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M1 Muscarinic Acetylcholine Receptor Signaling and Regulation of Amyloid Precursor Protein Processing

By

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Doctor of Philosophy

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ABSTRACT

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By Albert Augustus Davis

Alzheimer's disease (AD), a progressive neurological disorder characterized by memory loss, cognitive decline, and behavioral disturbances, is the leading cause of dementia among the elderly and affects nearly half of the population over the age of 85. Although AD is a complex disease and remains incompletely understood, multiple lines of evidence point to important roles of the neurotoxic amyloid beta peptide (Aβ) and the amyloid precursor protein (APP) from which Aβ is derived. Activation of several neurotransmitter receptors, including muscarinic acetylcholine receptors (mAChRs), has been shown to reduce Aβ production, but less is known about the specific mAChR subtypes that regulate APP processing in neurons.

Using primary neuron cultures from wildtype and M1 mAChR-deficient mice, we demonstrate that the M1 mAChR subtype is essential for cholinergic regulation of non-amyloidogenic APP processing. In wildtype neuron cultures, treatment with the muscarinic agonist carbachol stimulated non-amyloidogenic APP processing. In M1 knockout neurons, these responses were either abolished or reversed by carbachol treatment, and M1 overexpression restored the wildtype phenotype. *In vivo* experiments in APP-transgenic mice demonstrate that the loss of M1 receptors accelerates amyloid pathology. Complementary experiments in the neuronotypic PC12 cell line using recently developed, highly selective allosteric M1 agonists support this finding and provide proof

of principle that M1-selective drugs can regulate APP processing and are therefore good candidates for evaluation in more complex model systems.

We also investigated the activation and regulatory mechanisms of two structurally distinct allosteric M1 agonists. We show that allosteric agonists potently activate multiple signal transduction pathways linked to the M1 receptor but are significantly impaired in their ability to induce recruitment of arrestin-3, a protein involved in regulation of G-protein coupled receptor signaling. Consistent with their lack of arrestin recruitment, both allosteric agonists showed blunted responses in measurements of receptor desensitization, internalization, and downregulation. These results will both strengthen the understanding of basic receptor biology and help to shape the development of a new generation of drugs for the treatment of AD and other devastating neurological and neuropsychiatric diseases.

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Chapter I. INTRODUCTION

Alzheimer's Disease: Background and Significance

Initial Report and Prevalence of Disease

In 1901, the German psychiatrist Alois Alzheimer admitted a 51-year-old woman named Auguste D. to the hospital in Frankfurt am Main for symptoms of profound cognitive impairment, memory loss, aphasia, paranoid delusions, and auditory hallucinations. Auguste D.'s illness began with suspicious and jealous feelings towards her husband and progressed to include memory impairment, difficulty with language, disorientation, and fits of screaming, ultimately requiring her to be confined to an isolation room at times. Alzheimer followed his patient's deteriorating condition until her death in April of 1906, at which time he examined her brain using histochemical techniques recently pioneered by Franz Nissl and Max Bielschowsky (Maurer, Volk et al. 1997). Among his observations were the identification of thick and "impregnable" intracellular fibrils and "numerous small miliary foci," which he presciently attributed to "the storage of a peculiar material in the cortex." Alzheimer presented his findings in November of 1906, describing in precise detail the neurofibrillary tangles and amyloid plaques that remain the hallmarks of the disease (Alzheimer 1906) (Figure 1.1). While cases of dementia with similarly early onset continue to be very rare and were considered for decades to be a separate disease entity (termed "presenile dementia") from the majority of cases of senile dementia with onset in later life, the two forms are nearly indistinguisable from clinical and histological perspectives and it is now accepted that

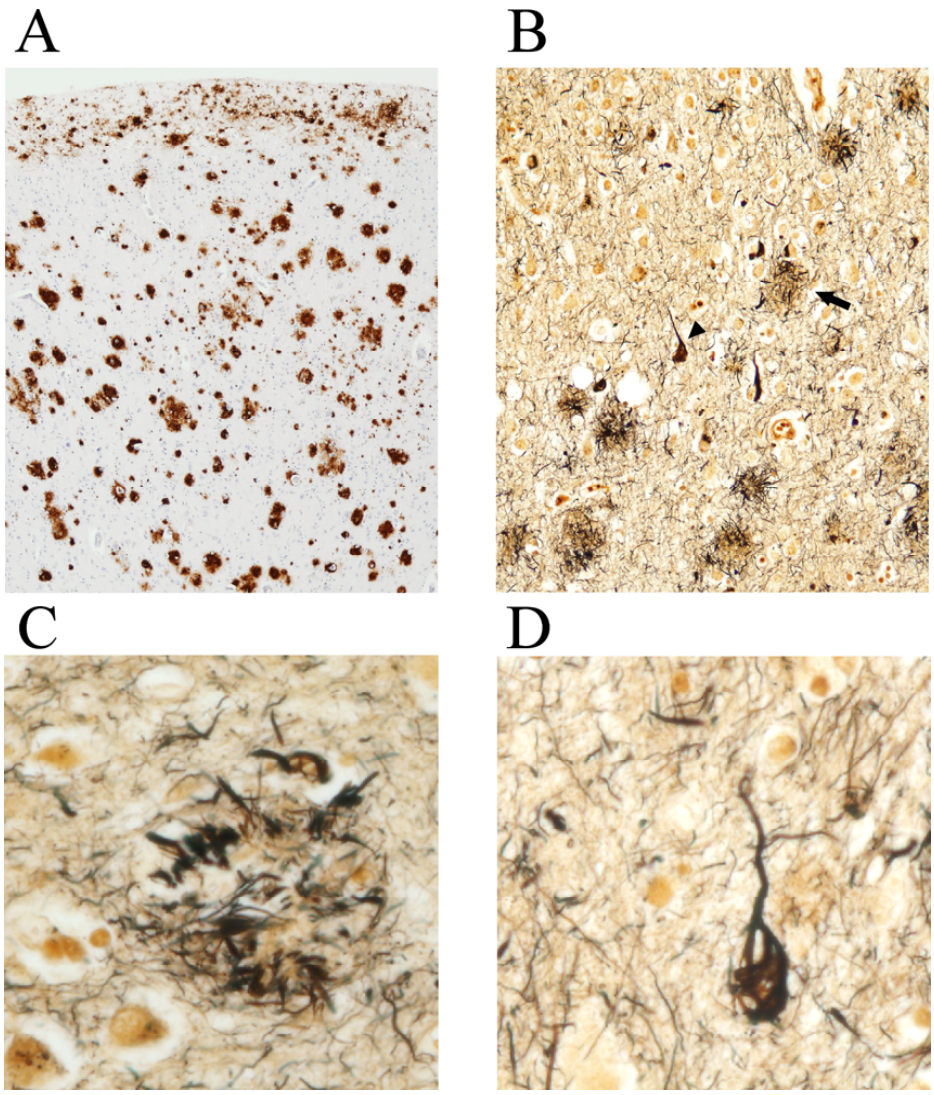


Figure 1.1. Pathological hallmarks of Alzheimer's disease.

(A) Low-magnification view of cerebral cortex showing multiple Aβ-immunopositive plaques. **(B)** Higher magnification of cerebral cortex showing argyrophilic amyloid plaques (arrow) and neurofibrillary tangles (arrowhead). **(C)** High-magnification view of a single neuritic plaque. The central core of the plaque contains amyloid peptides that are surrounded by swollen silver-positive dystrophic neurites. **(D)** High-magnification view of a neurofibrillary tangle.

"early onset" and "late onset" AD share common elements of pathophysiology (Selkoe 2001). Thus, Alzheimer's comments on the peculiar microscopic structures he observed in the brain of Auguste D. are directly relevant to the millions of patients who suffer from the disease which bears his name.

AD is one of many causes of dementia, a clinical diagnosis characterized by memory loss, cognitive impairment, language difficulties, and behavioral disturbances. For roughly 40 years, it has been recognized that AD is the most common cause of late-life dementia (Roth, Tomlinson et al. 1966; Roth, Tomlinson et al. 1967; Tomlinson, Blessed et al. 1970). Similar to most causes of dementia, AD typically presents in late adulthood, with the majority of patients over age 65. While the symptoms of dementia have been described since antiquity (Berchtold and Cotman 1998), the dramatic increase in human life expectancy during recent years has magnified the overall burden of dementing illnesses. In the United States, this trend is now compounded by the fact that a large segment of the population is nearing late adulthood. It is estimated that the prevalence of AD among individuals age 65-74 is less than 10%, while the prevalence among individuals age 85 years and older approaches 50% (Evans, Funkenstein et al. 1989). Statistical projections from census data indicate that the number of persons with AD will increase from between 4 and 5 million in the year 2000 to nearly 13 million by the year 2050 (Evans, Funkenstein et al. 1989; Hebert, Scherr et al. 2003). In addition to the physical and emotional devastation that AD inflicts on patients and their loved ones, there is an enormous and growing financial impact of the disease as more and more patients require attention and care from their adult children, many of whom are in their peak years of productivity. AD therefore represents a worsening threat to our society and

our economy, underscoring the urgent need for the development of effective interventions to prevent and slow the disease process.

Advances in Understanding the Pathophysiology of AD

In the decades following Alzheimer's original description of the disease, relatively little progress was made towards understanding its underlying causes. Some of the first major breakthroughs came in the late 1970s, when it was discovered that the activities of two key enzymes in the cholinergic neurotransmitter system, choline acetyltransferase and acetylcholinesterase, were reduced in brain samples from AD patients (Davies and Maloney 1976). The finding that decreased activity of these enzymes correlated with amyloid pathology and severity of dementia (Perry, Tomlinson et al. 1978) lent further support to the hypothesis that cholinergic abnormalities might play a central role in the development of AD. Complementing this biochemical data was the discovery that the cell bodies of cholinergic neurons in the basal forebrain appeared to be selectively vulnerable in AD (Whitehouse, Price et al. 1981). The intimate relationship between the cholinergic system and AD will be examined in greater detail below, but these advances bear mention here as early clues to the molecular basis of the disease.

While the association of cholinergic dysfunction and AD was pivotal and certainly helped to understand some of the basis of the intrinsic cognitive impairment in the disease, virtually nothing was known about the molecular identity of the aggregated proteinaceous structures that Alzheimer had identified using Bielschowsky's silver staining techniques. Several advances including Congo Red dye and electron microscopy

permitted the closer inspection and classification of the characteristic neuropathology (Kidd 1963; Terry, Gonatas et al. 1964; Howie and Brewer 2009), but still there were few clues as to the origin of the abnormal protein deposits. Then, in the mid 1980s, two independent groups reported the amino acid sequence analysis of a small peptide purified from amyloid-rich fractions of AD brain homogenate (Glenner and Wong 1984; Masters, Simms et al. 1985). The sequence of this peptide did not match that of any protein known at the time, suggesting that the novel peptide was a specific component of amyloid plaques and could hold promise for unraveling the molecular basis of the disorder. This peptide, termed A_4 because of its migration near 4000 Daltons on polyacrylamide gel electrophoresis, is now known to be the beta-amyloid, or Aβ, peptide that comprises the core of amyloid plaques. There are several forms of amyloid pathology in AD, ranging from diffuse deposits to fully-formed plaques with dense amyloid cores, that appear to involve increasing stages of aggregation of the Aβ peptide. Mature amyloid plaques are highly insoluble and consist of overlapping β-pleated sheets, typically surrounded by dystrophic neurites, reactive astrocytes, and activated microglia (Morgan, Colombres et al. 2004). The details concerning the generation of the Aβ peptide from its precursor molecule and the importance of Aβ in the pathogenic cascade of AD will be discussed in more detail, but it is worth noting its discovery as the predominant pathologic molecule in Alzheimer's disease.

Shortly after the identification of the beta amyloid peptide as the molecular constituent of senile plaques, a hyperphosphorylated form of the microtubule-associated protein tau was determined to be the primary element of neurofibrillary tangles (Grundke-Iqbal, Iqbal et al. 1986; Grundke-Iqbal, Iqbal et al. 1986; Kosik, Joachim et al.

1986; Wolozin, Pruchnicki et al. 1986; Wood, Mirra et al. 1986). Tau normally participates in the assembly and stabilization of microtubules, which are key protein structures in cells. The integrity of the microtubule system, including associated proteins such as tau, is believed to be critical for neurons, which can have long processes. A complete review of the structure and function of tau, as well as its involvement in the pathology of multiple neurodegenerative diseases is outside the scope of this thesis, but it should be mentioned that as the principal component of the paired helical filaments that make up neurofibrillary tangles, tau was considered to be a frontrunner for the molecular culprit of AD. Several compelling pieces of evidence, including correlation with dementia severity, contributed to the argument that tau dysfunction caused AD. A heated debate persisted for many years regarding whether tau or Aβ was the more important, and more proximal molecule in the cascade of AD pathogenesis, with proponents of each camp being labeled "tauists" and "baptists" (for β-amyloid protein). While perturbations in tau likely contribute to AD pathology and are also observed in several other neurodegenerative diseases including progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), frontotemporal lobar degeneration (FTLD), and the Parkinsonism-dementia complex of Guam (Hou, Carlin et al. 2004), it is becoming clear that Aβ supercedes tau in the pathophysiology of AD. Several lines of evidence point to a primal role for amyloid in AD. There are no mutations in the *tau* gene that cause familial AD, while mutations in *tau* are implicated in frontotemporal dementia and parkinsonism linked to chromosome 17, a non-AD form of dementia, as well as increased risk of other non-AD dementias (Cairns, Lee et al. 2004). In contrast, numerous mutations in the genes encoding the Aβ precursor protein as well as proteins that influence its metabolism

have been shown to cause autosomal-dominant inherited AD (examined in greater detail below). Additionally, recent experiments in a mouse model that develops both amyloid and tau pathology demonstrated that the onset of amyloid pathology precedes the development of tau pathology, that genetic manipulation of *tau* levels does not alter the onset or severity of amyloid pathology, and that immunization against the Aβ peptide can alleviate both amyloid and tau pathology (Oddo, Caccamo et al. 2003; Oddo, Billings et al. 2004; Oddo, Caccamo et al. 2007). These data should not be interpreted as evidence that tau is merely a bystander in neurodegenerative diseases, or even that tau plays no role in the development of AD symptoms. Rather, these findings support the hypothesis that changes in Aβ constitute the primary driving force in AD pathogenesis.

In addition to the major breakthroughs in amyloid and tau pathology, numerous other findings have increased the understanding of AD pathophysiology. There is a loss of large neurons, particularly in layer II of the entorhinal cortex and of pyramidal neurons in layers III and V of neocortex that likely explains the observed decrease in levels of the excitatory neurotransmitter glutamate in brains of AD patients (Davies, Mann et al. 1987; Young 1987; Lowe, Bowen et al. 1990; Francis 2003). In addition to frank degeneration of cortical neurons, much attention has focused on the loss of synapses and synaptic dysfunction that have been shown to precede cell loss and probably account for the onset of symptoms in early stages of disease (Terry, Masliah et al. 1991; Scheff and Price 2006; Scheff, Price et al. 2006; Knobloch and Mansuy 2008). Of all pathological alterations measured to date, synapse loss best correlates with cognitive deficits. There is also degeneration in several subcortical nuclei containing concentrations of neurons which utilize specific neurotransmitters and which project to brain regions involved in cognition,

memory, and emotion, all of which are profoundly affected in AD. Cholinergic neurons in the basal forebrain, serotonergic neurons in the raphe nucleus, and noradrenergic neurons in the locus coeruleus undergo degeneration in AD, and there is documented loss of pre- and post-synaptic enzymes, transporters, and receptors associated with these neurotransmitter circuits (Mossner, Schmitt et al. 2000; Mufson, Counts et al. 2008; Weinshenker 2008). There are also a variety of intracellular changes in endomembrane compartments. For example, the biosynthetic machinery, including the Golgi apparatus, is shrunken (Salehi, Lucassen et al. 1994), and there is an expansion of endosomal compartments (Cataldo, Barnett et al. 1995; Cataldo, Hamilton et al. 1996; Nixon 2005). Endosomal changes occur early in the disease, even at preclinical stages in vulnerable neurons, and appear to be intimately associated with genetic factors in AD.

Extensive evidence supports a role for oxidative damage in AD. Numerous studies have shown higher levels of oxidized lipids, proteins, and DNA in the brains of AD patients. Of significance, oxidative damage is highest in brain areas that are most heavily affected in AD (e.g., neocortex and hippocampus) and lowest in areas that are spared (e.g., cerebellum) (Pratico 2002; Pratico, Clark et al. 2002). Increased levels of oxidized lipid metabolites are detectable in the CSF, serum, and urine of patients with AD. Levels of one metabolite, an isomer of prostaglandin F2, are elevated in the CSF of patients with mild cognitive impairment, a condition that is believed to be a prodrome of AD (Pratico, Clark et al. 2002). Antioxidants may also play an important role in AD therapy. Observations from the Cache County Study Group have indicated reduced risk of AD among participants taking vitamin C and E supplements, and better cognitive performance was noted among participants who reported a diet rich in antioxidants

(Zandi, Anthony et al. 2004; Wengreen, Munger et al. 2007). However, data from controlled clinical trials have been mixed. The antioxidants vitamin E and selegiline have been shown to slow disease progression by 6–12 months in patients with moderate AD (Sano, Ernesto et al. 1997). However, another recent trial found no benefit from vitamin E in preventing the development of AD in patients with mild cognitive impairment (Petersen, Thomas et al. 2005).

Inflammation is a key component of AD (Eikelenboom, Rozemuller et al. 2000; McGeer and McGeer 2001; McGeer, Rogers et al. 2006). In fact, the presence of an inflammatory response is a key feature that distinguishes pathological neuritic plaques from more benign diffuse plaques. Neuritic plaques are surrounded by reactive astrocytes and activated microglia, the resident immune cells of the central nervous system. In epidemiological studies, the use of non-steroidal anti-inflammatory drugs (NSAIDs) initially appeared to prevent or delay AD (Stewart, Kawas et al. 1997; in t' Veld, Ruitenberg et al. 2001). However, randomized trials with anti-inflammatory drugs (including NSAIDs, cyclooxygenase–2 inhibitors, and steroids) have shown little benefit. Because the strongest association of NSAIDs in epidemiological studies is apparent with drug exposure that occurs at least 2–3 years before disease onset, it is possible that anti-inflammatory agents may have a protective role only at earlier preclinical stages of AD. Some mechanistic studies have suggested that certain NSAIDs may achieve their benefit by directly modulating enzymes involved in amyloid processing, instead of via an anti-inflammatory pathway, underscoring the importance of drug selection in future efforts to modulate AD pathology in humans using anti-inflammatory agents (Weggen, Eriksen et al. 2001; Eriksen, Sagi et al. 2003; Weggen, Eriksen et al. 2003).

There is a well-established and growing relationship between cholesterol and AD. The strongest genetic risk factor for "sporadic" AD is inheritance of the ε4 allele of the *APOE* gene, involved in cholesterol homeostasis (Corder, Saunders et al. 1993). Hypercholesterolemia and other cardiovascular risk factors also increase the risk of developing AD (Notkola, Sulkava et al. 1998; Kivipelto, Helkala et al. 2001), and there is fairly strong epidemiological evidence that the cholesterol lowering HMG Co-A reductase inhibitors (statins) reduce the risk of AD (Jick, Zornberg et al. 2000; Wolozin, Kellman et al. 2000). Epidemiological evidence also indicates a reduced risk of AD with increased consumption of the omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA) (Kalmijn, Launer et al. 1997; Morris, Evans et al. 2003; Kalmijn, van Boxtel et al. 2004), and animal model studies suggest that manipulations of dietary DHA can influence the development of AD pathology (Calon, Lim et al. 2004; Lim, Calon et al. 2005; Hooijmans, Van der Zee et al. 2009).

In the past decade, the lipoprotein/sorting receptor LR11/SorLA has been shown to play a fundamental role in the development of AD, particularly the late-onset "sporadic" form of the disease, further strengthening the connections between lipoproteins, cholesterol biology, and AD. LR11 mRNA and protein levels are reduced in sporadic AD but not in cases of familial AD (Scherzer, Offe et al. 2004; Dodson, Gearing et al. 2006), and deficiency of LR11 accelerates amyloid pathology in a mouse model (Dodson, Andersen et al. 2008). LR11 expression is also reduced in mild cognitive impairment, further supporting its involvement in early stages of human disease (Sager, Wuu et al. 2007). Recently, polymorphisms of the LR11 gene *SORL1* have been shown to associate with increased AD risk in some populations (Lee, Cheng et al. 2007;

Meng, Lee et al. 2007; Rogaeva, Meng et al. 2007). There are no treatment strategies that directly act through LR11 as of yet, but multiple avenues of basic science research are being actively pursued to further understand its mechanism of influencing AD pathogenesis and to reveal opportunities for therapeutic intervention.

AD Genetics

While the vast majority of AD cases are not inherited in a Mendelian fashion, there are well characterized mutations in three genes—*APP* on chromosome 21, *PSEN1* on chromosome 14, and *PSEN2* on chromosome 1—that cause an autosomal dominant form of the disease often referred to as familial AD or FAD. As previously mentioned, FAD shares many of the same symptoms observed in sporadic AD (SAD), and they are nearly identical from a pathologic standpoint. Estimates vary, but it is generally accepted that FAD accounts for less than 5% of total AD cases. FAD typically presents at an earlier age than the more common sporadic form, with some particularly aggressive mutations causing disease onset in the fourth or fifth decade of life (Kauwe, Wang et al. 2008). All three of the genes known to cause FAD are fundamentally involved in the amyloid cascade theory of pathogenesis. Disease-associated mutations in *APP*, the gene encoding the amyloid precursor protein (APP), were first reported in the early 1990s (Goate, Chartier-Harlin et al. 1991; Murrell, Farlow et al. 1991; Hendriks, van Duijn et al. 1992; Mullan, Crawford et al. 1992). Several years later, mutations were also identified in the *PSEN1* and *PSEN2* genes that code for the highly similar presenilin-1 (PS1) and presenilin-2 (PS2) proteins, members of the γ-secretase enzyme complex that participates in the release of the Aβ peptide from APP (Levy-Lahad, Wasco et al. 1995;

Rogaev, Sherrington et al. 1995; Sherrington, Rogaev et al. 1995). Most of the disease-associated mutations in APP and presenilin cause a shift in the processing of APP, resulting in either increased "β-secretase" cleavage of APP holoprotein or a higher ratio of $A\beta_{42}$: $A\beta_{40}$ peptides produced by "γ-secretase" cleavage (reviewed in detail below).

For the remaining >95% of AD cases termed "sporadic," only one gene has been consistently shown to increase risk across populations: *APOE*, which codes for an apolipoprotein involved in cholesterol metabolism. There are three alleles of *APOE*—ε2, ε3, and ε4. Homozygosity for the ε4 allele of confers an increased AD risk compared to individuals with no copies of ε4 (i.e. 2/2, 2/3, and 3/3), with one copy of ε4 passing on an intermediate risk (Corder, Saunders et al. 1993; Saunders, Strittmatter et al. 1993; Chartier-Harlin, Parfitt et al. 1994). Despite intense searches and investigation of hundreds of genes, few other candidates have been identified that have withstood rigorous association studies across populations. This is likely due to the complex, multifactorial disease process and the fact that many single gene polymorphisms may exert only a small increase in risk. A comprehensive online database was recently established to aid in the organization of studies of AD genetics and facilitate their interpretation through the use of meta-analysis (Bertram, McQueen et al. 2007), http://www.alzgene.org. Detailed family histories performed by clinicians reveal that there is a substantial genetic component in what was previously considered "sporadic" AD (Rosen, Steenland et al. 2007), and continued exploration of disease-linked polymorphisms holds promise for identifying underappreciated aspects of pathogenesis and novel targets for AD treatment.

Patients with trisomy 21 (Down syndrome, DS) who survive until about age 40 invariably develop AD pathology (Heston 1984). This phenomenon is believed to be because the *APP* gene resides on chromosome 21. Expression analysis has shown that DS subjects have four- to five-fold increases in APP mRNA and protein levels, higher than would be predicted by simply having an extra copy of chromosome 21, which may account for their strong predisposition for developing AD pathology. Significant effort has also been directed at understanding the regulation of APP transcription in the brain and other tissues and specifically at identifying polymorphisms in regulatory DNA elements that govern APP expression in healthy subjects and AD patients (Beyreuther, Pollwein et al. 1993; Lahiri and Ge 2004; Lahiri, Ge et al. 2005). It is interesting to note that there are presumably no mutations in *APP* in cases of DS; simply increasing the copy number of wildtype *APP* seems sufficient to trigger disease. This notion is supported by several kindreds affected by early-onset AD who harbor relatively small duplications of chromosome 21 loci containing the *APP* gene, and by a case report describing a DS subject whose trisomy 21 did not include duplication of the *APP* gene and who did not develop AD-like pathology (Prasher, Farrer et al. 1998; Rovelet-Lecrux, Hannequin et al. 2006).

APP, Proteolytic Processing, and the Amyloid Cascade Hypothesis

Following the purification and sequencing of the Aβ peptide, much attention focused on elucidating the precursor molecule from which it is derived. These efforts culminated in the identification of the amyloid precursor protein (APP), a 695-770 amino acid protein expressed throughout the body and at high levels in the brain (Kang, Lemaire

et al. 1987; Tanzi, Gusella et al. 1987). APP is closely related to two other proteins, APLP1 (APP-like Protein) and APLP2, and these family members seem to be at least partially redundant. APP is a type I transmembrane glycoprotein that exists in three major isoforms resulting from alternative splicing of exons. APP-695 (numbers refer to length in amino acids) is highly expressed in neural tissue, and is the major isoform in fetal brain. The longer APP-751 and APP-770 isoforms are mainly expressed in peripheral tissue early in life but may account for a large proportion of APP in the adult human brain. APP-751 and APP-770 contain a Kunitz protease inhibitor (KPI) domain in the N-terminal ectodomain of the protein. Levels of the two longer APP isoforms have been shown to be higher in AD brain as compared to control, implicating APP-KPI in the disease process, possibly by reducing clearance of the Aβ peptide (Moir, Lynch et al. 1998; Moir and Tanzi 2005).

Owing to its high degree of conservation throughout evolution and widespread expression in multiple tissues, much attention has been directed at elucidating the normal function of APP. APP knockout mice are viable and can reproduce, but have reduced body weight, display decreased locomotor activity and strength, and show evidence of reactive gliosis in the hippocampus and cortex (Zheng, Jiang et al. 1995). That APP null mice survive at all has been interpreted as evidence of the complementary and partially redundant function that the three family members serve. Mice deficient in either APLP1 or APLP2 alone show no overt phenotype, while most APP/APLP2 double knockout mice die within one week of birth (von Koch, Zheng et al. 1997). Multiple proteins have been shown to physically interact with APP and its metabolites, including Fe65, the Mint/X11 family, low density lipoprotein receptors, and multiple components of

extracellular matrix including F-spondin, laminin, collagen, and heparin (Small, Nurcombe et al. 1993; Hill, Li et al. 2003; Miller, McLoughlin et al. 2006; Hoe and Rebeck 2008; Jaeger and Pietrzik 2008; McLoughlin and Miller 2008; Shrivastava-Ranjan, Faundez et al. 2008). Studies in cultured cells and animal models have implicated APP in a variety of cellular processes, including transcriptional regulation (Cao and Sudhof 2001), neuronal adhesion and migration (Coulson, Paliga et al. 2000; Young-Pearse, Bai et al. 2007), neurite outgrowth (Perez, Zheng et al. 1997; Young-Pearse, Chen et al. 2008), and recently, axon pruning (Nikolaev, McLaughlin et al. 2009).

APP is proteolytically processed by multiple enzymes to yield several distinct membrane-bound and soluble derivatives (Sisodia 1992; Selkoe 1994; Selkoe, Yamazaki et al. 1996) (Figure 1.2). Two mutually exclusive pathways, termed "amyloidogenic" and "non-amyloidogenic" in reference to whether or not they ultimately produce the Aβ peptide, exist for the metabolism of full-length APP. The non-amyloidogenic pathway of APP processing is initiated by cleavage of the full length protein at a site 12 amino acids N-terminal to the transmembrane domain. This cleavage occurs within the Aβ sequence, thereby precluding its formation (Esch, Keim et al. 1990; Sisodia, Koo et al. 1990). There are several enzymes, referred to as "α-secretases" that have been shown to be able to cut APP at this site. The best characterized of these candidates are members of the A Disintegrin and Metalloprotease (ADAM) family, including ADAM-10 and ADAM-17, also called tumor necrosis factor alpha converting enzyme (TACE) (Buxbaum, Liu et al. 1998; Lammich, Kojro et al. 1999). α-secretase cleavage releases a large soluble ectodomain called "APPsα", leaving behind an 83 amino acid membrane-bound carboxyl-terminal fragment ("CTFα"). This membrane-anchored stub can then be

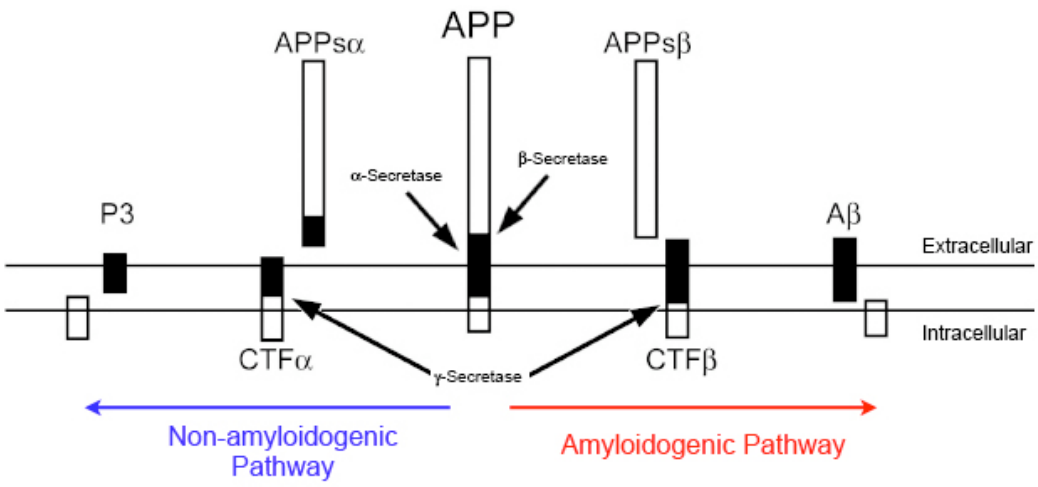


Figure 1.2. APP is processed by a series of enzymatic reactions.

In the amyloidogenic pathway (right), APP is first cleaved by β-secretase to generate the soluble ectodomain APPsβ and the membrane-anchored carboxy-terminal fragment CTFβ. γ-secretase cleavage of CTFβ releases the Aβ peptide and the APP intracellular domain (AICD). In the competing non-amyloidogenic pathway (left), α-secretase cuts APP within the Aβ domain, producing the slightly longer APPsα ectodomain and the slightly shorter CTFα. γ-secretase cleavage of CTFα releases AICD as well as the non-toxic fragment P3.

cleaved by an integral membrane enzyme complex called γ-secretase, which releases a non-toxic fragment called P3 and generates a small peptide called AICD (APP intracellular domain). γ-secretase is actually a complex of at least four proteins—presenilin, nicastrin, Aph-1 and Pen-2—that catalyzes the unusual intramembranous cleavage of membrane-anchored stubs of APP as well as a growing list of other type I transmembrane proteins including Notch, ErbB4, the epidermal growth factor receptor (EGFR), LR11/SorLA, and the $p75^{NTR}$ low affinity neurotrophin receptor, to name several (Edbauer, Winkler et al. 2003; Kimberly, LaVoie et al. 2003; Bohm, Seibel et al. 2006; Steiner, Fluhrer et al. 2008; Wolfe 2008).

In the amyloidogenic pathway, cleavage by an enzyme termed "β-secretase" cleaves APP between amino acid residues 671 and 672 (numbered from the APP-770 sequence), generating a large soluble ectodomain termed "APPsβ" and a 99 amino acid membrane-bound carboxyl-terminal fragment, "CTFβ". Just before the turn of the century, it was determined that the enzyme responsible for β-secretase cleavage is the transmembrane aspartyl protease BACE (β-site APP-cleaving enzyme) (Hussain, Powell et al. 1999; Sinha, Anderson et al. 1999; Vassar, Bennett et al. 1999; Yan, Bienkowski et al. 1999; Lin, Koelsch et al. 2000). Subsequent cleavage of CTFβ by γ-secretase releases the Aβ peptide and generates the same AICD produced in the non-amyloidogenic pathway. There does not appear to be a consensus cleavage site for γ-secretase; rather, the enzyme complex can cut at multiple sites within the transmembrane domain of APP, releasing Aβ peptides of various lengths from 37 to 43 amino acids in length (Steiner, Fluhrer et al. 2008). The two most abundant species are Aβ40 and Aβ42, with Aβ42 being highly fibrillogenic and considered to be the driving force behind aggregation of

amyloid into oligomeric complexes and ultimately plaques (reviewed in further detail below).

There is now a wealth of data characterizing the proteolytic processing of APP, the cellular organelles in which it takes place, the biochemical signals that regulate it, and its relevance to normal physiology and disease states (Gandy, Caporaso et al. 1993; Selkoe 1994; Selkoe 2001; Suzuki and Nakaya 2008; Thinakaran and Koo 2008). Like many transmembrane proteins, APP undergoes a dynamic redistribution among numerous cellular compartments during its lifespan. Following synthesis in the endoplasmic reticulum (ER) and posttranslational modification in the Golgi apparatus, APP traffics to the cell surface, is subsequently internalized to early endosomes, and is ultimately recycled or targeted for degradation (Caporaso, Takei et al. 1994; Selkoe, Yamazaki et al. 1996; Bayer, Wirths et al. 2001). During the course of this translocation, there are numerous opportunities for APP to interact with the secretase enzymes that cleave it. ADAM proteases have been localized to the cell surface as well as intracellular compartments including ER, Golgi, and lipid rafts (Lammich, Kojro et al. 1999; Schlondorff, Becherer et al. 2000; Hooper and Turner 2002; Gutwein, Mechtersheimer et al. 2003; Wakatsuki, Kurisaki et al. 2004). The β-secretase enzyme BACE is also expressed throughout the secretory and endocytic pathways (Walter, Fluhrer et al. 2001; Lee, Kao et al. 2003; Shiba, Kametaka et al. 2004). It is conceivable that the balance of amyloidogenic vs. non-amyloidogenic cleavage of APP could be determined in part by the traffic of APP, or the secretase enzymes that cleave it, to discrete cellular compartments where α- vs. β-secretase activity predominates. In particular, the trans-Golgi network (TGN) (Thinakaran, Teplow et al. 1996; Wild-Bode, Yamazaki et al.

1997; Xu, Sweeney et al. 1997; Skovronsky, Moore et al. 2000) and endosomal system (Cataldo, Barnett et al. 1997; Lah and Levey 2000; Cataldo, Petanceska et al. 2004) are emerging as critical intracellular locations for the regulated cleavage of APP.

APP is phosphorylated on both its carboxyl-terminus and amino-terminal ectodomain, but the implications of phosphorylation on regulated APP processing are unclear (Gandy, Czernik et al. 1988; Suzuki, Nairn et al. 1992; Knops, Gandy et al. 1993; Hung and Selkoe 1994). For instance, phosphorylation of APP itself does not seem to be required for regulation of APPs shedding by activation of protein kinase C (PKC) (Caporaso, Gandy et al. 1992; da Cruz e Silva, Iverfeldt et al. 1993; Hung, Haass et al. 1993; Hung and Selkoe 1994). Other possibilities include activation of α-secretase itself, or regulation of intracellular traffic that brings APP and α-secretase into contact with each other (Hung and Selkoe 1994; Xu, Greengard et al. 1995; Jolly-Tornetta and Wolf 2000; Palacino, Berechid et al. 2000; Skovronsky, Moore et al. 2000).

Some of the most compelling studies of regulated APP processing, especially with respect to the context of the disease, have examined the APP cleavage in response to activation of several distinct families of neurotransmitter receptors. This phenomenon first became evident in the early 1990s with the demonstration that activation of two subtypes of the muscarinic acetylcholine receptor could increase the release of APPs from cultured cells (Nitsch, Slack et al. 1992; Farber, Nitsch et al. 1995). Further studies have indicated that agonists for other G-protein coupled receptors, including glutamate, serotonin, and bradykinin, are capable of regulating APP processing as well (Nitsch, Slack et al. 1993; Nitsch, Deng et al. 1996; Nitsch, Deng et al. 1997; Ulus and Wurtman

1997). Muscarinic regulation of APP processing is a major theme of this thesis and will be explored at length in the following sections.

As previously mentioned, amyloid plaque formation exists on a continuum from individual monomers of Aβ peptide to fully formed, dense-core plaques surrounded by dystrophic neurites and other markers of cellular damage. The $A\beta_{42}$ peptide is more fibrillogenic than the shorter $A\beta_{40}$ peptide, and is thought to initiate the process of amyloid aggregation and plaque formation. Intermediate forms of aggregated amyloid, including dimers, trimers, higher order oligomers, and protofibrils, exist en route to the mature dense-core plaque. Of these, oligomers have come into focus as the species most likely to be responsible for the neurotoxicity and impaired synaptic function in AD (Walsh, Klyubin et al. 2002; Takahashi, Almeida et al. 2004; Glabe 2005; Walsh, Klyubin et al. 2005; Walsh, Townsend et al. 2005). The precise sequence of events leading from amyloid oligomerization to clinical dementia is not completely understood, but distinct molecular and cellular phenotypes are reproducibly observed, including activation of microglia, astrocytosis, impairment of long term potentiation, and neuronal cell death (Golde, Dickson et al. 2006). Recent attention has focused on defining and testing therapeutic approaches, including preventing Aβ formation by inhibiting β- and γ-secretase, dissolution and clearance of existing amyloid, and vaccination against the Aβ peptide.

The Cholinergic System: Normal Function and Involvement in AD

Historical Perspective

In 1906, the same year that Auguste D. finally succumbed to what is now called Alzheimer's disease, John Langley gave a lecture at the Royal Society of London in which he presented his findings that nicotine and curare had opposing actions on muscle contraction. In his remarks, Langley proposed that both nicotine and curare were exerting their actions by binding to a common material that he coined the "receptive substance" (Langley 1906; Langley 1907). This idea was similar to the concept of the "receptive side chain" that Paul Ehrlich had discussed several years previously for a protective interaction of cellular side chains with toxins but had nearly abandoned as a model for drug interaction. Langley's own elegant experiments and those of his student Thomas Elliott on the action of adrenaline ultimately convinced Ehrlich that such a receptive substance did exist for chemicals, and he contributed his own term to the idea: "chemoreceptor." The effects of curare on muscle contraction had been recognized as early as 1811, when Brodie used poison-tipped arrows to paralyze animals and found that they could be kept alive via respiratory support. Claude Bernard demonstrated that this paralysis occurred distal to the motor nerve, since electrical stimulation of the nerve could not overcome the curare block (Bernard 1883). Leading up to the development of his theory, Langley performed a series of experiments in which he characterized the opposing actions of atropine and pilocarpine on saliva secretion in submaxillary glands, prompting him to speculate on the existence of a common substance that could bind both drugs (Langley 1878). In 1906, concurrent with Langley's description of the receptive

substance, Hunt and Taveau reported the chemical synthesis of acetylcholine and noted that the actions of acetylcholine could be blocked by atropine, drawing similarities to the effects of vagus nerve innervation of the heart (Hunt and Taveau 1906). In a series of experiments, Henry Dale outlined the physiological impact of experimentally applied acetylcholine, highlighting its effects on heart rate, blood pressure, and intestinal motility (Dale 1914; Dale 1914). One major barrier to the widespread acceptance of acetylcholine as a native regulator of physiological systems was the inability of the scientific community to localize a source of acetylcholine or its precursor within the body. This debate was largely overcome in 1929 when Dale and a chemist named Harold Dudley isolated endogenous acetylcholine from ox and horse spleen (Dale and Dudley 1929). Several years earlier, Otto Loewi (who would go on to share the 1936 Nobel Prize for Physiology or Medicine with Dale for their work on acetylcholine) had made what is widely considered to be one of the seminal observations in the field of neuroscience and receptor biology when he determined that the nature of the signal from the vagus nerve controlling heart rate was chemical and not electrical. Loewi initially termed the chemical "Vagusstoff," which we now recognize is acetylcholine (Loewi 1921).

Following the establishment of acetylcholine as the first neurotransmitter and the descriptions of its effects on various physiological systems, scientists set out to uncover the mechanisms responsible for the regulation of its signaling. Based on observations that physostigmine could potentiate the actions of acetylcholine, Loewi had hypothesized that various tissues possess an intrinsic esterase that could degrade acetylcholine, a molecule which he named "cholinesterase" (Loewi and Navratil 1926). By the 1940s it had also been found that acetylcholine could be synthesized by an enzyme now called

choline acetyltransferase (ChAT) (Nachmansohn and Machado 1943; Nachmansohn and John 1945). Many of the second messenger systems that neurotransmitters including acetylcholine utilize to propagate signaling in tissues were characterized in detail in the 1960s and 1970s (Gilman 1987), but the existence of receptors as discrete molecular entities was not definitely proven until Robert Lefkowitz and colleagues isolated adrenergic receptors and then reconstituted functional receptor/G-protein/enzyme systems (Lefkowitz 2007). An era of molecular cloning then followed, during which many neurotransmitter receptors were identified at the genetic level. This progress has included many advances cholinergic research, which has provided insight and drug targets for many CNS and systemic diseases (Giacobini and Pepeu 2006).

Functional Neuroanatomy of the Cholinergic System

A detailed understanding of cholinergic synapses is now available as a result of diverse research techniques, including biochemical analysis, molecular genetics, and microscopy (Figure 1.3) (Cooper, Bloom et al. 1996). In the presynaptic terminal, acetylcholine (ACh) is synthesized from its precursors acetyl CoA and choline by the ChAT enzyme. ACh is packaged into synaptic vesicles by the vesicular acetylcholine transporter (VAChT) and subsequently released into the synaptic cleft. Released molecules of the neurotransmitter can bind and activate pre- and post-synaptic acetylcholine receptors only for a brief time until they are degraded by the acetylcholinesterase and related butyrylcholinesterase enzymes. Following degradation,

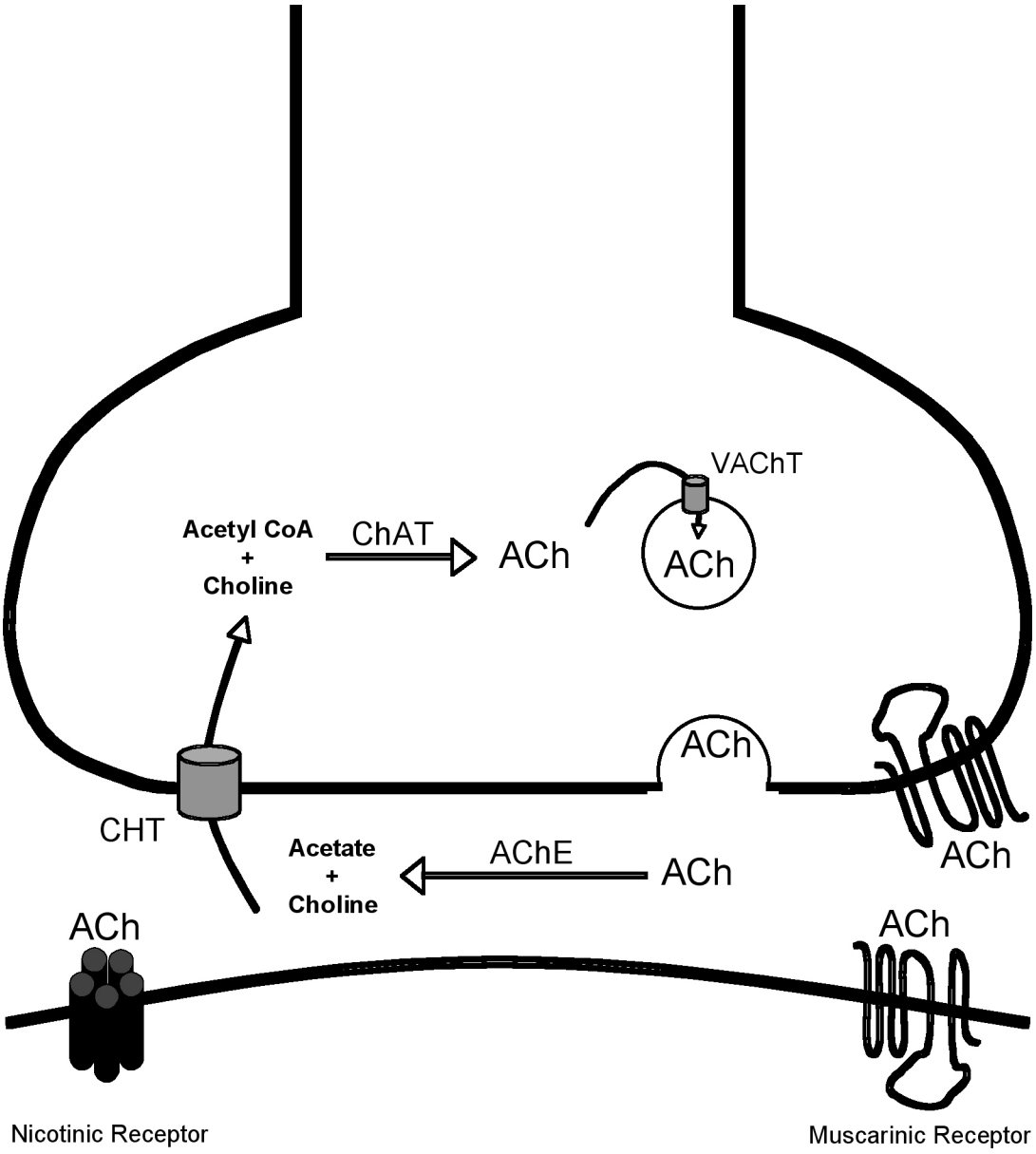

VAChT
Acetyl CoA
+
Choline
ChAT
ACh
ACh
ACh
CHT
ACh
Acetate
+
Choline
AChE
ACh
ACh
ACh
Nicotinic Receptor
Muscarinic Receptor

Figure 1.3. Model of cholinergic neurotransmission in the central nervous system. Acetylcholine (ACh) is synthesized in presynaptic nerve terminals by the enzyme choline acetyltransferase (ChAT), packaged into synaptic vesicles by the vesicular acetylcholine transporter (VAChT), and released into the synaptic cleft. Intact ACh can bind and activate nicotinic and muscarinic acetylcholine receptors on both pre- and post-synaptic membranes until it is hydrolyzed by the enzyme acetylcholinesterase (AChE). The precursor choline is then taken up by the presynaptic terminal by the high affinity choline transporter (CHT).

choline is taken up into the presynaptic axon terminal by a high affinity choline transporter (CHT). This reuptake process is the rate limiting step in acetylcholine biosynthesis.

Nuclei in the basal forebrain, a group of structures in the medial and ventral telencephalon, contain large numbers of neurons that project to the hippocampus, amygdala, and cerebral cortex and provide the majority of cholinergic innervation to these areas (Mufson and Kordower 2001). The basal forebrain can be divided into several constituent regions that send bundles of axons to their respective targets. Neurons in the medial septum and the diagonal band of Broca project to the hippocampus, while neurons in the nucleus basalis of Meynert project broadly to the neocortex as well as to the amygdala. Cholinergic neurons in the basal forebrain are distinguished among other features by the presence of ChAT, and the majority of these cells also express the neurotrophin receptors $p75^{NTR}$ and TrkA. These two receptors bind the neurotrophin NGF, and are believed to participate in the regulation of basal forebrain neuron development and survival. Congruent with this hypothesis, high levels of NGF are present in the projection fields of basal forebrain neurons, and it is thought that retrograde transport of NGF plays a role in the trophic support of these neurons.

Muscarinic Acetylcholine Receptors

Two families of receptors bind acetylcholine and mediate its action in target tissues: nicotinic receptors, which form ion channels and participate in rapid postsynaptic neurotransmission, and muscarinic receptors (mAChR), which are G-protein coupled receptors and play a role in modulating the activity of many circuits within the CNS.

These two receptor families were originally named for their activation by nicotine and muscarine, respectively, but have been extensively characterized since that time on a molecular basis. This thesis will focus primarily on muscarinic acetylcholine receptor subtypes. The diversity and complexity of muscarinic cholinergic signaling is facilitated in part by five distinct receptor subtypes, M1-M5, the genes for which were cloned in the mid to late 1980s (Bonner, Buckley et al. 1987; Peralta, Ashkenazi et al. 1987; Bonner, Young et al. 1988). Muscarinic receptors belong to the superfamily of seven transmembrane G-protein coupled receptors (GPCRs), the largest family of cell-surface receptors and key regulators of a wide variety of physiological processes (Lefkowitz 2007). The five mAChR subtypes are highly homologous, with some divergence occurring in the third intracellular loop. Their signaling properties are quite different, however. M1, M3, and M5 preferentially couple to G_q to activate phospholipase C, while M2 and M4 couple to G_i to inhibit adenylate cyclase.

Early binding studies using mAChR ligands revealed the presence of mAChRs in numerous brain regions (Yamamura and Snyder 1974; Yamamura and Snyder 1974; Kuhar and Yamamura 1975; Kuhar and Yamamura 1976; Mash, Flynn et al. 1985; Mash and Potter 1986). Subsequent *in situ* hybridization experiments following the cloning of mAChR subtype genes revealed that individual subtypes were expressed in partially overlapping tissues, with some regions, including the hippocampus, expressing all five mAChR subtypes (Buckley, Bonner et al. 1988; Weiner, Levey et al. 1990). A series of studies using subtype-selective antibodies has illustrated the distinct neuroanatomical localization of the mAChR subtypes in brain and have provided important clues as to their function in neural circuits (Levey, Kitt et al. 1991; Mrzljak, Levey et al. 1993;

Hersch, Gutekunst et al. 1994; Levey, Edmunds et al. 1994; Levey, Edmunds et al. 1995; Levey, Edmunds et al. 1995; Rouse and Levey 1996; Rouse, Gilmor et al. 1998; Rouse, Edmunds et al. 2000).

The M1 receptor is expressed at high levels in multiple brain regions, including cortex, hippocampus, and striatum. In cortex, M1 is localized to pyramidal cells and is prominent in the neuropil of layers II/III and VI (Levey, Kitt et al. 1991). M1 is expressed broadly throughout the hippocampus, including in pyramidal neuron cell bodies and dendritic processes in the stratum radiatum and stratum oriens, and in the molecular layer and granule cells of the dentate gyrus (Levey, Edmunds et al. 1995). In the striatum, M1 is found in the majority of neurons as well as in the neuropil. At the electron microscopy level, M1 can be visualized at the postsynaptic density of asymmetrical synapses, suggesting a role in modulating excitatory neurotransmission (Hersch, Gutekunst et al. 1994). Behavioral studies have suggested an important role for hippocampal M1 in consolidation of learning and memory, and it has been demonstrated that M1 potentiates NMDA currents in hippocampal pyramidal cells (Marino, Rouse et al. 1998). M1 is also the sole mAChR subtype in brain responsible for activation of extracellular signal-regulated kinase (ERK 1/2), an enzyme implicated in synaptic plasticity (Berkeley, Gomeza et al. 2001; Hamilton and Nathanson 2001). Careful observation of M1 knockout mice has revealed deficits in long term potentiation and in certain aspects of memory, including working memory and consolidation (Anagnostaras, Murphy et al. 2003).

M2 is the most widespread mAChR subtype in brain. It shows a distinct laminar distribution in the cortical neuropil of layer IV and the junction between layers V/VI, and

is also present in occasional interneuron somata (Levey, Kitt et al. 1991). Close inspection has revealed that M2 is expressed both pre-and post-synaptically (Mrzljak, Levey et al. 1993). In the hippocampus, M2 localizes to discrete bands of cell bodies and processes along the oriens/alveus border, and is also found in processes along the pyramidal cell layer, most prominently in the CA3 region (Levey, Edmunds et al. 1995). There is a high concentration of M2 in the basal forebrain, in both cholinergic and non-cholinergic cells, as well as in the neuropil (Levey, Edmunds et al. 1995). Presynaptic M2 appears to function as an autoreceptor in the cortex and hippocampus to regulate ACh release (Zhang, Basile et al. 2002) Studies in M2 knockout mice have demonstrated a physiological role for this protein in locomotion, regulation of body temperature, and response to pain (Gomeza, Shannon et al. 1999).

The M3 receptor is expressed at low levels in brain, accounting for only 5-10% of total mAChRs in various brain regions (Levey, Edmunds et al. 1994). By immunohistochemistry, M3 can be seen to localize to multiple brain regions, including cortex, hippocampus, olfactory bulb, amygdala, striatum, thalamus, and pons. Subcellularly, M3 appears in cell bodies and proximal dendrites, suggesting a postsynaptic localization, and also as a diffuse, punctate reaction product in the neuropil that may reflect presynaptic terminals or dendritic processes. In the CNS, M3 helps regulate the release of several neurotransmitters, including dopamine in the striatum, GABA and glycine in the dorsal horns of the spinal cord, and endocannabinoids (Zhang, Yamada et al. 2002; Ohno-Shosaku, Matsui et al. 2003; Zhang, Chen et al. 2006; Zhang, Zhou et al. 2007). Studies in M3 knockout mice have also implicated this subtype in multiple peripheral and autonomic functions, including arterial vasodilation, insulin

release, salivation, weight gain, and smooth muscle contraction in the stomach, trachea, and urinary bladder (Matsui, Motomura et al. 2000; Yamada, Miyakawa et al. 2001; Duttaroy, Zimliki et al. 2004; Khurana, Chacon et al. 2004).

The M4 receptor is expressed at somewhat lower levels than other mAChR subtypes in cortical laminae, and is localized to discrete layers in the hippocampus, including the stratum radiatum and stratum oriens in CA1 and the inner molecular layer of the dentate gyrus. In the striatum, dense patches of M4 are observed that correspond to postsynaptic sites on medium spiny neurons. M4 is also found in the islands of Calleja where it may play a role in reward behaviors (Levey, Kitt et al. 1991; Hersch, Gutekunst et al. 1994). In the striatum, M4 is believed to participate in regulating dopaminergic signaling, and M4 knockout mice show increased basal and dopamine-regulated locomotor responses (Gomeza, Zhang et al. 1999; Zhang, Yamada et al. 2002). Analogous to the inhibitory role that M2 plays in the hippocampus, M4 is the major autoreceptor in the striatum responsible for feedback regulation of neurotransmitter release from the presynaptic terminal (Zhang, Basile et al. 2002).

Levels of the M5 receptor approach the lower limits of specific detection in brain as determined by quantitative immunoprecipitation and immunohistochemistry, although M5 mRNA is detectable in multiple brain tissues. M5 knockout mice have revealed roles for M5 in dilation of cerebral blood vessels and in reward and reinforcement behaviors, specifically in response to drugs of abuse such as morphine and cocaine (Yamada, Lamping et al. 2001; Basile, Fedorova et al. 2002; Thomsen, Woldbye et al. 2005).

Cholinergic Involvement in AD

As previously mentioned, one of the earliest observations concerning molecular changes in AD was that cholinergic enzyme activity was reduced in AD brain (Davies and Maloney 1976; Perry, Perry et al. 1977; Perry, Tomlinson et al. 1978). Subsequent reports demonstrated that basal forebrain cholinergic neurons and projection fibers are lost in advanced AD (Whitehouse, Price et al. 1982; Mufson, Bothwell et al. 1989). Binding studies using non-selective and semi-selective mAChR ligands began to establish patterns of mAChR alterations in AD (Nordberg, Larsson et al. 1983; Mash, Flynn et al. 1985; Flynn, Weinstein et al. 1991), and immunoprecipitation studies using subtype-selective antibodies have further clarified our understanding of mAChR subtype changes in AD. Specifically, M1 is decreased in multiple regions of cortex and hippocampus, M2 is downregulated in cortex, hippocampus, and nucleus basalis, and M4 appears up-regulated in cortex (Flynn, Ferrari-DiLeo et al. 1995).

The observed deficits in cholinergic signaling in AD prompted the widespread clinical use of cholinesterase inhibitors in AD patients. Data from clinical trials have demonstrated somewhat improved clinical outcomes for patients receiving cholinomimetic therapy, strengthening the cholinergic hypothesis (Farlow 2002; Petersen, Thomas et al. 2005). Evidence from AD patients exposed to drugs with anti-cholinergic effects has suggested that cholinergic antagonism worsens clinical trajectory and may be associated with increased AD neuropathology (Lu and Tune 2003; Perry, Kilford et al. 2003). The basis for these effects may lie with mAChR regulation of APP processing and amyloid production. As mentioned, cell culture and brain tissue studies demonstrated a role for the G_q-coupled M1 and M3 mAChRs in promoting non-

amyloidogenic APP processing and suggested that the G_i-coupled M2 and M4 receptors may have an opposite effect (Nitsch, Slack et al. 1992; Farber, Nitsch et al. 1995). Data from AD patients have shown that muscarinic agonists can regulate Aβ production *in vivo* (Hock, Maddalena et al. 2000; Nitsch, Deng et al. 2000; Hock, Maddalena et al. 2003), and a recent study in a transgenic mouse model of AD demonstrated that a muscarinic agonist reduced amyloid and tau pathology as well as improved behavioral outcomes (Caccamo, Oddo et al. 2006). While these data are very exciting and reinforce the potential utility of cholinergic treatments for AD neuroprotection, less is known about the precise molecular basis of muscarinic regulation of APP processing and amyloidogenesis. Progress in understanding mAChR function in the brain and other physiological systems has long been hampered by the lack of subtype-selective mAChR drugs, and since many of the existing studies on mAChR-regulated APP processing have relied on non-selective drugs, the conclusions that can be drawn regarding the molecular basis of their effects are limited. These molecular details are becoming increasingly important now that the first truly subtype-selective mAChR agonists are available. For example, studies in M3 knockout mice have revealed a prominent role for this receptor subtype in regulating urinary bladder contraction as well as important metabolic processes including insulin release and gastrointestinal motility (Abrams, Andersson et al. 2006; Unno, Matsuyama et al. 2006; Gautam, Jeon et al. 2008), and M2 receptors are involved in regulating heart rate (Stengel, Gomeza et al. 2000). Therefore, dose-limiting and potentially life-threatening side effects would be predicted from pharmacologic activation or inactivation of peripheral M2 and M3 receptors. For these reasons, and the fact that M1 is the predominant mAChR subtype and is capable of regulating amyloid

processing in cultured cells, therapeutic attention has focused on the M1 receptor. Now that M1-selective agonists are becoming available (Spalding, Trotter et al. 2002; Jones, Brady et al. 2008), it is important to investigate whether M1 is capable of regulating amyloid production in neuronal systems.

Proposed Research

The goal of this dissertation is to determine the role of the genetically defined M1 mAChR in regulating APP processing in neuronal systems and to explore avenues of pharmacologic regulation of this process. The use of a genetic approach is essential because of the high level of similarity among mAChR family members and the lack of pharmacological compounds that can reliably activate specific subtypes. Understanding the precise contributions of receptor subtypes in neural systems is critical because particular receptors may signal in subtly different manners in different tissues and cell types. Drugs that activate GPCRs, including mAChRs, represent the majority of clinical therapeutics for treating disease. Further understanding of the mechanisms by which novel drugs influence receptor signaling will enhance our knowledge of basic cell biology and may help guide the development of therapies for neurodegenerative and other diseases.

Aim 1: To determine the role that the M1 mAChR plays in regulating APP processing in neurons. We used embryonic cortical neurons from wildtype and M1 knockout mice to investigate the role of the M1 receptor in cholinergic regulation of APP processing. We demonstrate that M1 is critical for cholinergic stimulation of non-amyloidogenic APP processing, and that M1 activation promotes APPsα secretion and limits Aβ formation. Additionally, we show that novel, highly-selective compounds activate M1 to regulate non-amyloidogenic APP processing in the neuronotypic PC12 cell line. We therefore propose that M1 is the major regulator of APP processing in neurons.

Aim 2: To evaluate the consequences of M1 deletion in an *in vivo* model of amyloidogenesis. Because of the complexity of the brain and the multifaceted nature of amyloidogenesis, it is important to study the regulation of APP processing and amyloid deposition in living organisms. To this end, we crossed APP transgenic mice with M1 knockout mice in order to study the impact of losing M1 signaling on amyloidogenesis in vivo. We show that $M1^{-/-}$ mice have increased levels of Aβ in the brain and more extensive amyloid plaque pathology than their $M1^{+/+}$ littermates. This finding indicates that M1 is an important regulator of amyloidogenesis *in vivo*.

Aim 3: To characterize novel signaling pathways activated by allosteric M1 agonists. Allosteric agonists bind to structurally distinct regions on the M1 receptor and may influence its conformation such that distinct signal transduction pathways are activated. In this study, we explored the patterns of signal transduction pathways activated by orthosteric vs. allosteric M1 agonists and the cell biological consequences of this divergence in signaling. Our results add to the knowledge of how allosteric agonists modulate GPCR signaling, and may help inform strategies to develop compounds with desirable clinical properties for use as drug targets.

Chapter II. MATERIALS AND METHODS

Primary Neuron Culture

Primary cortical neuron cultures were prepared from wildtype mice and M1 knockout mice (a generous gift of Dr. Jurgen Wess, National Institute of Diabetes and Digestive and Kidney Diseases) at embryonic day E18. The generation and characterization of these mice has been described previously (Miyakawa, Yamada et al. 2001). Time-pregnant dams were anesthetized with isoflurane and decapitated. Embryos were dissected and corical hemispheres were isolated in dissection buffer (Hanks Balanced Salt Solution (HBSS), 10 mM HEPES, 1% penicillin/streptomycin). Tissue was digested with 0.25% trypsin (Gibco) and 0.01% deoxyribonuclease in dissection buffer for 15 minutes at 37°C and rinsed twice with dissection buffer and twice with plating medium (buffered MEM (Gibco), 0.6% glucose (Gibco), 2 mM L-glutamine (Cellgro), 10% heat-inactivated horse serum (Gibco), 1% penicillin/streptomycin). Tissue was mechanically dissociated by trituration through a fire-polished Pasteur pipette and viable cells were determined by Trypan blue exclusion. Neurons were plated at a density of 80,000 cells/cm^2 on poly-L-lysine coated 60mm culture dishes. Cultures were maintained in Neurobasal medium (Gibco) containing B-27 supplement (Gibco), 2 mM L-glutamine, and 1% penicillin/streptomycin at 37°C, 5% CO_2. Lentivirus vectors encoding human APP695swe and human M1 were added at the time of plating at a multiplicity of infection ~1 and allowed to incubate for 72 hours before removal. Cytosine arabinoside was added at a final concentration of 5 μM on day 3 *in vitro* to control proliferation of non-neuronal cells.

Cell Culture

Parental Chinese Hamster Ovary (CHO-K1) cells and CHO-K1 cells overexpressing human M1 (M1-CHO) were a gift from Dr. P. Jeffrey Conn (Vanderbilt University). Cells were maintained in DMEM (BioWhittaker) containing 10% Fetal Bovine Serum (Gibco), 1% non-essential amino acids (BioWhittaker), and 1% penicillin/streptomycin at 37°C, 5% CO_2. M1-CHO cells were maintained in the presence of G418 (50 μg/mL, Calbiochem).

PC12 N21 cells (a gift from Dr. Richard Burry, Ohio State University) were maintained in DMEM containing 10% heat-inactivated horse serum, 5% FetalClone (HyClone), and 1% penicillin/streptomycin at 37°C, 5% CO_2.

HEK293T cells were transduced with a human M1 lentiviral vector. Cells were maintained in DMEM containing 10% Fetal Bovine Serum and 1% penicillin/streptomycin.

$APP_{Swedish/Indiana}$ x M1KO mice

Line J20 transgenic mice expressing human amyloid precursor protein incorporating the Swedish and Indiana mutations were generously donated by Dr. Lennart Mucke (Gladstone Institute, University of California, San Francisco) and have been previously described (Mucke, Masliah et al. 2000). J20 heterozygous mice were bred to M1 (-/-) mice to generate offspring according to the breeding scheme shown in Figure 4.1. M1 genotype was confirmed according to the protocol described in Figure 2.1.

Oligonucleotides

M1S1: 5'-CCA ACA TCA CCG TCT TGG CAC-3'

M1A1: 5'-AGT GCC AAT GAT GAG ATC AGC-3'

NEO-1: 5'-CAG CTC ATT CCT CCC ACT CAT GAT-3'

Reaction I

DNA	2 ul
H_2O	8 ul
Buffer(10 X)	4 ul
dNTP(2.5 mM)	4 ul
DMSO	2 ul
M1S1 (10 ng/ul)	10 ul
M1A1(10 ng/ul)	10 ul
AmpliTag Gold (5 U/ul)	0.5 ul
Total:	**40 ul**

Reaction II

DNA	2 ul
H_2O	8 ul
Buffer(10 X)	4 ul
dNTP(2.5 mM)	4 ul
DMSO	2 ul
NEO-1 (10 ng/ul)	10 ul
M1A1 (10 ng/ul)	10 ul
AmpliTag Gold (5 U/ul)	0.5 ul
Total:	**40 ul**

Polymerase Chain Reaction Conditions:

94 º C	10 min	
94 º C	30 sec	**30 cycles**
55 º C	30 sec	
72 º C	2 min	
72 º C	10 min	

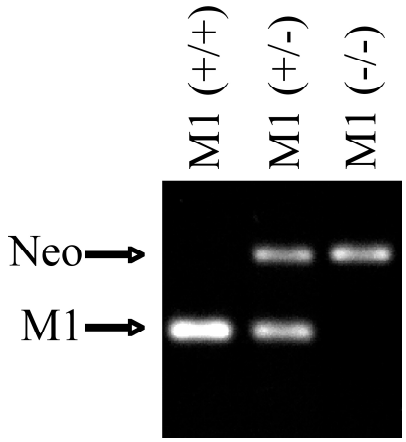


Figure 2.1. Genotyping of M1 knockout mice.

Genomic DNA was amplified by polymerase chain reaction using oligonucleotide probes specific for regions of the *M1* gene (M1S1 and M1A1) and the neomycin resistance cassette used to disrupt *M1* (NEO-1). A representative agarose gel stained with ethidium bromide showing the PCR products for M1 and neo at the expected molecular weights is shown.

Antibodies, Plasmids, and Chemicals

Antibodies used in this study included: 6E10 (APP Aβ domain, Signet, Dedham, MA), C8 (APP C-terminus, gift from Dr. Dennis Selkoe, Harvard Medical School, Boston, MA), β-actin (goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA), Aβ42 (rabbit polyclonal, BioSource (Invitrogen), Carlsbad, CA), phospho-ERK 1/2 (rabbit polyclonal, Cell Signaling, Danvers, MA), total ERK 1/2 (mouse monoclonal, Cell Signaling), M1 (i3 loop, in house rabbit polyclonal), Na/K ATPase (mouse monoclonal, Upstate (Millipore), Billerica, MA), V5 epitope tag (mouse monoclonal, Invitrogen, Carlsbad, CA).

Transient transfections were performed using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. A plasmid containing Arrestin-3 tagged with green fluorescent protein (GFP) (cloned into pEGFP-N) was a generous gift from Dr. Vsevolod Gurevich (Vanderbilt University, Nashville, TN). Humanized Swedish mutation (KM670/671/NL) APP, V5 epitope-tagged LR11, and human M1 muscarinic receptor sequences were individually cloned in place of GFP in the FUGW backbone (Lois, Hong et al. 2002). Lentiviruses were packaged by calcium phosphate triple transfection of HEK293FT cells with the transgene/FUW cassette, Δ8.9 HIV-1 packaging vector, and pVSVG envelope glycoprotein. Conditioned media was collected and virus was concentrated by ultracentrifugation. High titer virus (~1 x 10^9 infectious particles per mL) was used to transduce primary neurons and PC12 N21 cells. AC260584 was kindly provided by Acadia Pharmaceuticals (San Diego, CA). TBPB was provided by P. Jeffrey Conn and Craig Lindsley (Vanderbilt University, Nashville, TN).

Unless otherwise noted, all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Ectodomain shedding assays

Cells were plated at 50,000 cells/cm^2 in 60 mm culture dishes 4 days before the experiment. On the day of the experiment, the medium was replaced with 1.5 mL serum free DMEM containing the vehicle (DMSO) or the indicated drugs. Cells were incubated at 37°C for the indicated times, after which 1 mL of conditioned medium was collected and centrifuged at 17,000 x g for 5 minutes to remove any cellular debris. Cells were placed on ice, rinsed with cold phosphate-buffered saline, and harvested in phosphate-buffered saline containing protease inhibitor cocktail (Roche).

Western blotting

Cell lysates and conditioned media were prepared in Laemmli sample buffer, separated by SDS-PAGE, and transferred to PVDF Immobilon-P membranes (Millipore, Billerica, MA). Membranes were blocked at room temperature and incubated with primary antibodies overnight at 4°C. Blots were rinsed, incubated with fluorophore-conjugated secondary antibodies (Molecular Probes, Eugene, OR and Rockland, Gilbertsville, PA) for one hour at room temperature. Blots were imaged and band intensities were quantified using an Odyssey Image Station (LI-COR, Lincoln, NE).

ELISA measurement of Aβ40 and Aβ42 peptides

Aβ40 and Aβ42 levels in conditioned media and tissue homogenates were measured using hAmyloid ELISA (HS) kits (The Genetics Company, Schlieren, Switzerland) according to the manufacturer's instructions. Plates were read at 450 nm on a Spectra Max Plus plate reader (Molecular Devices, Sunnyvale, CA).

Tissue Collection

Animals were euthanized by sodium pentobarbital overdose and perfused with normal saline. Brains were rapidly removed and sectioned along the sagittal plane. One hemibrain was immersion fixed in 4% buffered paraformaldehyde, and cerebral cortex and hippocampus were isolated from the other hemibrain, snap-frozen in liquid nitrogen, and stored at -80°C until analysis. Individual tissue fractions were not subjected to more than one freeze/thaw cycle.

Histochemical Amyloid Plaque Analysis

Sagittal hemibrains were immersion fixed with 4% paraformaldehyde for 2 hours at 4°C, cryoprotected in 30% sucrose, and sectioned at 50 μm on a freezing-sliding microtome. For Thioflavin-S plaque staining, sections were mounted on glass slides, treated with 1% Thioflavin-S solution for 10 minutes, and rinsed in 80% ethanol and water. For Aβ42 immunohistochemistry, free floating sections were fixed with 2% glutaraldehyde, treated with sodium borohydride to quench unreacted glutaraldehyde, and incubated with 70% formic acid to retrieve antigens. Following treatment with hydrogen peroxide, sections were blocked with normal serum and incubated with an anti-Aβ42 antibody overnight at 4°C. Sections were then incubated with a biotinylated secondary

antibody and signal was visualized using the avidin-biotin-peroxidase complex method (Vector Laboratories, Burlingame, CA) with diaminobenzidine. Mounted sections were dehydrated with sequential ethanol and Histoclear and all images were captured using an Olympus BX51 microscope and Olympus software. Thioflavin-stained plaques were manually counted in a blinded fashion using Metamorph image analysis software (Molecular Devices). Total amyloid burden was quantified by measuring Aβ42 immunopositive surface area in a blinded manner using Metamorph image analysis software.

Sequential amyloid extraction

Cortical hemispheres and hippocampi were homogenized using a Konte's Dounce tissue grinder in phosphate-buffered saline with protease inhibitor cocktail (Roche, Indianapolis, IN) and sonicated (~30 seconds at level 7 using a Branson Sonifier 250, Krackeler Scientific, Inc., Albany, NY) in the presence of 2% sodium dodecyl sulfate, then pelleted by centrifugation for 1 hour at 100,000 x g at 8°C (Optima TLX Ultracentrifuge, Beckman-Coulter, Fullerton, CA). The supernatant was collected and the pellet resuspended in an equal volume of 70% formic acid and re-sonicated. Formic acid soluble fractions were neutralized using 1.0 M Tris (pH 11). SDS-soluble and neutralized formic acid-soluble fractions were diluted in ELISA sample diluent (50 mM Tris base, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.1 mg/mL phenylmethylsulfonyl fluoride, protease inhibitor cocktail, pH 7.4).

Measurement of ERK 1/2 phosphorylation

Cells were plated at a density of 50,000/cm^2 in 6-well culture dishes 3 days before use. On the day before the experiment, the culture medium was replaced with 2 mL of serum-free DMEM. Prior to beginning the experiment, the cells were rinsed with 2 mL serum-free DMEM. Cells were treated with vehicle or the drug concentrations listed in the figure legends. Atropine control conditions were pre-treated for 30 minutes with 1 μM atropine sulfate (Calbiochem, Gibbstown, NJ). Following treatment, cells were collected in phosphate-buffered saline containing protease inhibitor cocktail (Roche), 1 mM sodium orthovanadate, and 0.1 mM ammonium molybdate. Fifty micrograms of protein per sample was separated by SDS-PAGE on 12% acrylamide gels, transferred to PVDF membranes and probed with phospho- and total-ERK 1/2 antibodies. Following primary antibody incubation, blots were rinsed and incubated with Alexa 680 (Molecular Probes, Eugene, OR) and IR Dye 800 (Rockland, Gilbertsville, PA) conjugated secondary antibodies. Blots were scanned and band intensities quantified on an Odyssey Infrared Imager (Li Cor, Lincoln, NE).

Intracellular calcium mobilization assays

M1-CHO cells were plated on poly-lysine coated glass coverslips and loaded with 5 μM Fura-2 AM in 1 μM pluronic acid (Invitrogen, Carlsbad, CA) for 1 hour at 37°C in buffer containing 150 mM NaCl, 10 mM HEPES, 3 mM KCl, 22 mM sucrose, 10 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 2.5 mM probenecid, pH 7.4. Following excitation at 340 and 380 nm, Fura-2 emission was detected at 510 nm and ratiometric images were captured using Imaging Workbench software (INDEC Biosystems, Santa Clara, CA) in conjunction with an Olympus BX51WI microscope and a PTI IC200

intensified camera. Data are represented as the ratio of fluorescence intensity from 340 nm/380 nm excitation normalized to baseline.

Binding assays

M1-CHO cells were treated for 24 hours with the drug concentrations indicated in the figure legends. All drug treatments were carried out in the presence of 20 μg/mL cycloheximide. Following treatment, cells were suspended and incubated at 37°C for 90 minutes with [^{3}H]-quinuclidinyl benzilate (QNB) to label total mAChRs. [^{3}H]-N-methylscopolamine (NMS) internalization assays were performed using M1-CHO cells as previously described (Volpicelli, Lah et al. 2001). Radioligand binding was quantified by liquid scintillation spectroscopy. Nonspecific binding was determined using 1 μM atropine.

Immunocytochemistry

Cells were treated with the indicated agonists, fixed with 2% paraformaldehyde for 30 minutes at room temperature, blocked and permeabilized with 5% normal serum, 1% bovine serum albumin, and 0.1% Triton X-100 in phosphate buffered saline containing 0.05% saponin, and incubated with primary antibodies overnight. Staining was visualized with Alexa 488- and Alexa 594-conjugated secondary antibodies raised against the host species of the primary antibody (Invitrogen). Images were captured on a Zeiss LSM 510 laser scanning confocal microscope and analyzed using MetaMorph image analysis software (Molecular Devices).

Arrestin recruitment

CHO-K1 cells were co-transfected with Arrestin-3-GFP and M1-FUW using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions and replated 1 day later onto glass coverslips coated with Matrigel extracellular matrix (BD Biosciences, Franklin Lakes, NJ). Cells were incubated for 5 minutes at 37°C with the indicated agonists, immediately fixed with 2% paraformaldehyde, and processed by immunocytochemistry to verify M1 expression. Cells expressing both Arrestin-3-GFP and M1 were imaged on a Zeiss LSM 510 confocal microscope.

Statistical Analysis

Data were analyzed with GraphPad Prism 4.0 software (La Jolla, CA). Unless otherwise indicated, quantification is presented as mean ± SEM.

Chapter III. APP PROCESSING IN PRIMARY NEURON CULTURES FROM WILDTYPE AND M1 MUSCARINIC RECEPTOR SUBTYPE KNOCKOUT MICE

Introduction

There is a large amount of evidence indicating that alterations in cholinergic signaling are intimately involved in AD pathophysiology. Furthermore, muscarinic receptor signaling has been shown to regulate the processing of the amyloid precursor protein. However, less is understood about the precise impact that muscarinic receptor activation may have on the process of amyloidogenesis in neurons. Many of the studies that support a role for mAChR activation in regulated APP processing were carried out in transformed cell lines using transient transfection methods that result in massive gene overexpression. There are numerous reports, including mAChR-specific literature, citing examples of signal transduction pathways that are regulated by GPCRs in neuron-specific modes (Berkeley and Levey 2003). Therefore, testing the effects of mAChR activation on APP processing in neurons is necessary to clarify the potential role that mAChR subtypes might play in regulating amyloidogenesis in the brain.

Another fundamental limitation of previous studies is the reliance on agonists and antagonists that are not selective for individual mAChR subtypes. Given the diversity in expression patterns of mAChR subtypes in various cell types throughout the brain, cholinergic regulation of APP processing has the potential to be highly subtype specific. While many exciting studies have reported effects of mAChR agonists, including a recent report that mAChR activation modulates pathology in a transgenic mouse model of AD,

the conclusions that can be drawn from such experiments are limited by the non-selective nature of the agonists used (Caccamo, Oddo et al. 2006).

Because the accumulation of pathogenic Aβ species is implicated as a proximal event in AD, it is important to understand the regulatory mechanisms governing the processing of APP in the brain. To this end, we designed experiments to examine the regulation of APP processing by mAChR activation in primary neuron cultures. Primary cultures of neurons from embryonic and postnatal rodents have been well established as a useful tool for addressing hypotheses in a physiologically relevant model system, and have been previously used to study the regulated processing of APP (Hama, Shirotani et al. 2004; Kimberly, Zheng et al. 2005; Patil and Chan 2005; Kienlen-Campard, Feyt et al. 2006; Patil, Sheng et al. 2006). Furthermore, experiments in cultured neurons from mice facilitate the comparison of APP processing in wild type neurons with those derived from mice deficient in specific genes that are hypothesized to regulate APP processing. This opportunity is ideal for the study of mAChRs, as the genetic deletion of specific mAChR subtypes is much more conclusive than using semi-selective agonists and antagonists. By comparing regulated APP processing in wildtype mouse cortical neurons to those cultured from M1KO mice, we establish a role for the genetically defined M1 receptor in regulating non-amyloidogenic APP processing in neurons.

Results

Cortical neurons from E18 embryonic wildtype and M1KO mice were cultured *in vitro* and infected with a lentivirus vector to achieve expression of human sequence APP. As shown in Figure 3.1, lentiviral transduction of mouse cortical neuron cultures results in

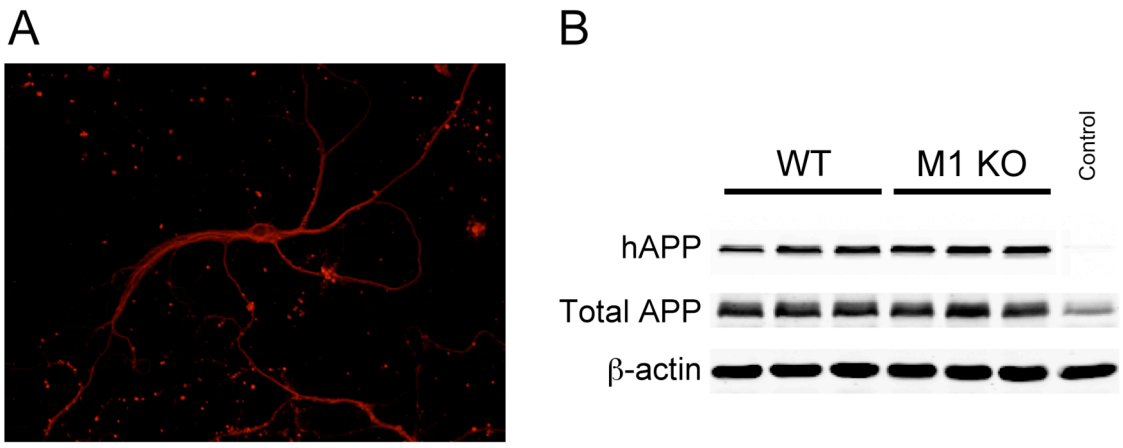


Figure 3.1. Lentivirus transduction of human APP in mouse primary neuron cultures.

(A) Immunocytochemistry showing expression of human sequence APP in a mouse cortical neuron in culture. **(B)** Western blot of individually transduced neuron cultures from wildtype (WT) and M1 knockout (M1KO) demonstrating consistent overexpression of the human sequence protein (hAPP). Infection with FUGW virus alone (no APP) is shown as a control. Both the overexpressed hAPP and endogenous murine APP are detected by a C-terminal antibody (Total APP), demonstrating ~2-3 fold overexpression using the hAPP lentivirus vector.

efficient and consistent APP expression. Owing to the high efficiency of retroviral gene delivery, a low copy number of transgene per target cell can still achieve a high percentage of transduced cells (>90% efficiency for multiple independent viruses tested in our laboratory, data not shown) but is less likely to interfere with normal cell biology and introduce overexpression artifacts. We have reproducibly observed modest levels of overexpression in cortical neurons (~2-3 fold over basal) of APP and other lentivirus-delivered genes using this system (Figure 3.1). To measure mAChR-regulated APP processing, neurons were allowed to condition 1.5 mL of medium for eight hours in the presence or absence of the non-selective mAChR agonist carbachol (CCh). Western blot analysis of secreted APP derivatives showed a significant increase (60%) in the release of APPsα following carbachol stimulation (Figure 3.2). This result is consistent with a number of other reports in cultured cells and brain slices (Nitsch, Slack et al. 1992; Farber, Nitsch et al. 1995), and indicates that the cellular machinery required for mAChR-mediated signaling and APP processing is intact and functional in our primary neuron culture system. Measurement of Aβ peptides in the conditioned medium by ELISA also revealed a small but significant decrease in the secretion of total Aβ peptides in CCh-treated cultures (Figure 3.4).

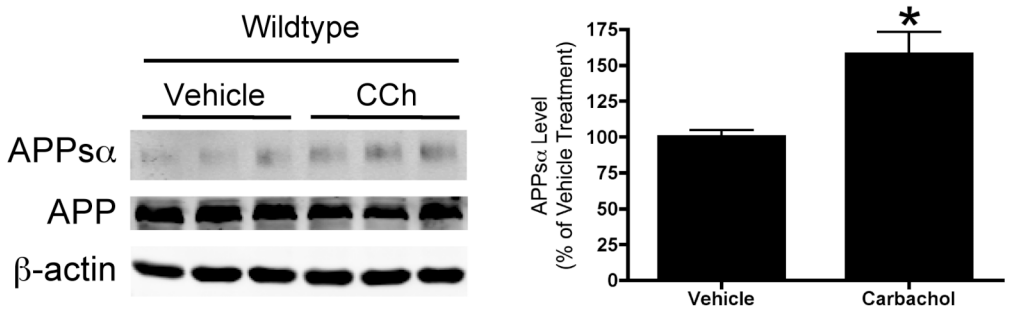


Figure 3.2. mAChR stimulation increases APPsα release in wildtype neuron cultures.

Western blots of conditioned media demonstrate increased secretion of APPsα from wildtype cortical neuron cultures treated with the mAChR agonist carbachol (CCh). Quantitation of APPsα band intensity shows a significant increase in APPsα release in CCh-treated cultures ($p<0.05$). ELISA measurements from conditioned medium show a small but significant decrease in Aβ40 secretion from cultures stimulated with CCh ($p<0.05$). Data are shown as the percent of vehicle control and represent mean ± SEM from three to five independent experiments.

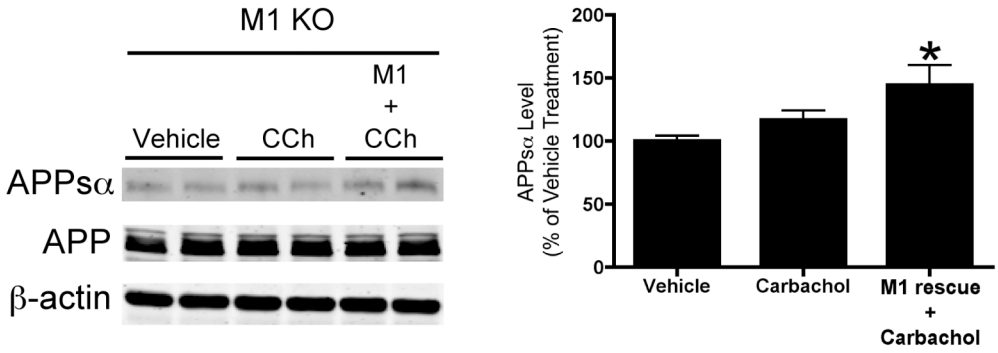


Figure 3.3. mAChR-regulated APPsα release is lost in M1 knockout neuron cultures and rescued by M1 overexpression.

Western blots of conditioned media demonstrate a loss of carbachol-stimulated APPsα release in neuron cultures from M1KO mice. Lentivirus expression of M1 restores the effect of carbachol on APPsα secretion. Quantitation of APPsα band intensity shows a significant increase in APPsα release in M1-lentivirus rescued, CCh-treated cultures ($p<0.05$). ELISA measurements show a significant increase in Aβ40 secretion in M1KO cultures treated with CCh ($p<0.05$). Data are shown as the percent of vehicle control and represent mean ± SEM from three to four independent experiments.

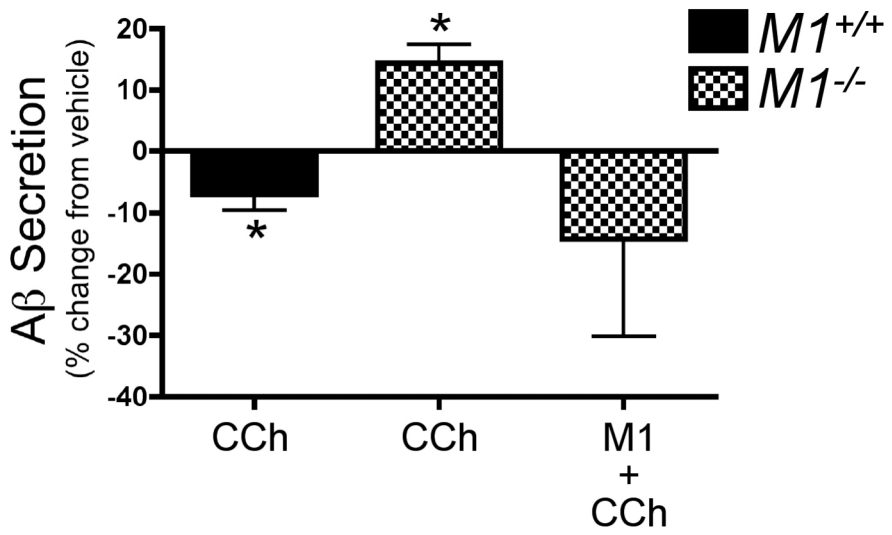


Figure 3.4. Regulated secretion of Aβ peptide in wildtype and M1 knockout primary neuron cultures.

Measurement of total Aβ levels by ELISA show a significant increase in Aβ40 secretion in M1KO cultures treated with CCh ($p<0.05$). Data are shown as the percent of vehicle control and represent mean ± SEM from three to four independent experiments.

Having established regulated APP cleavage in wildtype neurons, we performed the same experiment using neurons from M1KO mice. As shown in Figure 3.3, deletion of the M1 receptor results in a loss of significant APPsα shedding following carbachol stimulation. However, in M1KO cultures transduced with both APP and human M1, carbachol-mediated APPsα shedding was restored, indicating that the lentivirus delivery of M1 was able to rescue the M1KO phenotype. Measurement of Aβ by ELISA in conditioned media samples demonstrated that CCh-treatment actually increased secretion of Aβ40 in M1KO neurons ($p<0.05$). Neuron cultures rescued with M1 lentivirus and stimulated with CCh showed a trend towards reduction of Aβ40, although this result did not reach statistical significance. A similar pattern was observed for measurement of Aβ42, but again, the effect was not statistically significant. Taken together, these data indicate that M1 is essential for carbachol-mediated APPsα release, and therefore represents the major mAChR subtype responsible for regulation of non-amyloidogenic APP processing in cultured neurons. The fact that CCh stimulation caused a decrease in Aβ40 secretion in wildtype neurons but an increase in secretion in M1KO neurons indicates that not only is M1 activation necessary for the prevention of Aβ formation, but that there are also other mAChR subtypes capable of promoting amyloidogenic APP processing.

Discussion

The data from this study provide the first assessment of regulation of APP processing by genetically defined mAChR subtypes in neurons. Our results indicate that M1 is indispensable for regulating non-amyloidogenic APP processing, and likely play a critical

role in influencing amyloidogenesis in the brain. There are several implications for this finding. We observed the largest changes in levels of secreted APPsα, the ectodomain released from full length APP by the action of α-secretase. APPsα has been shown to be neuroprotective in some systems, and may play a role in memory enhancement, possibly by facilitating synapse formation (Mattson, Cheng et al. 1993; Meziane, Dodart et al. 1998; Bell, Zheng et al. 2008). A recent study has also proposed a role for APPsα in the disruption of APP dimers on the cell surface, which the authors argue is important for regulating cell survival (Gralle, Botelho et al. 2009). Regardless of the combination of mechanisms by which APPsα exerts a beneficial effect in the CNS, it is logical to conclude that signaling pathways that promote its secretion may be important for normal physiological brain function.

We also found that M1 activation by CCh mediates decreased Aβ40 secretion in wildtype neurons, and that CCh actually promotes increased Aβ40 secretion in M1KO neurons. This finding suggests that other mAChR subtypes can regulate amyloidogenic APP processing and agrees with a previous report showing that an M2/M4 antagonist can potentiate CCh-stimulated APPs release from brain slices (Farber, Nitsch et al. 1995). These data indicate that mAChR signaling may be important for regulating multiple aspects of APP processing and amyloidogenesis in neurons, and therefore, that a loss of M1 signaling may have multiple deleterious consequences in the context of AD pathogenesis.

Chapter IV. IMPACT OF M1 MUSCARINIC RECEPTOR DELETION ON PATHOLOGY IN A MOUSE MODEL OF AMYLOIDOSIS

Introduction

The biochemical and cell biological mechanisms that regulate APP processing have been studied in detail, and much is known about the factors that bias cleavage of APP towards non-amyloidogenic vs. amyloidogenic pathways. Cholinergic regulation of APP processing is a good example from the vast AD literature where an initial observation in human patients of decreased cholinergic system markers was followed by basic science studies utilizing biochemistry, gene overexpression, and pharmacological manipulation to elucidate the molecular mechanism of cholinergic influence on APP cleavage. This reductionist approach has yielded many valuable clues to the biology of AD, and several efforts to translate these findings into modulation of disease pathology in animal models and humans subjects have shown some encouraging results (Nitsch, Deng et al. 2000; Beach, Walker et al. 2001; Hock, Maddalena et al. 2003; Caccamo, Oddo et al. 2006).

However, the precise molecular mechanism of cholinergic regulation of amyloidogenesis *in vivo* remains unclear, and therefore, definitive conclusions regarding the optimal strategy for moving forward with candidates for cholinergic therapy are premature. For example, many of the experiments supporting a role for the M1 mAChR receptor in regulating non-amyloidogenic APP cleavage and the reduction of amyloid pathology have relied on non-selective pharmacologic agents. Specifically, both

talsaclidine and AF267B, mAChR agonists that have shown effects on amyloidogenesis, activate both M1 and M3. In fact, AF267B was recently shown to be nearly five times more potent at M3 than at M1 (Jones, Brady et al. 2008). Nevertheless, a considerable amount of data from multiple model systems, including our own experiments in wildtype and M1 knockout neuron cultures, indicate that M1 activation is critical for regulating APP processing. This should not be construed as evidence that M3 plays no role in AD pathogenesis, as M3 was equally effective in promoting APPs release in the initial report of mAChR-regulated APP cleavage (Nitsch, Slack et al. 1992). Rather, since M1 and M3 show non-overlapping anatomical distribution and subcellular localization in the brain, identifying the molecular subtypes of muscarinic receptors responsible for cholinergic regulation of amyloidogenesis *in vivo* is of paramount importance for the efficacy of future cholinergic therapies for AD. As new and highly selective allosteric agonists for mAChR subtypes are becoming available, this knowledge would allow more precise targeting of the relevant mAChR subtypes and could theoretically permit the use of higher, more effective drug doses. Furthermore, since cholinomimetic drugs have a considerable liability for dose-limiting side effects (including gastrointestinal side effects that are likely mediated in part by M3 activation), precise targeting with highly selective M1 agonists and/or potentiators could avoid these adverse effects and might afford better tolerance.

In order to address this important question, we crossed M1 knockout mice with mice expressing a mutant form of APP that results in the development of AD-like pathology. The use of M1 knockout mice circumvents any ambiguity associated with

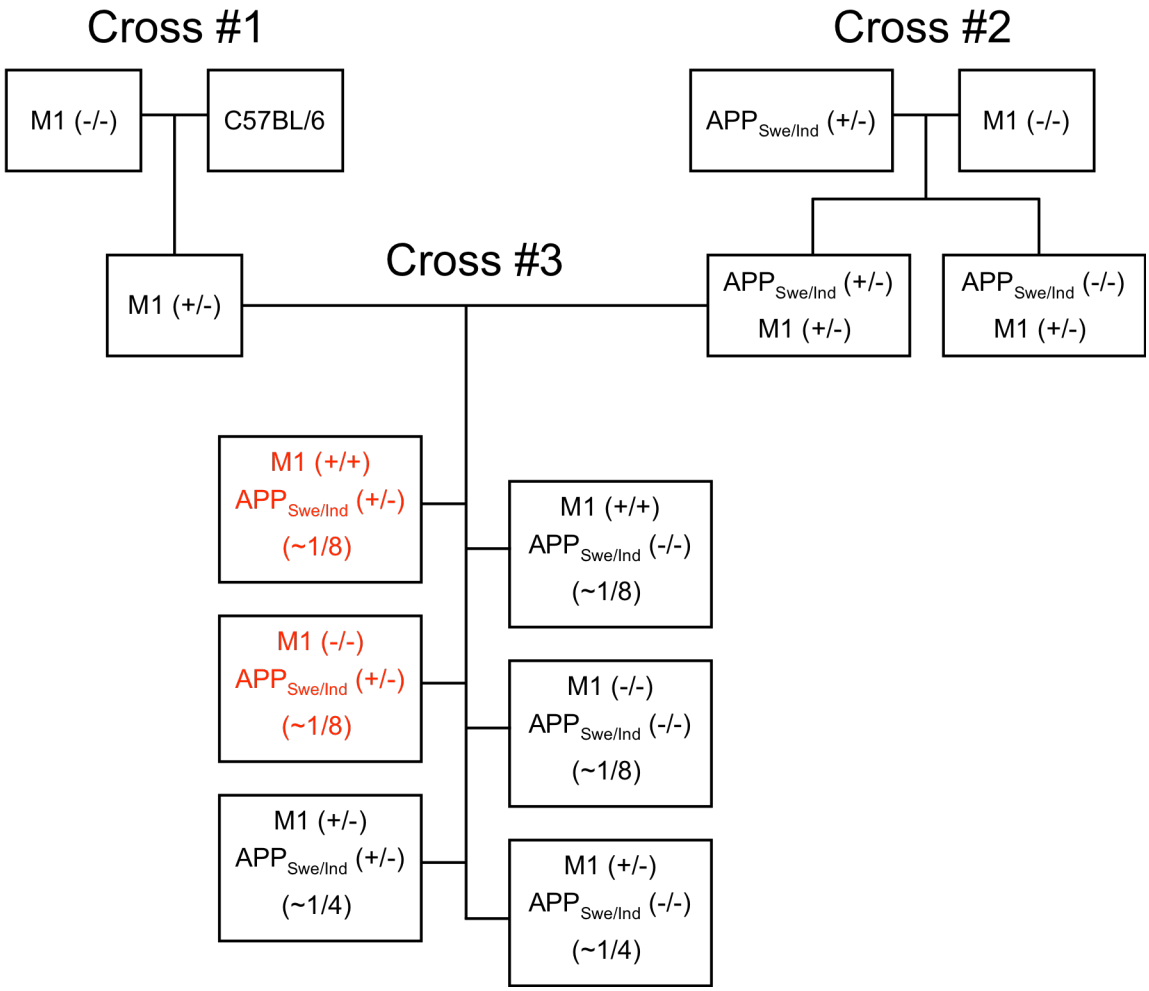


Figure 4.1. Breeding scheme for generating M1-deficient APP-transgenic mice and littermate controls. The genotypes compared in this study are highlighted in red.

non-selective drugs, and the *in vivo* design of this experiment allows the direct examination of amyloid production in the brain.

Results

$M1^{+/+}$ and $M1^{-/-}$ littermates carrying the $APP_{Swe/Ind}$ transgene were generated according to the breeding scheme depicted in Figure 4.1. In order to examine the potential role of M1 in regulating the development and progression of amyloidogenesis *in vivo*, we quantified measures of amyloid pathology at multiple ages. At 3 months of age, total Aβ peptide levels in cerebral cortex are quite low and are predominantly found in SDS-soluble fractions (Figure 4.2). There were no differences in Aβ levels between $M1^{+/+}$ and $M1^{-/-}$ mice. There were also no visible thioflavin-S-positive plaques at this age, indicating that amyloid has not yet deposited into cored plaques (data not shown). At 6 months, brain Aβ levels were not significantly increased (Figure 4.2), but we did observe the formation of scattered thioflavin-S plaques, primarily in the hippocampus (Figure 4.3). This finding is consistent with the initial reports characterizing the development and progression of amyloid pathology in this transgenic mouse strain (Mucke, Masliah et al. 2000). By 12 months of age, there was a substantial age-associated increase in the number of thioflavin-S plaques, indicating that amyloid has begun to be deposited as insoluble plaques (Figure 4.3). There were no differences in the numbers of thioflavin-S plaques between $M1^{+/+}$ and $M1^{-/-}$ mice at 12 months, nor did we detect significant differences between genotypes in brain Aβ levels, although soluble and insoluble levels of Aβ, particularly Aβ42, began to rise exponentially by 12 months (Figure 4.2 and data not shown).

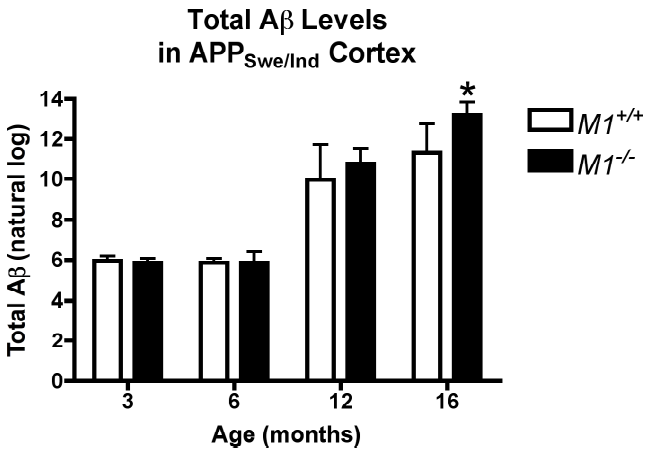


Figure 4.2. ELISA measurement of Aβ levels in *M1*$^{+/+}$ and *M1*$^{-/-}$ brain.

Sandwich ELISA measurement of Aβ40 and Aβ42 peptide levels (total level is shown) in *M1*$^{+/+}$ and *M1*$^{-/-}$ cortex at 3, 6, 12, and 16 months of age. At 16 months of age, Aβ levels are significantly higher in *M1*$^{-/-}$ cortex.

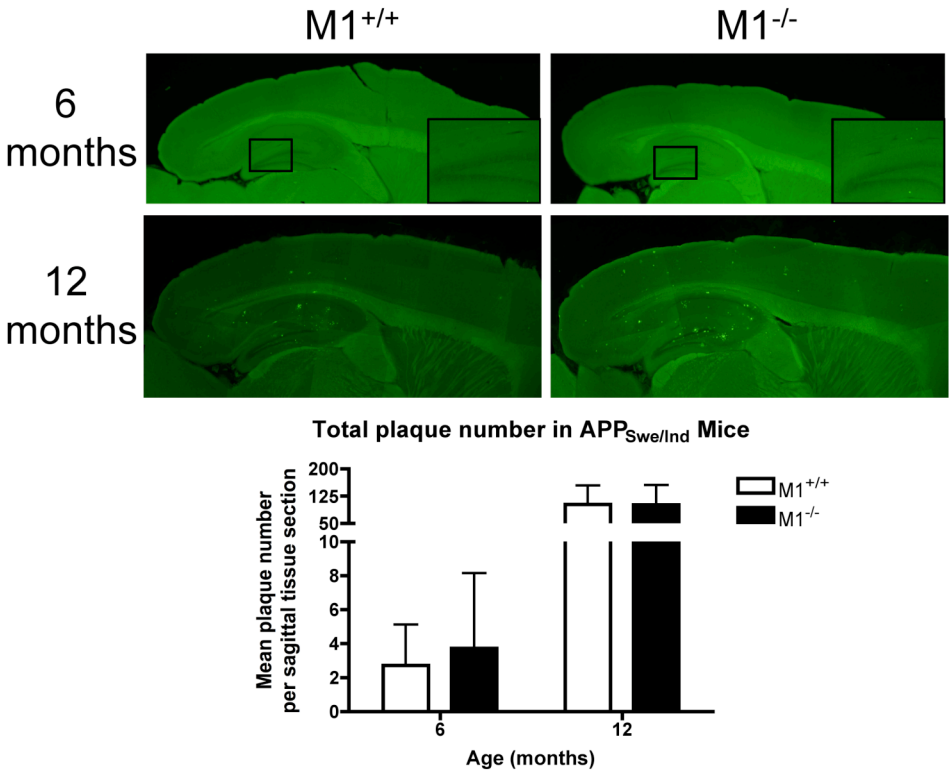


Figure 4.3. Amyloid plaque counts in *M1*$^{+/+}$ and *M1*$^{-/-}$, APP$_{Swe/Ind}$ mice.

Thioflavin-S positive amyloid plaques in the cortex and hippocampus of *M1*$^{+/+}$ and *M1*$^{-/-}$, APP$_{Swe/Ind}$ mice at 6 and 12 months of age. There are no significant differences between genotypes at these ages.

At 16 months, we observed a further increase in total brain Aβ, with levels in *M1*$^{-/-}$ mice significantly higher than their *M1*$^{+/+}$ littermates (Figure 4.2). To examine the effect of loss of M1 signaling on amyloid plaque pathology, we performed immunohistochemical staining for Aβ42 on brain sections from 16 month-old *M1*$^{+/+}$ and *M1*$^{-/-}$ mice. As shown in Figure 4.4, *M1*$^{-/-}$ mice have increased accumulation of amyloid plaque pathology, particularly in cerebral cortex. Quantification of amyloid plaque burden by measuring the surface area of Aβ42 staining revealed a ~140% increase in plaque load in *M1*$^{-/-}$ mice compared to *M1*$^{+/+}$ littermates (Figure 4.4).

Discussion

Our data from *M1*$^{-/-}$ x $APP_{Swe/Ind}$ mice represent the first assessment of M1 loss on the development of amyloid pathology *in vivo*. We demonstrate that M1 deletion results in increased levels of pathogenic Aβ peptides in brain, as well as increased accumulation of amyloid plaque pathology. These findings are consistent with the important role that M1 plays in regulating APP processing as well as reports from several model systems, including human data, demonstrating that manipulation of mAChR signaling can modulate the development of amyloid pathology *in vivo* (Beach, Potter et al. 2000; Nitsch, Deng et al. 2000; Beach, Kuo et al. 2001; Beach, Walker et al. 2001; Perry, Kilford et al. 2003; Caccamo, Oddo et al. 2006).

Further research, including follow-up studies in APP transgenic mice, will be required to more fully understand M1 regulation of amyloidogenesis in the brain. In addition to the observed effects on amyloidogenesis, it will be important to investigate whether loss of M1 has an effect on learning and memory impairment in APP-transgenic

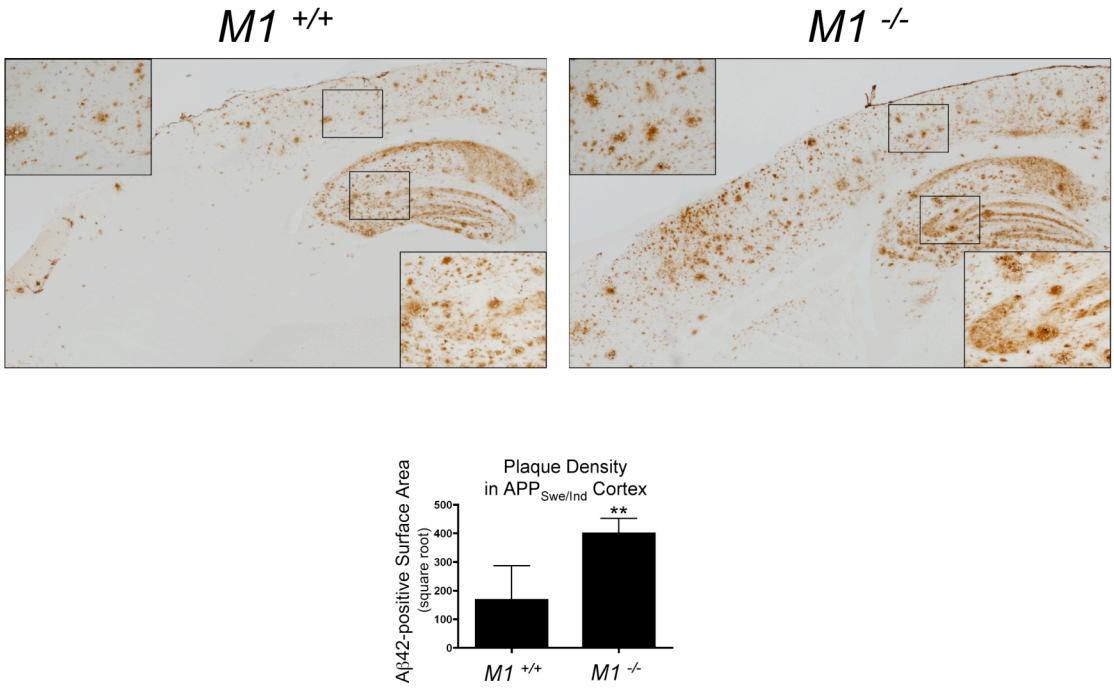


Figure 4.4. Amyloid plaque density in 16 month-old $APP_{Swe/Ind}$ mice.

Aβ42-immunoreactivity (mean surface area (pixels) per section) is significantly increased in 16 month-old $M1^{-/-}$ $APP_{Swe/Ind}$ mice. Quantification is shown for cerebral cortex.

mice. Accumulation of neurotoxic Aβ species impairs synaptic function (Walsh, Klyubin et al. 2005) and multiple lines of APP-transgenic mice show deficits in learning and memory tasks (Woodruff-Pak 2008), so it is logical to hypothesize that the increase in amyloid pathology induced by deletion of M1 would exacerbate cognitive deficits. Given the role that M1 plays in certain aspects of learning and memory, the loss of M1 signaling accompanied by increased accumulation of amyloid pathology may have an additive detrimental effect on cognition.

In conjunction with studies examining the effects of M1 deletion on amyloid pathology and memory impairment, it will be important to evaluate the potential for M1-selective agonists in reducing amyloid pathology and promoting cognitive processes. None of the mAChR-targeted therapies that have been tried to date have been successful enough to progress to the final stages of clinical trials and use in humans, but it remains to be seen whether newer generations of M1-selective agonists will be able to offer more meaningful therapeutic benefit than the modest effects currently achievable with cholinesterase inhibitors. Our data from cultured cells indicates that M1-selective agonists and PAMs are effective at promoting non-amyloidogenic APP processing and are therefore excellent candidates for therapies aimed at reducing amyloid pathology *in vivo*.

Chapter V. REGULATION OF APP PROCESSING BY M1-SELECTIVE AGONISTS

Introduction

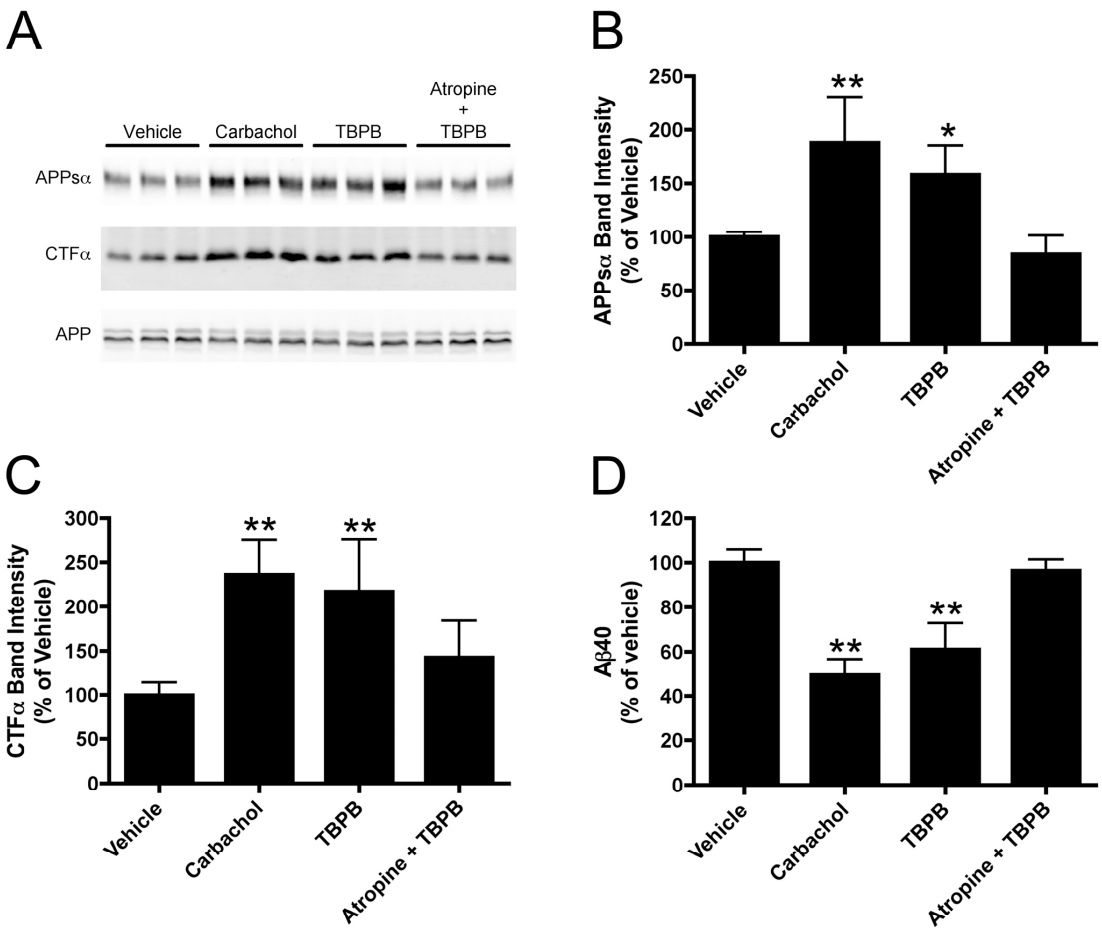
Converging lines of evidence from cell culture studies and animal model experiments, including our own data from primary neuron cultures, support the hypothesis that M1 is an important regulator of non-amyloidogenic APP processing. Currently, the most feasible approach to translate this basic observation into real differences in clinical settings is by pharmacologically activating M1 in the central nervous system. For more than two decades, cholinesterase inhibitors have been used as the front-line therapy for Alzheimer's disease, and while these drugs can achieve some stabilization of symptoms, their efficacy is probably limited by their non-selective mechanism of action and side effects. Another limitation of these drugs is their modest level of acetylcholinesterase inhibition in the brain (Shinotoh, Aotsuka et al. 2001; Bohnen, Kaufer et al. 2005; Darreh-Shori, Kadir et al. 2008; Kadir, Darreh-Shori et al. 2008). Therefore, new avenues of more effective and precisely targeted cholinomimetic therapy warrant exploration for the treatment of AD and other CNS disorders, especially given the advances in our understanding of the tissue distribution and functional roles of mAChR subtypes.

Modulation of GPCRs with centrally acting ligands has been very successful for treating a wide range of clinical disorders. In fact, it is estimated that the majority of all prescribed drugs target GPCR systems (Pierce, Premont et al. 2002). While numerous semi-selective mAChR compounds have been developed over the last twenty-plus years,

efforts to develop truly subtype selective mAChR agonists have largely failed. This failure is likely explained by the high degree of homology across mAChR subtypes, particularly at the acetylcholine binding pocket. More recent efforts based on large-scale functional screening of small molecule libraries has finally paid dividends with the identification of multiple structurally distinct agonists that display unprecedented selectivity for the M1 mAChR (Spalding, Trotter et al. 2002; Bridges, Brady et al. 2008; Lewis, Sheffler et al. 2008; Miller, Daniels et al. 2008). The selectivity of these novel compounds is presumably owed to their binding to an allosteric site on the M1 receptor, spatially distinct from the orthosteric site where acetylcholine and its related analogues bind. This property has been confirmed by antagonist titration curves and mutation analysis demonstrating that M1 allosteric agonists can still activate M1 mAChR constructs harboring point mutations that block activation by orthosteric ligands (Spalding, Trotter et al. 2002; Jones, Brady et al. 2008). These chemicals represent a huge theoretical advantage over previous generations of mAChR-directed pharmaceuticals, but their efficacy in regulating disease-relevant biochemical and physiological processes must be directly measured before they can proceed to the next phase of development. As a first step towards evaluating M1-selective agonists for potential utility in treating AD, we have examined the abilities of two structurally distinct compounds to regulate non-amyloidogenic APP processing. Using an established PC12 cell model of APP processing, we found that both agents were effective in promoting non-amyloidogenic APP processing. These studies provide proof of principle that M1-selective compounds can modulate APP processing in physiologically relevant systems and are therefore good candidates for further evaluation in more complex models.

Results

Based on functional screening of a large library of small molecules, Dr. P. Jeffrey Conn and colleagues at Vanderbilt University identified TBPB [1-(1'-2-methylbenzyl)-1,4'-bipiperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one] as an allosteric M1 agonist (Bridges, Brady et al. 2008; Miller, Daniels et al. 2008). In order to test whether a functionally-selective M1 agonist could regulate APP processing, we treated PC12 cells over-expressing human sequence, Swedish mutation APP and human M1 with vehicle, CCh, or TBPB, and then analyzed the APP derivatives produced. Treatment with 1 μM TBPB increased the shedding of APPsα, the ectodomain released by α -secretase cleavage, by 58% as compared to vehicle-treated cells (Figure 5.1, A, B). The magnitude of the TBPB response was comparable to that of the CCh positive control and blocked by atropine. It should be noted that the antibody used to detect APP_s in these experiments recognizes an epitope contained within APPsα but not APPsβ, indicating that the shedding of APPsα is specifically increased. Consistent with these data, TBPB also increased the production of CTFα (also called C83), the carboxyl-terminal fragment of APP derived from alpha-secretase cleavage, in an atropine-sensitive manner (Figure 5.1 A, C). We also analyzed conditioned media from these cells for Aβ40 by ELISA (Figure 5.1, D). In TBPB treated cells, Aβ40 levels were reduced to 61% of the vehicle control and this effect was blocked by atropine. Together, these results are consistent with the hypothesis that selective activation of M1 could regulate APP processing and indicate that activation of M1 with TBPB shifts the processing of APP toward the non-amyloidogenic pathway, resulting in increased shedding of APPsα and decreased production of Aβ.


A
Vehicle
Carbachol
TBPB
Atropine + TBPB
APPsα
CTFα
APP
B
APPsα Band Intensity (% of Vehicle)
250
200
150
100
50
0
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*
Vehicle
Carbachol
TBPB
Atropine + TBPB
C
CTFα Band Intensity (% of Vehicle)
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Vehicle
Carbachol
TBPB
Atropine + TBPB

Figure 5.1. TBPB regulates non-amyloidogenic APP processing.

(A) Western blots of conditioned media and cell lysates demonstrate increased production of APPsα and CTFα in cells treated with CCh and TBPB. **(B)** Quantitation of APPsα band intensity shows a significant increase in APPsα shedding from CCh and TBPB treated cells (**, $p<0.001$, n=7 across 3 separate experiments for CCh; *, $p<0.01$, n=7 across 3 separate experiments for TBPB). **(C)** Quantitation of CTFα band intensity demonstrates a significant increase in production of CTFα in CCh and TBPB treated cells (**, $p<0.001$, n=16 across 6 separate experiments). **(D)** ELISA measurements from conditioned media show a significant decrease in secreted Aβ40 from cells treated with either CCh or TBPB (**, $p<0.001$, n=6 across 2 separate experiments for CCh and **, $p<0.001$, n=9 across 3 separate experiments for TBPB).

We next examined the effects of a second compound called BQCA, a positive allosteric modulator (PAM) that is highly selective for the M1 mAChR. In order to test whether BQCA can potentiate the APP processing effect of a low concentration of the mAChR agonist carbachol (CCh), we treated PC12 cells over-expressing humanized Swedish mutation APP and human M1 with an approximate EC20 concentration (50 nM) of CCh in the presence of increasing concentrations of BQCA and measured the levels of APP metabolites in the conditioned media and cell extracts. BQCA caused a dose-dependent increase in the shedding of APPsα, the amino-terminal ectodomain of APP released by α-secretase cleavage (Figure 5.2, A, B). The highest concentration of BQCA tested (30 μM) increased APPsα levels to 244% of vehicle-treated cells ($p<0.05$). BQCA treatment also resulted in the accumulation of CTFα (C83), the corresponding carboxyl-terminal fragment generated by α-secretase (Figure 5.2, A, C; increased to 245% of vehicle, $p<0.05$). Finally, consistent with the observed increases in non-amyloidogenic APP fragments, 30 μM BQCA treatment resulted in a 30% decrease ($p<0.01$) in the secretion of the β-secretase derived $A\beta_{40}$ peptide (Figure 5.2, D). Taken together, these results indicate that BQCA can effectively regulate non-amyloidogenic APP processing, strengthening the hypothesis that M1 activation may be beneficial in AD.

Discussion

The demonstration that a highly selective M1 agonist can promote non-amyloidogenic APP processing is very encouraging. As previously mentioned, cholinesterase inhibitors are the most commonly prescribed medications for AD, but their non-selective mechanism and side effects limit their overall efficacy. Direct targeting of post-synaptic

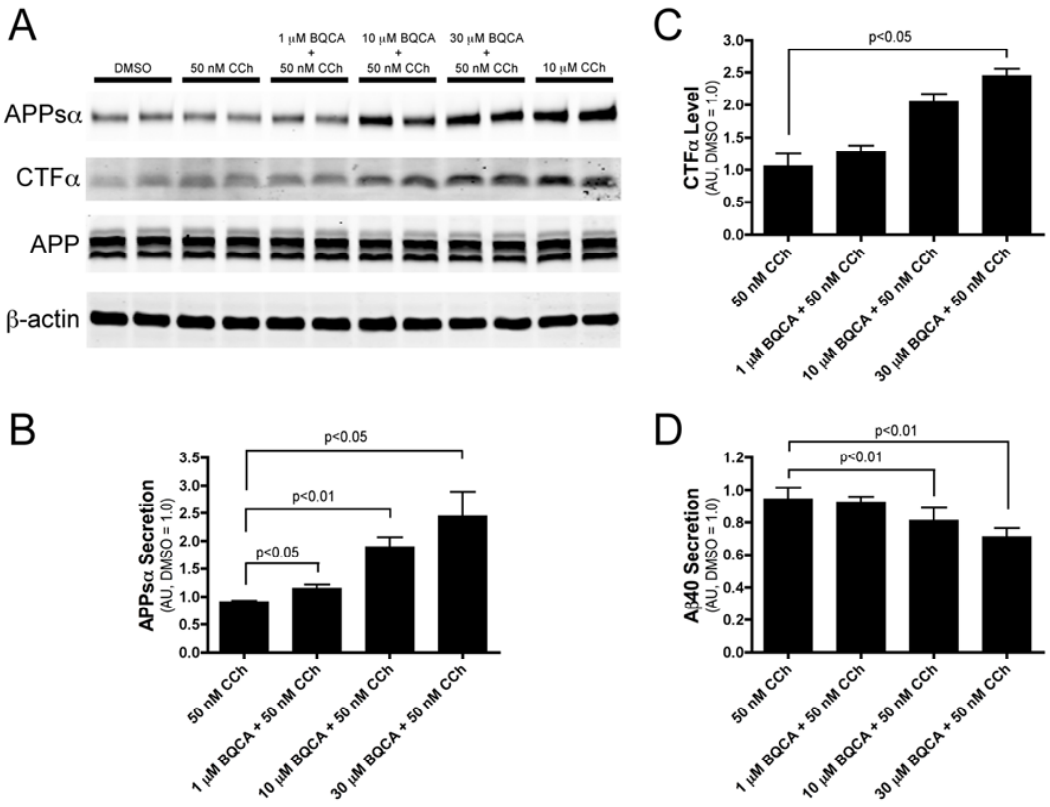

A
DMSO
50 nM CCh
1 μM BQCA + 50 nM CCh
10 μM BQCA + 50 nM CCh
30 μM BQCA + 50 nM CCh
10 μM CCh
APPsα
CTFα
APP
β-actin
B
APPsα Secretion (AU, DMSO = 1.0)
p<0.05
p<0.01
p<0.05
C
CTFα Level (AU, DMSO = 1.0)
p<0.05
D
Aβ40 Secretion (AU, DMSO = 1.0)
p<0.01
p<0.01
50 nM CCh
1 μM BQCA + 50 nM CCh
10 μM BQCA + 50 nM CCh
30 μM BQCA + 50 nM CCh

Figure 5.2. BQCA regulates non-amyloidogenic APP processing.

(A) Western blot analysis of APP metabolites from conditioned media and cell lysates demonstrates increased generation of APPsα and CTFα with increasing concentrations of BQCA as compared to the submaximal concentration of 50 nM CCh. 10 μM CCh is shown as a maximum concentration. β-actin is shown as a loading control. **(B)** Quantitation of APPsα band intensity from conditioned media demonstrates a dose-dependent effect of BQCA on the shedding of APPsα (repeated measures ANOVA, p=0.0271), and pairwise comparisons revealed significant differences at all concentrations of BQCA compared to 50 nM CCh alone (p values for paired t-tests are shown). **(C)** Quantitation of CTFα band intensity from cell lysates shows a dose-dependent effect of BQCA on the production of CTFα (repeated measures ANOVA, p=0.0017) and a significant difference (paired t-test) between 30 μM BQCA plus 50 nM CCh as compared to 50 nM CCh alone. **(D)** ELISA measurements from conditioned media demonstrate that BQCA decreases the secretion of $A\beta_{40}$ peptide in a dose-dependent manner (repeated measures ANOVA, p=0.0019), with significant differences between 50 nM CCh alone and the two highest concentrations of BQCA (paired t-tests). Mean values are shown from three or four independent experiments performed in duplicate. All values are normalized to DMSO-treated cells. CCh, carbachol; DMSO, dimethylsulfoxide.

M1 receptors is likely to achieve better outcomes for addressing cognitive symptoms. Semi-selective mAChR agonists have been tried in AD, and some drug candidates have shown promise in early clinical studies, but their lack of selectivity and side effects ultimately makes them unsuitable for widespread clinical use (Sramek, Hurley et al. 1995; Bodick, Offen et al. 1997; Nitsch, Deng et al. 2000). Nevertheless, these previous reports have demonstrated benefits in some cognitive measures and, coupled with the findings in these studies, indicate that M1 activation may have disease-modifying potential as well. The importance of subtype-selectivity is further underscored in the example of AD since it has been shown that the M2 and/or M4 mAChR subtypes may have an antagonistic effect on the non-amyloidogenic APP processing shown to be promoted by M1 activation (Farber, Nitsch et al. 1995). The full effects of M1 stimulation on AD disease progression and symptomatology will have to be evaluated directly in humans. Given the continuum of changes in the cholinergic system during the course of mild cognitive impairment and AD, it may prove useful to closely examine the effects of M1 activation at multiple stages of clinical disease in order to determine the most effective window of drug intervention.

The finding that BQCA promotes non-amyloidogenic APP processing has important implications for the potential of subtype-selective PAMs as clinical therapeutic agents. PAMs would theoretically avoid the limitations of cholinesterase inhibitors by selectively activating a single neurotransmitter subtype and might achieve better clinical efficacy by permitting a higher degree of activation of the target receptor subtype. Furthermore, by potentiating the action of native ligands, PAMs have the ability to increase signaling through specific receptor subtypes in response to the release of

endogenous neurotransmitter molecules. This strategy has the potential to be more physiologically relevant than exogenous administration of a full agonist that is always "active." Thus, selective activation of M1 by PAMs may not only prevent dose-limiting side effects, but may also be central to the disease modifying potential of M1 activation in AD. It will be important to evaluate the utility of PAMs in animal models and human subjects, with special attention paid to their ability to modulate signaling through the normal release of endogenous neurotransmitter.

Chapter VI. REGULATED SHEDDING OF THE LR11/SORLA ECTODOMAIN BY THE M1 MUSCARINIC RECEPTOR

Introduction

Signal transduction initiated by GPCRs can have a wide range of endpoint effects and is involved in regulating many physiological processes. The sequence of events involved in GPCR signaling is often confined within single cells, but a new paradigm involving GPCR regulation of cross talk between cells has emerged. By promoting the activity of metalloproteases(including the ADAM family enzymes known to function as α-secretases for the amyloid precursor protein), GPCR signaling can regulate the shedding of transmembrane protein ectodomains which can go on to transactivate receptors on adjacent cells. This phenomenon was first described for GPCR-regulated transactivation of the epidermal growth factor receptor (EGFR), a tyrosine kinase receptor involved in multiple cellular functions including differentiation, proliferation, and survival (Prenzel, Zwick et al. 1999; Ohtsu, Dempsey et al. 2006). While this process has been implicated in brain tumor formation including glioblastoma, it is possible that GPCR regulation of EGFR transactivation may regulate normal physiological functions in the developing and adult CNS. It is also possible that GPCR-regulated ectodomain shedding may have other effects besides transactivation of EGFR. Multiple GPCRs, including muscarinic receptors, appear to be able to regulate shedding of protein ectodomains. The best studied example in the context of AD is the stimulation of APP cleavage by mAChRs and other neurotransmitter receptors. We were curious to see whether mAChR signaling could regulate the shedding of other protein ectodomains

that may be relevant to AD. The mosaic lipoprotein and sorting receptor LR11/SorLA emerged as an intriguing candidate, in part because of the presence of an EGF homology domain present in the luminal N-terminus of the protein. LR11 ectodomain shedding has been reported by be stimulated by head activator, a neuropeptide involved in head regeneration in *Hydra*, and is associated with cell proliferation (Hampe, Riedel et al. 2000).

Results

In order to test whether mAChR signaling might regulate LR11 shedding, we designed a series of experiments in PC12 cells analogous to those used to demonstrate M1 regulation of APP cleavage. In cells overexpressing LR11, CCh stimulation had no effect on the release of the soluble LR11 ectodomain (LR11s) into conditioned media (Figure 6.1). Treatment with the phorbol ester phorbol 12-myristate 13-acetate (PMA) resulted in increased shedding of LR11, consistent with the known role that protein kinase C (activated by PMA) plays in regulating metalloprotease activation. When M1 was over-expressed along with LR11, carbachol stimulation increased the level of LR11s in conditioned media to levels comparable to PMA treatment (Figure 6.1). This result indicates that M1 is capable of regulating the shedding of LR11. To test whether M1-mediated LR11 ectodomain shedding is dependent on the activation of a metalloprotease, we treated cells with carbachol in the presence and absence of GM6001, a hydroxamic acid compound that inhibits a broad range of metalloprotease enzymes. In cells pre-treated with GM6001, carbachol-stimulated release of LR11s was abolished, indicating

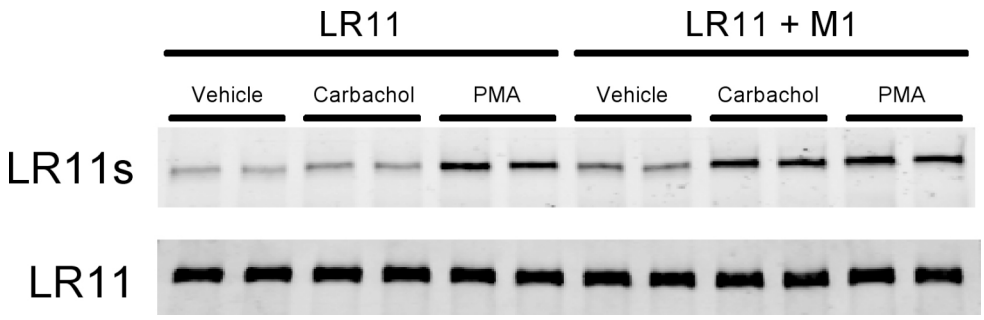


Figure 6.1. M1 regulation of LR11 ectodomain shedding.

In PC12 cells overexpressing LR11 only (left), carbachol stimulation does not substantially increase shedding of the LR11 ectodomain (LR11s, blotted from conditioned media). In cells where M1 is co-expressed with LR11 (right), carbachol stimulation results in a substantial increase in LR11 ectodomain shedding. Stimulation with the phorbol ester PMA is shown for reference.

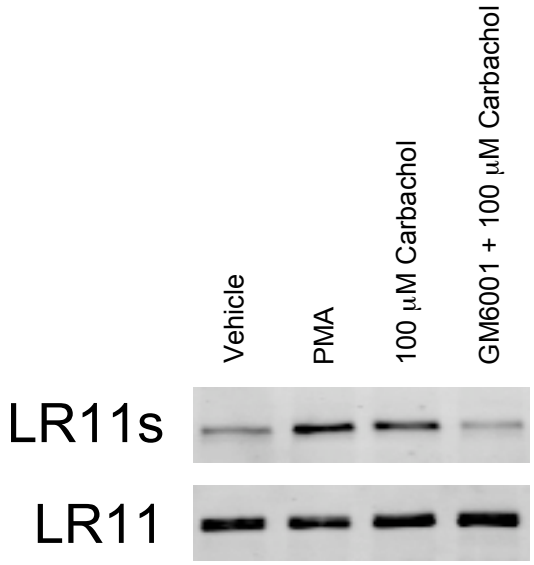


Figure 6.2. M1-regulated LR11 ectodomain shedding depends on the activation of a metalloprotease.

In PC12 cells co-expressing LR11 and M1, carbachol-regulated shedding of the LR11 ectodomain is completely blocked by the metalloprotease inhibitor GM6001, indicating that M1-regulated LR11 shedding proceeds through a metalloprotease-dependent mechanism.

that the mechanism of M1-regulation of LR11 ectodomain shedding is metalloprotease-dependent (Figure 6.2).

Discussion

Based on these data, the implications of M1-regulation of LR11 ectodomain shedding are not immediately clear. Given that the LR11 ectodomain contains an EGF homology domain, it is attractive to speculate that LR11 shedding might induce transactivation of the EGFR. As of this writing, there are no published reports of LR11 involvement in EGFR transactivation, but low density lipoprotein receptor (LDLR) family members, including LR11, have been reported to be associated with cell proliferation (Dong, Lathrop et al. 1998; Chen, Lathrop et al. 1999; Kanaki, Bujo et al. 1999; Hampe, Riedel et al. 2000; Zhao and Michaely 2008). While there are multiple signaling pathways that can lead to cellular proliferation, it would be possible to test whether purified recombinant LR11s can transactivate the EGFR and whether LR11s application results in any EGFR-linked phenotypes such as cell proliferation or survival. However, demonstrating that mAChR-regulated shedding of LR11 controls any of these effects would be more difficult. There are multiple proteins whose ectodomains have been shown to transactivate EGFR, and many of them are shed in GPCR-activated, metalloprotease-dependent fashions through what appears to be a fairly generic mechanism (Pierce, Luttrell et al. 2001). The LR11 ectodomain contains multiple other functional motifs, including a VPS10 homology domain, LDLR type A ligand binding repeats, and fibronectin type III repeats, and it is certainly possible that one or more of these motifs is involved in a physiological event downstream of LR11 ectodomain

shedding. Further experiments are required to elucidate a physiological role for LR11 ectodomain shedding in the CNS, and it will be interesting to see how signaling through GPCRs such as M1 might contribute to the regulation of this biology.

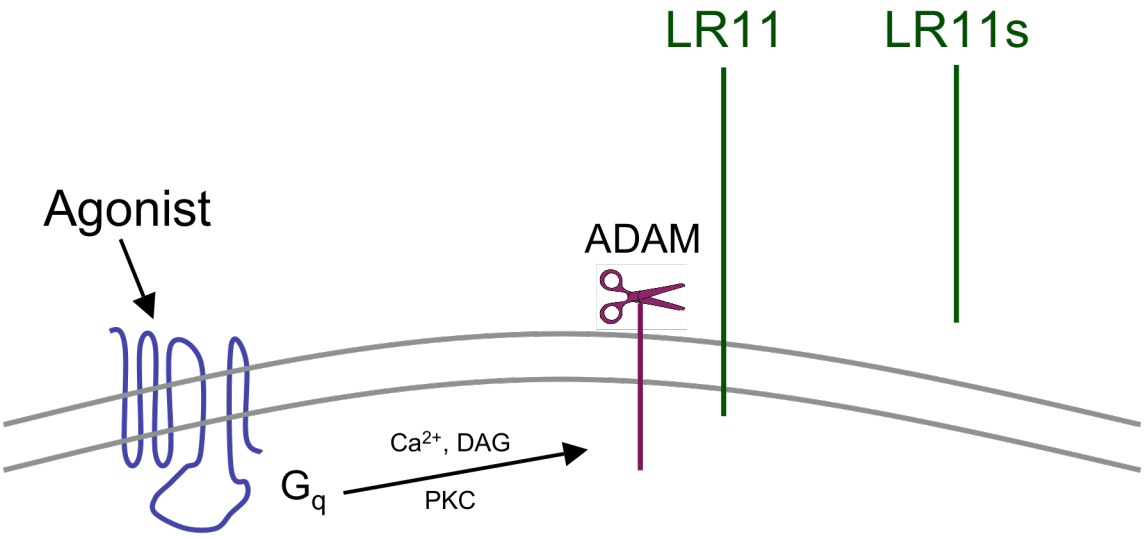


Figure 6.3. Proposed model for metalloprotease-dependent M1 mAChR regulation of LR11 ectodomain shedding.

In response to agonist stimulation, second messengers including calcium and diacylglycerol (DAG) activate protein kinase, which in turn activates members of the ADAM family of metalloprotease enzymes. Metalloproteases liberate the ectodomain of the transmembrane protein LR11 (termed LR11s for "secreted" LR11), which may then diffuse locally to exert autocrine and/or paracrine effects.

Chapter VII. ALLOSTERIC AGONISTS DIFFERENTIALLY REGULATE M1 MUSCARINIC RECEPTOR SIGNALING AND HOMEOSTASIS MECHANISMS

Introduction

Following agonist binding and activation of GPCRs, a series of well characterized homeostatic mechanisms act to terminate signaling (for recent reviews, see (DeWire, Ahn et al. 2007) and (Moore, Milano et al. 2007)). Typically, activated receptors are rapidly phosphorylated, serving as a site of recruitment for a family of regulatory proteins called arrestins. Arrestins attenuate GPCR signaling by uncoupling the receptor from its cognate G-protein, and also promote receptor internalization by facilitating interactions with the endocytic proteins clathrin and AP2. Internalized GPCRs can either be recycled back to the cell surface, or following continuous agonist stimulation, may be targeted to the lysosome for degradation. However, not all GPCR agonists activate these homeostatic mechanisms equally (Whistler, Chuang et al. 1999), and an emerging paradigm suggests that, for a given receptor, distinct agonists can have differential actions on G-protein and arrestin-linked signaling pathways, a phenomenon recently termed “biased agonism” (DeWire, Ahn et al. 2007; Violin and Lefkowitz 2007).

In this study, we examined activation and regulatory mechanisms of the M1 mAChR in response to the orthosteric agonist carbachol (CCh) and two allosteric agonists, AC260584 and TBPB. All three agonists produced robust activation of M1 in calcium mobilization and ERK 1/2 phosphorylation assays, but in contrast to carbachol, neither

allosteric agonist induced M1 internalization and degradation. AC260584 and TBPB also failed to significantly recruit arrestin-3, which likely explains their lack of effect on M1 internalization. Finally, in contrast to carbachol, M1 receptors pre-treated with both allosteric agonists remained sensitive to subsequent agonist stimulation. Taken together, these results indicate that allosteric and orthosteric agonists fundamentally differ in their mechanism of M1 activation, with these allosteric agonists displaying a "biased agonism" toward G_q-coupled signaling. Subtype-selective allosteric agonists represent a major step forward in cholinergic pharmacology, and will likely have a significant impact on the understanding of basic receptor biology and on the ability to modulate cholinergic receptors in clinical settings.

Results

Activation of the M1 mAChR by Orthosteric and Allosteric agonists

As previously reported, AC260584 and TBPB are potent and highly selective M1 agonists (Spalding, Ma et al. 2006; Jones, Brady et al. 2008). In order to more extensively characterize the signal transduction pathways activated by allosteric vs. orthosteric M1 agonists, we compared functional responses in two separate assays known to be activated by M1. The M1 receptor couples to the G_q G-protein, which initiates multiple signaling cascades including the mobilization of intracellular calcium. In M1-CHO cells loaded with the calcium-sensitive dye Fura-2, CCh, AC260584, and TBPB all caused a rapid release of intracellular calcium (Figure 7.1). The CCh-evoked response returned to baseline within four to five minutes, while cells treated with AC260584 and

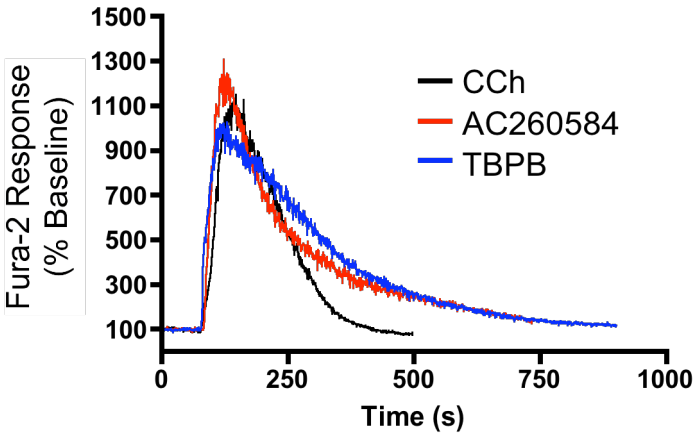


Figure 7.1. Intracellular calcium mobilization induced by orthosteric and allosteric M1 agonists. Ratiometric measurement of intracellular calcium concentration in M1-CHO cells loaded with Fura-2 AM and perfused with loading buffer. One minute into the protocol, cells were stimulated with the indicated agonists (100 μM CCh, 320 nM AC260584, or 1 μM TBPB) for 15 seconds (arrowhead) after which agonists were washed out and recording was continued in the prescence of buffer alone until calcium concentrations returned to baseline. Data is shown as the average response from 8-12 cells per treatment group and is representative of three independent experiments.

TBPB showed a slightly more prolonged response, returning to baseline by eight to ten minutes.

M1 has also been linked to activation of the extracellular signal regulated kinase (ERK 1/2) in neurons, which plays a key role in synaptic plasticity, learning, and memory. ERK 1/2 is activated by phosphorylation, which can be measured using phospho-specific antibodies. CCh, AC260584, and TBPB all produced concentration dependent increases in the phosphorylation of ERK 1/2 in HEK293T cells over-expressing human M1 (Figure 7.2). Atropine completely blocked ERK 1/2 phosphorylation by all three agonists, but the response was not changed by pre-incubation of cells with AG1478, an tyrosine kinase inhibitor that blocks activation of the epidermal growth factor receptor (EGFR) (Figure 7.3). ERK phosphorylation has been shown to be dependent on EGFR activation in some systems, and carbachol stimulation is known to induce EGFR transactivation in specific cell lines (Prenzel, Zwick et al. 1999) but the results from the current experiments demonstrate that M1-regulated ERK 1/2 phosphorylation proceeds in an EGFR-independent manner in HEK293 cells.

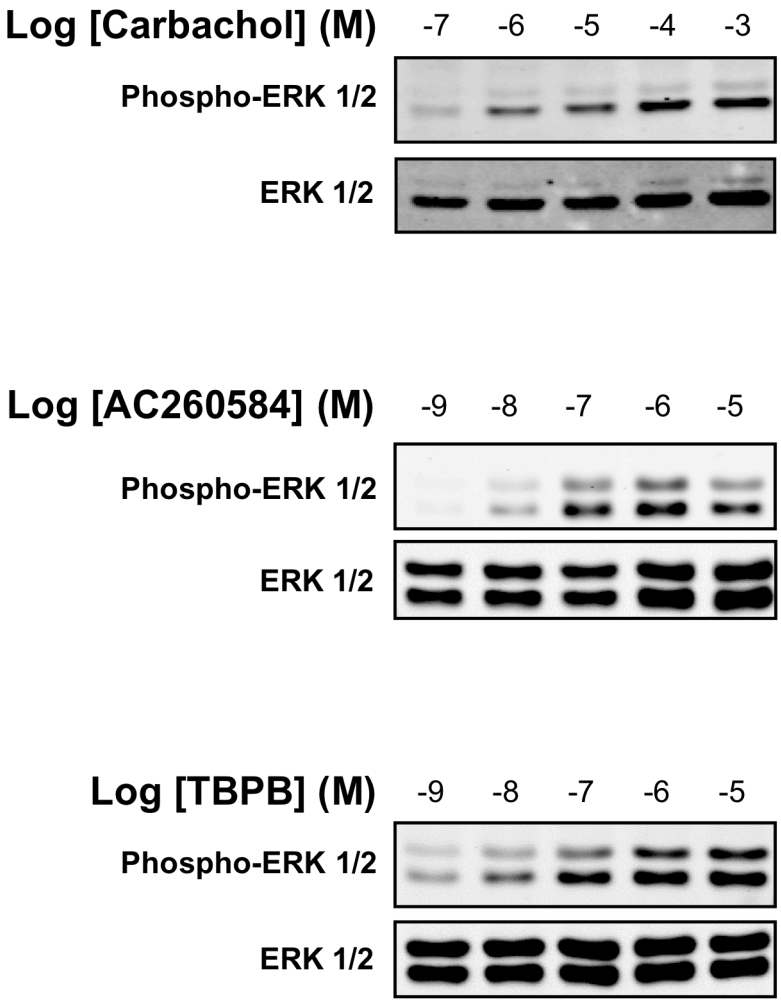


Figure 7.2. Phosphorylation of ERK 1/2 regulated by orthosteric and allosteric M1 agonists.

In HEK293T cells overexpressiong the human M1 mAChR, both orthosteric (CCh) and allosteric (AC260584, TBPB) agonists promote concentration-dependent increases in the phosphorylation of the mitogen activated protein kinase ERK 1/2. For each panel, detection of phospho-specific ERK 1/2 bands are shown above, and bands corresponding to total (non-phospho-specific) ERK 1/2 are shown below.

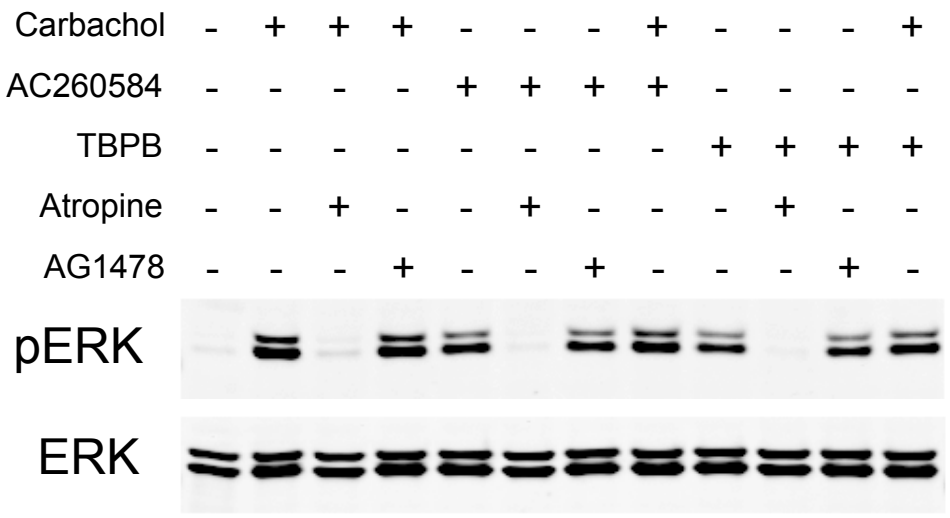


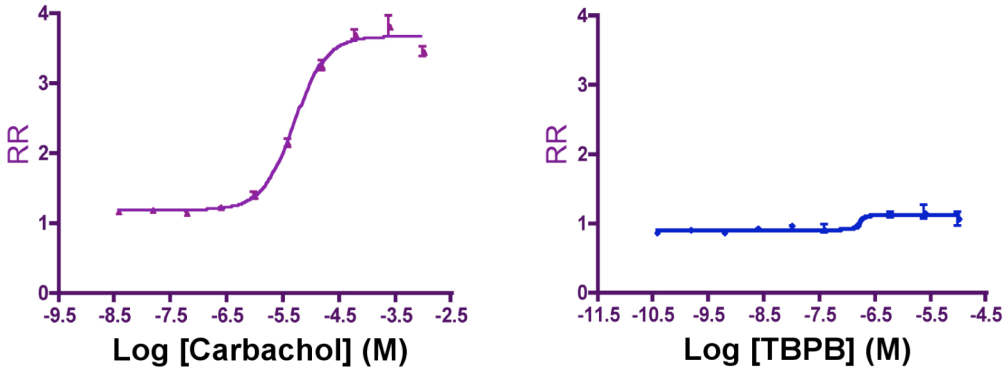
Figure 7.3. Specificity of ERK 1/2 signaling by orthosteric and allosteric M1 agonists.

In HEK293T cells expressing the human M1 receptor, all three agonists tested induced ERK 1/2 phosphorylation that was completely blocked by atropine. Activation was not affected by the tyrosine kinase inhibitor AG1478, indicating that M1-regulated ERK 1/2 phosphorylation is not dependent on EGFR activation in this cell type.

TBPB does not Cause Arrestin-3 Recruitment

Because GPCR signaling is commonly regulated by recruitment of members of the arrestin family (Moore, Milano et al. 2007), we investigated whether orthosteric and allosteric M1 agonists differed in their ability to recruit arrestin. Of the four mammalian arrestin subtypes (for a review, see (Gurevich and Gurevich 2006)), arrestin 1 and arrestin 4 are restricted to the visual system; thus, we focused our attention on arrestin 2 (also called β-arrestin 1) and arrestin 3 (also called β-arrestin 2). In a recombinant cell-based assay in which physical association of recruited arrestin with activated receptors drives transcription of an enzyme that cleaves two fluorophores and disrupts fluorescence resonance energy transfer (FRET) between them, CCh induced a robust concentration-dependent recruitment of Arrestin-3 (Arr3), but TBPB was nearly ineffective at recruiting Arr3 (Figure 7.4, A). When M1 and GFP-tagged Arr3 were co-expressed in CHO-K1 cells, CCh produced a striking translocation of Arr3 from its cytoplasmic reservoir to discrete puncta within five minutes. In contrast, Arr3 recruitment induced by both AC260584 and TBPB was severely blunted, with no significant change in depletion of cytoplasmic Arr3 and only occasional formation of puncta (Figure 7.4, B). These data suggest that distinct conformations stabilized by agonists binding to separate regions on the M1 receptor can promote multiple signaling mechanisms. Because arrestins have been shown to mediate specific signaling cascades independent of G-proteins (Luttrell, Ferguson et al. 1999; McDonald, Chow et al. 2000), this finding may have additional implications for the potential diversity of signaling regulated by allosteric mAChR agonists.

A


RR
4
3
2
1
0
-9.5 -8.5 -7.5 -6.5 -5.5 -4.5 -3.5 -2.5
Log [Carbachol] (M)
RR
4
3
2
1
0
-11.5 -10.5 -9.5 -8.5 -7.5 -6.5 -5.5 -4.5
Log [TBPB] (M)

B

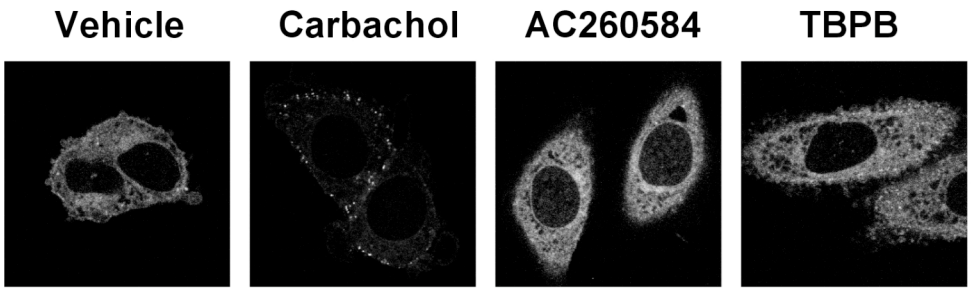

Vehicle
Carbachol
AC260584
TBPB

Figure 7.4. Allosteric agonists fail to induce recruitment of Arr3.

(A) Concentration-response curve showing carbachol-induced recruitment of Arr3 in a recombinant cell-based reporter assay in which Arr3 recruitment disrupts fluorescence resonance energy transfer (FRET). TBPB shows only minimal recruitment of Arr3. Data are shown as a reponse ratio (RR) of the FRET fluorophores where a higher ratio indicates increased Arr3 recruitment. **(B)** GFP-tagged Arr3 is localized diffusely throughout the cytoplasm at baseline,

M1 Receptors Exposed to Allosteric Agonists Remain on the Cell Surface

As arrestin recruitment is tightly linked with receptor endocytosis (Zhang, Ferguson et al. 1997),we next asked whether stimulation with allosteric agonists causes measurable internalization of M1 from the cell surface. In the case of orthosteric agonists (e.g. CCh), initial exposure (minutes to hours) causes internalization from the plasma membrane and traffic to endosomal compartments, from which receptors can either be recycled to the cell surface or targeted to lysosomes for degradation. In CHO-K1 cells expressing human M1, 60 minutes of CCh treatment induced only minimal internalization of M1 as measured by radioligand binding with membrane-impermeant [^{3}H]-NMS. Co-expression of Arr3 with M1 significantly accentuated this CCh-mediated internalization, with a ~25% reduction in [^{3}H]-NMS binding following 60 minutes CCh stimulation. However, the same duration of exposure to AC260584 or TBPB failed to cause significant internalization of M1 (Figure 7.5). In order to directly visualize agonist effects on M1 internalization, we performed double label immunocytochemistry and confocal microscopy to colocalize M1 with Na^+/K^+ ATPase, a marker of the cell surface. In HEK293 cells expressing human M1, 60 minute CCh treatment caused a ~60% decrease in colocalization between M1 and Na^+/K^+ ATPase. Treatment with AC260584 and TBPB appeared to result in a shift towards internalization, but neither allosteric agonist caused a statistically significant loss of M1 colocalization with Na^+/K^+ ATPase (Figure 7.6). Together, these results demonstrate that M1 activation by allosteric agonists produces significantly less internalization of M1 than that induced by the orthosteric agonist CCh.

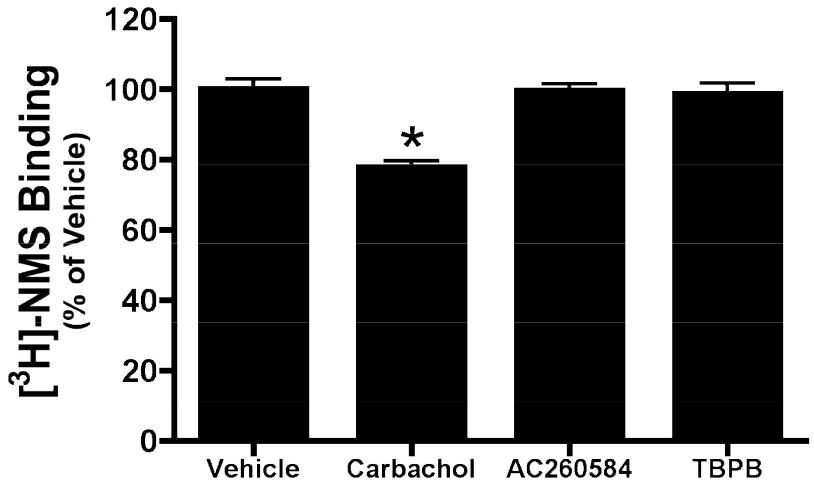
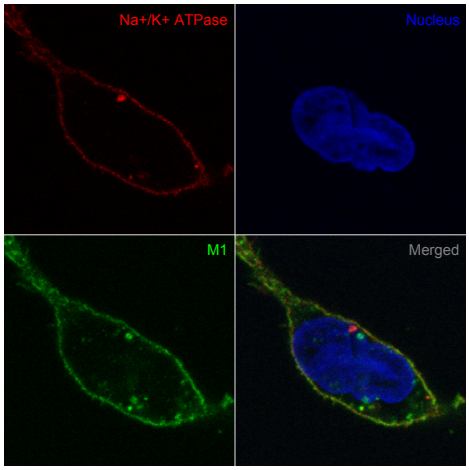


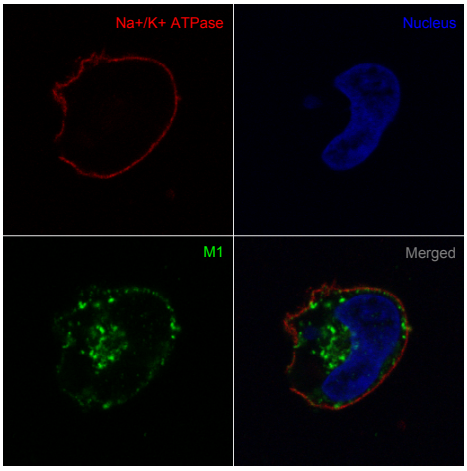
Figure 7.5. Measurement of agonist-induced M1 internalization by radioligand binding.

CHO-K1 cells expressing M1 and Arrestin-3 were treated for 60 minutes with the indicated drug, and surface M1 receptors were quantified by membrane-impermeant [^{3}H]-NMS radioligand binding. Specific binding (determined by substracting background from atropine pre-treated samples) is expressed as a percentage of binding in vehicle treated cells from three independent experiments. Binding is significantly reduced in carbachol treated cells as compared to vehicle (paired t-test, $p<0.0001$).

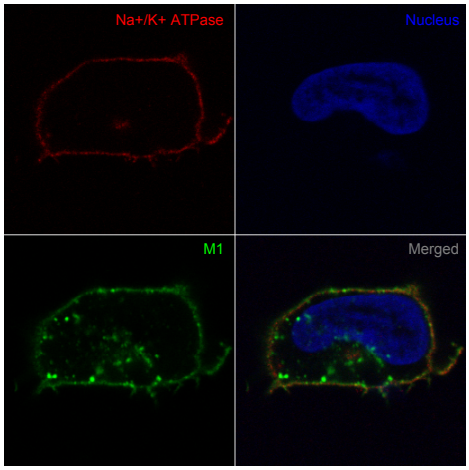
Vehicle



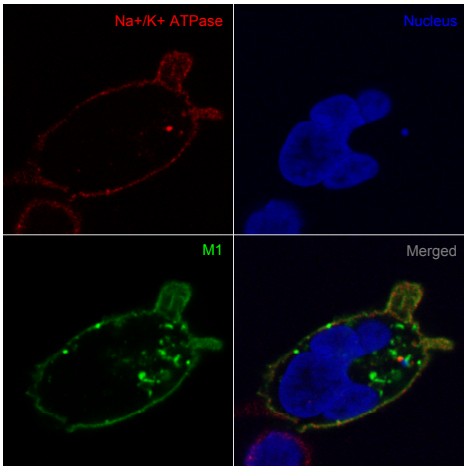
Carbachol



AC260584



TBPB



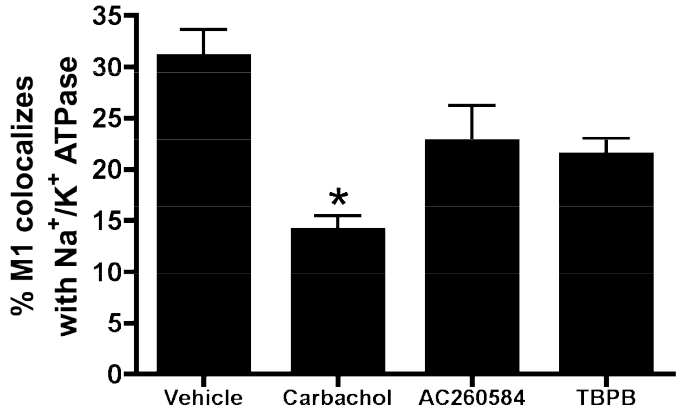


Figure 7.6. Measurement of agonist-induced M1 internalization by immunocytochemistry and confocal microscopy.

HEK293 cells expressing human M1 were treated with the indicated drugs for 60 minutes, and cells were double-labeled by immunocytochemistry for M1 and Na^+/K^+ ATPase, a marker of the cell surface. Colocalization of M1 and Na^+/K^+ ATPase was determined from confocal microscopy images and is expressed as the percentage of specific M1 pixels that colocalize with Na^+/K^+ ATPase pixels. Data represent three independent experiments, with 10 cells imaged per experiment for each drug condition. One-way ANOVA is significant ($p=0.0083$), and Tukey's multiple comparison post-test demonstrates a significant difference in colocalization between vehicle and carbachol-treated cells ($p<0.01$).

TBPB does not Induce M1 Degradation

Prolonged agonist exposure typically induces lysosomal degradation of GPCRs within hours to days (Tsao, Cao et al. 2001). Given the markedly blunted arrestin recruitment and receptor internalization observed following stimulation with allosteric M1 agonists, one would predict that these compounds would not induce appreciable receptor degradation either. In order to assess whether TBPB induces M1 receptor degradation, CHO-K1 cells expressing M1 and Arr3 were exposed to CCh or TBPB for 24 hours, and total-cell receptors were measured using the lipophilic muscarinic ligand [^{3}H]-QNB. As shown in Figure 7.7, 24-hr exposure to CCh resulted in the loss of ~25% of M1 binding sites, but neither AC260584 nor TBPB caused significant loss of M1 receptors.

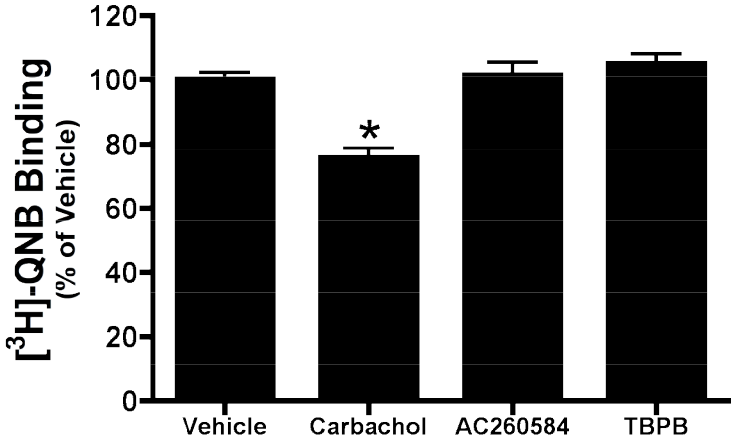


Figure 7.7. Allosteric agonists do not induce M1 downregulation.

CHO-K1 cells expressing M1 and Arrestin-3 were treated for 24 hours with the indicated drugs, and total-cell M1 was measured using the lipophilic radioligand [^{3}H]-QNB. Specific binding (determined by subtracting background from atropine controls) is shown as a percent of vehicle-treated cells from five independent experiments. Binding is significantly reduced in carbachol-treated cells as compared to vehicle (paired t-test, $p<0.0001$).

M1 Receptors Exposed to Allosteric Agonists Remain Functionally Sensitive

Although the lack of arrestin recruitment and receptor internalization predict that M1 receptors may not undergo functional desensitization following activation by allosteric agonists, we directly tested the effects of AC260584 and TBPB exposure on the ability of M1 to respond to subsequent agonist stimulation. Pre-treatment of M1-HEK cells with CCh strongly attenuated the ERK 1/2 phosphorylation response to a subsequent CCh challenge, but cells pre-treated with AC260584 and TBPB did respond to subsequent CCh stimulation, indicating that M1 remains sensitive to stimulation following exposure to allosteric agonists (Figure 7.8).

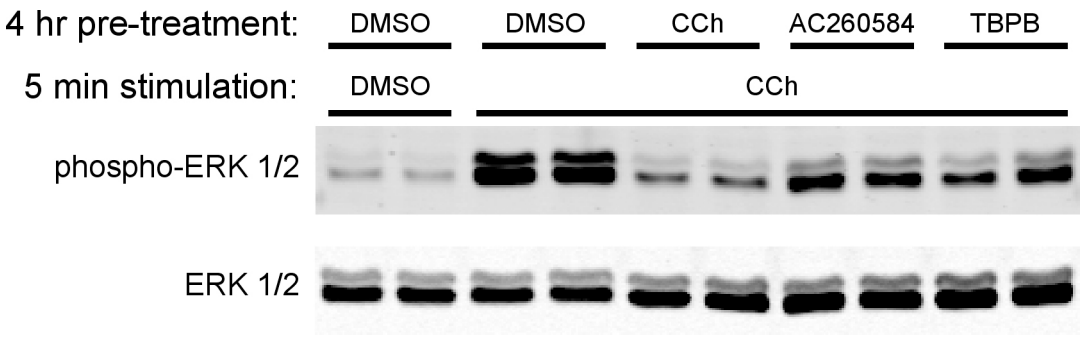


Figure 7.8. M1 receptors exposed to allosteric agonists remain sensitive to carbachol-stimulation.

In HEK293T cells expressing M1, pre-treatment (4 hours) with CCh virtually abolishes a secondary ERK 1/2 phosphorylation response to a 5-minute CCh stimulation. In contrast, cells pre-treated with AC260584 and TBPB show a measurable, though slightly blunted, response to carbachol stimulation.

Discussion

As the understanding of GPCR signaling and regulation has been refined, much attention has focused on the role of arrestins. While originally characterized as proteins that mediate receptor desensitization and endocytosis, it is now known that arrestins can directly regulate signaling events independent of G-proteins (Luttrell, Ferguson et al. 1999; McDonald, Chow et al. 2000) and participate in several cell biological processes including chemotaxis (Fong, Premont et al. 2002), stress fiber formation (Barnes, Reiter et al. 2005), and protein synthesis (DeWire, Kim et al. 2008). Further investigation has shown that specific agonists for the β_2-adrenergic receptor display efficacy for arrestin-based signaling that is disproportionately higher than their efficacy for G-protein-based signaling would have predicted, leading to the coining of the term "biased agonism" to describe selective or preferential activation of arrestin-mediated signaling (Drake, Violin et al. 2008). In this study, we present data demonstrating that the allosteric M1 agonists AC260584 and TBPB are unable to effectively recruit arrestin, yet potently stimulate Ca^{2+} release and ERK 1/2 activation. This allosteric agonist-induced signaling presumably utilizes classical G-protein mechanisms, indicating that GPCR agonists can be "G-protein biased" as well as "arrestin biased." This possibility should open new avenues of research into the signaling pathways and clinical utility of allosteric agonists for cholinergic pharmacotherapy.

Previous reports have established a tight correlation between the intrinsic activity of a GPCR agonist and its efficacy for promoting receptor endocytosis (Kallal, Gagnon et al. 1998; Szekeres, Koenig et al. 1998), providing support for the model that GPCR

activation is directly linked to regulatory mechanisms that attenuate signaling and lead to receptor sequestration and down-regulation. While the majority of agonists display this pattern, it has been shown that certain GPCR agonists activate receptors without promoting receptor desensitization or endocytosis (Whistler and von Zastrow 1998). Here we demonstrate that two structurally distinct allosteric agonists activate M1 without inducing receptor endocytosis and downregulation.

In previous studies by our laboratory on carbachol-induced M4 muscarinic receptor traffic, a tight correlation was found between radioligand and immunocytochemical measurements of receptor endocytosis (Volpicelli, Lah et al. 2001). In the present study, we observed carbachol stimulation to produce a greater loss of colocalization between M1 and Na^+/K^+ ATPase than would be predicted based upon [^{3}H]-NMS binding. There are several potential interpretations for this apparent discrepancy. There appears to be a greater amount of intracellular staining for the M1 receptor as compared to M4, which could affect measurements of internalization as determined by the percentage of M1 that colocalizes with Na^+/K^+ ATPase. There could also be differences in endocytic machinery between the CHO-K1 cells used in the [^{3}H]-NMS binding assays and the HEK293 cells used for immunocytochemistry that might impact the efficiency of internalization. Finally, there are differences in ligand vs. antibody recognition of mAChRs, which could have an effect on what is measured to be "M1" in each assay. Despite these differences, though, the central point remains that the two allosteric M1 agonists examined in this study cause significantly less receptor endocytosis than an orthosteric agonist.

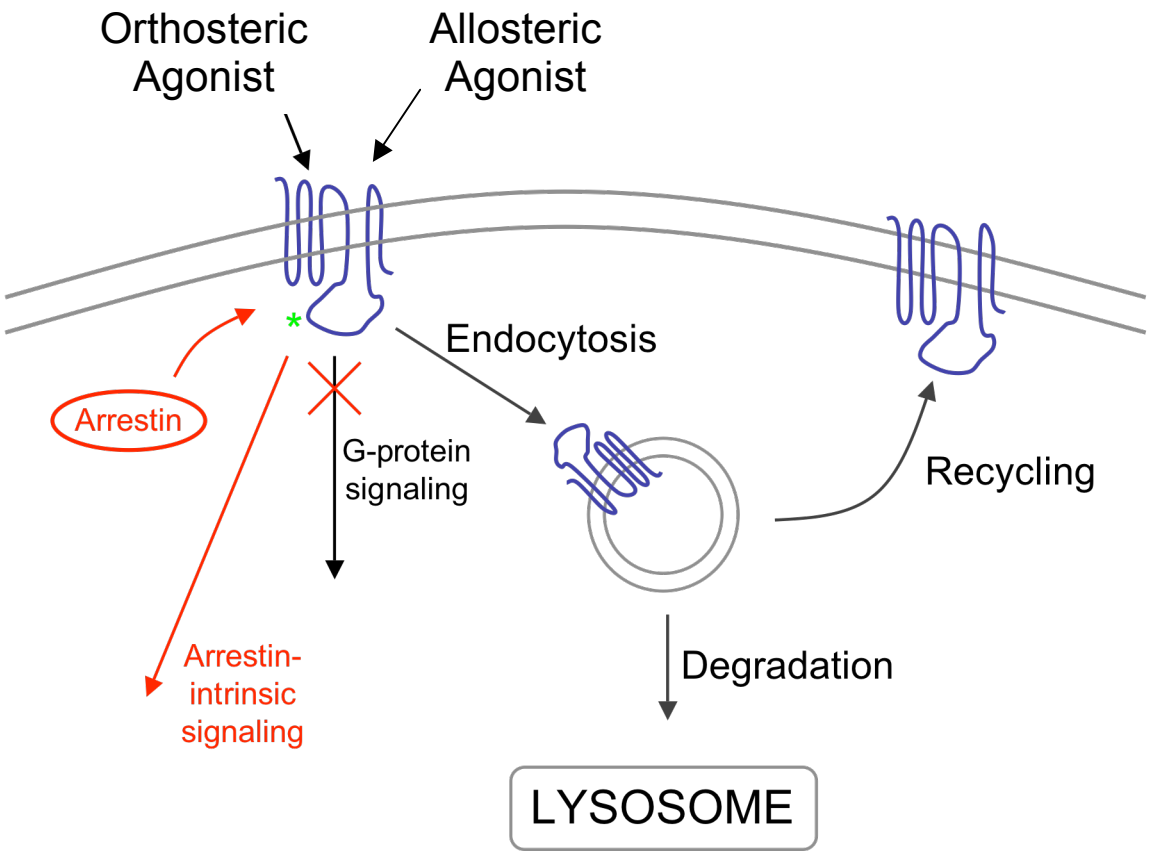


Figure 7.9. Diversity and regulation of GPCR signaling.

Agonist stimulation of GPCRs can result in receptor phosphorylation (green asterisk), which serves as a scaffold for the recruitment of arrestin proteins. Arrestins can terminate signaling through G-protein pathways associated with the receptor, and activate distinct pathways independent of G-proteins. Arrestin recruitment is linked to receptor endocytosis, after which receptors can be recycled or targeted to lysosomal compartments for degradation. Both G-protein and arrestin pathways are activated by orthosteric agonists, while the allosteric M1 agonists AC260584 and TBPB display a bias towards G-protein signaling.

The finding that these allosteric agonists elicit only a subset of the responses produced by orthosteric agonists may indicate that allosteric agonist binding puts the M1 receptor in a conformation in which it interacts with certain intracellular signaling and/or scaffolding proteins but not others. In two recent papers, Li and colleagues demonstrated that different classes of agonists induce distinct structural changes in the M3 mAChR subtype (Li, Han et al. 2007; Li, Hamdan et al. 2008), providing evidence for a molecular basis by which distinct agonists acting on a single receptor can differentially regulate signaling pathways. It is possible that in addition to activating signaling cascades shared by orthosteric agonists, allosteric agonists could also regulate additional pathways. Privileged signaling regulated by allosteric agonists is beginning to be explored for a variety of GPCRs including metabotropic glutamate receptors (Sheffler and Conn 2008), and will likely develop into an intriguing and clinically useful aspect of GPCR signaling.

There are important pharmacological and cell biological implications of AC260584 and TBPB's failure to recruit arrestin and induce M1 endocytosis. Agonist-induced receptor endocytosis and lysosomal degradation would appear to limit efficacy over extended periods of time, making allosteric agonists that do not induce these compensatory changes attractive targets for chronic therapeutic applications. Indeed, studies in acetylcholinesterase knockout mice have revealed that the loss of this enzyme, which regulates attenuation of signaling at cholinergic synapses, results in significant down-regulation of mAChRs, aberrant receptor traffic, and blunted response to agonist stimulation (Volpicelli-Daley, Duysen et al. 2003; Volpicelli-Daley, Hrabovska et al. 2003). These perturbations in the cholinergic system serve as a model for the alterations

that likely occur following chronic administration of cholinesterase inhibitors, the predominant therapy for Alzheimer's disease (AD), and may account for the limited clinical efficacy of these drugs (Raina, Santaguida et al. 2008). It is worth noting, however, that there is evidence supporting a role for arrestin-mediated endocytosis in maintaining the ability of a GPCR to respond to repeated agonist stimulation. Whistler et al. showed that morphine, an agonist at the μ-opioid receptor, fails to promote arrestin recruitment and receptor internalization, in contrast to the μ-opioid receptor agonist etorphine (Whistler and von Zastrow 1998; Whistler, Chuang et al. 1999). Interestingly, morphine causes more physiological tolerance and dependence than etorphine, and the authors hypothesize that persistent receptor activation in the absence of desensitization, endocytosis, and recycling triggers alternative mechanisms of compensation that lead to tolerance. The effects of chronic *in vivo* administration of allosteric M1 agonists need to be investigated directly in order to determine whether they induces functional changes *in vivo* following repeated administration.

Subtype-selective allosteric agonists represent a tremendous advance in cholinergic pharmacology, and will likely have a major impact on cholinergic-based therapies for neurological and neuropsychiatric disorders. The findings of this study complement a growing body of literature indicating that GPCR signaling is remarkably diverse and that structurally distinct agonists differ with respect to the profiles of responses they elicit. Ongoing investigation in this exciting field should continue to enhance both the understanding of basic receptor biology and the utility of clinical pharmacotherapy.

Chapter VIII. SUMMARY AND FUTURE DIRECTIONS

A. M1 mAChR regulation of APP processing and amyloidogenesis

In this dissertation I have characterized M1 mAChR regulation of APP processing and amyloidogenesis *in vitro* and *in vivo*. The relationship between AD pathogenesis and cholinergic dysfunction is one of the longest-standing associations in the field, but despite this history, and the widespread use of cholinomimetic drugs as therapies for AD, comprehension of the precise molecular underpinnings of this relationship has lagged. Multiple lines of evidence point toward a pivotal role for the M1 receptor in regulating amyloidogenesis, but the high degree of homology among mAChR family members has made it difficult to test this hypothesis directly. In order to isolate the M1 mAChR subtype, I used primary neurons cultured from wildtype and M1 mAChR knockout mice. Cholinergic stimulation increased secretion of the non-amyloidogenic APP metabolite APPsα in wildtype neurons, but this regulated secretion was lost in neurons lacking M1. Measurement of Aβ peptide generation in wildtype and M1KO neuron cultures indicated that M1 activation reduces Aβ production while activation of other mAChR subtypes increases its production. Experiments in transgenic mice confirmed that the loss of M1 increases amyloid pathology *in vivo*. Complementary studies using an M1-selective agonist and an M1-selective potentiator demonstrated that pharmacologic activation of M1 is capable of promoting non-amyloidogenic APP processing, a finding that has important implications for cholinergic-based therapies for AD. Together, these data highlight the M1 mAChR as an important regulator of non-amyloidogenic APP processing in neurons and indicate that M1-based therapeutics may be effective in reducing amyloid pathology in AD. This relationship is depicted in Figure 8.1.

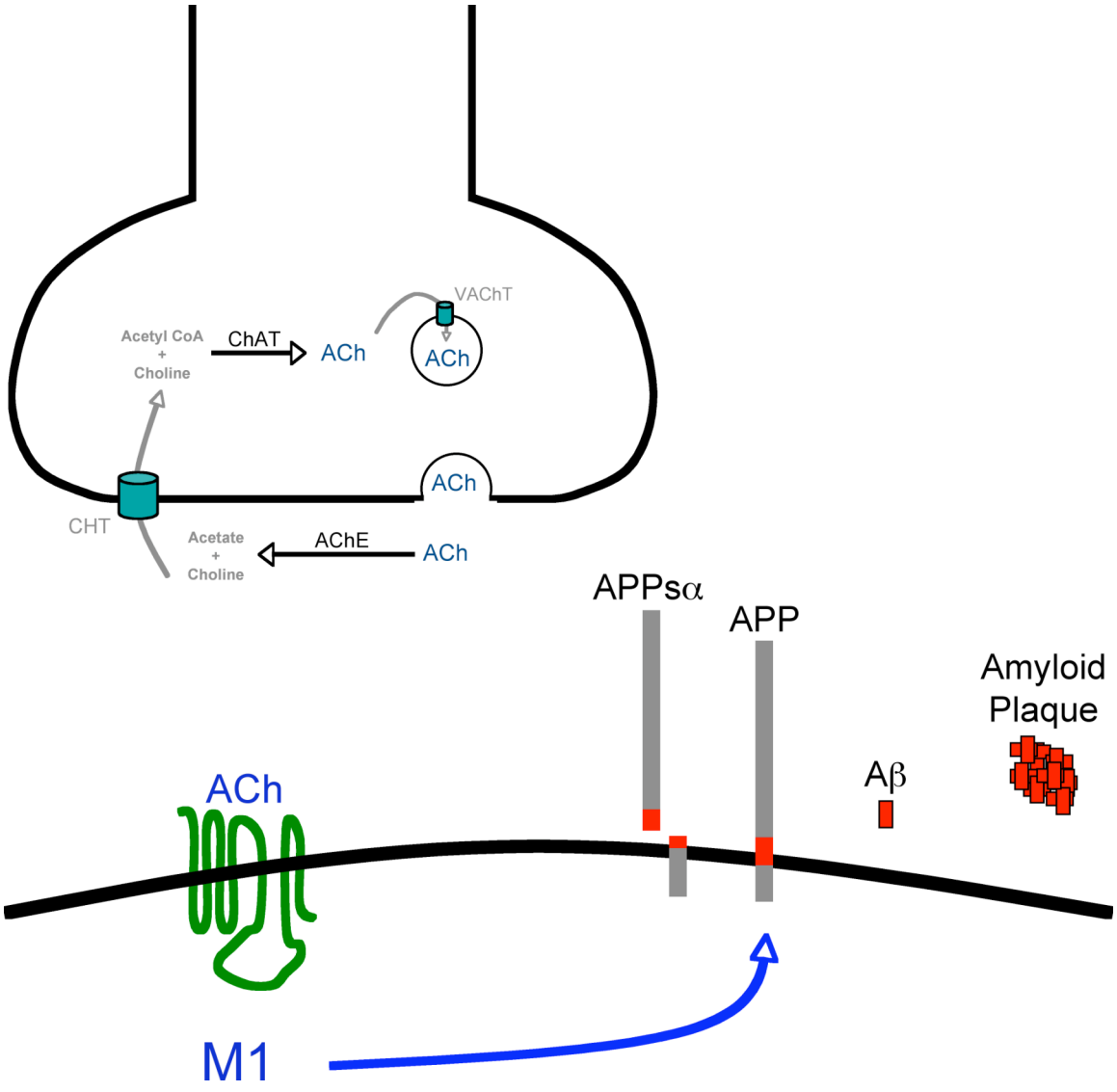


Figure 8.1. M1 mAChR signaling regulates non-amyloidogenic APP processing in neurons. Activation of post-synaptic M1 receptors initiates signal transduction mechanisms that stimulate non-amyloidogenic APP processing, resulting in increased shedding of APPsα and decreased production of Aβ peptides. In vivo, this signaling limits amyloid plaque formation, as evidenced by the increased amyloid plaque pathology observed in *M1*$^{-/-}$ mice.

The use of mice lacking specific genes has proved to be a tremendous advance in biological research. In recent years, gene targeting approaches have been successful in creating mice deficient in one or more mAChR subtypes. These valuable reagents permit the study of mAChR function *in vivo*, and have been instrumental in defining the roles of individual receptor subtypes in a wide range of physiological processes (Wess 2003; Wess, Duttaroy et al. 2003; Wess 2004). It is worth noting that deletion of a single mAChR subtype does not appear to result in compensatory overexpression of other members of the receptor family, a potential concern when using this type of technology (Hamilton, Loose et al. 1997; Gainetdinov, Bohn et al. 1999; Gomeza, Shannon et al. 1999; Gomeza, Zhang et al. 1999; Yamada, Miyakawa et al. 2001).

In this dissertation, I describe for the first time the use of mAChR knockout mice to address cholinergic regulation of APP processing in neurons. The use of primary neuron cultures is of vital importance, since the cell biological processes that control APP processing are likely variable from cell type to cell type. Because AD is a disease that is intrinsic to the nervous system, it follows that studies in neurons will be the most relevant to the underlying physiology. In a series of experiments in primary neuron cultures from mouse cortex, I established that cholinergic signaling can promote non-amyloidogenic APP processing, a pathway that directly competes with the amyloidogenic pathway responsible for producing the Aβ peptide that forms the core of amyloid plaques in AD. This cholinergic regulation is lost in neurons derived from mice lacking the M1 receptor, providing the first evidence that the genetically defined M1 receptor is critical for regulating non-amyloidogenic processing in neurons. Furthermore, I demonstrated that

"rescue" of M1 knockout neuron cultures by overexpression of the genetically-defined M1 receptor is sufficient to restore the deficits resulting from the loss of M1. Together, these data indicate that M1 is critical for the cholinergic regulation of APP processing in neurons.

In order to extend the examination of M1 regulation of APP processing from cultured neurons to an *in vivo* model, I crossed $APP_{Swe/Ind}$ transgenic mice with M1 knockout mice. In this model of amyloidogensis, M1 deficiency increased levels of pathogenic Aβ peptides and exacerbated the accumulation of amyloid plaque pathology in cerebral cortex and hippocampus, brain regions vulnerable to AD pathology. This experiment confirms the importance of the M1 mAChR as an important regulator of amyloidogenesis in the brain and is a compelling factor for M1-focused therapeutics.

One factor that has hindered the pace of research in cholinergic systems has been the absence, until recently, of highly subtype-selective mAChR ligands. We have taken advantage of recent advances in cholinergic drug discovery by using a highly selective M1 agonist to show that M1 activation regulates non-amyloidogenic APP processing in the neuronotypic PC12 cell line. These experiments establish that pharmacologic activation of M1 is effective in modulating APP processing pathways that would be beneficial in the context of AD, indicating that M1-selective agonists may be useful in the treatment of AD. It should be noted that regulation of amyloidogenesis is only one potential mechanism by which M1 agonists could exert a beneficial effect in AD. M1 is fundamentally involved in cognition, learning, and memory, and it is reasonable to

assume that direct activation of M1 may have a positive impact on the cognitive symptoms of AD independent of its effect on amyloidogenesis. This hypothesis was not tested in this thesis, but the prospect remains intriguing and warrants further exploration in animal models and human patients.

In addition to the M1 agonist studies, we demonstrated that an M1-selective positive allosteric modulator (PAM) is capable of regulating non-amyloidogenic APP processing. PAMs work by potentiating the response of a receptor to a sub-maximal concentration of a full agonist. Highly selective PAMs such as the one used in this study can increase the signaling through one receptor subtype at concentrations of the full agonist that are unlikely to activate other subtypes. In experiments in PC12 cells, we demonstrate that an M1-selective PAM is capable of significantly increasing the shedding of APPsα when applied in conjunction with a low concentration of the agonist carbachol. This finding is particularly exciting because it suggests that it may be possible to modulate existing neurotransmitter circuits in a more physiologically relevant manner than could be achieved with exogenous administration of a full agonist. The discovery and characterization of novel selective agonists, antagonists, and modulators of mAChR subtypes should accelerate progress in basic research, and will hopefully translate into more effective therapies for AD as well as other disorders of the CNS (Conn, Christopoulos et al. 2009; Conn, Jones et al. 2009).

One major question concerning the cholinergic dysfunction observed in AD centers around causation. That is, does faulty cholinergic signaling trigger

amyloidogenesis that subsequently leads to AD, or does accumulation of pathogenic Aβ and other toxic molecules precede, and potentially cause, the damage to the cholinergic system? This question is not a simple one to approach, and the answer is not absolutely clear. For example, profound impairment in cholinergic enzyme activity and frank loss of cholinergic cells in the basal forebrain are not typically observed until later stages of AD (Gilmor, Erickson et al. 1999), but early phenotypic changes in cholinergic neurons can be observed in earlier stages, including mild cognitive impairment (Mufson, Ma et al. 2002; Herholz 2008). Studies in sporadic and familial cases of AD suggest that cholinergic dysfunction is present in both forms of the disease, a finding that argues for amyloid pathology being fundamentally capable of affecting the cholinergic system and therefore representing the primary alteration (Rasool, Svendsen et al. 1986). Experiments in transgenic animals have also shed light on this question. In several mouse models that incorporate mutations in APP that cause familial AD, derangements in cholinergic anatomy and physiology have been observed, including dystrophic fibers, signs of denervation, reduced transport of neurotrophic molecules such as NGF, and some loss of cholinergic neurons in the basal forebrain and other brain regions (Apelt, Kumar et al. 2002; Aucoin, Jiang et al. 2005; Bellucci, Luccarini et al. 2006; Christensen, Bayer et al. 2008). Most notably, cholinergic deficits in a line of mutant APP transgenic mice were alleviated by crossing this mouse with a *BACE1*$^{-/-}$ mouse, indicating that Aβ overproduction causes dysfunction in cholinergic systems (Ohno, Sametsky et al. 2004). Despite the evidence arguing for a causal role of neurotoxic Aβ driving changes in cholinergic neurons, there are clear data showing that experimental lesions to the cholinergic system can directly lead to increased amyloid pathology, and correlative

studies suggest that medications with anticholinergic effects can exacerbate and accelerate amyloid pathology in humans (Beach, Potter et al. 2000; Perry, Kilford et al. 2003; Liskowsky and Schliebs 2006). Furthermore, treatment with cholinergic agonists decreases amyloid pathology in several model systems, including in human patients, as previously mentioned. In light of these data, it is perhaps most appropriate to view the interplay between cholinergic dysfunction and amyloid pathology not as a linear cause-effect relationship, but rather as a vicious cycle in which disease-causing accumulation of toxic Aβ damages the cholinergic system (as well as other neurotransmitter systems), resulting in impaired cholinergic signaling that can further exacerbate pathology and accelerate the disease process. If this hypothesis is correct, it underscores the urgent need for improvement in early detection techniques for AD, so that patients at highest risk for developing the disease can be treated as early as possible once effective therapies, including those that might target the cholinergic system, become available.

The finding that genetic and pharmacologic modulation of M1 signaling has a pronounced effect on APPsα secretion has interesting implications for its mechanism of action. APP is known to be cleaved at the α-secretase site by members of the ADAM family of metalloproteases, and numerous pieces of data using a variety of approaches have highlighted the involvement of ADAM proteases in the regulation of APP processing by signaling pathways that M1 can activate (Hung, Haass et al. 1993; Slack, Nitsch et al. 1993; Xu, Greengard et al. 1995; Xu, Sweeney et al. 1996; Mills, Laurent Charest et al. 1997; Haring, Fisher et al. 1998; Lammich, Kojro et al. 1999; Skovronsky, Moore et al. 2000). As previously mentioned, APPsα has been proposed to have multiple

beneficial effects on its own, and it remains an intriguing possibility that M1-mediated APPsα shedding could have a mechanistic role in AD pathogenesis independent of a potential role in regulating amyloid pathology.

The possibilities for physiologically-relevant M1-regulated ectodomain shedding may not be limited to the amyloid precursor protein. In this thesis, we present evidence of metalloprotease-dependent, M1-regulated shedding of the LR11 ectodomain. LR11 has already been established as an important molecule in AD pathophysiology, but much of the focus of LR11 research has centered on its involvement in intracellular traffic of APP and influence on APP processing (Andersen, Reiche et al. 2005; Andersen, Schmidt et al. 2006; Offe, Dodson et al. 2006; Spoelgen, von Arnim et al. 2006; Schmidt, Sporbert et al. 2007). LR11 contains several functional motifs in its amino-terminal ectodomain, including EGF homology domains. Hence, while it is speculation at this point, it is interesting to imagine the LR11 ectodomain as a trans-activating factor for molecules like the EGF receptor. The EGFR, its related family members, and their various ligands are involved in a wide variety of biological processes including cell survival, development, and signaling, including several prominent functions in the developing and adult CNS, and it is possible that LR11 may functionally interact with this system in the brain in the context of normal biology and/or disease (Gschwind, Zwick et al. 2001; Falls 2003; Ohtsu, Dempsey et al. 2006).

B. Specificity of signaling mechanisms initiated by allosteric M1 agonists

We have also characterized an unexpected divergence in the signal transduction pathways activated by orthosteric vs. allosteric agonists for the M1 receptor. In a series of experiments in cultured cells expressing the M1 receptor, we demonstrated that two structurally distinct allosteric M1 agonists are capable of activating signal transduction pathways linked to M1 but are much less effective at inducing a series of regulatory steps including arrestin recruitment, receptor endocytosis, and down-regulation of receptors following chronic treatment. This finding underscores the complexity of receptor-ligand binding and emphasizes the diversity of intracellular signaling mediated by discrete interactions between receptors and specific binding partners within the cell. This diversity of signaling properties can have profound consequences *in vivo*, and better understanding of the fundamental biology may provide a foundation for exploiting specific ligands to achieve desirable clinical outcomes.

CNS modulation of cholinergic systems represents an attractive target for not only AD, but for several other neurodegenerative and neuropsychiatric disorders. For this reason, it is important to understand the cell biological consequences of pharmacologically manipulating this system. In this dissertation, I describe a divergence in the signaling pathways activated by orthosteric vs. allosteric agonists for the M1 mAChR. I demonstrate that two structurally distinct allosteric agonists do not effectively recruit Arr3 following M1 activation, and do not trigger receptor desensitization, endocytosis, or down-regulation. Similar studies have demonstrated that agonists for the μ-opioid receptor that fail to induce arrestin recruitment and endocytosis are associated with increased tolerance and addiction (Martini and Whistler 2007). The implications of

reduced arrestin recruitment and endocytosis on physiological homeostasis and *in vivo* effects in cholinergic systems are unknown, but it will be important to keep this phenomenon in mind as new generations of cholinergic agonists are developed.

In summary, this thesis presents the first evidence that the genetically defined M1 mAChR plays a critical role in regulating APP processing and amyloidogenesis *in vitro* and *in vivo*, and demonstrates that highly selective M1 agonists and potentiators are valuable tools for modulating amyloidogenesis. Furthermore, this work demonstrates that allosteric M1 agonists activate a precise subset of signaling pathways. This discovery has basic implications for the biochemistry and cell biology of mAChR signaling and traffic, and may have an impact on the utility of mAChR-based therapeutics in clinical settings as well.

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