NOVEL STRATEGIES TO MODULATE SYNAPTIC COMMUNICATION AND INVESTIGATE THE ROLE OF HDAC6 IN ALZHEIMER’S DISEASE

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NOVEL STRATEGIES TO MODULATE SYNAPTIC COMMUNICATION AND INVESTIGATE THE ROLE OF HDAC6 IN ALZHEIMER’S DISEASE

A Dissertation Presented

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DEDICATION

To my family:

Linda L. Medeiros
Walter R. Medeiros
Elizabeth A. Medeiros
Kenneth G. Ventura

Thank you for being my inspiration, biggest supporters, and critics.
ACKNOWLEDGMENTS

This work would not have been possible without the support and constructive feedback of others.

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I would also like to thank my committee members, Dr. Min Chen, Dr. Dan Hebert, and Dr. Pat Wadsworth for their helpful comments and suggestions in my research.
ABSTRACT

NOVEL STRATEGIES TO MODULATE SYNAPTIC COMMUNICATION AND INVESTIGATE THE ROLE OF HDAC6 IN ALZHEIMER’S DISEASE

MAY 2014

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Neuronal communication is mediated by chemical signaling at the synapse. The underlying molecular mechanisms of learning and memory are poorly understood. Very few tools are available to study how memories are formed in the mammalian brain. This dissertation focuses on developing novel strategies to study neural activity. Here we develop and use a chemical-genetic approach to enable target-specific photocontrol of inhibitory synaptic neurotransmission of GABA_A receptor subtypes. The tools developed here selectively photocontrolled GABA_A receptor subtypes. This enabled the investigation of the functional role these receptor subtypes have in inhibitory synaptic neurotransmission. This dissertation also focuses on identifying the role of HDAC6 in Alzheimer’s disease. Increased expression of HDAC6 was identified as an underlying molecular factor that led to pathological tau accumulation and early changes that correlate with synaptic dysfunction, hallmarks of Alzheimer’s disease.
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1.1 Neurons

Neurons are post-mitotic polarized cells in the brain specialized for communication via chemical signaling. The main morphological characteristics of a neuron are a cell body with a single axon and multiple dendrites extending from the cell body (Figure 1.1A). Axon morphology and function is dependent on microtubules (Conde and Caceres, 2009). Dendrites have branched processes of micron-sized protrusions called dendritic spines (Perez et al., 2009; Penzes et al., 2011; Shirao and Gonzalez-Billault, 2013; Jaworski et al., 2009). The morphology and function of dendritic spines is dependent on cytoskeletal actin filaments (Sala and Segal, 2014; Hotulainen and Hoogenraad, 2010; Dent et al., 2011; Gu and Zheng, 2009). Both microtubules and actin dynamics are critical for the development of neuronal polarity and synaptic communication (Conde and Caceres, 2009). The polymerization dynamics of actin and microtubules play an important role in neuron morphology, communication, and survival.

The axon contains a parallel bundle of microtubules with the plus ends oriented towards the cell periphery and the minus end oriented towards the cell body (Dixit et al., 2008; Conde and Caceres, 2009; Shirao and Gonzalez-Billault, 2013). Microtubules are integral structural components of the cell composed of \(\alpha\) and \(\beta\) tubulin heterodimers that are necessary for cytoskeleton support serving as a track for directional axonal transport of cargos such as proteins, vesicles, and organelles (Buee et al., 2000; Conde and Caceres, 2009). Neuronal survival depends on highly regulated functional axonal transport of cargos to and from the cell body to distal portions of the cell.
Figure 1.1. Neuron Morphology

A) Cartoon depiction of a healthy polarized neuron illustrating the main features of a neuron including axon, soma, presynaptic axon terminal, dendrites, and postsynaptic dendritic spines.

B) Cartoon depiction of morphological characteristics of postsynaptic dendritic spines.
The axon transmits chemical signals via neurotransmitter release at the presynaptic terminal. Dendrites have multiple branched processes of dendritic spines that receive these chemical signals at a site called the postsynaptic terminal. Communication between the presynaptic terminal and the postsynaptic terminal form a specialized site of chemical signaling responsible for neurotransmission as well as learning and memory and is called the synapse. Trafficking of cargo to presynaptic and postsynaptic terminals contribute to the development of mature synapses (Niesmann et al., 2011). Changes in size and shape of the dendritic spine correlate with the strength of the synaptic connection and the remodeling of the underlying actin cytoskeleton (Hotulainen and Hoogenraad, 2010). Dendritic spines are highly dynamic structures that change size and shape at timescales that range from seconds to minutes to hours (Hering and Sheng, 2001; Kasai et al., 2010). Dendritic spines are often categorized by morphology into three categories: thin spines, stubby spines, and mushroom spines (Figure 1.1B) (Hotulainen and Hoogenraad, 2010; Jaworski et al., 2009; Harris and Kater, 1994; Hering and Sheng, 2001). These classifications of dendritic spine morphology are critical for neuronal communication and believed to be snapshots of underlying synaptic plasticity (Shirao and Gonzalez-Billault, 2013).

1.2 Synaptic Plasticity

The ability of a neuron to modify communication at the synapse in response to changes at the synapse is referred to as synaptic plasticity. Synaptic plasticity is the underlying mechanism of learning and memory. This change in synaptic strength is regulated at the presynaptic terminal by neurotransmitter release and by receptors at the postsynaptic terminal. Homeostasis of neuronal communication is maintained by a balance between strengthening synaptic communication by long-term potentiation (LTP)
and reducing the strength of synaptic communication by long-term depression (LTD) (Figure 1.2).

LTP is an increase in synaptic strength and associated with dendritic spine enlargement (Kasai et al., 2010; Shepherd and Huganir, 2007; Pontrello et al., 2012; Collingridge et al., 2004). Enlargements of dendritic spines and increased receptor trafficking to the postsynaptic sites are associated with LTP. Changes in the functional strength of synapses, the size and shape of dendritic spines, as well as the physical connectivity of neural networks all contribute to LTP (Bingol and Sheng, 2011). LTD is a decrease in synaptic strength associated with a reduction in the number and size of dendritic spines (Pontrello et al., 2012; Shepherd and Huganir, 2007). This reduction in dendritic spine size and number as well as decreased receptor trafficking are associated with LTD. Altered neuronal communication at the synapse and a decreased number of postsynaptic receptors correlate with a reduction in synaptic strength and plasticity (Bingol and Sheng, 2011). Actin polymerization is involved in LTP and actin depolymerization is involved in LTD (Matsuzaki et al., 2004; Okamoto et al., 2004; Zhou et al., 2004). Synaptic remodeling and cellular homeostasis requires proper protein turnover that is coordinated by protein synthesis and protein degradation (Bingol and Sheng, 2011).

Impaired LTP and induced LTD are associated with disease and defects in learning and memory (Hering and Sheng, 2001; Keifer and Zheng, 2010; Shepherd and Huganir, 2007). Abnormal synaptic communication resulting from changes in dendritic spine density and shape has been observed in a number of neurological disorders (Penzes et al., 2011). Defects in protein degradation lead to the accumulation of toxic
Figure 1.2. Synaptic Plasticity

Cartoon illustration of mechanisms underlying synaptic plasticity. During LTP there is an increase in synaptic strength and receptors at the synapse where as during LTD there is a decrease in synaptic strength and receptors at the synapse. Excitatory neurotransmission at the synapse is regulated by glutamate neurotransmitter release and two main types of glutamate-gated ion channels: AMPA and NMDA. Inhibitory neurotransmission at the synapse is regulated by γ-aminobutyric acid (GABA) neurotransmitter release and ionotropic GABA\(\_A\) receptors.
protein aggregates and are associated with neurological diseases. Reduced protein degradation is the result of defective protein clearance. Defects in the ubiquitin proteasome system (UPS), lysosomes, and autophagy have been associated with defective protein clearance and neurological disease (Bingol and Sheng, 2011). Protein aggregation, accumulation, and deposition are hallmarks of neurodegenerative diseases and are believed to be the result of impaired protein degradation pathways. Impaired synaptic plasticity is the result of degeneration of axons and dendritic spines leading to loss of synapses.

1.3 Excitatory and Inhibitory Neurotransmission

Communication at the synapse is mediated by neurotransmitter release from the presynaptic terminal and receptor activation at the postsynaptic terminal. Excitatory neurotransmission at the synapse is regulated by glutamate neurotransmitter release and two main types of glutamate-gated ion channels: \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and \(N\)-methyl-D-aspartate (NMDA) receptors (Collingridge et al., 2004; Keifer and Zheng, 2010; Pinheiro and Mulle, 2008; Rao and Finkbeiner, 2007). AMPA receptors are composed of combinations of four subunits (GluR1, GluR2, GluR3, GluR4) (Rao and Finkbeiner, 2007; Shepherd and Huganir, 2007). NMDA receptors are heterotetramers composed of combinations of NR1 subunit, NR2 subunits (NR2A, NR2B, NR2C, NR2D), and NR3 subunits (NR3A, NR3B) (Paoletti et al., 2013). An important scaffolding protein, postsynaptic density protein 95 (PSD-95) plays a pivotal role in LTP and LTD (Bhattacharyya et al., 2009; Chen et al., 2011). PSD-95 plays a functional role in maintaining molecular organization of excitatory dendritic spines. PSD-95 is the most-abundant scaffolding protein in postsynaptic densities, and is one of the most stable proteins in dendritic spines (Chen et al., 2011;
Kasai et al., 2010). Inhibitory neurotransmission at the synapse is regulated by $\gamma$-aminobutyric acid (GABA) neurotransmitter release and ionotropic GABAA receptors (D'Hulst et al., 2009). GABAA receptors are heteropentamers containing the common stoichiometry of two subunits of $\alpha$ and two $\beta$ and an additional subunit of $\gamma$ or $\delta$ (Belelli and Lambert, 2005). Variation of subunit composition of receptors allows for differential expression patterns, localization (extrasynaptic vs. synaptic), pharmacology and function. The role that subunit composition, differential expression patterns, extrasynaptic and synaptic localization plays in synaptic communication, function and dysfunction in neurological diseases has been elusive.

1.4 Controlling Synaptic Neurotransmission

Mammalian cells have ion channels that are activated by ligand binding, voltage, and temperature but they are not activated by light (Banghart et al., 2004). To control neural circuits, we can engineer ion channels to be activated or inhibited by light. Engineering ion-channels to respond to light enables exploration and manipulation of synaptic neurotransmission. Light is a non-invasive method that enables spatial and temporal resolution to control neural activity (Boyden et al., 2005; Fortin et al., 2008; Kramer et al., 2005; Kramer et al., 2009; Thompson et al., 2005). Advances in microscopy, optical physics and laser instrumentation allows for a beam of light of a certain wavelength to be focused on a subcellular region of a cell, on a single cell, or projected diffusely to regulate a network of cells thus activating or inhibiting ion channels enabling photocontrol of synaptic neurotransmission that regulates synaptic plasticity and behavior using light (Fortin et al., 2008; Kramer et al., 2005; Thompson et al., 2005).

Optical methods for controlling synaptic neurotransmission using light include: caged neurotransmitters, natural photosensitive proteins, and synthetic small molecule
photoswitches (Chambers and Kramer, 2008; Kramer et al., 2005; Thompson et al., 2005). These optical light sensitive tools offer precise, non-invasive control of neural activity. Caged neurotransmitters have a photolabile protecting group that renders them biologically inactive until the protecting group is removed or ‘uncaged’ upon exposure to light releasing the neurotransmitter (Ellis-Davies, 2007; Fortin et al., 2008; Kramer et al., 2009; Thompson et al., 2005). Caged neurotransmitters have several caveats: light-scattering limits the precision of uncaging, the uncaging of neurotransmitter is irreversible, prolonged uncaging can lead to receptor desensitization and local depletion of the caged neurotransmitter can limit long-term experiments (Fortin et al., 2008; Kramer et al., 2009). Advances in molecular biology led to gene delivery of the natural photosensitive protein Channelrhodopsin-2 (ChR2), a light-gated cation channel that contains the photoisomerizable molecule retinal to neuronal subtypes (Boyden et al., 2005; Zhang et al., 2006). Exposure to light triggers retinal isomerization leading to a conformational change in the photosensitive protein, which opens the channel thus enabling photocontrol of neurons. A caveat to natural photosensitive proteins is that the gene encoding the photosensitive protein needs to be delivered to the neurons of interest and the protein needs to be expressed and trafficked to the plasma membrane. Alternatively, photocontrol of neurons can be achieved through synthetic chemistry and the use of small molecule photoswitches.

Small molecule photoswitches have been used to convert ion channels to light-sensitive ion channels. Small molecule photoswitches are photoisomerizable molecules in which the conformation of the molecule changes in response to specific wavelengths of light. Azobenzenes as a small molecule photoswitch that have been commonly used to develop synthetically-photosensitive ion channels. Azobenzenes are often the small molecule photoswitch of choice because of their biological suitability, low phototoxicity,
ease of synthesis, and photochemical stability (Kramer et al., 2009). Azobenzenes photoswitch from an extended *trans* isomer conformation in response to illumination with visible light >460 nm and upon illumination with ultraviolet (UV) light at 380 nm convert to a *cis* bent isomer conformation approximately 7Å shorter than the *trans* isomer conformation (Figure 1.3) (Banghart et al., 2006; Feliciano et al., 2010). A ligand can then be coupled to the small molecule photoswitch to target the photoswitchable ligand to a receptor of interest by affinity labeling. In order to prevent the limitations of using a freely diffusible light-sensitive photoswitchable ligand often a molecule that can covalently bind the photoswitchable ligand to the receptor of interest is employed. Maleimide molecules are commonly used to covalently attach photoswitchable ligands to receptors by cysteine-reactivity to residues on the surface of the target protein. Specificity can be achieved by engineering a cysteine to a specific location on the receptor of interest rather than relying on endogenous cysteines. Genetic engineering of a cysteine to the appropriate location for target photoswitch tethering prevents diffusion of photo-tethered ligand and off target effects. Attachment of the small molecule photoswitch to the ion channel of interest enables photocontrol of receptor activity.

By employing a photoswitchable tethered ligand (PTL) to photocontrol ion channels the activity of a neuron can be modulated by light without diffusion of the PTL thus increasing the local concentration of the ligand near the target protein. This “drugs” activity can be turned on and off using specific wavelengths of light to control chemical signaling of receptors involved in learning and memory at synapses thus creating a light-gated ion channel that can be reversibly photocontrolled in response to irradiation with ultraviolet or visible light by altering the location of the ligand in relation to the ligand binding site (Figure 1.4). This PTL strategy has been used to photocontrol the activity of
Figure 1.3. Azobenzene Photoswitch

Illustration showing azobenzene isoform configuration to extended *trans* isomer in response to irradiation with visible light (>460 nm) and to bent *cis* isomer in response to irradiation with ultraviolet light (360-380 nm). Figure adapted from (Banghart et al., 2006).
Figure 1.4. Photocontrol of Ion Channels

Cartoon illustration of the photocontrol of ion channels. Tethered small molecule photoswitches enable remote photocontrol of neuron ion channels using specific wavelengths of light. Shown here photocontrol of neuronal firing of synthetic photoisomerizable azobenzene-regulated K⁺ (SPARK) channels. In green (illumination with visible light) spontaneous action potential firing occurs, and in purple (illumination with ultraviolet light) action potentials are silenced. Figure adapted from (Kramer et al., 2005).
glutamate receptors and shaker K⁺ channels (Banghart et al., 2006; Chambers et al., 2006; Volgraf et al., 2006). The PTL approach allows reversible photocontrol of neuronal receptors and can be applied to a variety of receptors using both genetic and chemical engineering. This technology allows for investigation of the role receptor subtypes play in synaptic communication and their function.

1.5 Alzheimer's disease

Alzheimer's disease (AD) is a chronic, progressive, neurodegenerative disease with no cure. AD is the most common form of dementia and the 6th leading cause of death in the United States (Thies et al., 2013). According to the 2010 US Census and the Chicago Health and Aging Project (CHAP) the estimated prevalence of AD is 11% of individuals older than 65 years of age have AD, and approximately 32% of people aged 85 and older have AD (Thies et al., 2013). Memory impairment is the earliest symptom of AD (Nestler et al., 2009; Thies et al., 2013). As disease progression occurs, cognitive impairment increases and eventually patients exhibit complete loss of independence (Nestler et al., 2009). It is estimated that by 2040, there will be 80 million people worldwide with AD (Nestler et al., 2009). The underlying cause or causes and the precise sequence of changes in the human brain that trigger the development of sporadic AD are unknown (Thies et al., 2013). Sporadic AD is believed to be a multifactoral, slow progressing disease in which the sequence of changes in the brain, and the accumulation of pathological proteins, leads to neuronal dysfunction that ultimately leads to neuronal death correlating with defects in memory and cognition, the symptoms of AD. These underlying changes are believed to occur between 10 and 20 years before any memory or cognitive impairment occurs (Gravitz, 2011; Thies et al., 2013).
In 1907, Alois Alzheimer first discovered and characterized the two pathological hallmarks of AD: neurofibrillary tangles (NFTs) and amyloid-beta (Aβ) plaques from the brain of a diseased patient (Spires-Jones et al., 2009). NFTs are intracellular aggregates of insoluble hyperphosphorylated tau whereas Aβ plaques are extracellular aggregates of Aβ produced from the proteolytic cleavage of the amyloid-β precursor protein (APP) by β and γ secretases (Figure 1.5) (Andorfer et al., 2003; Bordji et al., 2010; Mazanetz and Fischer, 2007). The accumulation of both of these aggregates correlate with loss of synapses and neuronal death, which leads to memory impairment, cognitive decline, and disease progression. Intracellular NFTs are believed to impair cellular transport of nutrients, cargos, and neurotransmitters whereas extracellular Aβ plaques are believed to interfere with synaptic communication (Thies et al., 2013). Additionally, it has been shown that neuron death leads to brain atrophy, reducing the size of an AD brain compared to a normal healthy brain (Santacruz et al., 2005).

Significant reduction in postsynaptic density protein 95 (PSD-95), AMPARs, and NMDARs are found in the post-mortem brains of AD patients (Keifer and Zheng, 2010; Proctor et al., 2011). This reduction in PSD-95, AMPARs, and NMDARs are associated with LTD and deficits in LTP. The reduction of postsynaptic scaffolding protein, PSD-95, is believed to contribute to AMPA receptor dependent LTP deficits in AD (Proctor et al., 2011). Loss of synapses and dendritic spines are a common pathology in human postmortem brains (Tackenberg et al., 2009). Additionally, synapse loss precedes neuron death indicating disruption of neuronal communication at the synapse is an early event in disease progression (Selkoe, 2002). The extent of disease progression and cognitive impairment in AD patients correlates with degree of synaptic loss (Terry et al., 1991). Synaptic dysfunction, LTP deficits and memory impairment have been observed
Figure 1.5. Pathological Hallmarks of Alzheimer’s Disease

Cartoon illustration of degenerating neuron in Alzheimer’s disease with intracellular neurofibrillary tangles (NFTs) accumulating in the soma and extracellular amyloid-beta (Aβ) plaques. The presynaptic axon terminal shows shrinkage representative of synaptic dysfunction in the degenerating neuron.
in AD mouse models before the accumulation of Aβ plaques and NFTs indicating synaptic dysfunction underlies the initial development of the disease (Oddo et al., 2003). Impaired synaptic plasticity is believed to be one of the earliest and prominent pathologies in an AD brain mediating cognitive defects and memory impairment (Penzes et al., 2011). Changes in postsynaptic strength contribute to synaptic dysfunction in AD. Decreased synaptic communication and synaptic dysfunction precedes neuronal death.

Abnormal accumulation of protein aggregates composed of Aβ plaques and NFTs in AD is believed to contribute to synaptic dysfunction and neuron death in late stages of AD progression. Impaired synaptic plasticity and spine loss is associated with accumulation of Aβ plaques and NFTs (Tackenberg et al., 2009). Defects in synaptic plasticity, impaired LTP and induced LTD have been associated with Aβ plaque and NFT accumulation (Ittner and Gotz, 2011; Malinow, 2012). Dysfunction of protein degradation clearance mechanisms is believed to contribute to AD progression. Ubiquitin-proteasome system (UPS) dysfunction, impaired lysosomal degradation, and impaired autophagy have been found in AD (Keller et al., 2000; Ling et al., 2009; Min et al., 2010; Wolfe et al., 2013). Since the discovery of these two pathological hallmarks, research has focused on trying to prevent the accumulation of aggregated Aβ plaques and NFTs in an effort to prevent the progression of AD (Gravitz, 2011). Each hallmark has been extensively studied individually; the focus has been on their separate modes of toxicity (Ittner and Gotz, 2011). Recent evidence has suggested synergistic roles in mediating AD progression and possible interactions between Aβ plaques and NFTs (Ittner and Gotz, 2011). Additionally it is believed that both Aβ plaques and NFTs directly contribute to defects in synaptic plasticity by impairing LTP and LTD.

NFTs are aggregates of hyperphosphorylated tau that are mistargeted to both the soma and dendritic spines (somatodendritic compartment). NFTs are implicated in
mediating Aβ toxicity and synaptic dysfunction in AD. Hyperphosphorylated tau in the somatodendritic compartment modulates the activity of the src-tyrosine kinase, Fyn, via the amino-terminal projection domain of tau (Ittner and Gotz, 2011; Hoover et al., 2010; Ballatore et al., 2007; Roberson et al., 2011). This Fyn-tau interaction is believed to target Fyn to dendritic spines (Ittner and Gotz, 2011). Fyn phosphorylates excitatory ionotropic glutamate N-methyl-D-aspartate receptors (NMDAR) subunit 2B (NR2B) facilitating interaction with postsynaptic density protein 95 (PSD-95) in the postsynaptic compartment (Ittner and Gotz, 2011). Phosphorylation of NMDAR can result in excitotoxic downstream signaling (Ittner et al., 2010). Hyperphosphorylated tau exhibits increased affinity for Fyn, resulting in increased localization of postsynaptic Fyn, phosphorylated NMDARs and potentially results in excitotoxic signaling (Bhaskar et al., 2005; Ittner and Gotz, 2011). This excitotoxic downstream signaling of NMDARs results in neurotoxicity, Ca²⁺ dysregulation, synapse loss, and increased Aβ toxicity in AD (Alberdi et al., 2010; Hoover et al., 2010; Ittner and Gotz, 2011; Malinow, 2012; Roberson et al., 2011).

Multiple studies have shown that Aβ plaques alter synapse formation, reduces surface expression of NMDAR and AMPARs, promotes NMDAR endocytosis, loss of dendritic spines, and induces Ca²⁺ dysregulation and neuronal death by activation of NMDARs and AMPARs (Alberdi et al., 2010; Hsieh et al., 2006; Liu et al., 2010; Penzes et al., 2011; Snyder et al., 2005). NMDARs at the synapse are neuroprotective whereas extrasynaptic NMDAR are neurotoxic (Hardingham and Bading, 2010). Compelling recent evidence from AD mouse models have implicated that in the absence of tau the postsynaptic targeting of Fyn is disrupted, decreasing both NMDAR-mediated excitotoxicity and Aβ postsynaptic toxicity (Ittner et al., 2010; Roberson et al., 2011). Additionally, hyperphosphorylated aggregated tau has increased association with Fyn
and this interaction has been implicated in mediating Aβ toxicity and synaptic dysfunction in AD (Bhaskar et al., 2005; Ittner et al., 2010; Roberson et al., 2011).

Clearly, these synergistic roles of Aβ plaques and NFTs enhance late stage AD progression. The degree of accumulation of both of these aggregates is an indicator of the level of synaptic dysfunction, memory impairment, cognitive decline, and disease progression. Targeting the accumulation of Aβ plaques and NFTs remains controversial. Many researchers disagree on which pathological hallmark is key to preventing AD progression and focus on targeting proteins involved in the accumulation Aβ plaques and NFTs. Other researchers believe Aβ plaques and NFTs are not the right target because they appear after synaptic dysfunction has occurred. AD is a complex multifactoral disease of dying neurons. The earliest known pathology contributing to AD progression is synaptic dysfunction (Oddo et al., 2003; Penzes et al., 2011). Some researchers have recently shifted to identifying and targeting underlying molecular factors leading to synaptic dysfunction, and ultimately Aβ plaques and NFTs accumulation.

A unique class IIb histone deacetylase (HDAC) with cytoplasmic localization, HDAC6 (Li et al., 2013; Xu et al., 2011), has emerged as a potential key player in a number of neurodegenerative diseases such as Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, and Amyotrophic lateral sclerosis (ALS). HDAC6 may be an underlying factor leading to synaptic dysfunction in these neurodegenerative diseases. Unlike other HDACs, HDAC6 has two catalytic deacetylase domains, a Ser Glu-peptide repeat domain (SE14), which acts as a cytoplasmic retention signal, and a BUZ domain that binds polyubiquitinated proteins (Ding et al., 2008; Li et al., 2013). HDAC6 is a versatile multifunctional enzyme responsible for deacetylating key proteins involved in protein degradation pathways, cellular stress, and cytoskeletal dynamics.
The role of HDAC6 in neurodegenerative disease is controversial as it is unclear if HDAC6 functions as neuroprotective or neurotoxic.

HDAC6 deacetylates two important cytoskeletal proteins, α-tubulin subunits of microtubules and cortactin, a filamentous actin binding partner (Hubbert et al., 2002; Zhang et al., 2007). Deacetylation of α-tubulin renders microtubules less stable and leads to depolymerization (Hubbert et al., 2002; Matsuyama et al., 2002). Deacetylation of cortactin inhibits binding to filamentous actin leading to actin depolymerization, impairing cell motility (Kaluza et al., 2011; Zhang et al., 2007). HDAC6 alters cytoskeletal dynamics by influencing depolymerization of key proteins involved in synaptic remodeling.

HDAC6 is an integral protein with diverse functions in mediating and coordinating cellular response to the cytotoxic accumulation of misfolded and aggregated proteins by interacting with polyubiquitinated proteins when UPS degradation is impaired by activating both autophagy and heat-shock proteins (Kalveram et al., 2008; Matthias et al., 2008; Pandey et al., 2007). Thus, HDAC6 is an important protein for coordinating the sequestration of protein aggregates and their clearance. Another cellular stress response that HDAC6 has is in redox regulation. Cellular stress leading to accumulation of reactive oxygen species can be detrimental to cell survival and are common in neurodegenerative diseases. HDAC6 deacetylates two redox regulatory antioxidant enzymes peroxiredoxin I (PRXI) and II (PRXII), which when deacetylated function to reduce H₂O₂ (Parmigiani et al., 2008).

Evidence from post-mortem AD human brains, AD animal models, and AD studies suggest that HDAC6 may be an underlying molecular factor that becomes neurotoxic when upregulated and thus contributes to AD pathology and progression. In
fact, immunoblots from post-mortem human AD brains have shown that HDAC6 protein levels are increased 52% in the cortex and 91% in the hippocampus compared to age-matched control brains (Ding et al., 2008). HDAC6 is known to mediate and coordinate cellular response to the cytotoxic accumulation of misfolded and aggregated proteins and in AD misfolded aggregated proteins accumulate yet UPS dysfunction, impaired lysosomal degradation, and impaired autophagy have been shown in AD (Keller et al., 2000; Ling et al., 2009; Min et al., 2010; Wolfe et al., 2013). A number of recent publications have illustrated that reduction or loss of HDAC6 reduces cognitive deficits in a mouse model for AD, alleviates abnormal tau accumulation, and rescues phosphorylated tau-mediated microtubule instability (Cook et al., 2012; Govindarajan et al., 2013; Xiong et al., 2013). This data suggest that increased expression of HDAC6 is neurotoxic in AD and that targeting HDAC6 may be a potential strategy for preventing the progression of AD. The question remains whether HDAC6 is an underlying molecular factor leading to synaptic dysfunction, and ultimately NFTs and Aβ plaques accumulation.

HDAC6 has previously been shown to interact with a key protein phosphatase, protein phosphatase 1 (PP1), via HDAC6’s second catalytic domain (Brush et al., 2004). PP1 is an important phosphatase responsible for removing phosphate groups from proteins, regulating their function. One protein regulated by PP1 is tau. Tau is a microtubule-associated protein (MAP), primarily expressed in neurons that promote microtubule assembly and stabilization (Iqbal et al., 2005). PP1 has been shown to interact and dephosphorylate tau, increasing the affinity of tau for microtubules and stabilizing them (Liao et al., 1998). Dysregulated tau homeostasis results in increased tau phosphorylation and subsequently abnormally hyperphosphorylated tau with reduced affinity for microtubules and this form of hyperphosphorylated tau forms NFT in AD.
(Andorfer et al., 2003; Spires-Jones et al., 2009). Interestingly, a domain-selective small-molecule enzyme inhibitor tubacin, targets the second catalytic domain of HDAC6 preventing HDAC6 from deacetylating α-tubulin (Haggarty et al., 2003). In vitro inhibition of HDAC6 by tubacin was shown to attenuate tau phosphorylation, however, it is unclear if tubacin disrupted an HDAC6-PP1 interaction enabling PP1 to dephosphorylate tau (Ding et al., 2008). Additionally, shRNA-mediated knockdowns of HDAC6 were shown to attenuate tau phosphorylation suggesting HDAC6 regulates PP1 activity (Ding et al., 2008). These data suggest that overexpression of HDAC6 alters PP1 enzymatic activity thus creating an imbalance of kinase and phosphatase activity that leads to the aberrant hyperphosphorylation of tau. A proposed model of HDAC6 mediated AD pathology and synaptic dysfunction is shown in Figure 1.6.
Figure 1.6. Model of HDAC6 Mediated Alzheimer's Disease Pathology

Cartoon illustration of HDAC6 mediated AD pathology and synaptic dysfunction in degenerating neuron. An increase in HDAC6 protein levels leads to decreased PP1 activity. The changes in HDAC6 protein levels and PP1 activity lead to an increase in pathological hyperphosphorylated tau assembling into intracellular neurofibrillary tangles (NFTs) which accumulates in the soma of the degenerating neuron along with extracellular accumulation of amyloid-beta (Aβ) plaques. These changes ultimately lead to synaptic dysfunction in the degenerating neuron.
1.6 Motivation

The overall goals of this thesis are to develop novel strategies to understand underlying mechanisms of synaptic communication, and learning and memory. The mammalian brain is a complex system with very few tools to study how memories are formed, the function of receptor subtypes, and changes that lead to disease. By developing novel strategies to study neural activity using pharmacology tools and genetics, we can perturb the system and discover the role of receptor subtypes in controlling neural activity and the role of specific proteins in disease progression. By developing novel photoswitchable tethered ligands photocontrol of specific ion channels can be achieved, allowing the discovery of the role those receptors have in regulating learning and memory at the synapse.

Efforts by scientists and pharmaceutical companies to target AD pathology and progression late in the disease have been unsuccessful in preventing disease progression in humans. Early changes that lead to AD pathology and the discovery of underlying molecular factors leading to synaptic dysfunction and AD progression have been elusive. Most of what is known about AD progression and pathology comes from post-mortem adult late-stage AD brains and animal models developed to investigate plaque and tangle modes of toxicity, disease progression and memory impairment and cognitive decline. Both models are only snapshots of the disease pathology. The goal here is to identify key changes that lead to the pathological hallmarks of AD and early changes that correlate with synaptic dysfunction by developing a neuron cell culture model system.
CHAPTER 2
PHOTOCONTROL OF GABA\textsubscript{A} RECEPTORS

The work presented in this chapter was an interdisciplinary collaboration project between members of James J. Chambers laboratory at UMASS Amherst, which included Devaiah Vytla and members of Richard H. Kramer’s laboratory at University of California Berkeley, which included Wan-Chen Lin, Christopher M. Davenport, Alexandre Mourot, and Caleb M. Smith. Parts of this chapter are from several manuscripts in preparation or under current review.

2.1 Introduction

Controlling synaptic neurotransmission with a phototethered ligand (PTL) has been utilized to photosensitize glutamate channels, K\textsuperscript{+} channels, and nicotinic acetylcholine receptors (Banghart et al., 2004; Lester et al., 1980; Volgraf et al., 2006). The major inhibitory neurotransmission in the brain is controlled by activation of the GABA receptor by GABA neurotransmitter. The GABA receptor is an untouched target for a PTL. Ionotropic GABA\textsubscript{A} receptors are important drug targets in the treatment of anxiety disorders, sleep disorders, epilepsy and many drugs of abuse, including alcohol, barbiturates, and benzodiazepines (Jones-Davis and Macdonald, 2003; Atack, 2005; Mohler, 2006; Korpi et al., 2002; Rudolph and Knoflach, 2011; Smith and Rudolph, 2012). Nineteen genes encoding GABA\textsubscript{A} receptor subunits have been identified: \(\alpha\textsubscript{1-6}, \beta\textsubscript{1-3}, \gamma\textsubscript{1-3}, \delta, \epsilon, \theta, \pi, \) and \(\rho\textsubscript{1-3}\) (Barnard et al., 1998; Olsen and Sieghart, 2008). Subunit composition of GABA\textsubscript{A} receptor subtypes determines subcellular localization, expression patterns, function and pharmacology (Olsen and Sieghart, 2008). The most common subunit composition for GABA\textsubscript{A} receptors are two subunits of \(\alpha\) and two \(\beta\) and an additional subunit of \(\gamma\) or \(\delta\) (Belelli and Lambert, 2005). The \(\alpha\)-subunit is important for
receptor localization and channel gating kinetics, and with the β-subunit forms the
GABA-binding site (Mohler, 2006; Rudolph and Knoflach, 2011). GABA\textsubscript{A} receptors
containing the α1-subunit are the most common α-isoform in the brain (Olsen and
Sieghart, 2008). The function of two α-isoforms, α1 and α5 subunits, were of particular
interests to us due to their distinct expression patterns in the hippocampus and
pharmacology. GABA\textsubscript{A} receptors containing α1-subunits are believed to detect
presynaptically released GABA and mediate transient "phasic" inhibition, where as
GABA\textsubscript{A} receptors containing α5-subunits are believed to mediate persistent “tonic"
inhibition (Farrant and Nusser, 2005).

Identifying the physiological function of receptor subtypes in synaptic
neurotransmission has been elusive due to the paucity of current pharmacological tools
that target specific subtypes and inability to prevent compensatory changes in gene
expression of other isoforms or related proteins in subtype specific gene-knockout
animals (Kralic et al., 2002; Mohler, 2006; Ortinski et al., 2006; Rudolph and Mohler,
2004; Rudolph and Knoflach, 2011). To overcome these obstacles in discovering the
function of GABA\textsubscript{A} receptor subtypes in synaptic neurotransmission, we developed a
series of photochemical tools for precise and selective manipulation of specific subtypes
of GABA\textsubscript{A} receptors. We designed a chemical-genetic approach to enable target-
specific photocontrol of neuronal inhibition. We engineered light-regulated GABA\textsubscript{A}
receptors (LiGABA\textsubscript{A}R) by employing a PTL strategy for creating light-sensitive α1- and
α5-GABA\textsubscript{A} receptors.
2.2 Results

2.2.1 PTL design and photoisomerization of MAM-6

The design of a PTL that targeted GABA$_A$ receptor was based on previous GABA$_A$R probes in which the amino group of a potent GABA$_A$ receptor agonist muscimol was linked to biotin or a fluorophore (Vu et al., 2005). The PTL design contained an azobenzene photoswitch, which allowed photoisomerization from a trans confirmation upon irradiation with 500 nm to cis conformation upon irradiation with 380 nm. The azobenzene photoswitch enabled the conformation of the PTL to change from an extended trans conformation or retracted cis conformation. Additionally, our PTL design contains a maleimide group for covalent attachment to genetically engineered cysteine mutant GABA$_A$ receptors. The PTL was named MAM-6 for maleimide, azobenzene, and muscimol linked by a 6-carbon spacer. This spacer enables muscimol to enter the GABA-binding pocket. An illustration of the photoisomerization of MAM-6 is shown in Figure 2.1.
Figure 2.1. Photoisomerization of MAM-6

Scheme depicting the photoisomerization of MAM-6. The PTL reversibly photoisomerizes between elongated trans confirmation upon irradiation with 500 nm visible light (green) and retracted cis confirmation upon irradiation with 380 nm ultraviolet light (violet).
2.2.2 Attachment sites and photoregulation of MAM-6 in $\alpha_1$- and $\alpha_5$-subunit

Potential PTL attachment sites for optimal photoregulation were screened using the $\alpha_1$-containing GABA$_A$ receptor, the most common isoform in the brain. We focused on a region of the $\alpha_1$ subunit that is close to the GABA-binding pocket, a region between the $\alpha$- and $\beta$-subunits. Residues facing towards the GABA-binding site were chosen as PTL attachment sites, residues in yellow were cysteine substituted to make a library of single-cysteine mutant $\alpha_1$-subunits (Figure 2.2A). Initial testing of the activity of the PTL was done in an exogenous system where cysteine mutant receptors of interest were co-expressed with wild-type $\beta_2$-subunit to form functional receptors in Xenopus oocytes. Muscimol is an agonist of GABA$_A$ receptors, yet we found mutant receptors tethered with MAM-6 were not activated by irradiation with either 380 nm or 500 nm light but were inhibited (Figure 2.2B). The amplitude of the GABA response decreased under 500 nm visible light conditions when MAM-6 was in an extended trans conformation and the current increased when MAM-6 was in a retracted cis conformation under 380 nm ultraviolet light conditions (Figure 2.2B). Thus, tethered MAM-6 functioned as an antagonist under 500 nm visible light conditions.

Next, we made a library of single-cysteine mutant $\alpha_5$-subunits that were homologous to screened residues of $\alpha_1$-subunits facing towards the GABA-binding site (Figure 2.2A, Figure 2.2C). We screened for photoregulation to identify the optimal PTL attachment site in $\alpha_1$- and $\alpha_5$ cysteine mutant receptors. Tethered MAM-6 enabled photoregulation of both $\alpha_1$- and $\alpha_5$-cysteine mutant receptors (Figure 2.2D) as the photoregulation index, which is a ratio of GABA-elicited currents in 380 nm vs. 500 nm ($I_{380}/I_{500}$). The identified PTL attachment sites that substantially photoregulated GABA-elicited current were ($\alpha_1$: S68C, T121C, T125C and $\alpha_5$: S72C).
Figure 2.2. PTL Attachment Sites and Photoregulation of Engineered $\alpha_1$- and $\alpha_5$ Receptors

A) Homology model of $\alpha_1\beta_2$ subunits showing MAM-6 attachment sites with cysteine mutated residues in yellow and the GABA binding site with docked muscimol in red.

B) Representative traces from *Xenopus oocytes* expressing $\alpha_1$(S68C)$\beta_2$ treated with MAM-6 showing reversible photoregulation of GABA-elicited current.

C) Sequence alignment with residues chosen for cysteine substitution mutation sites shown in red for $\alpha_1$ and $\alpha_5$. Residues homologous to tested $\alpha_1$ cysteine-mutants were chosen as sites for cysteine substitution in $\alpha_5$.

D) Photoregulation after MAM-6 treatment of $\alpha_1$ or $\alpha_5$ mutant receptors expressed in *Xenopus oocytes*. Each mutant was co-expressed with wild-type $\beta_2$ subunit to form the GABA-binding site. Cysteine-mutated residues for $\alpha_1$ or $\alpha_5$ mutant receptors indicated in blue. The ratio (mean ± SEM) of current amplitude (from 3 $\mu$M GABA, -80 mV) elicited from irradiation with 380 nm vs. 500 nm ($I_{380}/I_{500}$) light was used as an index of photoregulation, where a ratio of 1 indicates no light sensitivity of the tested cysteine-mutant receptor.
2.2.3  *Trans* MAM-6 inhibits LiGABA<sub>α</sub>R after PTL treatment

Next, we determined if binding of the PTL altered receptor sensitivity to GABA-elicited current. Whole-cell patch clamp electrophysiology of GABA-elicited current from *Xenopus oocytes* expressing α1(T125C)β2 showed that GABA responses before and after PTL attachment are similar to GABA responses in the retracted *cis* conformation whereas in the extended *trans* conformation GABA responses are reduced after PTL attachment (Figure 2.3). The GABA responses to 300 µM GABA before and after PTL attachment indicate negligible changes in receptor activity during the experiment. Only in the presence of externally applied GABA was the receptor activated, irradiation with light did not trigger receptor activation. Attachment of the PTL to genetically engineered cysteine mutant receptors created light-regulated GABA<sub>α</sub> receptors (LiGABARs).

To determine if cysteine substitution or PTL attachment affected receptor sensitivity to GABA we investigated GABA sensitivity of untreated, MAM-6 treated α-cysteine engineered mutant receptors to that of untreated wild-type α1β2 receptors or wild-type α5β2 receptors. The effect of MAM-6 attachment or engineered cysteine residue substitution on receptor function was evaluated by quantifying the change in current ratio elicited from responses to 3 µM to 3 mM GABA (Table 1). We determined that the following cysteine mutation sites for α1: 41, 68, 171 had lower sensitivity to GABA compared to wild-type and therefore these cysteine mutants were not optimal sites for PTL attachment to study receptor subtype function in neurons because receptor sensitivity was altered (Table 1). Further, the α1(T125C) mutation was chosen as the optimal PTL attachment site because it allowed substantial photoregulation of α1 GABA receptors without altering receptor sensitivity to GABA before or after PTL attachment (Table 1). For α5 GABA receptors we determined the α5(S72C) mutation as the optimal PTL attachment site.
Figure 2.3. Attachment of *Trans* MAM-6 Inhibits Engineered GABA<sub>A</sub> Receptors

Representative trace of GABA responses from a *Xenopus* oocyte expressing α1(T125C)β2. The oocyte was first exposed to 3 µM GABA followed by 300 µM GABA, then treated with 50 µM MAM-6 for 10 minutes to allow for cysteine tethering of the PTL. After MAM-6 treatment the oocyte was irradiated with 500 nm light during 3 µM GABA exposure followed by irradiation with 380 nm light during 3 µM GABA exposure, and finally treatment with 300µM GABA during irradiation with 380 nm light.
Table 1: GABA sensitivity of untreated and MAM-6 treated engineered α-mutants

<table>
<thead>
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<th>mutation site</th>
<th>MAM-6 attached receptor</th>
<th>untreated receptor</th>
<th>comments</th>
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</thead>
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<tr>
<td></td>
<td>$I_{300}/I_{380}$ (mean ± SEM)</td>
<td>$I_{3µM}/I_{3mM}$ (380 nm) (mean ± SEM)</td>
<td>trials</td>
</tr>
<tr>
<td>α1wild-type</td>
<td>1.08 ± 0.02 (untreated)</td>
<td>-- --</td>
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</tr>
<tr>
<td>41</td>
<td>0.62 ± 0.03</td>
<td>0.41 ± 0.03</td>
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</tr>
<tr>
<td>171</td>
<td>0.63 ± 0.03</td>
<td>0.20 ± 0.01</td>
<td>4</td>
</tr>
<tr>
<td>α3wild-type (untreated)</td>
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<td>--</td>
<td>3</td>
</tr>
<tr>
<td>92</td>
<td>0.95 ± 0.10</td>
<td>0.22 ± 0.02</td>
<td>11</td>
</tr>
</tbody>
</table>

a. Current ratio was measured at 3 µM GABA (500 nm vs. 380 nm).
b. The effect of MAM-6 attachment or cysteine substitution on receptor function was evaluated by the change in current ratio at 3 µM GABA vs. 3 mM GABA. The EC$_{50}$ of wild-type α1β2 receptors is ~3 µM.
c. The mutant receptor’s sensitivity to GABA is lower than that of the wild-type α1β2 receptors (indicated by $I_{3µM}/I_{3mM}$).
d. The receptor’s sensitivity to GABA is reduced in both 500 nm and 380 nm lights after MAM-6 attachment.
e. The receptor’s sensitivity to GABA is unaltered in 380 nm light after MAM-6 attachment. This feature makes α1(T125C) the best mutant for downstream applications in neurons.
2.2.4 Identification of critical PTL components for LiGABA\textsubscript{AR} control

To determine the critical components required for the PTL to photoregulate GABA receptors of interest we altered the structure of the MAM-6 PTL design. Both the muscimol ligand and spacer moieties of MAM-6 were altered and the resulting PTLs were tested for their ability to photoregulate 10 \( \mu \text{M} \) GABA sensitivity of \( \alpha_1(\text{T125C}) \) GABA\textsubscript{A} receptors or \( \alpha_5(\text{S68C}) \) GABA\textsubscript{A} receptors. Muscimol is negatively charged at physiological pH, enabling receptor binding. Consistent with this mechanism, replacing muscimol with a neutral analogue 4-hydroxylbenzylamine reduced the photoregulation effect in \( \alpha_1(\text{T125C}) \) GABA\textsubscript{A} receptors; this PTL was named MAB-6 (Figure 2.4A, Figure 2.4B). Photoregulation was nearly completely suppressed in \( \alpha_1(\text{T125C}) \) GABA\textsubscript{A} receptors when the 6-carbon spacer of MAM-6 was removed; this PTL was named MAM-0 (Figure 2.4A, Figure 2.4B). Surprisingly, removal of the 6-carbon spacer and substituting muscimol with neutral analogue 4-hydroxylbenzylamine (MAB-0), increased the photoregulation effect in \( \alpha_1(\text{T125C}) \) GABA\textsubscript{A} receptors, resulting in an effect greater than that by MAM-6, suggesting that the muscimol ligand was not a required component of the PTL design for photoregulation (Figure 2.4A, Figure 2.4B). Interestingly, \( \alpha_5(\text{S68C}) \) GABA\textsubscript{A} receptors were only photoregulated with MAM-6 attachment, suggesting both a flexible linker and a negatively charged ligand are required for photoregulation of \( \alpha_5 \) receptors (Figure 2.4C). In summary, screening of engineered cysteine-mutant receptors and PTLs revealed the best combination for creating light-regulated GABA\textsubscript{A} receptors (LiGABARs) was \( \alpha_1(\text{T125C}) \) tethered to MAB-0 and \( \alpha_5(\text{S68C}) \) tethered to MAM-6.

The strong photoregulation by MAB-0 suggests that its ligand binds strongly to the GABA-binding pocket when the azobenzene is photoswitched to the \textit{trans} isomer.
suggesting MAB-0 prevents GABA binding by blocking the GABA-binding site. To investigate the increased photoregulation effect of MAB-0 in α1(T125C) GABA\textsubscript{A} receptors, we used molecular modeling to dock *trans* and *cis* MAB-0. Docking *trans* MAB-0 in a homology model of α1(T125C)β2 dimer suggested that the PTL spans the GABA binding site and the binding pocket (Figure 2.4D, top). Aromatic residues in the GABA-binding pocket are indicated in yellow (Figure 2.4D). *Trans-to-cis* isomerization of azobenzene retracts MAB-0 from the GABA binding site subsequently relieving receptor antagonism (Figure 2.4D, bottom).
Figure 2.4. Critical Components of PTL for Photocontrol of LiGABA\_\text{Rs}

A) Series of PTLs

B) Photoregulation of $\alpha_1$(T125C)$\beta_2$ receptors with MAM-6, MAB-6, MAM-0 and MAB-0. The ratio (mean ± SEM) of current amplitude (elicited from 10 $\mu$M GABA, -70 mV, HEK293T cells) elicited from irradiation with 380 nm vs. 500 nm ($I_{380}/I_{500}$) light was used as an index of photoregulation, where a ratio of 1 indicates no light sensitivity of the tested cysteine-mutant receptor.

C) Photoregulation of $\alpha_5$(S68C)$\beta_2$ receptors with MAM-6, MAB-6, MAM-0 and MAB-0. The ratio (mean ± SEM) of current amplitude (elicited from 10 $\mu$M GABA, -80 mV, Xenopus oocytes) elicited from irradiation with 380 nm vs. 500 nm ($I_{380}/I_{500}$) light was used as an index of photoregulation, where a ratio of 1 indicates no light sensitivity of the tested cysteine-mutant receptor.

D) Docking pose of trans or cis MAB-0 in a homology model of $\alpha_1\beta_2$ dimer. A positional constraint was used to mimic tethering at $\alpha_1$(T125C) shown in orange. Aromatic residues in the GABA-binding pocket are in yellow.
2.2.5 Photoregulation of mIPSC of $\alpha_1$- and $\alpha_5$-GABA$\alpha$ receptors

Once the optimal PTL design and attachment site for $\alpha_1$- and $\alpha_5$-GABA$\alpha$ receptors was determined, we investigated if synaptic inhibition in primary neurons could be controlled optically with these LiGABA$\alpha$Rs. These experiments were to test if $\alpha_1$- and $\alpha_5$-GABA$\alpha$ enable photoregulation of phasic and tonic inhibition. Primary dissociated cultures of hippocampal neurons isolated from rat brains were transfected with a bicistronic construct encoding either $\alpha_1$(T125C) or $\alpha_5$(S72C), along with eGFP as a transfection marker. When these neurons were synaptically active, between DIV 17-21 days, they were treated with MAB-0($\alpha_1$) or MAM-6($\alpha_5$) for 15 minutes. To investigate phasic inhibition, we acquired whole-cell patch clamp electrophysiology recordings of endogenous mini inhibitory postsynaptic currents (mIPSCs) from neurons expressing $\alpha_1$(T125C) or $\alpha_5$(S72C) after PTL treatment (Figure 2.5A). Both $\alpha_1$(T125C) or $\alpha_5$(S72C) LiGABA$\alpha$Rs enabled photoregulation of endogenous mIPSCs. The amplitude of the mIPSC were increased upon irradiation with 380 nm light when the PTL was in the retracted cis conformation whereas the amplitude decreased upon irradiation with 500 nm light when the PTL was in the extended trans conformation (Figure 2.5A, Figure 2.5B). To quantify the amount of photoregulation, the average mIPSC when the PTL was in cis or trans conformation was calculated. Neurons with $\alpha_1$(T125C)-LiGABA$\alpha$Rs, cis-to-trans photoisomerization had a 38 ± 2% decrease in peak amplitude and a 57 ± 4% decrease in total charge transfer ($n = 6$, $P < 0.001$; Figure 2.5B, Figure 2.5C). Neurons with $\alpha_5$(S72C) LiGABA$\alpha$Rs cis-to-trans photoisomerization had a 29 ± 3% decrease in peak amplitude and 34 ± 4% decrease in total charge transfer ($n = 6$, $P < 0.001$; Figure 2.5B, Figure 2.5C).
Figure 2.5. LiGABA_4R Mediated Photoregulation of mIPSCs

A) Representative traces of mIPSC recordings from a cultured hippocampal neuron containing MAB-0 tethered to α1(T125C) subunits (top) or MAM-6 tethered to α1(S68C) subunits (bottom). Neurons were held at -60 mV. To convert the PTL to its cis isomer, cells were exposed to 380 nm light for 5 seconds, then no light where the majority of the PTL remains in the cis conformation.

B) Representative average mIPSC traces when the PTLs were in the trans (green trace) or cis (violet trace) conformation from the cells shown in A. MAB-0 tethered to α1(T125C) subunits (top panel) or MAM-6 tethered to α1(S68C) subunits (bottom panel).

C) Group data (mean ± S.E.M.) of mIPSC photoregulation in cultured hippocampal neurons (n = 6 for each isoform). The degree of photoregulation was quantified as the percent decrease (in peak amplitude or charge transfer) when the PTL was photoswitched from cis to trans.
2.2.6 MAB-O selectivity

Control experiments were completed to confirm that PTL were target-specific and did not photoregulate or confer light sensitivity on wild-type neurons. These control experiments were completed to confirm off-target receptors were not altering their function in response to light. These controls were necessary because the PTL contains a maleimide moiety could potentially allow for cysteine tethering to receptors containing endogenous cysteine residues. Treatment of PTL on wild-type neurons did not photoregulate wild-type GABA$_A$ receptors, ionotropic glutamate receptors, or voltage-gated channels, confirming that photocontrol is specific to the engineered LiGABA$_A$R (Figure 2.6). The designed PTL only photosensitizes the mutant receptor containing the $\alpha$ subunit of interest.
Figure 2.6. MAB-0 Treatment Does Not Photosensitize Endogenous Ligand-gated or Voltage-gated Ion Channels

A) Average mIPSC traces from representative neuron following MAB-0 treatment does not photosensitize endogenous GABA\(_A\)Rs in cultured hippocampal neurons. \(I_{500}/I_{380} = 1.00 \pm 0.02 (V_{\text{hold}} = -60 \text{ mV}; n = 3, P = 0.87)\).

B) Average mEPSC from representative neuron following MAB-0 treatment does not photosensitize endogenous GABA\(_A\)Rs in cultured hippocampal neurons. \(I_{500}/I_{380} = 0.97 \pm 0.10 (V_{\text{hold}} = -70 \text{ mV}; n = 4, P = 0.64)\).

C) MAB-0 treatment does not photosensitize endogenous AMPARs in a hippocampal slice. Evoked EPSC traces from a CA1 pyramidal neuron are shown. \(I_{500}/I_{380} = 1.02 \pm 0.01 (V_{\text{hold}} = -70 \text{ mV}; n = 4, P = 0.2)\).

D) MAB-0 treatment does not photosensitize endogenous NMDARs in a hippocampal slice. Evoked EPSC traces from a CA1 pyramidal neuron are shown. Currents were measured 100ms after the stimulus. \(I_{500}/I_{380} = 0.95 \pm 0.04 (V_{\text{hold}} = +40 \text{ mV}; n = 3, P = 0.3)\).

E) MAB-0 treatment does not photosensitize endogenous voltage-gated Na\(^+\) channels. Inward currents (elicited by a 200-ms depolarization to \(-10 \text{ mV}\)) from a representative neuron are shown. \(I_{500}/I_{380} = 0.99 \pm 0.05 (V_{\text{hold}} = -70 \text{ mV}; n = 4, P = 0.87)\).

F) MAB-0 treatment does not photosensitize native voltage-gated K\(^+\) channels. Voltage-gated K\(^+\) currents (elicited by a 200-ms depolarization to +40 mV) from a representative neuron are shown. \(I_{500}/I_{380} = 0.98 \pm 0.05 (V_{\text{hold}} = -70 \text{ mV}; n = 4, P = 0.43)\).

All traces in 380 nm and 500 nm are shown in violet and green colors, respectively.
2.2.7 Photocontrol of neuron excitability

The goal of designing PTL to target subtype specific α receptors was to discover the function of GABA\textsubscript{A} receptor subtypes in synaptic neurotransmission using photochemical tools for precise and selective manipulation of specific subtypes of GABA\textsubscript{A} receptors. GABA\textsubscript{A} receptors have a crucial function in preventing runaway neuronal excitation. Pharmacological inhibition or knock-out of GABA\textsubscript{A} receptors are known to alter GABA\textsubscript{A} function and result in epileptic brain activity. To determine if epileptic activity can be induced by photo-antagonizing α1(T125C) LiGABA\textsubscript{A}R in intact brain tissue we introduced α1(T125C) LiGABA\textsubscript{A}R into hippocampal brain slices using AAV. After MAB-0 treatment we applied a train of presynaptic stimuli and recorded action potential firing of neurons irradiated with 380 nm and 500 nm light. Photocontrol of neural inhibition of α1(T125C) LiGABA\textsubscript{A}R irradiated with 500 nm light where the PTL was in an extended confirmation led to photocontrol α1(T125C) LiGABA\textsubscript{A}R that prevented of action potential firing, a characteristic of epileptiform activity (Figure 2.7). Irradiation of the α1(T125C) LiGABA\textsubscript{A}R with 380 nm reversed this effect enabling action potential firing and prevention of epileptic activity (Figure 2.7).
Figure 2.7. LiGABA_4 Receptors Enable Photocontrol of Neuron Excitability

Whole-cell current-clamp recording of CA1 pyramidal neuron expressing α1(T125C) receptors treated with MAB-0 enabled photocontrol of neuron excitability. Irradiation with 500 nm light resulted in an “epileptic” plateau preventing action potential firing that was reversed upon irradiation with 380 nm light. Traces in 380 nm shown in violet and 500 nm are shown in green.
2.3 Discussion

We developed a series of LiGABAₐRs that enable light-controlled inhibitory neurotransmission. The α₁- and α₅-LiGABAₐRs developed here are distinct from previously developed photochemical tools for manipulating neural inhibition such as caged GABA in that LiGABAₐRs allow specificity and for native forms of inhibitory neurotransmission to be controlled by light. The LiGABAₐRs developed here provide a unique opportunity for spatiotemporal precision to investigate the underlying mechanisms of subtype-specific neural inhibition. Designing PTLs and genetically-engineering GABAₐ receptors enabled specific spatiotemporal precision to investigate and manipulate receptor-subtype contributions in inhibitory neurotransmission. We have developed a method for photocontrolling GABAₐ receptors that is fast, reversible and subtype-specific.

PTLs have the ability to target surface exposed cysteines on endogenous receptors and engineered-cysteine substituted receptors. The results of our experiments showed that our PTLs only conferred light-sensitivity and photocontrol on engineered receptors. Wild-type GABAₐ receptors and other off-target ion-channels were not photoregulated by these PTLs demonstrating that the PTLs in our experiments were specific to receptor subtypes of interest. Further, cysteine substitutions of α₁ and α₅ GABAₐ receptors with subsequent PTL attachment enabled photocontrol of GABAₐ receptors. PTL attachment sites had varying degrees of photocontrol. The optimal PTL attachment site and subsequent PTL attachment enabled substantial photocontrol of inhibitory neurotransmission without altering the receptors GABA sensitivity.

The results illustrate tethered muscimol acts as an antagonist when the PTL is irradiated with 500 nm light on genetically-engineered cysteine substituted GABAₐ.
receptors. The PTL design was based on reports that muscimol conjugates in which the amino group was linked to biotin or a fluorophore remained a GABA<sub>A</sub> receptor agonists (Vu et al., 2005). Receptor antagonism by tethered muscimol may be the result of changes to the moiety that render unable to induce protein conformation changes required for channel gating. Loss of the critical ammonium group of agonists are know to prevent hydrogen bonding which prevents changes required for channel gating (Cromer et al., 2002). The tethering of muscimol due to N-acylation leads to the loss of an ammonium group that may disrupt conformational changes required for channel gating. Additionally, the PTL may disrupt binding at the α-β interface by blocking or restricting binding at the GABA-binding site. In fact, removal of the muscimol ligand and the flexible linker in MAB-0 enabled precise photocontrol of genetically engineered α1 receptors suggesting delivery of a terminal phenol group to the GABA-binding site can disrupt channel gating. Further modifications to PTL designs that provide functional groups that interact more favorably with the GABA-binding site have the potential to enhance the photoregulation effect of PTLs.

Hippocampal neurons expressing LiGABA<sub>A</sub>R enabled photoregulation of endogenous mIPSCs. Subtype-specific LiGABA<sub>A</sub>R enable investigation into the role α1 and α5 have in mediating phasic and tonic inhibition. LiGABA<sub>A</sub>R photoregulation will provide a precise method to investigate the distribution of sub-type specific receptors and examine their functional role in leaning and memory. Additionally LiGABA<sub>A</sub>R in intact hippocampal brain slices enabled photocontrol of action potential firing. This result suggests that LiGABA<sub>A</sub>R can be utilized to exert light-switchable control on native receptors in the brain to investigate the role specific subtypes of GABA<sub>A</sub> receptors have in preventing epilepsy.
2.4 Methods

2.4.1 Plasmid preparation

2.4.1.1 Wild-type and mutant cDNAs

The cDNAs of wild-type rat GABA\(_{A}\)R \(\alpha1\) (in pGH19), \(\beta2\) (in pGH19), and \(\gamma2S\) (in pUNIV) were obtained from Professor Cynthia Czajkowski (University of Wisconsin, USA). The \(\alpha5\) wildtype subunit was obtained in vector pRK7 from Dr. Hartmut Luddens (University of Mainz, Germany). The genes of \(\alpha\) and \(\beta\) subunits were sub-cloned into vector pCDNA3.1(-) for expression in HEK293T cells and *Xenopus oocytes*. Mutants \(\alpha1(K70C)\) and \(\alpha1(D123C)\) (in pGH19) were obtained from Professor Cynthia Czajkowski (University of Wisconsin-Madison). Other cysteine mutants were prepared by site-directed mutagenesis of the wild-type \(\alpha1\) and \(\alpha5\) clones in pCDNA3.1(-) using oligonucleotide primers under the following PCR profile: 1 cycle (95°C for 30s); 18 cycles (95°C for 50s, 60°C for 50s, 68°C for 9min); 1 cycle (68°C for 7min). Mutations were confirmed by sequencing.
2.4.1.2 Adeno-associated virus construction of pAAV-hSyn-EGFP-2A-myc-α1(T125C)

The construct is designed to express GFP and α1(T125C) in a 1:1 ratio using the 2A protein sequence (GSGATNFSLKQAGDVEENPGP) (Provost et al., 2007) and to restrict expression to neurons using the human synapsin promoter (Kugler et al., 2003). The vector also contains terminal repeats (TR) and woodchuck hepatitis post-transcriptional regulatory element (WPRE) for packaging into the adeno-associated virus (AAV). The human synapsin promoter was chosen because it allows powerful neuron-specific expression of genes, and is easily delivered via an adenoviral vector. Human synapsin 1 gene promoter confers highly neuron-specific long-term transgene expression from an adenoviral vector in the adult rat brain depending on the transduced area (Kugler et al., 2003). The myc epitope (protein sequence EQKLISEEDL) was inserted into the α1(T125C) subunit as previously described (Connolly et al., 1996). The pAAV vector backbone was digested with NheI and HindIII and ligated with the 2A-α1 and GFP-2A fragments. The final construct was confirmed with sequencing.

2.4.1.3 Preparation of Adeno Associated Virus (AAV)

AAV9 encoding GFP-2A-α1(T125C) produced by plasmid co-transfection into HEK293T cells as described previously (Choi et al., 2007). After ultracentrifugation, the interphase between the 54 and 40% iodixanol fraction, and the lower three-quarters of the 40% iodixanol fraction, were extracted and diluted with an equal volume of phosphate-buffered saline (PBS) plus 0.001% Tween 20. This fraction was then buffer exchanged and concentrated with Amicon Ultra-15 Centrifugal Filter Units to a final volume of ~100 µL. The α1(T125C) AAV titered was $7.5 \times 10^{12}$ vg/mL.
2.4.2 Cell culture, Mutant Expression and PTL treatment

2.4.2.1 Xenopus oocytes

RNA was prepared with the mMessage mMachine T7 kit (Ambion, Austin, TX). Defolliculated *Xenopus laevis* oocytes were injected with 2.5–20 ng of mRNA in 50 nL H$_2$O in a 1:1 ratio of $\alpha$ to $\beta$ subunit RNA. The injected oocytes were incubated at 18°C (in OR1 buffer) and subjected to two-electrode voltage-clamp recording 1–3 days after RNA injection. The OR1 buffer comprised (in mM): 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES; pH 7.4. For testing MAM-6 mediated photo-control of receptor activities, oocytes expressing cysteine substituted $\alpha$1 or $\alpha$5 mutants were incubated with 50 µM MAM-6 for 0.5–1.5 hr prior to recording.

2.4.2.2 HEK293T cells

HEK293T cells were maintained in Dulbecco’s Minimum Essential Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO$_2$. Cells were plated at 18–24 $\times$ 10$^3$ cells/cm$^2$ on poly-L-lysine coated coverslips and transfected by calcium phosphate precipitation. A total of 0.9 µg DNA per coverslip ($\alpha$1:$\beta$2:eGFP at 4:4:1 ratios) was added to the cells. Recordings were performed 36 to 48 hours post-transfection. For attaching PTL to the mutant receptors, cells were treated with dithiothreitol (DTT; 1 mM, 5 min), incubated in fresh external recording solution (5 min), and then treated with PTL (25 µM, 20 min, pH 8) at 37°C in the dark. Whole-cell voltage-clamp recordings were performed thereafter.
2.4.2.3 Dissociated hippocampal neurons

Primary cultures of neonatal rat hippocampal neurons were performed using standard procedures, as previously described (Fortin et al., 2008). Cells were grown in minimum essential medium (MEM) containing 5% FBS, 20 mM glucose, B27 (Invitrogen), 2 mM glutamine and Mito+ Serum Extender (BD Biosciences). Transfection was performed via calcium phosphate precipitation 6–7 days after plating. Whole-cell electrophysiology recordings at 3 weeks after plating.

2.4.2.4 Organotypic hippocampal slice cultures

Postnatal day 8 Sprague-Dawley rat pups were anaesthetized and decapitated. Hippocampi were removed and sliced into 350 µm thick sections using a tissue chopper (Stoelting, Wood Dale, IL). Slices were maintained at 34°C on cell culture inserts (Milipore, Billerica, MA) in Neurobasal-A medium (Invitrogen) supplemented with 20% horse serum (Thermo Scientific, Waltham, MA), 0.03 units/ml insulin (Sigma, St. Louis, MO), 0.5 mM ascorbic acid (Sigma), 1X Gluta-Max (Invitrogen), 80 units/ml penicillin, 80 µg/ml streptomycin (Invitrogen), and 25 mM HEPES. One day after preparation, slices were injected with AAV9 encoding GFP-2A-α1(T125C). The CA1 pyramidal cell body layer was injected at 3–7 sites/slice with 100 nl of 10^{12}-10^{13} vg/ml virus with a fine glass pipette. 5–14 days post-injection, slices were incubated for 2 min with 1 mM Tris(2-carboxyethyl)phosphine hydrochloride in artificial cerebrospinal fluid (ACSF) containing in mM: 126 NaCl, 2.5 KCl, 1.25 NaH_{2}PO_{4}, 10 Glucose, 1.3 MgCl_{2}, 26 NaHCO_{3} and 2.5 CaCl_{2} equilibrated with 95% O_{2}/5% CO_{2}, washed, and incubated with 25 µM MAB-0 in ACSF for 30 min at room temperature. The PTL treated slices were subjected to whole-cell current-clamp recording.
2.4.3 Electrophysiology

2.4.3.1 Two-electrode voltage-clamp of *Xenopus oocytes*

Single oocytes were placed in a 0.2-mL perfusion chamber and impaled with two glass microelectrodes (1–2.5 MΩ resistance) filled with 3M KCl and voltage clamped with an OC-725C amplifier (Warner Instruments, Hamden, CT) in OR1 solution containing (in mM): 96 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$, 5 HEPES; pH 7.4. Oocytes were held at -80 mV. Drug perfusion, data acquisition, and analysis were carried out with pClamp software and a Digidata 1200 interface (Molecular Devices, Sunnyvale, CA). Oocytes were illuminated by a Lambda DG-4 (Sutter Instruments, Novato, CA) using 383 nm and 497 nm filters.

2.4.3.2 Whole-cell voltage-clamp of HEK293T cells

Recordings were performed using pipettes with 2.5–4 MΩ resistance. Pipettes were pulled from filamented borosilicate pipettes (G150TF-3, Sutter Instruments). Extracellular solution contained (in mM): 140 NaCl, 5 KCl, 1 MgCl$_2$, 10 HEPES, 2 CaCl$_2$, 10 glucose; pH 7.4. Intracellular solution contained (in mM): 140 CsCl, 4 NaCl, 10 HEPES, 2 MgCl$_2$, 2 Mg-ATP, 10 EGTA; pH 7.2. Cells were held at –70 mV. Signals were amplified using a Patch Clamp PC-505A amplifier (Warner Instruments), digitized by a Digidata 1322A converter (Molecular Devices), and acquired with software Clampex 10 (Molecular Devices). Illumination for PTL photoisomerization was provided by a Lambda-LS xenon lamp (Sutter Instruments) with band pass filters (379 ± 17 nm and 500 ± 8 nm).
2.4.3.3  Whole-cell voltage-clamp of dissociated hippocampal neurons

Voltage-clamp recordings were performed at room temperature using standard whole cell patch-clamp techniques. Signals were amplified using a Patch Clamp PC-505B amplifier (Warner Instruments), filtered at 2kHz, digitized at 10kHz using a Digidata 1200 converter (Molecular Devices), and acquired using Clampex 8 (Molecular Devices).

To record spontaneous mIPSCs, cells were held at –60 mV. The extracellular solution contained (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 5 HEPES, 10 Glucose, 0.001 TTX (to block voltage-dependent Na⁺ channels), 0.025 DNQX (to block AMPA receptors), and 0.05 APV (to block NMDA receptors); pH 7.4. The internal solution contained (in mM): 140 CsCl, 4 NaCl, 2 MgCl₂, 10 HEPES, 10 EGTA, 2 Mg-ATP; pH 7.4

To record spontaneous mEPSCs, cells were held at –70 mV. The extracellular solution contained (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 5 HEPES, 10 Glucose, 0.001 TTX, 0.02 bicuculline; pH 7.4. The internal solution contained (in mM): 8 NaCl, 135 K-gluconate, 4 MgCl₂, 10 HEPES, 2 Mg-ATP, 1 EGTA; pH 7.4. All miniature events were detected using the software MiniAnalysis (Synaptosoft, Decatur, GA) and were verified visually. The search protocol threshold was set at fivefold the root-mean-square (RMS) noise level, which typically was 2–5 pA.

For recording voltage-gated currents, extracellular solution contained (in mM): 138 NaCl, 1.5 KCl, 1.2 MgCl₂, 2.5 CaCl₂, 5 HEPES, 10 Glucose, 0.02 bicuculine, 0.025 DNQX, and 0.05 APV; pH 7.4. The internal solution contained (in mM): 10 NaCl, 135 K-gluconate, 2 MgCl₂, 10 HEPES, 1 EGTA, 2 Mg-ATP; pH 7.4. Cells were held at –70 mV and stepped for 200ms to values between –100 and +40 mV, with increment steps of 10 mV.
2.4.3.4 Whole-cell current-clamp recording of hippocampal slices

Slices were placed in a recording chamber mounted on an Olympus BX51WI microscope and perfused with ACSF at RT at 1–2 ml/min. Whole-cell current clamp recordings were made from CA1 pyramidal cells with glass microelectrodes (R = 4–7 MΩ) filled with internal solution containing (in mM): 116 K-gluconate, 20 HEPES, 6 KCl, 2 NaCl, 0.5 EGTA, 4 ATP-Mg, 0.3 GTP-Na, ~ 7.2 pH, ~290mOsm. A glass stimulating electrode was placed in stratum radiatum 300–500 µm away from the recorded cell. Synaptic responses were evoked by a 0.2 ms, 10–100 µA current pulse delivered via a stimulus isolation unit (AMPI, Jerusalem, Israel). To record evoked IPSPs cells were depolarized to -45mV by somatic current injection. Signals were amplified (Axopatch, Molecular Devices), digitized (Digidata, Molecular Devices) and recorded (pClamp, Molecular Devices) to computer. Light was delivered through the microscope optical port by a Polychrome (Till Photonics, Munich, Germany) controlled by pClamp.
2.4.4 Molecular Modeling

Docking of trans- and cis-MAB-0 was carried out in the docking program GLIDE (Shrödinger Inc., Portland, OR) (Korpi et al., 2002), implemented in Maestro (Shrödinger Inc.). A dimer of α1 and β2 subunits from a previously published homology model for GABA\textsubscript{A} receptor (α1β2γ2) was used (O’Mara et al., 2005). Threonine residue 125 of the α1 subunit was replaced by a cysteine residue, and a receptor grid was created based on a dimmer with this α1 mutation. Trans- and cis-MAB-0 were individually docked in the grid using the Standard Precision algorithm. To mimic MAB-0 tethering, a positional constraint was applied during docking. This constraint enforced at least one of the reactive carbons (of the maleimide) of MAB-0 to locate within 4.5 Å from the cysteine side-chain carbon. A maximum of 100 poses were listed. The docking poses were further inspected visually to remove unreasonable results. All of the docking poses for trans-MAB-0 insert the azobenzene moiety in the αβ interface. The predominant docking mode of trans-MAB-0 is shown in Figure 2.4D top, in which the terminal phenol group of trans-MAB-0 reaches deeply into the GABA-binding site (surrounded by several aromatic residues represented in yellow). The docking poses for cis-MAB-0 are fewer and more diversely oriented. The majority of the cis poses place the terminal phenol group outside of the aromatic region of the GABA-binding site. The bottom of Figure 2D bottom presents a docking pose of cis-MAB-0 that has a similarly positioned maleimide group to that of the trans pose, showing the change in the molecular shape and the accessibility of the phenol group when MAB-0 is switched from trans to cis conformation.
CHAPTER 3
INCREASED HDAC6 EXPRESSION ALTERS TAU AND PP1 PROTEIN LEVELS

3.1 Introduction

HDAC6 is a protein that has multiple roles in the cytosol. This protein is a versatile multifunctional enzyme responsible for deacetylating key proteins involved in protein degradation pathways, cellular stress, and cytoskeletal dynamics (d'Ydewalle et al., 2012; Li et al., 2013; Parmigiani et al., 2008; Zhang et al., 2013). The role of HDAC6 in neurodegenerative disease is controversial. It is unclear if the observed increases in HDAC6 protein levels in the AD brain are the result of a neuroprotective response to eliminate protein aggregates or an underlying causative agent in AD pathology and progression. The experiments described here were devised to investigate the role of HDAC6 in AD tau pathology. A heterologous mammalian cell culture system was utilized to assess the role of HDAC6 in a simplified system in which genes can be easily delivered and expressed. The goal of this chapter is to determine if increased HDAC6 protein levels lead to increased pathological tau accumulation and if disrupting an HDAC6-PP1 interaction decreases pathological tau.

Tau is a microtubule-associated protein (MAP), primarily expressed in neurons that promotes microtubule assembly and stabilization (Iqbal et al., 2005). Tau plays a key role in regulating cytoskeletal microtubule dynamics, axonal transport and neurite outgrowth and all of these functions are modulated by site-specific phosphorylation (Johnson and Stoothoff, 2004). Under normal physiological conditions, tau is in a constant equilibrium, on and off the microtubules (Ballatore et al., 2007; Dixit et al., 2008). The enzymatic activity of phosphatases and kinases control this equilibrium of microtubule-binding affinity by regulating the phosphorylation state of tau at serine and
threonine residues (Ballatore et al., 2007). Tau phosphorylation is catalyzed by protein kinases and dephosphorylated by protein phosphatases. An imbalance of protein kinase and protein phosphatase activity can lead to tau hyperphosphorylation. One key phosphatase, protein phosphatase 1 (PP1) has been shown to interact with HDAC6 and this interaction is believed to disrupt the ability of PP1 to dephosphorylate tau leading to an increase in the accumulation of pathological hyperphosphorylated tau (Brush et al., 2004; Ding et al., 2008).

The human tau gene, located on chromosome 17, is alternatively spliced to generate six different isoforms. The tau isoforms differ by having three repeat (3R) or four repeat (4R) microtubule-binding domains in the C-terminal and the presence or absence of exons 2 and 3 in the N-terminal (Buee et al., 2000; Johnson and Stoothoff, 2004). Healthy adult human brains contain approximately a 1:1 ratio of the 3R tau and 4R tau isoforms. Deviations from this 1:1 ratio are associated with neurodegenerative disease (Andorfer et al., 2003; Ballatore et al., 2007). Since tau is primarily expressed in the brain, an established heterologous system that stably expresses tau was required for these experiments.

Human embryonic kidney cells (HEK) stably expressing tau (HEK-tau) were chosen as the cell-line for these experiments because they stably express human tau isoforms with four microtubule binding repeats without exon 2 and 3 (Ding et al., 2008; Cho and Johnson, 2003). These HEK-tau cells have been used to investigate tau phosphorylation in the presence of endogenous HDAC6 and disruption of the catalytic activity of HDAC6 by domain-selective small-molecule inhibitor tubacin or shRNA knockdown of HDAC6 in these cells was found to attenuate tau phosphorylation (Ding et al., 2008). The experiments in this chapter were designed to assess if HDAC6 overexpression altered tau pathology, and to examine PP1 levels in HEK-tau cells.
expressing catalytically inactive HDAC6 or catalytically active HDAC6. In order to target
the catalytic domains of HDAC6 without altering protein folding or truncating the protein,
established single-point mutations to render HDAC6 catalytically inactive were used
(Brush et al., 2004; Ding et al., 2008; Haggarty et al., 2003).

3.2 Results

3.2.1 Overexpression HDAC6 alters tau protein levels

To assess if HDAC6 overexpression had an effect on the accumulation of pathological tau in a heterologous expression system, HEK-tau cells were transfected with human HDAC6. Additionally single point mutations that rendered each of the catalytic domains of HDAC6 inactive, and a double mutant with both catalytic domains inactive were created. Mutant HDAC6 constructs were created to assess if functional HDAC6 was responsible for increased tau phosphorylation and if disruption of an HDAC6-PP1 interaction attenuated tau phosphorylation. Cells were lysed 36 hrs post-transfection, total protein was separated by SDS-PAGE and then immunoblotted. The monoclonal Tau-5 antibody was used to detect total tau (both phosphorylated and non-phosphorylated forms of tau) (Tau-5, Invitrogen), a monoclonal FLAG antibody to verify HDAC6 expression (FLAG, Stratagene) and GAPDH as a loading control (GAPDH, SantaCruz). HEK-tau cells overexpressing HDAC6 were found to have increased total tau protein levels of high-molecular weight compared to wild-type HEK-tau control cells (Figure 3.1). HDAC6 overexpression in HEK-tau cells led to a 2.4 fold increase in total tau compared to wild-type HEK-tau control cells. Unexpectedly, HEK-tau cells overexpressing catalytically inactive HDAC6 mutants: H216A, H611A, and double mutant H216AH611A all had increased total tau protein levels of high-molecular weight (Figure 3.1) suggesting increased accumulation of hyperphosphorylated tau
accumulation compared to wild-type HEK-tau control cells. Mutant HDAC6 overexpression in HEK-tau cells led to an total tau increase of 2.2 fold in H216A mutant expressing cells, a 2.0 fold increase in total tau in H611A mutant expressing cells, and a 2.3 fold increase of total tau in catalytically inactive double mutant H216AH611A expressing cells compared to wild-type HEK-tau control cells.
Figure 3.1. Increased HDAC6 Expression Alters Tau Protein Levels

A) Schematic of FLAG-tagged HDAC6 constructs.
B) Proteins isolated from cell lysates of wild-type non-transfected HEK-tau control cells and HEK-tau cells transiently expressing HDAC6 constructs were separated by SDS-PAGE and immunoblotted with mouse anti-FLAG (top-panel), mouse anti-Tau-5 (middle-panel), and rabbit anti-GAPDH (bottom-panel). Approximate molecular mass in kDa are shown next to representative blots.
C) Quantification of Tau-5 from wild-type non-transfected HEK-tau control cells and HEK-tau cells transiently expressing HDAC6 constructs plotted as mean ± S.E.M from at least 4 separate experiments are indicated.
3.2.2 Overexpression HDAC6 reduces PP1 protein levels

Next, HEK-tau cells overexpressing wild-type and mutant HDAC6 constructs were utilized to determine if the increase in hyperphosphorylated high-molecular weight tau was the result of altered PP1 protein levels or the enzymatic activity of PP1. The monoclonal PP1 E-9 antibody was used to detect PP1 (PP1, SantaCruz) and GAPDH as a loading control (GAPDH, SantaCruz). HEK-tau cells overexpressing HDAC6 had decreased PP1 protein levels compared to wild-type HEK-tau control cells (Figure 3.2). HDAC6 overexpression in HEK-tau cells led to a 1.55 fold decrease in PP1 protein levels compared to wild-type HEK-tau control cells. Additionally, HEK-tau cells overexpressing catalytically inactive HDAC6 mutants: H216A, H611A, and double mutant H216AH611A all revealed decreased PP1 protein levels compared to wild-type HEK-tau cells (Figure 3.2). Mutant HDAC6 overexpression in HEK-tau cells led to decreased PP1 levels of 1.19 fold in H216A mutant expressing cells, a 1.32 fold decrease in PP1 in H611A mutant expressing cells, and a 1.61 fold decrease in PP1 in catalytically inactive double mutant H216AH611A expressing cells compared to wild-type HEK-tau control cells. These decreases in PP1 protein levels correlated with the increased accumulation of hyperphosphorylated high-molecular weight tau accumulation in these cells suggesting reduction in the amount of available PP1 to dephosphorylate tau leading to the pathological accumulation of tau in cells overexpressing both wild-type and catalytically inactive HDAC6. Interestingly, overexpression of either catalytically inactive or wild-type HDAC6 led to decreased PP1 protein levels, revealing HDAC6 catalytic activity was not necessary for altering PP1 homeostasis.
Figure 3.2. Increased HDAC6 Expression Alters PP1 Protein Levels

A) Schematic of FLAG-tagged HDAC6 constructs.

B) Proteins isolated from cell lysates of wild-type non-transfected HEK-tau control cells and HEK-tau cells transiently expressing HDAC6 constructs were separated by SDS-PAGE and immunoblotted with mouse anti-PP1 (top-panel), and rabbit anti-GAPDH (bottom-panel). Approximate molecular mass in kDa are shown next to representative blots.

C) Quantification of PP1 from wild-type non-transfected HEK-tau control cells and HEK-tau cells transiently expressing HDAC6 constructs plotted as mean ± S.E.M from at least 4 separate experiments are indicated.
3.2.3 Catalytically inactive HDAC6 does not deacetylate α-tubulin

The second catalytic domain of HDAC6 has been shown to be responsible for cytosolic deacetylase functions of HDAC6. Therefore, to confirm that mutations to the second catalytic domain rendered HDAC6 catalytically inactive HEK-tau cells overexpressing wild-type and mutant HDAC6 constructs were used to assess levels of a well-known protein substrate, α-tubulin. The monoclonal acetylated α-tubulin 6-11B-1 antibody was used to detect levels of acetylated tubulin (Acetylated-tubulin, Sigma) and GAPDH as a loading control (GAPDH, SantaCruz). As expected, the single-point mutation H611A of the second catalytic domain of HDAC6 rendered the enzyme inactive and thus unable to deacetylate α-tubulin (Figure 3.3). This result confirmed that only mutations to the second catalytic domain rendered HDAC6 catalytically inactive.
Figure 3.3. Mutations to the Second Catalytic Domain of HDAC6 Render HDAC6 Catalytically Inactive and Unable to Deacetylate Tubulin

A) Schematic of FLAG-tagged HDAC6 constructs.  

B) Proteins isolated from cell lysates of wild-type non-transfected HEK-tau control cells and HEK-tau cells transiently expressing HDAC6 constructs were separated by SDS-PAGE and immunoblotted with anti-Acetylated Tubulin (top-panel), and anti-GAPDH (bottom-panel).  Approximate molecular mass in kDa are shown next to representative blots.
3.3 Discussion

In the present study, overexpression of HDAC6 was identified as a potential underlying molecular factor altering both tau and PP1 protein levels. The data presented here show that increased HDAC6 expression in a heterologous expression system leads to increased tau accumulation (Figure 3.1) that correlates with decreased PP1 (Figure 3.2). Further, recent publications have shown reduction or loss of HDAC6 alleviates abnormal tau accumulation and reduces cognitive deficits in a mouse model for Alzheimer's disease (AD) (Cook et al., 2012; Govindarajan et al., 2013; Xiong et al., 2013). These data suggest that HDAC6 may be a novel target for potential therapeutic of AD.

To date, the available broad-spectrum HDAC inhibitors and HDAC6 specific inhibitors target the deacetylase enzymatic activity (Dallavalle et al., 2012; Haggarty et al., 2003; Inks et al., 2012; Xu et al., 2011). Therefore experiments were designed to determine the effect of catalytically inactive HDAC6 on tau and PP1 protein levels. Inactivation of HDAC6 catalytic domain did not reverse the increase in pathological tau accumulation (Figure 3.1). Further, HEK-tau cells expressing catalytically inactive HDAC6 resulted in reduced PP1 protein levels that were comparable to cells overexpressing functional HDAC6 thus revealing enzymatic activity of either catalytic domain of HDAC6 is not required for altering PP1 levels. These data suggest that inhibitors that target the catalytic domain of HDAC6 will not be an effective therapeutic strategy in altering the accumulation pathological tau in AD.

Interestingly, Brush et al. reported that increased HDAC6 led to decreased phosphatase activity in a dose-dependent manner (Brush et al., 2004). The second catalytic domain of HDAC6 has been shown to be responsible for interacting with the
catalytic domain of PP1 suggesting that HDAC6 deacetylates PP1 and regulates its enzymatic activity (Brush et al., 2004). It was unexpected that PP1 levels were reduced in HEK-tau cells overexpressing both catalytically active and inactive HDAC6. The data presented here suggest that an HDAC6-PP1 interaction at the second catalytic domain of HDAC6 is not responsible for the reduction in PP1 activity. Previously, Ding et al. reported that by selectively inhibiting the enzymatic activity of the second catalytic domain of HDAC6 with tubacin or shRNA knockdown of HDAC6 decreased the amount of phosphorylated tau and suggested this was the result of the disruption of an HDAC6-PP1 interaction (Ding et al., 2008). The data reported here suggests that increased expression of HDAC6 leads to decreased PP1 protein levels and that this reduction in PP1 may lead to increased accumulation of pathological phosphorylated tau. Both pathological tau accumulation and reduction in phosphatase levels are hallmarks of AD.

Acetylated tubulin is a marker of stable microtubules. HDAC6 deacetylates α-tubulin thus decreasing the level of acetylated tubulin and reducing the stability of microtubules. Disruption of the microtubule network is a central pathological mechanism leading to neuronal dysfunction in neurons (Janke and Bulinski, 2011; Zhang et al., 2013). Interestingly, tau has been reported to be an inhibitor of the deacetylase function of HDAC6 (Perez et al., 2009). Therefore experiments were designed to determine if HEK-tau cells that stably express tau inhibit the deacetylase function of HDAC6. HEK-tau cells expressing HDAC6 with a functional second catalytic domain (HDAC6 and H216A mutant HDAC6) had deacetylated tubulin levels confirming tau expression did not inhibit the deacetylase function of HDAC6 in these cells (Figure 3.3). Additionally, HEK-tau cells expressing HDAC6 with a non-functional second catalytic domain (H611A mutant and H216AH611A) had acetylated tubulin levels comparable to wild-type control
HEK-tau cells (Figure 3.3). As expected, targeting the α-tubulin deacetylase function of HDAC6 led to an increase in acetylated tubulin.

Analysis of the phosphorylation status of tau was not confirmed in HEK-tau cells stably expressing tau because HEK cells do not normally express tau therefore post-translational modifications in these cells may not reflect the regulation of tau homeostasis in neurons. Further, only tau isoforms with four microtubule-binding repeats without exon 2 and exon 3 are stably expressed in HEK-tau cells. Normal adult human brain contains approximately a 1:1 ratio of the 3R tau and 4R tau isoforms and deviation from this ratio has been associated with neurodegenerative disease (Andorfer et al., 2003; Ballatore et al., 2007). Further experiments to determine if increased HDAC6 expression alters tau phosphorylation in an endogenous system were required to address changes to tau pathology in neurons.

3.4 Methods

3.4.1 Cell culture

3.4.1.1 HEK-tau cells

HEK-tau cells (Dr. Gail V. W. Johnson, University of Rochester Medical Center) were maintained in Dulbecco’s Minimum Essential Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂. Cells were transfected at 70% confluency in t-25 flask 24 hrs after plating with LipoD293™ according to manufactures instructions (SignaGen®).
3.4.2 Constructs and site-directed mutagenesis of HDAC6

The cDNA of human HDAC6 was obtained from pcDNA3.1(+)HDAC6FLAG (Addgene plasmid #13823) and used to create catalytically inactive HDAC6. Cysteine mutants were prepared by site-directed mutagenesis of wild-type HDAC6 using oligonucleotide primers to target the first catalytic domain (H216A), or the second catalytic domain (H611A) under the following PCR profile: 1 cycle (95 °C for 1 min); 18 cycles (95 °C for 50 s, 68 °C for 50 s, 68 °C for 9.5 min); 1 cycle (68 °C for 7 min) (Agilent Technologies, QuickChangeII XL Site-directed Mutagenesis kit #200521). The catalytically inactive double mutant (H216AH611A) was created by creating a single catalytic domain mutant and then targeting the remaining catalytic domain. Mutations were confirmed by sequencing. The H216A primer was 5'-cattaggcctcttgacatgccgcccagcacc-3' and the H216A antisense primer was 3'-gtatccggagacgtacgccggtcgtg-5'. The H611A primer was 5'-gtcggccagcccgctccgagcaggatg-3' and the H611A antisense primer was 3'-caggggtctgtgcgtctcgtctac-5'.

3.4.3 Cell lysis, immunoblotting and analysis

HEK-tau cells were collected in lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM Na3VO4, 1 mM NaF, and Protease Inhibitor Cocktail (Sigma P8340) containing 1.04 mM AEBSF, 15 µM Pepstatin A, 14 µM E-64, 2 µM Leupeptin, 4 µM Bestatin, 80 nM Aprotinin), incubated at 4 °C shaking for 30 minutes and centrifuged at 4 °C at 12,000 g for 10 minutes. Protein concentrations were determined by bicinchoninic (BCA) assay (Pierce). Total protein lysates (20 µg per sample) were separated by SDS-PAGE, transferred to nitrocellulose membrane with CAPS transfer buffer (10 mM 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), 10 % Methanol, pH 11) and immunoblotted. The membrane was blocked with 5 % bovine
serum albumin (BSA) in 1X Tris Tween Buffered Saline (1X TTBS; 20 mM Tris, 137 mM NaCl, 0.1 % Tween-20, pH 7.6), and antibody incubations in 1 % BSA 1X TTBS and washes with 1 % BSA 1X TTBS. The primary antibodies used in this study were: Mouse Anti-Tau5 (Invitrogen #AHB0042), Mouse Anti-FLAG (Stratagene #200471), Mouse Anti-PP1 E-9 (Santa Cruz, sc-7482), Mouse Anti-Acetylated Tubulin 6-11B-1 (Sigma, T6793-2ML), Rabbit Anti-GAPDH (Sigma, G9545-25UL). The secondary antibodies used in this study were: Goat Anti-Mouse IgG, HRP (Millipore, AP308P), Goat Anti-Rabbit IgG, HRP (Millipore, AP307P). The membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Pierce, 34080) and imaged on a G:box with cooled-CCD camera (Syngene). HRP inactivation by excess hydrogen peroxide (blot incubation in 30 % H₂O₂ for 30 min shaking at 37 °C) was used to enable multiple membrane probing of different species (rabbit vs. mouse) without the need for stripping blots. Signal from GAPDH was used as a loading control for normalization. Signal from wild-type non-transfected HEK-tau cells were used as basis for the change in protein signal due to increased expression of HDAC6. Band quantification was carried out using G:box gene tools analysis software. All figures were prepared with GraphPad Prism ® version 6.
CHAPTER 4
INCREASED HDAC6 EXPRESSION IS AN EARLY MARKER OF AD PROGRESSION

4.1 Introduction

A major hurdle in studying AD is identifying novel targets that alter disease progression and pathology. Once it was established that HDAC6 could be a potential underlying molecular factor altering tau pathology in a heterologuous expression system experiments were designed to investigate the neuropathological role of HDAC6 in AD in primary dissociated hippocampal neurons. The experiments described in this chapter were performed to assess if increased HDAC6 protein levels in primary dissociated hippocampal neurons leads to the missorting of pathological phosphorylated tau to the soma and early synaptic dysfunction of these neurons. Primary dissociated neurons are the simplest neuron culture system for evaluating changes in synaptic proteins and neuronal function. The hippocampus was chosen because of its prominent role in learning and memory in the mammalian brain, the hippocampus is greatly decreased in size in AD due to neuron death, and HDAC6 protein levels are increased by 91% in the hippocampus of human post-mortem AD brains compared to age-matched control brains (Ding et al., 2008; Thies et al., 2013).

Another major hurdle in developing a neuron cell culture model system to study underlying molecular mechanisms in AD progression is that neurons are notoriously difficult to deliver genes of interest to using standard mammalian gene delivery techniques. Neurons are post-mitotic and very sensitive to physical stress, and to changes in temperature, pH and osmolarity (Karra and Dahm, 2010). Calcium phosphate (CaPO₄) transfection results in low gene delivery (>5%), low expression, and few viable transfected cells where as lipid based transfection methods (Lipofectamine)
are toxic to neurons (Karra and Dahm, 2010). Additionally, CaPO$_4$ transfections have the highest gene delivery efficiency between day \textit{in vitro} (DIV) 7 and DIV 10. After this small-time frame, >1% of neurons are typically transfected. Alternatively, nucleofection is a method similar to electroporation in that a series of high voltage pulses enable gene delivery. Nucleofection is a proprietary technique requiring expensive reagents and equipment that have been modified for efficient (>60%) primary neuron gene delivery (Lonza Amaza Nucleofector™). Nucleofection requires very high concentrations of cells and the gene of interest to be delivered to neurons while in suspension before plating at DIV 0. Methods that require gene delivery before DIV 16 have limited success in evaluating changes in synaptic proteins and neuronal function if the transfected neurons do not survive to DIV 18 to DIV 24 when they are synaptically active.

The only established technology to deliver genes of interest to neurons at any age with high efficiency is recombinant DNA technology via recombinant virus. Recombinant viruses are the method of choice for gene delivery in neurons because gene delivery efficiency has been reported to achieve 90 to 99% transduction, depending on the virus. Recombinant viruses are not the first choice in gene delivery for neurons because they are a labor intensive, expensive, and demand safety precautions, typically requiring biosafety level 2+ practices. For these experiments, recombinant adenovirus technology was chosen because the recombinant virus does not integrate into the genome eliminating the risk of insertional mutagenesis and ease of cloning and generating recombinant adenovirus (Karra and Dahm, 2010; Luo et al., 2007).
4.2 Results

4.2.1 HDAC6 Gene Delivery in Neurons

Several methods were employed to deliver HDAC6 to primary neurons including CaPO$_4$, and nucleofection. Each of these methods were unsuccessful in transfecting neurons after DIV 10. Each method resulted in less than 1% transfection efficiency and transfected neurons overexpressing HDAC6 were found to not be viable 4 days post-transfection (data not shown). A collaboration with Dr. Gregory Tew’s lab (UMASS Amherst, Polymer Science and Engineering) was initiated to evaluate novel polymers in mediating gene delivery in neurons. These novel polymers had less than 1% transfection efficiency, and were unsuccessful in transfecting neurons past DIV 10 (data not shown). In order to evaluate if increased HDAC6 protein levels in primary dissociated hippocampal neurons is an underlying molecular factor that leads to the missorting of pathological phosphorylated tau to the soma and early synaptic dysfunction of these neurons, recombinant HDAC6-adenovirus was generated and used to deliver FLAG-tagged HDAC6 to neurons. Recombinant HDAC6_FLAG-Adenovirus (HFA) infection resulted in robust gene expression and >70% transfection efficiency in neurons at MOI 100. Representative widefield images taken at 40X magnification of neurons infected at DIV 10 and fixed 36 hr post-transfection at DIV 12 show high transfection efficiency and expression (Figure 4.1B). Fixed neuron cultures are autofluorescent and as expected, control neurons show non-specific autofluorescence (Figure 4.1A). Representative phase contrast images of wild-type control neurons vs. HFA infected neurons show healthy cultures (Figure 4.1C, Figure 4.1D). These experiments established HFA infection as a reproducible and effective method for gene delivery of flag-tagged HDAC6 to primary hippocampal neurons.
Figure 4.1. Primary Hippocampal Neurons at DIV 12

Scale bars, 100 µM
A) Autofluorescence of FLAG stained wild-type control neurons, DIV 12 at 40X.
B) Distribution HDAC6 expression of FLAG stained HFA infected neurons, 36 hr post-infection DIV 12 at 40X.
C) Phase contrast image of control neurons from A, DIV 12 at 40X.
D) Phase contrast image of HFA infected neurons from B, DIV 12 at 40X.
4.2.2 **HDAC6 Overexpression Missorts Total Tau, Early and Late Pathological Tau to the Soma of Neurons 36 hr Post-Infection at DIV 12.**

To assess if increased HDAC6 expression had an effect on tau localization, primary hippocampal neurons containing immature synapses were infected with HFA at an MOI of 100 and fixed 36 hrs post-infection at DIV 12. Established tau markers were used to determine the effect of HDAC6 overexpression on the distribution of total tau, as well as early and late pathological tau. The monoclonal Tau-5 antibody was used to detect total tau (both phosphorylated and non-phosphorylated forms of tau) (Tau-5, Invitrogen). The pTau231 antibody was used to detect phosphorylation at Threonine 231, a site important for regulating the ability of tau to bind microtubules and the pTau202 antibody specific to phosphorylation at Serine 202 was used to detect tau pathology in early and late stages of NFT formation (Andorfer et al., 2003).

In normal primary hippocampal neurons DIV 12 containing immature synapses, tau has distinct puncta staining (Figure 4.2A). Within 36 hr of HDAC6 overexpression, neurons infected with HFA, total tau appears in the entire neuron, axon and somatodendritic compartment (Figure 4.2A). HDAC6 overexpression led to a shift in the localization of total tau, a 1.7 fold increase of Tau-5 in soma of HDAC6 overexpressing neurons vs. control neurons.

To assess if the missorted tau was phosphorylated, a phospho-specific antibody (pTau231) that detects tau unbound from the microtubules was used. Within 36 hr of HDAC6 overexpression, neurons infected with HFA, increased pTau231 immunostaining appears in the entire neuron, axon and somatodendritic compartment whereas control cells showed very little pTau231 immunostaining (Figure 4.3). HDAC6 overexpression led to a increase in phosphorylated tau and a 1.39 fold increase in pTau231 in soma of HDAC6 overexpressing neurons compared to control neurons.
Next, a phospho-specific antibody (pTau202, phosphorylated Serine 202) that detects early and late pathological tau was used to determine if missorted tau was in fact NFTs. Within 36 hr of HDAC6 overexpression, neurons infected with HFA, increased pTau202 immunostaining appears in the entire neuron, axon and somatodendritic compartment whereas control cells have distinct puncta staining restricted from the soma (Figure 4.4). HDAC6 overexpression led to an increase in phosphorylated pathological tau, a 1.4 fold increase in the accumulation of pathological tau in the soma of HDAC6 overexpressing neurons compared to control neurons. HDAC6 overexpression in primary hippocampal neurons containing immature synapses led to the missorting of total tau, regulatory tau, and early and late pathological tau to the somatodendritic compartment.
Figure 4.2. Increased Tau-5 in Soma of Neurons Overexpressing HDAC6

A) Tau-5 distribution in wild-type control neurons (top) at DIV 12 and neurons overexpressing HDAC6 (bottom) 36 hrs post-HFA MOI 100 infection at DIV 12. Scale bars 100µM.

B) Quantification of Tau-5 fluorescence intensity in soma plotted as mean ± S.E.M. Control, n = 143 cells (14 FOV); HDAC6 overexpressing, n = 109 cells (14 FOV). **** P value < .0001. FOV= fields of view
Figure 4.3. Increased pTau231 in Soma of Neurons Overexpressing HDAC6

A) pTau231 distribution in wild-type control neurons (top) at DIV 12 and neurons overexpressing HDAC6 (bottom) 36 hrs post-HFA MOI 100 infection at DIV 12. Scale bars 100µM.

B) Quantification of pTau231 fluorescence intensity in soma plotted as mean ± S.E.M. Control, n = 146 cells (10 FOV); HDAC6 overexpressing, n = 150 cells (15 FOV). *** P value = 0.0003. FOV= fields of view
Figure 4.4. Increased pTau202 in Soma of Neurons Overexpressing HDAC6

A) Pathological pTau202 distribution in wild-type control neurons (top) at DIV 12 and neurons overexpressing HDAC6 (bottom) 36 hrs post-HFA MOI 100 infection at DIV 12. Scale bars 100µM.

B) Quantification of pTau202 fluorescence intensity in soma plotted as mean ± S.E.M. Control, n = 195 cells (16 FOV); HDAC6 overexpressing, n = 139 cells (21 FOV). *** P value = 0.0003. FOV = fields of view
4.2.3 Increase in Pathological pTau202 in Soma of Synaptically Active HDAC6 Overexpressing Neurons.

To assess if HDAC6 overexpression had an effect on tau localization in synaptically active neurons, primary hippocampal neurons containing mature synapses were infected with HFA at an MOI of 100 at DIV 18 and fixed 36 hrs post-infection at DIV 20 and 72 hrs post-infection at DIV 21. Within 36 hr of HDAC6 overexpression, synaptically active neurons infected with HFA at DIV 20 have increased pTau202 in the entire neuron, axon and somatodendritic compartment whereas control cells have distinct tau puncta staining restricted from the soma (Figure 4.5A). HDAC6 overexpression 36 hrs post-infection led to a 1.22 fold increase in the accumulation of phosphorylated pathological tau in the soma of HDAC6 overexpressing neurons compared to control neurons at DIV 20 (Figure 4.5B). By 72 hrs of HDAC6 overexpression in synaptically active neurons at DIV 21 there is a distinct increase in the accumulation of phosphorylated pathological tau in the soma and puncta whereas control cells have tau puncta staining and tau restricted from the soma (Figure 4.6A). Interestingly, HDAC6 overexpression 72 hrs post-infection led to a 1.33 fold increase in the accumulation of phosphorylated pathological tau in the soma of HDAC6 overexpressing neurons compared to control neurons at DIV 21 (Figure 4.6B). Comparison of pTau202 in the soma of control neurons at 36 hr vs. 72 hr was not significantly different. There is an age-dependent 1.13 fold increase in the amount of pTau202 accumulating in the soma of HDAC6 overexpressing neurons at 36 hr at DIV 20 vs. 72 hrs at DIV 21 and this increase is significant (P value = 0.0409).
Figure 4.5. Increased pTau202 in Soma of Synaptically Active Neurons Overexpressing HDAC6 36 hrs post-infection at DIV 20

A) Pathological pTau202 distribution in wild-type control neurons (top) at DIV 20 and neurons overexpressing HDAC6 (bottom) 36 hrs post-HFA MOI 100 infection at DIV 20. Max projection images of laser scanning confocal z-stacks, 63X oil-immersion, scale bars 50µM.

B) Quantification of pTau202 fluorescence intensity in soma plotted as mean ± S.E.M. Control, n = 113 cells (33 FOV); HDAC6 overexpressing, n = 114 cells (29 FOV). ** P value = 0.0019.
Figure 4.6. Increased pTau202 in Soma of Synaptically Active Neurons Overexpressing HDAC6 72 hrs post-infection at DIV 21

A) Pathological pTau202 distribution in wild-type control neurons (top) at DIV 21 and neurons overexpressing HDAC6 (bottom) 72 hrs post-HFA MOI 100 infection at DIV 21. Max projection images of laser scanning confocal z-stacks, 63X oil-immersion, scale bars 50µM.

B) Quantification of pTau202 fluorescence intensity in soma plotted as mean ± S.E.M. Control, n = 146 cells (40 FOV); HDAC6 overexpressing, n = 140 cells (43 FOV). **** P value < 0.0001.
4.2.4 Decreased pTau202 Puncta in Synaptically Active HDAC6 Overexpressing Neurons at DIV 20

To assess if HDAC6 overexpression had an effect on the size or number of tau puncta in synaptically active neurons, primary hippocampal neurons containing mature synapses were infected with HFA at an MOI of 100 at DIV 18 and fixed 36 hrs post-infection at DIV 20. Puncta size ranges are typically characterized into three size ranges based on their role in synaptic plasticity: 0.2 – 0.5µM², 0.5 – 1µM², and 1 - 2µM². Analysis of the average pTau202 puncta overall size for control compared to HDAC6 overexpressing neurons at 36 hrs post-infection at DIV 20 was not significant (control = 0.612 ± 0.003, n=15,876; HDAC6 overexpressing = 0.621 ± 0.003, n=12,312). At 36 hr post-infection, neurons overexpressing HDAC6 at DIV 20 had an overall decrease in total number of puncta at each size range compared to wild-type control neurons at DIV 20 (Figure 4.7). Although this reduction in average number of pTau202 puncta per FOV in each size range was not significant, the decrease in total number of puncta was 22.45% (total pTau202 puncta detected 0.2 to 2µM²: control = 15,876; HDAC6 overexpressing = 12,312). Comparison of images of control neurons compared to HDAC6 overexpressing neurons at 36 hr post-infection at DIV 20 show an increased overall fluorescence intensity of pTau202 puncta (Figure 4.5A) but the size of pTau202 puncta was decreased at each size range, which led to investigation of the average fluorescence intensity of the detected puncta at each size range. Indeed, at each size range the fluorescence intensity of pTau202 puncta was significantly increased in neurons overexpressing HDAC6 at 36 hr post-infection at DIV 20 vs. control neurons (Figure 4.8).
Figure 4.7. Average Number of pTau202 Puncta per FOV at DIV 20

The wild-type control neuron data was from 113 cells (33 FOV) at DIV 20. HDAC6 overexpressing neuron 36 hrs post-infection at DIV 20 data was from 114 cells (29 FOV). The total number of pTau202 puncta detected (0.2 to 2µM²) from 113 control cells = 15,876; 114 HDAC6 overexpressing cells = 12,312.
Figure 4.8. Average Fluorescence Intensity of pTau202 puncta in DIV 20 neurons

The wild-type control neuron data was from 113 cells (33 FOV) at DIV 20. HDAC6 overexpressing neuron 36 hrs post-infection at DIV 20 data was from 114 cells (29 FOV). Data plotted as mean ± SEM, n = total puncta detected. At 0.2 to 0.5µM \( \text{pTau}^202 \): Control = 2,536 ± 2.941, n=8,426; HDAC6 = 2,574 ± 3.750, n=6,521. At 0.5 to 1µM \( \text{pTau}^202 \): Control = 2,732 ± 4.386, n=4,873; HDAC6 = 2,768 ± 5.533, n=3,664. At 1 to 2µM \( \text{pTau}^202 \): Control = 2,844 ± 6.059, n=2,577; HDAC6 = 2,867 ± 6.886, n=2,127.
4.2.5 Decreased pTau202 Puncta in Synaptically Active HDAC6 Overexpressing Neurons at DIV 21

Next, to assess if HDAC6 overexpression had an effect on the size or number of tau puncta in synaptically active neurons, primary hippocampal neurons containing mature synapses were infected with HFA at an MOI of 100 at DIV 18 and fixed 72 hrs post-infection at DIV 21. Analysis of the average size of pTau202 puncta for control vs. HDAC6 overexpressing neurons at 72 hrs post-infection at DIV 21 was significant (control = 0.596 ± 0.002, n=31,156; HDAC6 overexpressing = 0.607 ± 0.003, n=16,884).

At 72 hr post-infection, neurons overexpressing HDAC6 at DIV 21 had a decrease in total number of puncta at each size range compared to wild-type control neurons at DIV 21 (Figure 4.9). The decrease in total number of pTau202 puncta was 45.8% (total pTau202 puncta detected 0.2 to 2μM²: control = 31,156; HDAC6 overexpressing = 16,884). Images of control neurons compared to HDAC6 overexpressing neurons at 72 hr post-infection at DIV 21 appeared to have increased overall fluorescence intensity of pTau202 puncta (Figure 4.6A) but the actual size of pTau202 puncta was decreased at each size range, which led to investigation of the average fluorescence intensity of the detected puncta at each size range. Indeed, at each size range the fluorescence intensity of pTau202 puncta was increased and statistically significant in neurons overexpressing HDAC6 at 72 hr post-infection at DIV 21 compared to control neurons (Figure 4.10).
Figure 4.9. Average Number of pTau202 Puncta per FOV at DIV 21

The wild-type control neuron data was from 146 cells (40 FOV) at DIV 21. HDAC6 overexpressing neuron 72 hrs post-infection at DIV 21 data was from 140 cells (43 FOV). The number of total pTau202 puncta detected (0.2 to 2µM²) from 146 control cells = 31,156; 140 HDAC6 overexpressing cells = 16,884. Data plotted as mean ± SEM, n = total FOV. At 0.2 to 0.5µM²: Control = 432.6 ± 34.83, n=40; HDAC6 = 212.4 ± 20.69, n=43. At 0.5 to 1µM²: Control = 225.7 ± 19.55, n=40; HDAC6 = 116.9 ± 11.85, n=43. At 1 to 2µM²: Control = 120.6 ± 11.62, n=40; HDAC6 = 63.12 ± 7.273, n=43.
Average Fluorescence of pTau202 puncta at DIV 21

**Figure 4.10. Average Fluorescence Intensity of pTau202 puncta in DIV 21 neurons**

The wild-type control neuron data was from 146 cells (40 FOV) at DIV 21 and HDAC6 overexpressing neuron 72 hrs post-infection at DIV 21 data was from 140 cells (43 FOV). Data plotted as mean ± SEM, n = total puncta detected. At 0.2 to 0.5µM²: Control = 2,570 ± 2.132, n=17,304; HDAC6 = 2,600 ± 3.356, n=9,128. At 0.5 to 1µM²: Control = 2,744 ± 3.158, n=9,029; HDAC6 = 2,796 ± 4.837, n=5,028. At 1 to 2µM²: Control = 2,822 ± 4.135, n=4,823; HDAC6 = 2,875 ± 6.295, n=2,714.
4.2.6 Decreased PSD-95 Puncta in Synaptically Active Neurons Overexpressing HDAC6

To assess if HDAC6 overexpression had an effect on the size or number of PSD-95 puncta in synaptically active neurons, primary hippocampal neurons containing mature synapses were infected with HFA at an MOI of 100 at DIV 18 and fixed at 36 hr post-infection at DIV 20 and 72 hrs post-infection at DIV 21. PSD-95 is an important scaffolding protein that has a functional role in maintaining molecular organization of postsynaptic dendritic spines and synaptic plasticity. Significant reduction of PSD-95 is an indicator of synaptic dysfunction in post-mortem AD brains (Proctor et al., 2011) therefore PSD-95 was used as a marker for synaptic dysfunction in neurons overexpressing HDAC6.

Representative images of PSD-95 puncta from DIV 20 for control and HDAC6 overexpressing at 36 hr post-infection neurons are shown in Figure 4.11A. Quantification of PSD-95 puncta from DIV 20 for control compared to HDAC6 overexpressing at 36 hr post-infection revealed neurons overexpressing HDAC6 at DIV 20 had significant decreases in total number of puncta at each size range compared to wild-type control neurons at DIV 20 (Figure 4.11). Within 36 hrs of HDAC6 overexpression the average PSD-95 puncta size (0.2 to 2μM^2) from DIV 20 neurons decreased by 1.15 fold compared to wild-type control neurons (Figure 4.12). The percent decrease of the total number of PSD-95 puncta was 77.9% (total pTau202 puncta detected 0.2 to 2μM^2: control = 2,697; HDAC6 overexpressing = 596).
Figure 4.11. Average Number of PSD-95 Puncta per FOV at DIV 20

The wild-type control neuron data was from 113 cells (33 FOV) at DIV 20. HDAC6 overexpressing neuron 36 hrs post-infection at DIV 20 data was from 114 cells (29 FOV).

A) Representative PSD-95 images showing puncta distribution in control neurons (top) at DIV 20 and neurons overexpressing HDAC6 infected with HFA MOI 100 (bottom) and fixed at 36 hrs post-infection at DIV 20. Max projection images of laser scanning confocal z-stacks, 63X oil-immersion, scale bars 50µM.

B) Data plotted as mean ± SEM, n = total FOV. At 0.2 to 0.5µM²: Control = 55.21 ± 8.285, n=33; HDAC6 = 15.97 ± 3.814, n=29. At 0.5 to 1µM²: Control = 20.33 ± 3.624, n=33; HDAC6 = 3.586 ± 0.9023, n=29. At 1 to 2µM²: Control = 6.152 ± 1.501, n=33; HDAC6 = 1.000 ± 0.5012, n=29. The total number pTau202 puncta detected (0.2 to 2µM²) from 113 control cells= 2,697; 114 HDAC6 overexpressing cells = 596.
The wild-type control neuron data was from 113 cells (33 FOV) at DIV 20. HDAC6 overexpressing neuron 36 hrs post-infection at DIV 20 data was from 114 cells (29 FOV).

Data plotted as mean ± SEM, n = total number puncta: Control = 0.483 ± 0.006, n=2,697; HDAC6 overexpressing = 0.418 ± 0.011, n=596.

Figure 4.12. Average Size of PSD-95 Puncta from DIV 20 Neurons
Next, analysis of the effect of HDAC6 overexpression 72 hrs post-infection of DIV 21 on PSD-95 puncta number and size from primary hippocampal neurons with mature synapses was compared to wild-type control DIV 21 neurons. Representative images of PSD-95 puncta from DIV 21 for control vs. HDAC6 overexpressing at 72 hr post-infection neurons are shown in Figure 4.13A. Quantification of PSD-95 puncta from DIV 21 for control and HDAC6 overexpressing at 72 hr post-infection revealed neurons overexpressing HDAC6 at DIV 21 had significant decreases in total number of puncta at both 0.2 – 0.5µM² and 0.5 – 1µM² ranges compared to wild-type control neurons at DIV 21 (Figure 4.13B). The percent decrease of the total number of PSD-95 puncta was 53.44% (total pTau202 puncta detected 0.2 to 2µM²: control = 3,052; HDAC6 overexpressing = 1,421). Analysis of the average PSD-95 puncta size (0.2 to 2µM²) for HDAC6 overexpressing neurons at 72 hrs post-infection at DIV 21 increased slightly by 1.08 fold compared to wild-type control neurons DIV 21 (Figure 4.14).
**Figure 4.13. Average Number of PSD-95 Puncta per FOV at DIV 21**

The wild-type control neuron data was from 146 cells (40 FOV) at DIV 21. HDAC6 overexpressing neuron 72 hrs post-infection at DIV 21 data was from 140 cells (43 FOV). **A**) Representative PSD-95 images showing puncta distribution in control neurons (top) at DIV 21 and neurons overexpressing HDAC6 infected with HFA MOI 100 (bottom) and fixed at 72 hrs post-infection at DIV 21. Max projection images of laser scanning confocal z-stacks, 63X oil-immersion, scale bars 50µM.

**B** Data plotted as mean ± SEM, n = total FOV. At 0.2 to 0.5µM²: Control = 53.83 ± 9.032, n=40; HDAC6 = 21.72 ± 4.217, n=43. At 0.5 to 1µM²: Control = 17.28 ± 3.175, n=40; HDAC6 = 8.302 ± 2.376, n=43. At 1 to 2µM²: Control = 5.200 ± 1.133, n=40; HDAC6 = 3.023 ± 0.973, n=43. The total number pTau202 puncta detected (0.2 to 2µM²) from 146 control cells= 3,052; 140 HDAC6 overexpressing cells = 1,421.
The wild-type control neuron data was from 146 cells (40 FOV) at DIV 21. HDAC6 overexpressing neuron 72 hrs post-infection at DIV 21 data was from 140 cells (43 FOV). Data plotted as mean ± SEM, n = total number puncta: Control = 0.465 ± 0.005, n=3,052; HDAC6 overexpressing = 0.500 ± 0.009, n=1,421.

**Figure 4.14. Average Size of PSD-95 Puncta from DIV 21 Neurons**

The wild-type control neuron data was from 146 cells (40 FOV) at DIV 21. HDAC6 overexpressing neuron 72 hrs post-infection at DIV 21 data was from 140 cells (43 FOV). Data plotted as mean ± SEM, n = total number puncta: Control = 0.465 ± 0.005, n=3,052; HDAC6 overexpressing = 0.500 ± 0.009, n=1,421.
4.3 Discussion

Tau homeostasis is regulated by a series of post-translational modifications including phosphorylation, acetylation, glycosylation, etc (Spires-Jones et al., 2009). It is well established that tau is a microtubule-associated protein primarily expressed in neurons and localized to the axon (Ballatore et al., 2007; Iqbal et al., 2005; Johnson and Stoothoff, 2004). This study focused solely on changes in tau phosphorylation because of its implicated role in tau dysregulation, AD pathology, and disease progression.

Phosphorylation of tau is a post-translational modification that regulates the affinity of tau for microtubules. In healthy neurons, the phosphorylation of tau is tightly regulated enabling tau to bind to microtubules stabilizing them whereas and phosphorylated tau with reduced affinity for microtubules enables axonal transport. Site-specific phosphorylation of tau enables tau to regulate cytoskeletal microtubule dynamics, axonal transport and neurite outgrowth (Johnson and Stoothoff, 2004). Specifically, phosphorylation of tau at threonine 231 has been observed to reduce the affinity of tau for microtubules (Cho and Johnson, 2003; Cho and Johnson, 2004; Goedert et al., 1994). However, phosphorylation alone is not an indicator of AD tau pathology as healthy fetal and adult brains have phosphorylated tau (Buee et al., 2000).

Abnormal hyperphosphorylation that leads to tau aggregation and missorting is an indicator of tau pathology. In degenerating neurons, hyperphosphorylation of tau leads to intracellular tau aggregation and the formation of neurofibrillary tangles (NFTs) containing both hyperphosphorylated and non-phosphorylated tau isoforms that are missorted to the somatodendritic compartment. Interestingly, the largest tau isoform has seventy-nine threonine and serine residues that are potential phosphorylation sites (Buee et al., 2000) of which more than half have been found to be phosphorylated and
implicated in tau pathology in AD brains (Hanger et al., 2007). Tau dysregulation, mislocalization of tau to soma and dendritic spines, the role of tau in dendritic spines, and tau dysfunction are implicated in AD pathology and progression (Haass and Mandelkow, 2010; Hoover et al., 2010; Ittner et al., 2010). Both missorting of tau and synapse loss is an early change in AD brains that precedes Aβ accumulation, NFTs and cognitive dysfunction.

HDAC6 recombinant adenovirus was developed to deliver HDAC6 to primary dissociated hippocampal neurons isolated from post-natal rat brains for the investigation of the neuroprotective vs. neuropathological role of increased HDAC6 protein levels in AD pathology and synaptic dysfunction. Early changes that mediate tau dysregulation and the accumulation and missorting of hyperphosphorylated pathological tau have remained elusive, therefore initial experiments were designed to investigate if HDAC6 overexpression led to any change in tau pathology including phosphorylation and missorting to the somatodendritic compartment in developing neurons. The data presented here indicates that HDAC6 as an underlying molecular factor in AD and a key player in mediating the missorting of pathological tau and synaptic dysfunction.

HDAC6_FLAG-Adenovirus enabled reproducible, efficient HDAC6 gene delivery and subsequent overexpression in primary dissociated hippocampal neurons (Figure 4.1). Increased expression of HDAC6 resulted in AD tau pathology and synaptic dysfunction. Increased HDAC6 was neuropathological in primary hippocampal neurons. HDAC6 overexpression missorted total tau to the soma of developing neurons (Figure 4.2). Since NFTs are composed of aggregates of phosphorylated and non-phosphorylated forms of tau that missort to the soma of neurons markers to determine the phosphorylation state of missorted tau was used. The phospho-specific antibody pTau231 detects tau with disrupted affinity for microtubules. Wild-type control neurons
had minimal staining of pTau231 suggesting these neurons have stable microtubules (Figure 4.3). Neurons overexpressing HDAC6 had distinct pTau231 staining of the entire neuron, axon and somatodendritic compartment suggesting a potentially destabilized microtubule network. Disruption of the microtubule network is a central mechanism leading to neuronal dysfunction (Zhang et al., 2013).

Further, the pTau202 antibody that detects phosphorylated Serine 202 indicative of NFTs (aggregated hyperphosphorylated tau) was used to confirm early and late tau pathology. Surprisingly, wild-type control neurons stained with pTau202 had puncta staining restricted from the soma suggesting phosphorylation of Serine 202 may have a functional role in mediating synaptic organization in healthy developing neurons (Figure 4.4). Neurons overexpressing HDAC6 show elevated pTau202 staining in the soma, indicating pathological tau accumulation in the soma (Figure 4.4). Dysfunction of hyperphosphorylated tau is well-studied and implicated in microtubule destabilization, aggregation, missorting of tau to the soma and dendritic spines, and synaptic dysfunction in neurons.

Experiments in synaptically active neurons were performed to investigate the impact of HDAC6 overexpression on tau pathology and synaptic dysfunction. Tau mislocalization in dendritic spines has been implicated in disrupting glutamate receptor (AMPA and NMDA receptors) trafficking and hence synaptic function (Hoover et al., 2010). The phosphospecific antibody pTau202 was used to recognize aggregated phosphorylated tau. Synaptically active neurons overexpressing HDAC6 show increased pTau202 staining in the soma, indicating pathological tau accumulation in the soma compared to wild-type control neurons (Figure 4.5, Figure 4.6). Synaptically active, wild-type control neurons showed puncta staining of phosphorylated tau restricted from the soma suggesting phosphorylation of Serine 202 may have a
functional role in mediating synaptic organization in healthy synaptically active neurons (Figure 4.5, Figure 4.6). Additionally, synaptically active neurons overexpressing HDAC6 had decreased total number of phosphorylated tau puncta suggesting changes in synaptic organization in these neurons (Figure 4.7, Figure 4.9).

Missorting of tau to soma in neurons correlates with the degeneration of synapses (Thies and Mandelkow, 2007), therefore I investigated changes in the size, number and fluorescence intensity of tau puncta to determine potential synaptic regulation mediated by HDAC6 expression. Compared to age-matched wild-type control neurons, neurons overexpressing HDAC6 had decreased tau puncta. Interestingly, the fluorescence intensity of size-matched tau puncta was increased in neurons overexpressing HDAC6 indicating increases in the amount of phosphorylated tau protein in puncta of the same size compared to wild-type neurons. This change in fluorescence intensity of phosphorylated tau suggests increased pathological tau in puncta leads to disruption of synaptic puncta organization and tau missorting in neurons overexpressing HDAC6.

The shift in tau homeostasis that mediates functional tau and pathological tau is regulated by a series of post-translational modifications. Recently, a number of studies have implicated acetylated tau as contributing to tau dysfunction, aggregation and accumulation by prevention of its degradation in AD (Cohen et al., 2011; Irwin et al., 2012; Min et al., 2010). It is likely that a combination of post-translational modifications (i.e. phosphorylation, acetylation) lead to hyperphosphorylated tau and accumulation of aggregated tau that is missorted to soma of neurons.

Altered neuronal communication at the synapse and a decreased number of postsynaptic receptors correlates with changes in synaptic strength and plasticity (Bingol et al. 2011). Experiments were designed to investigate any changes in the synaptic
organization of an important scaffolding protein, postsynaptic density protein 95 (PSD-95) mediated by HDAC6 overexpression. PSD-95 is the most-abundant scaffolding protein in postsynaptic densities, and is one of the most stable proteins in dendritic spines (Chen et al., 2011; Kasai et al., 2010). PSD-95 plays a functional role in maintaining molecular organization of excitatory dendritic spines and plays a pivotal role in LTD (Bhattacharyya et al., 2009; Chen et al., 2011). Interestingly, synaptically active neurons overexpressing HDAC6 had significant decreases in the total amount of PSD-95 puncta compared to wild-type control neurons (Figure 4.11, Figure 4.13). These reductions in the total number of PSD-95 puncta in neurons overexpressing HDAC6 suggest HDAC6 has a neuropathological role in disrupting the molecular organization of dendritic spines and impairing synaptic neurotransmission. Additionally, loss of postsynaptic scaffolding protein PSD is believed to contribute to AMPA receptor dependent LTP deficits in AD (Proctor et al., 2011). The size of synaptic puncta has been reported to correlate with changes synaptic neurotransmission and LTD and LTP. The average size of PSD-95 puncta was reduced at 36 hr post-infection in neurons overexpressing HDAC6 at DIV 20 (Figure 4.12), yet at neurons overexpressing HDAC6 for 72 hrs at DIV 21 the average size of PSD-95 puncta was increased (Figure 4.13). The PSD-95 data of neurons overexpressing HDAC6 for 36 hrs and 72 hrs compared to age-matched wild-type control neurons suggests a potential compensatory mechanism altering the total number of PSD-95 puncta and average size of PSD-95 when synaptic communication is disrupted by tau missorting and changes to PSD-95 during disease progression.
4.4 Methods

4.4.1 Cell Culture

4.4.1.1 HEK293T cells

HEK293T cells were maintained in Dulbecco’s Minimum Essential Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) at 37°C and 5% CO2. Cells were plated at 70% confluency in T75-flask and transfected 24 hrs after plating with LipoD293™ according to manufactures instructions (SignaGen®).

4.4.1.2 HeLa cells

HeLa cells were maintained in Eagle’s Minimum Essential Medium (EMEM) (ATCC Catalog #30-2003) supplemented with 10% FBS at 37°C and 5% CO2. For Adenoviral titer, cells were plated at 85% confluency.

4.4.1.3 Dissociated hippocampal neurons

All experiments involving rats were performed in accordance with local institutional and government regulation for animal welfare. Primary cultures of post-natal rat hippocampal neurons isolated from Sprague-Dawley rat pups (Charles Rivers Laboratories) were performed using standard procedures. Neurons were plated at 62–75 × 10^3 cells/cm² on poly-L-lysine coated glass coverslips and were grown in minimum essential medium (MEM) containing 5% FBS, 25 mM glucose, B27 (Invitrogen), 2 mM glutamine and Mito+ Serum Extender (BD Biosciences). Neurons were maintained in culture with ½ media changes every three days. Gene delivery was performed by HDAC6_FLAG-Adenovirus at a 100-fold multiplicity of infection (MOI).
4.4.2 Generation of HDAC6 recombinant adenovirus

Recombinant adenovirus was generated following the AdEasy method for generation and production similarly to previously described (Luo et al., 2007). Briefly, the following plasmids were used pcDNA3.1(+)HDAC6FLAG (Addgene plasmid #13823), pShuttleCMV (Addgene plasmid #16403), pAdEasy-1 (Addgene plasmid #16400). The cDNA of human HDAC6 with an engineered c-terminal FLAG tag was PCR amplified from pcDNA3.1(+)HDAC6FLAG subcloned into pShuttleCMV using 5’ BglII and 3’ HindIII sites. The resulting pShuttleCMV_HDAC6FLAG construct was linearized with PmeI and co-transformed with pAdEasy-1 into Escherichia coli BJ5183 cells (Addgene strain # 16398) for homologous recombination. Recombinant adenoviral plasmids were verified by PacI digestion. The recombinant adenoviral plasmid isolated from verified recombinant clones was transformed into recombination-defective Novablue strain. The recombinant adenoviral plasmid was then digested with PacI and used to transfect HEK293T cells (which contain E1 genes for adenoviral packaging) using LipoD293. Media was changed 16 hours post-transfection following BSL-2+ practices, and cells were maintained at 37°C and 5% CO₂ for 42 days until the primary HDAC6_FLAG-adenoviral stock was harvested by freeze-thaw.

4.4.3 Recombinant Adenovirus titer using immunocytochemistry

HeLa cells were infected with crude HDAC6_FLAG-Adenoviral stocks titrated from 10⁻⁵ to 10⁻¹⁶ in biological replicates of 4. Cells were fixed with 4% paraformaldehyde 24 hrs post-infection. Fixed cells were permeabilized with 0.1% TritonX-100. Cells were blocked with 5% goat serum and immunostained with a 1° antibody that detects Adenovirus type 5 Hexon protein (DSHB catalog # TC31-9C12.C9) followed by 2° Goat Anti-mouse IgG Dylight 488 (Pierce #35502). Widefield fluorescent
images were acquired and the average number of infected cells per area was calculated. The equation used to calculate viral titer was \( \frac{\text{(average number of infected cells/fields of view)} \times \text{(total number of fields of view possible/well)} \times \text{(dilution factor)}}{\text{(virus volume added per well)}} \). This equation calculated the number of viral infection units per mL in the crude viral stock. This method for adenovirus viral titer was designed with adenovirus expert Dr. Gary Ketner at John Hopkins University School of Medicine by adapting a protocol from his laboratory.

### 4.4.4 Neuron Immunocytochemistry

Neurons were fixed with 4% paraformaldehyde at the appropriate time-point. Fixed cells were permeabilized with 0.1% TritonX-100. Cells were blocked with 5% goat serum and immunostained with the appropriate primary and secondary antibodies. The primary antibodies used were Mouse Anti-PSD-95 (Millipore #MAB1596), Mouse Anti-Tau5 (Invitrogen #AHB0042), Mouse Anti-FLAG (Stratagene #200471), Rabbit Anti-pTau202 (Anaspec #28017-025), Rabbit Anti-pTau231 (Anaspec #55313-025), Chicken Anti-FLAG (AFLAG-5). The secondary antibodies used were Goat Anti-mouse IgG Dylight 488 (Pierce #35502), Goat Anti-rabbit IgG Dylight 549 (Pierce #35557), Goat Anti-Chicken Alexa Fluor 633 (Invitrogen #A21103). Images were acquired using a Zeis Laser Scanning 510 Meta confocal microscope at 63X (Plan-Apochromat 63X/1.4NA) oil-immersion using the appropriate lasers (488nm, 543nm, 633nm) and filters. Four channel (488nm, 549nm, 633nm, DIC) Z-stack 12 bit (1024 X 1024) images were acquired as 26 stacks at .1µM intervals. Images were acquired with settings to avoid pixel saturation and these settings were the same for all image acquisition for control and HDAC6 overexpressing cells.
4.4.4 Neuron Image analysis

4.4.4.1 FLAG Analysis

Images of at least 10 fields of view were taken for both control and HFA infected neurons. Using ImageJ software (NIH), z-stacks images split into 4 channels and MAX-intensity projection images of the appropriate channel were used. All neuron somas in these images were chosen as region of interest (ROI), and three background areas were chosen as background ROI for each image. The background ROI were averaged and subtracted from the average FLAG fluorescence of the soma ROI. Neurons were identified as HDAC6 overexpressing neurons or positive infected cells if they had FLAG fluorescence above the average control FLAG autofluorescence.

4.4.4.2 Tau Soma Analysis

Images of at least 10 fields of view were taken for both control and HFA infected neurons. Using ImageJ software (NIH), z-stacks images were split into 4 channels and MAX-intensity projection images of the appropriate channel were used. All neuron somas in these images were chosen as a ROI, and three background areas were chosen as background ROI for each image. The background ROI were averaged and subtracted from the average Tau fluorescence of the soma ROI. All figures were prepared with GraphPad Prism ® version 6.
4.4.4.3 Tau puncta analysis

Images of at least 10 fields of view were taken for both control and HFA infected neurons. Using ImageJ software (NIH), z-stacks images were split into 4 channels and MAX-intensity projection images of the appropriate channel were used. To determine Tau puncta size or the fluorescence intensity of puncta, images were analyzed using the ‘background subtracted’ function followed by ‘3D object counter’ function using a threshold of 2000. The threshold value was determined manually and then applied to all images, tau fluorescence detected was at least 5X above background. The object maps containing the coordinates of detected objects were saved and manually verified. The output data was also saved and then compiled for all FOV analyzed. All figures were prepared with GraphPad Prism ® version 6.

4.4.4.4 PSD-95 puncta analysis

Images of at least 10 fields of view were taken for both control and HFA infected neurons. Using ImageJ software (NIH), z-stacks images were split into 4 channels and MAX-intensity projection images of the appropriate channel were used. To determine PSD puncta size, images were analyzed using the ‘background subtracted’ function followed by ‘3D object counter’ function using a threshold of 1700. The threshold value was determined manually and then applied to all images, this allowed PSD-95 fluorescence detected to be at least 5X above background. The object maps containing the coordinates of detected objects were saved and manually verified. The output data was also saved and then compiled for all FOV analyzed. All figures were prepared with GraphPad Prism ® version 6.
CHAPTER 5
CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Conclusions

The mammalian brain is complex and progress in the field is limited by the availability of tools to probe the underlying molecular basis of synaptic communication and dysfunction. The cell culture models used throughout this dissertation have both advantages and disadvantages. Heterologous expression systems offer the most simplistic system to investigate and modulate synaptic communication. Dissociated primary hippocampal neuron culture allows for investigation and modulation of synaptic communication in a simplified system compared to intact brain slice cultures whereas slice cultures are a simplified system compared to animal models. Data generated from any of these model systems need to be validated against knowledge generated from human brain tissue to ensure global conclusions are representative of synaptic communication and dysfunction in the human brain.

In this dissertation, I have investigated neuronal communication using novel molecules to photocontrol receptors at the synapse, and identified HDAC6 as an underlying molecular factor in Alzheimer’s disease (AD) tau pathology and synaptic dysfunction. This research has used techniques in chemical neurobiology, and molecular and cellular biology including patch-clamp electrophysiology, immunoblotting, and confocal microscopy. By employing these techniques I have answered questions about the function of inhibitory receptor subtypes at synapses, and the role of HDAC6 in AD in both heterologous expression systems and primary hippocampal neurons. This research has given insight on the underlying molecular mechanisms of learning and
memory by deciphering the role of receptor subtypes in synaptic communication, and changes that indicate early synaptic dysfunction in AD.

I have described how we have successfully developed a series of LiGABA\textsubscript{\alpha}Rs that enable light-controlled inhibitory neurotransmission of subtype specific GABA receptors. Specifically, we developed \( \alpha 1 \)- and \( \alpha 5 \)-LiGABA\textsubscript{\alpha}Rs. These LiGABA\textsubscript{\alpha}Rs enabled spatiotemporal precision control of ion channels at the synapse thus allowing us to investigate the underlying mechanisms of subtype-specific neural inhibition. The PTLs we developed enabled a method for photocontrolling engineered GABA\textsubscript{\alpha} receptors that was fast, reversible photoregulation, and subtype-specific. Our experiments revealed that tethered muscimol acts as an antagonist to engineered GABA\textsubscript{\alpha} receptors when irradiated with 500 nm light. This method enabled photoregulation of endogenous mIPSCs from dissociated hippocampal neurons expressing LiGABA\textsubscript{\alpha}R. Further, this method also enabled the photocontrol of action potential firing of intact brain slices.

I have used an \textit{in vitro} heterologous cell culture system to investigate the outcome of the overexpression of HDAC6 on tau and protein levels in HEK-tau cells. The data from this study suggest that an increase in HDAC6 expression leads to increased pathological tau accumulation and may be the result of decreased PP1 levels in cells overexpressing HDAC6. Further, the expression of catalytically inactive HDAC6 in HEK-tau cells did not prevent the accumulation of increased pathological tau or the decrease in PP1 levels. This data suggests that HDAC6 is a underlying molecular factor altering both tau and PP1 protein levels, which may contribute to AD progression. I also verified that HEK-tau cells overexpressing HDAC6 with a catalytically inactive second deacetylase domain were in fact catalytically inactive by using a known substrate acetylated tubulin as a reporter of cytosolic functional HDAC6. As expected, HDAC6 with catalytically inactive second deacetylase domain were unable to deacetylate tubulin.
Additionally, this study revealed that the expression of tau does not inhibit the deacetylase function of cells overexpressing catalytically active HDAC6.

Studies using dissociated primary hippocampal neurons were used to further investigate the potential underlying molecular role of HDAC6 in altering key proteins involved in AD progression. For this project, I designed and developed recombinant HDAC6 adenovirus for efficient and reproducible HDAC6 gene delivery in neurons. This method resulted in the development of a primary hippocampal neuron cell culture model to study hallmarks of AD progression. The results presented in this dissertation reveal increased expression of HDAC6 in dissociated primary hippocampal neurons as an underlying molecular factor that contributes to the pathological missorting of phosphorylated tau to the soma of affected neurons, reduction of tau puncta, and reduction in PSD-95 puncta. These changes suggest that increased HDAC6 in neurons leads to changes in the molecular organization of dendritic spines that suggest HDAC6 is involved in regulating changes in excitatory neurotransmission and LTD. The results from these experiments suggest dissociated primary hippocampal neurons overexpressing HDAC6 can be used as a tissue culture model system to study changes correlate with human AD progression and pathology.

5.2 Future Directions

Using the PTL approach, we developed a series of LiGABA<sub>A</sub>Rs. This technology enables investigation into the roles that receptor subtypes play in mediating and altering synaptic neurotransmission. Additional studies with these LiGABA<sub>A</sub>Rs provide a method for precise, reversible photocontrol of sub-type specific receptors to investigate their distribution, and examine their functional role in leaning and memory. By exerting photoswitchable control on engineered receptor subtypes in brain slices, we can
investigate the role specific subtypes of GABA<sub>A</sub> receptors in intact brain tissue have in preventing epilepsy. Further, the PTLs we developed can be employed to exert photocontrol on future engineered GABA<sub>A</sub> receptor subtypes of interest. Additionally, ‘knock-in’ mice expressing LiGABA<sub>A</sub>Rs in place of endogenous subunits could be used to study genetically engineered receptors in the absence of wild-type background. The photocontrol of GABA receptor study presented in this dissertation begins to address how receptor subtypes contribute to inhibitory synaptic neurotransmission. The suggested future studies would enable further investigation into the role of receptor subtypes have in synaptic communication. This knowledge would further the current understanding of the molecular basis of learning and memory.

Alzheimer’s disease is a complex, multifactoral, slow progressing disease with no cure and little understanding of the underlying molecular basis leading to the pathological hallmarks of AD and synaptic dysfunction. By using a cell culture model, I was able to investigate the neuropathological role of HDAC6 and underlying molecular contributions that HDAC6 overexpression appears to have in AD tau pathology and synaptic dysfunction. The main advantages of this system are a single gene is altered enabling fast, efficient and reproducible pathological hallmarks representative of changes in post-mortem AD brains and animal models. Further studies that investigate early synaptic changes such as decreases in excitatory receptors (AMPA and NMDA) are possible using dissociated hippocampal neurons overexpressing HDAC6. Future studies using this system can investigate early changes that lead to defects in protein degradation and the subsequent accumulation of aggregated proteins. Additionally, this system can potentially be used for a high-throughput drug screen to determine if targeting HDAC6 reverses or slows the progression of AD tau pathology and synaptic dysfunction. Alternatively, this system can potentially be used for a high-throughput drug
screens to determine if targeting kinases upregulated in AD will reverse or slows the progression of AD tau pathology and synaptic dysfunction. The investigation and identification of increased HDAC6 expression as having an underlying neuropathological role in AD tau pathology and synaptic dysfunction presented in this dissertation begins to investigate early changes in AD that potentially could be targeted to prevent disease progression. The suggested future studies would enable further investigation of early changes in AD that may be the result of increased protein levels of HDAC6. Investigation of early synaptic changes that contribute to memory and cognitive impairment will greatly advancing our understanding of AD progression.
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