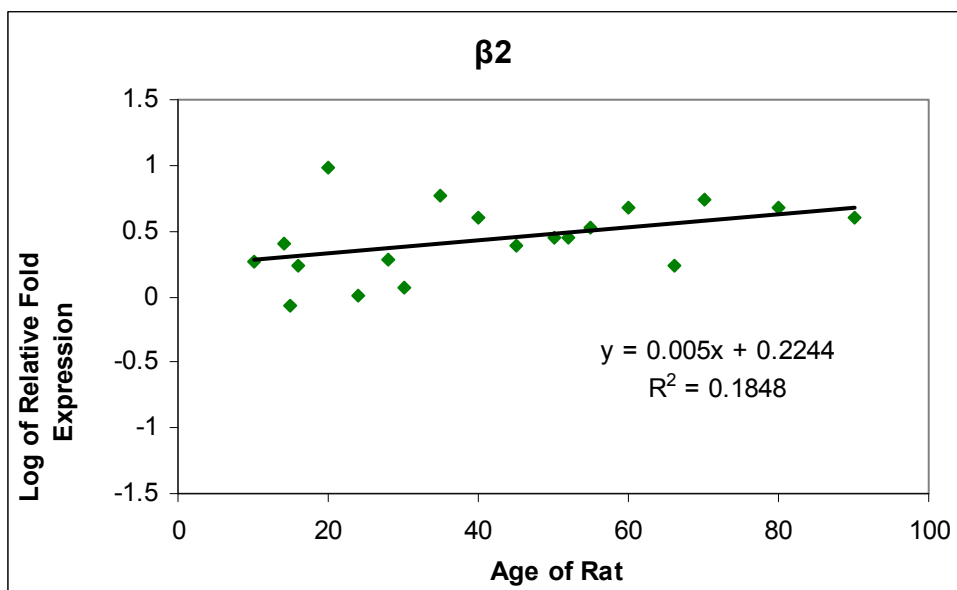


Figure 9- Graph of the values generated from samples tested for β2. The Green dot is the log of the values for the average expression for that day compared to the average expression of all subunits from every day. Linear regression and R<sup>2</sup> Value are to help visualize possible trends.

β2- (figure 9) β2 is expressed in 100% of the samples in the study. The subunit was not only expressed every sample it was also expressed at a high level, only α5 was expressed at a higher level. Like other subunits the variability of the expression seems to decrease as the animal ages. Again giving a slope of only 0.005 but an R<sup>2</sup> of 0.18. Calculations showed that statistical power was in the 70%-75% range (Statmate 2.0, Graphpad software, San Diego, CA.) indicating a slightly higher risk than normal (power <80%) for having a type II error (false negative).

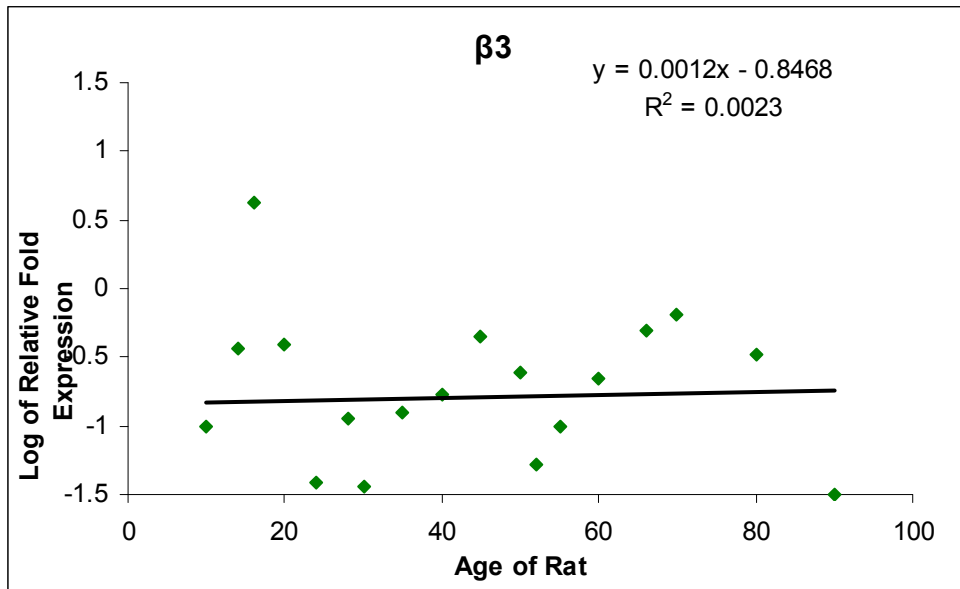


Figure 10- Graph of the values generated from samples tested for  $\beta 3$ . The Green dot is the log of the values for the average expression for that day compared to the average expression of all subunits from every day. Linear regression and  $R^2$  Value are to help visualize possible trends.

$\beta 3$ - (figure 10) This subunit was found in 97 of 106 for another over 90% expression ratio. The trend for this subunit was not well established. It seemed to bounce around and never really settle. The combination of the slope of 0.0012 and the  $R^2$  of 0.0023 was the lowest of all the subunits suggesting the data was well dispersed. Calculations showed that statistical power was in the 90%-95% range (Statmate 2.0, Graphpad software, San Diego, CA.).

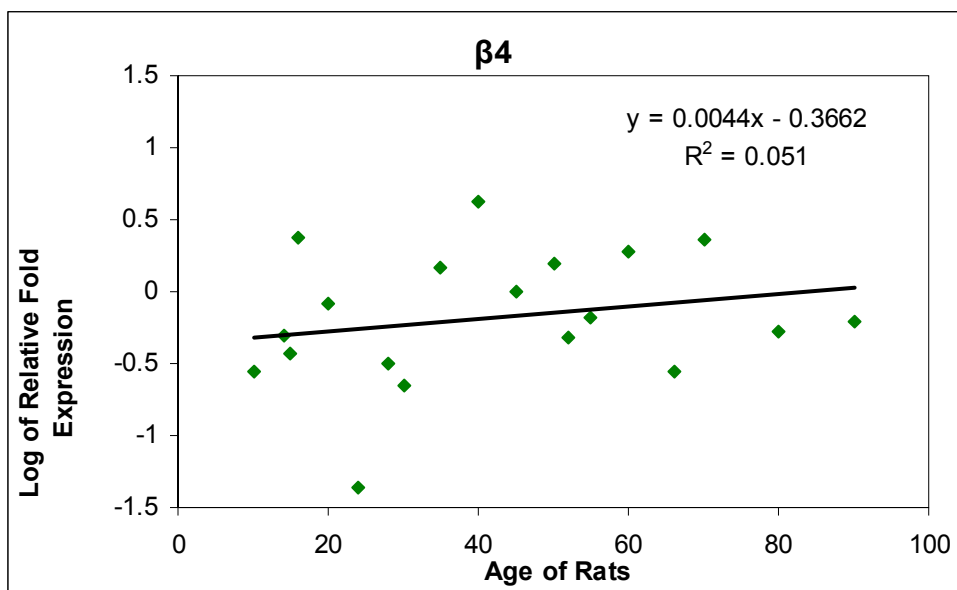


Figure 11- Graph of the values generated from samples tested for β4. The Green dot is the log of the values for the average expression for that day compared to the average expression of all subunits from every day. Linear regression and R<sup>2</sup> Value are to help visualize possible trends.

β4- (figure 11) This subunit like the others was highly expressed but was detected in 92 of 106 samples which gives a frequency of 87% which is the lowest of the subunits. The slight upward slope of .0044 and the low R<sup>2</sup> value of 0.051 again do not offer much in the way of concrete evidence for the linear regression. The average fold expression for this subunit is higher than two other subunits so should not be considered to be unimportant in future consideration. Calculations showed that statistical power was in the 50%-60% range (Statmate 2.0, Graphpad software, San Diego, CA.) indicating a substantial risk of having a type II error (false negative).

## Discussion

We do know that all eight subunits tested were expressed throughout the ages examined (10-90 days). This means that there is still a large array of possible receptor subtypes that could be expressed by hippocampal interneurons in adult animals. Of course, one caveat of this analysis is that this study only looked at the mRNA expression, we did not look at the production of proteins or the expression of the proteins in the cell membrane where they can function, both could be steps that could be affected post mRNA. Some of the more popular subunits that are studied at present, like  $\alpha 4, \beta 2$  or  $\alpha 7$ , were not found to be expressed at higher amounts of mRNA than the other less studied subunits, like  $\alpha 2$  or  $\alpha 5$ .

Because the mRNA expression was analyzed from tissue homogenates, it is impossible to know if the individual neurons maintain the same expression over time or if there are various populations that express specific subunit combinations. The significance in the difference between subunit expressions could be caused by all neurons simply expressing more of a subunit, or that a population of interneurons is more numerous in the hippocampus. We sampled the whole hippocampus so we cannot exclude the possibility that there are different populations that might express certain subunits and that the combined populations express all subunits. The uniformity of the data however seems to suggest that there is a baseline expression and that as the brain ages the individual neuron's expression would change based on adaptations caused by experiences. It is

interesting to note that in the older rats the expression seems to take on a less variable profile indicating that the brain may settle into a more constant level of neuronal nAChR subunit expression. Since the hippocampus is highly involved in learning and memory procession, it would be interesting to see if the levels of neuronal nAChR subunit mRNA change with varying the level of hippocampal activity, say by treating the rats with some kind of activity that would actively engage the hippocampus above simply living in a simple cage. Now that a baseline for subunit expression has been determined, there are future possibilities to study how things can affect the expression profiles. Future directions could be to isolate more specific regions of the hippocampus and test them for expression, testing the whole hippocampus again but treating the rats with a treatment to see how profiles may change, or looking to see if there is a difference in expression profile between sexes, hemispheres of the brain, or even species. There is also evidence that continued research to find specific agonists or antagonists to specific subunits could be very helpful in characterizing functional channels and even treatment for diseases that arise from hippocampal malfunction. Studies could possibly extend the period out and see if changes occur later in life. This study provides evidence that all the subunits that are initially expressed in the rat are expressed throughout life at least into day 90, which is into adulthood for rats. Most importantly sustained presence of all subunits validates the importance of characterizing all subunits and learning how each one plays a role in the functioning of interneurons in the hippocampus.

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