Chapter 6

Conclusions

Nicotinic acetylcholine receptors (nAChRs) are widespread throughout the body in both the peripheral and central nervous systems. In the brain, nAChRs are ubiquitously expressed. However, layers of regulation are involved in the highly specific localization of these important receptors [3]. Individual brain regions may express only certain subtypes of the receptors, and this expression may even be sub-region specific. For instance, α4β2 receptors are expressed in many regions of the brain, including the pre-frontal cortex (PFC). However, α5α4β2 receptors are highly concentrated only in cortical layer VI [7, 10]. Furthermore, cell types within sub-region offer an additional layer of regional specificity. In the mid-brain ventral tegmental area (VTA) glutamatergic neurons express α7 receptors, GABA neurons are known to express α4β2 and α6* receptors on dopaminergic neurons in the same region are important for dopamine release [59, 61].

Receptor composition and stoichiometry confers unique biophysical and pharmacological properties to each receptor sub-type. This variation in receptor function influences which brain regions are active in response to agonists, the concentrations at which certain cell-types are activated, and the nature of that activation. Accessory subunits such as α5 and β3 act as modulators to amplify or attenuate a chemical signal either by changing agonist affinity or increasing ionic permeability, and β subunits can affect surface occupancy of receptors on the plasma membrane [13, 23, 56]. Stochiometry is the key to many of the diverse effects of nAChR activation. Preferential upregulation of $(\alpha4)_2(\beta2)_3$ stochiometry receptors after nicotine exposure may contribute to the development of

nicotine tolerance and the development of disease states such as autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) [1, 6, 36].

Determination of nAChR composition and stochiometry is a challenging one. Many receptor subtypes have been identified, and the regions of their distribution elucidated; however, the number of specific subunits within a nAChR is not always known. Additionally, isolation of specific receptor populations for individual study is difficult, especially if unique agonists or antagonists do not exist, as in the case of the accessory subunits [13]. Recently, direct visualization of receptor composition has become possible using fluorescent microscopy techniques. Creation of fluorescently labeled nAChR subunits by fusion with fluorescent protein variants (FPs) has provided exciting alternatives to electrophysiology for the study of selective pharmacological effects on nAChR number, stoichiometry, and subcellular behavior [36]. Additionally, the ability to visualize nascent nAChRs, still in intracellular compartments, yields an advantage over immunohistochemistry, which require cell permiabilization, or electrophysiological assays that are restricted to measuring function of mature receptors. Advanced imaging techniques have been applied to fluorescent nAChRs including confocal and TIRF microscopy, FRET measurements, single molecule visualization, and FLIM. Combined, these methods form a powerful tool box for elucidation of intracellular assembly, processing, and trafficking of nAChRs [6, 21-23, 26, 46]. However, these methods are not without their own limitations.

The data presented in this thesis make a strong case for systematic optimization of the methods for studying fluorescent nAChRs and suggest that caution must be taken when interpreting fluorescence data. The placement of FP labels within the M3-M4 loop of the nAChR is crucial to satisfy the distance requirements of FRET and avoids steric

interference with ligand binding domains, which may be important for interactions with agonists or molecular chaperones, but the M3-M4 loop also contains sequencing motifs important for assembly of the nAChR pentamer and interaction with vesicular trafficking machinery [23, 35]. Chapter 3 has demonstrated that perturbation of the α5 M3-M4 region with a large fluorescent protein ≥ 4 times the size of the loop itself may make wild-type behavior impossible for such an encumbered subunit. Less invasive labeling methods to ensure wild-type behavior of receptors and their subunits are available but few utilize completely non-invasive methods. Site specific incorporation of fluorescent unnatural amino acids (UAAs) would be ideal for such a purpose. UAAs have been used in preliminary experiments, but use of these systems is tricky, and the technique has not been sufficiently compatible for expression in mammalian cells [62-63].

NFRET analysis has been proposed in the literature as a technique for elucidation of nAChR stoichiometries. However, findings presented in chapter 4 of this thesis suggest that these results may have been over interpreted. While it is likely that intracellular changes in pre-receptor assemblies are detectable after exposure to drugs such as nicotine or dihydro-β-erythroidine, these changes may not directly correlate to differences in stochiometry at the plasma membrane. The statistical similarity of NFRET histograms generated from cell populations with and without the ability to form mature receptor complexes is not encouraging. Further experiments could be performed to examine changes to NFRET% in the same population of cells after drug exposure to determine whether any meaningful conclusions about receptor stoichiometry can be drawn from NFRET histograms, but it remains a reliable measure of intracellular interaction of fluorescent nAChR subunits.

The combination of TIRF and FLIM techniques offers a unique opportunity for high resolution detection of FRET interactions in a selective population of mature nAChRs. It is possible that further optimization of the fluorescent nAChR constructs used in the experiments presented in chapter 5 will allow rigorous characterization of E_{FRET} values for individual geometric relationships within the nAChR pentamer. Once standard measurements are performed for the mouse muscle nAChR, the same FRET relationships can be applied to measurements of neuronal nAChR pentamers of unknown stoichiometric composition. If the trafficking restrictions can be overcome, TIRF-FLIM-FRET (or single molecule photo-bleaching as performed by Richards et al.) remains the most direct method for stochiometric determination of nAChRs.

Without exact knowledge of nAChR composition and stochiometry, design of unique pharmacological agents for selective activation or inactivation of many receptor sub-types is unlikely. nAChRs are notoriously hard to crystallize due to their multi-transmembrane nature and many subunits. This lack of detailed structural information contributes to a disparity of effective pharmaceuticals for the treatment of nAChR related diseases and nicotine addiction [64-65]. Understanding subtle differences in distributions of receptor composition and stochiometry will move us closer to a more complete picture of the molecular mechanisms underlying nAChR interactions within networks of neuronal function, disease, and addiction. Judicious application of fluorescent microscopy techniques will certainly be an important tool in elucidation of these mechanisms. The data presented here represents one small step towards this understanding.