Chapter 2

Investigation into miR-346 Regulation of the nAChR α5 Subunit

MicroRNA's (miRNAs) are small (< 25 base pairs), single stranded, non-coding RNAs that regulate gene expression at the post transcriptional level. Mature miRNAs form loose base pair interactions with the UTR regions of their target mRNA transcripts. This can lead to degradation and impaired translation. Because the base pair interaction between miRNA and mRNA target are imperfect, one miRNA may regulate many gene products [30]. Many miRNA's are expressed in spatially and temporally restricted patterns, and miRNA regulation has been associated with almost every cellular process, including cancer, immune response, and cellular degeneration [30]. miRNAs are also known to play a large part in development, acting as "switches" to control critical timing dependent processes such as organogenesis and neural development [30].

A few nAChRs are thought to be regulated by miRNAs, but no in-depth investigation has been undertaken. In *C. elegans,* a conserved miRNA, miR-1, regulates the expression of two nAChR muscle subtypes that are important for synaptic transmission at the neuromuscular junction [31]. Preliminary screening of a commercially available library of orphan miRNAs against luciferase-based reporter constructs with murine nAChR 3'UTRs have revealed several candidate miRNA-nAChR interactions (unpublished work by Eric Hogan, University of Massachusetts). A single miRNA, miR-346 was shown to inhibit luminescence when luciferase was coupled to the α5 3'UTR or α10 3'UTR (see figure 2.1).

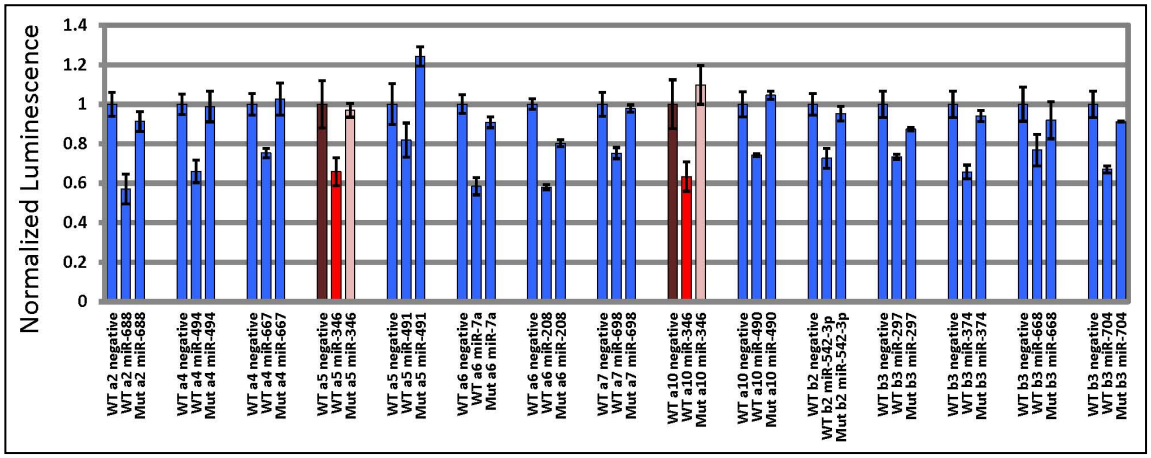

Figure 2.1. Results of luciferase screen of orphan miRNA library against nAChR 3'UTRs. Subunits affected by miR-346 are highlighted in red. Both α5 and α10 3'UTR constructs displayed a 30% reduction of luminescence in cells co-expressed with miR-346, but not when the 3'UTR contained mutated recognition sequences. (data from Hogan et al.)

Follow-up studies identified the miRNA recognition element recognized by miR-346 in the α5 and α10 3' UTR as ACAGGCAGACA. Mutations to this sequence in both the α5 and α10 3' UTR resulted in no effect on luciferase activity when miR-346 was present (figure 2.2). With the interaction between miR-346 and α5 and α10 3' UTRs firmly established, we next examined miR-346 regulation of the α5 protein as expressed in clonal cells transiently transfected with α5 subunits.

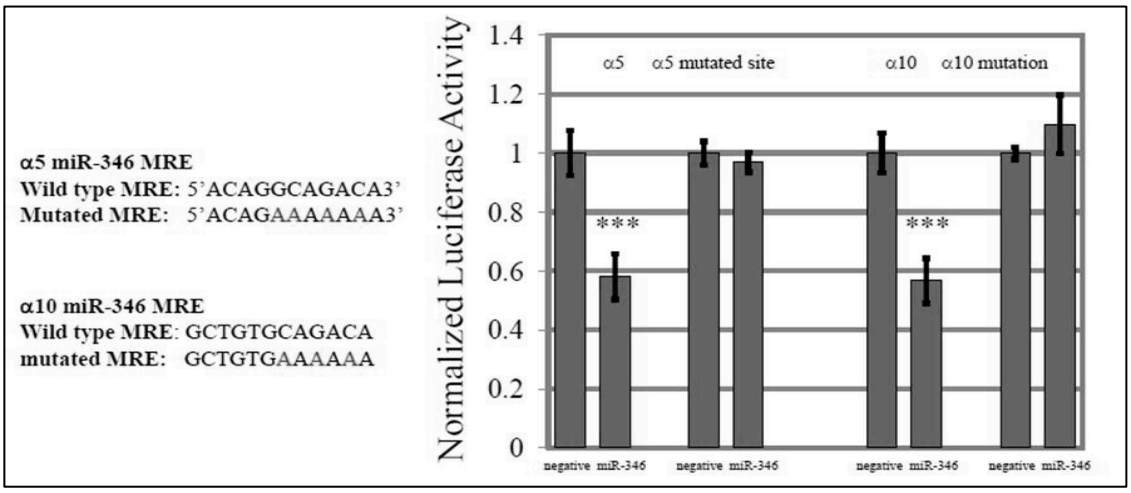

Figure 2.2
Site directed mutagenesis of predicted miR-346 binding sites in the 3' UTR of α5 and α10 was carried out in the context of MIR-REPORT-Luciferase. Mutated sequences are shown on the right. MRE = miRNA recognition element. Bars indicate mean ± SEM. Student t-test ***$p \leq 0.001$ (data from Hogan et al.)

In collaboration with Hogan et al. in the Tapper Lab at the University of Massachusetts, α5-mEGFP was selected as the reporter construct for further investigations. The complete mouse α5 wt 3'UTR (3'UTR) or 3'UTR with mutated miR-346 MRE (3'MUT) were subcloned into the α5-mEGFP378 and α5D/N-mEGFP378 plasmid constructs (see table 1). Expression analysis of the constructed α5-mEGFP fusion proteins was performed using HEK293-T cells transiently transfected with α5-mEGFP, α5-mEGFP-3'UTR, α5-mEGFP-3'MUT, α5D/N-mEGFP, α5D/N-mEGFP-3'UTR, or α5D/N-mEGFP-3'MUT (figure 2.3). Statistical differences in total fluorescence were detected between α5-mEGFP and α5D/N-mEGFP, and also between α5-mEGFP and α5D/N-mEGFP-3'MUT (t-test, $p = 0.05$). Interestingly, there was no significant difference in fluorescence between α5-mEGFP and α5D/N-mEGFP-3'UTR.

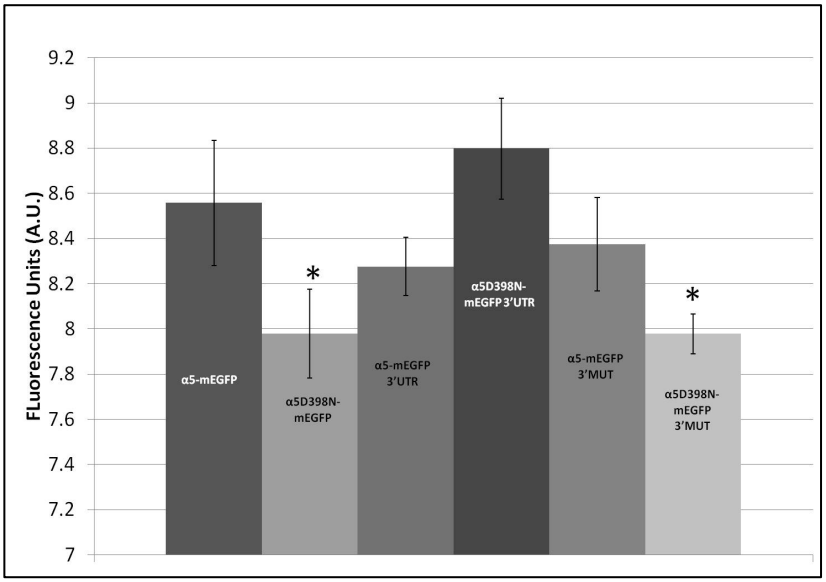

Figure 2.3
Relative fluorescene of
α5-mEGFP
α5D398N-mEGFP
α5-mEGFP3'UTR
α5D398N-mEGFP3'UTR
α5-mEGFP3'MUT
α5D398N-mEGFP 3'MUT
expressed in HEK293T cells and assayed for fluorescence using a standard fluoremeter.

Asterisks indicate significant reduction in expression compared to α5-mEGFP, by t-test, $p \leq 0.05$

Due to their small size and single stranded nature, miRNAs are highly unstable [30]. A precursor form of the desired miRNA is used for transfection to prevent degradation of the desired miRNA. Once inside the cell, the Pre-miR is processed by

endogenous proteins to create a mature miRNA strand. At high concentrations, non specific interactions of miRNA with mRNA transcripts can occur. These interactions may lead to regulatory effects on non-target sequences. We sought to optimize the concentration of miR-346 precursor (Pre-miR-346) necessary for regulation of α5-mEGFP-3'UTR. α5-mEGFP-3'UTR or α5-mEGFP-3'MUT were co-transfected with varying concentrations of Pre-miR346 for expression in HEK293-T cells. Results show that 1 nM Pre-miR-346 is the optimum concentration for use in further experiments (figure 2.4).

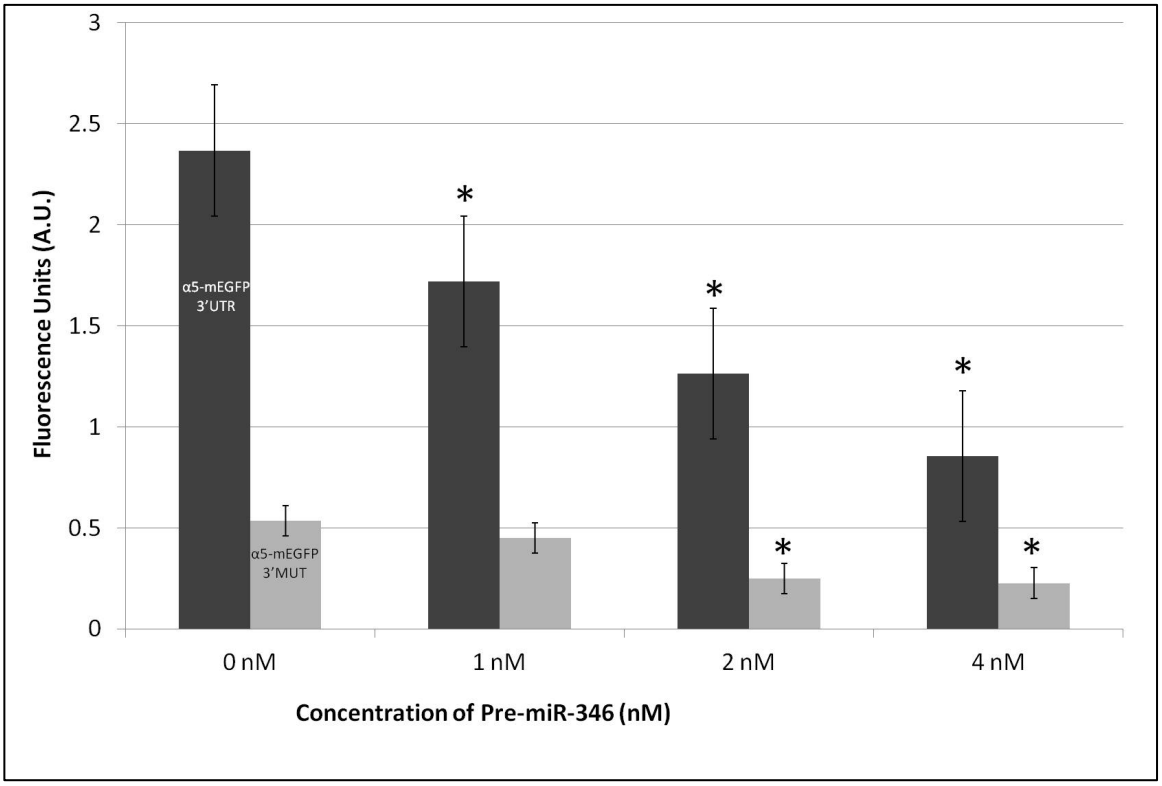

Figure 2.4
Bar graph illustrating resultant fluorescence from transfections of α5-mEGFP-3'UTR (dark grey) or α5-mEGFP-3'MUT (light grey) with varying concentrations of Pre-miR-346. Asterisk indicates significant variation ($p \leq 0.05$) from 0nM values. 1 nM was selected as the optimal concentration of Pre-miR346 because at that concentration significant reduction in α5-mEGFP-3'UTR but not in α5-mEGFP-3'MUT was observed.

α5-mEGFP-3'UTR was then co-transfected with 1 nM of Pre-miR346, a scrambled miRNA precursor, or α5 siRNA. Cells were assayed for fluorescence intensity using a

fluorescent plate reader 24 h post-transfection. Preliminary results from Hogan et al. showed a 50% reduction in α5-mEGFP-3'UTR expression when co-transfected with Pre-miR-346 (figure 2.5).

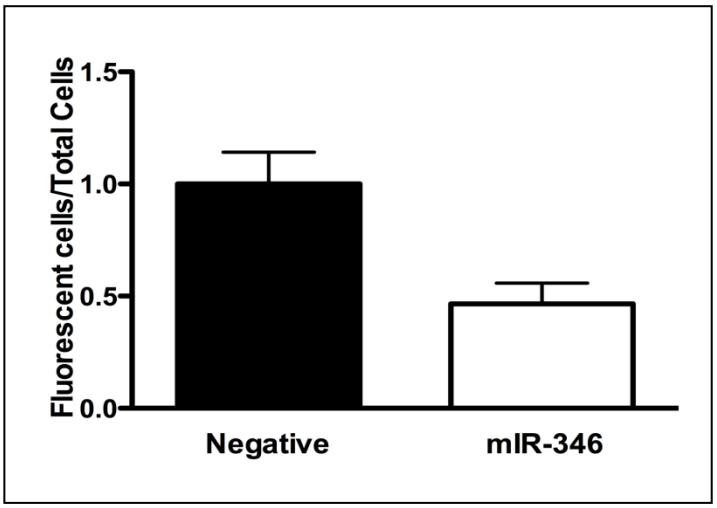

Figure 2.5
Results of fluorescence assay of HEK293-T cells transiently transfected with α5-mEGFP-3'UTR and either 1nM of scrambled negative control or 1nM Pre-miR-346.
Fluorescence is reduced by almost 50% when Pre-miR-346 is transfected with α5-mEGFP-3'UTR
(data from Hogan et al.)

However, additional experiments using α5-mEGFP and α5-mEGFP-3'MUT as positive and negative control, respectively, were unable to repeat these results. Figure 2.6 shows the consistent lack of response of the various α5-mEGFP constructs to different transfection conditions. Reduction in fluorescence of α5-mEGFP3'UTR upon addition of miR-346 was not significant and additionally, greater reduction of α5-mEGFP3'UTR expression was seen when α5-mEGFP3'UTR was co-transfected with a scrambled Pre-miR sequence than in the Pre-miR-346 condition. Obviously, more experiments are needed to determine specific regulation of α5-mEGFP3'UTR via direct, sequence-specific interaction between miR-346 and the α5 3'UTR.

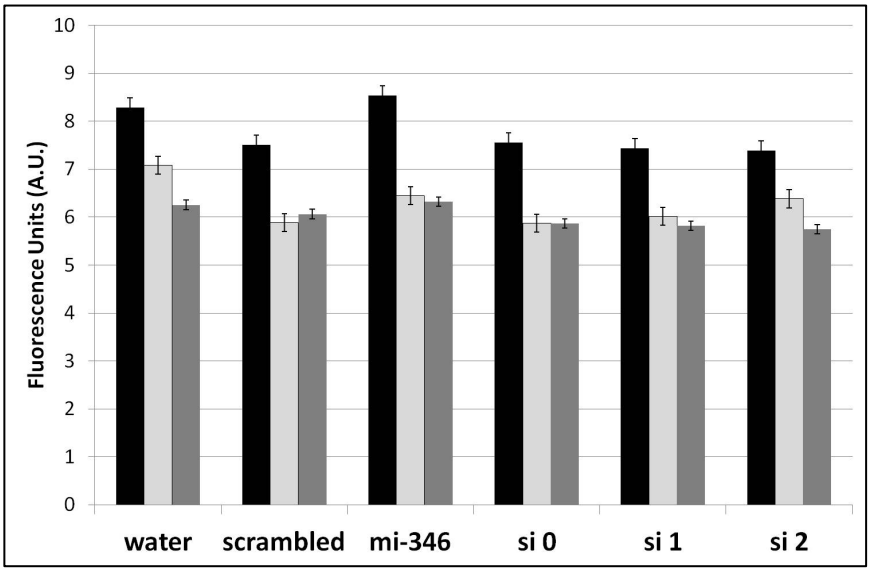

Figure 2.5
Bar graph representing relative fluorescence of α5-mEGFP (black) α5-mEGFP 3'UTR (light grey) α5-mEGFP 3'MUT (dark grey) after co-transfection with (right to left) water, scrambled Pre-miR, Pre-miR-346, or commercially available siRNAs to α5 siRNA0, siRNA2, siRNA.

α5* receptors have been implicated in developmental changes in activation and morphology of the medial prefrontal cortex (mPFC). The mPFC is responsible for decision making and attention as well as other higher-order cognitive processes. Cholinergic inputs to the cortex appear early in brain development and are widespread in rats by the third week of post-natal life [32]. There is a corresponding developmental peak in nicotinic current response to ACh in rodent mPFC layer VI neurons that is mediated by α5* receptors and occurs during a crucial period of cortical circuit refinement [10, 32-33]. Nicotinic stimulation during this period can modulate the retraction and maturation of neuronal processes [32]. α5* receptors are known to be enriched in layer VI of the mPFC and it has recently been discovered that proper expression of the α5 subunit in this area during development plays an important role in normal attention behavior in adult mice [10]. Work by Leslie et al. examining expression of α5 mRNA in rats indicates that there may be a global reduction in α5 mRNA after post-natal day 10 [34]. We hypothesize that this down regulation of α5 mRNA may be due to regulation by miRNAs such as miR-346.

In this chapter we have identified a miRNA, miR-346, that may be involved in regulation of nAChR expression. This miRNA is upregulated in adult mouse brain after chronic nicotine exposure, and a reduction in luminescence of a luciferase reporter construct fused to the α5 3'UTR in the presence of miR-346 is reported. More investigation is necessary to determine the nature of the biological interaction between miR-346 and α5. However, preliminary experiments suggest that fluorescence of α5-mEGFP-3'UTR is reduced in the presence of 1 nM Pre-miR-346. Data presented here lays the foundation for investigation of miRNA regulation of nAChRs and specific regulation of α5 by miR-346. Elucidating the role of miR-346 in regulation of α5 expression could have implications for brain development and disease states.