α-Melanocyte Stimulating Hormone Prevents GABAergic Loss and Improves Cognitive Function in Alzheimer’s Disease

by

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In Alzheimer’s disease (AD), the appropriate excitatory/inhibitory balance required for memory formation is impaired. In order to elucidate deficits in the inhibitory $\gamma$-aminobutyric acid (GABA)-ergic system in AD and to establish a link between GABAergic dysfunction and cognition, the TgCRND8 mouse model of AD was utilized. TgCRND8 mice with established amyloid beta peptide (A\textbeta) pathology exhibit spatial memory deficits and altered anxiety. Concomitant with behavioural changes, GABAergic deficits are observed in the hippocampus. The GABAergic marker glutamic acid decarboxylase 67 (GAD67) mRNA and protein levels in the hippocampus as well as the number of GAD67+ GABAergic cells in the CA1 region of the hippocampus are significantly decreased. However, at this stage of A\textbeta pathology, the number of ChAT+ cholinergic cells in the septum remains unchanged, suggesting that cholinergic cells may not underlie behavioural deficits. The GABAergic loss may represent an early target in AD disease progression. By increasing inhibition, the physiological excitatory/inhibitory balance in the brain may be restored resulting in normal function. I found that the neuropeptide, $\alpha$-melanocyte stimulating hormone ($\alpha$-MSH), prevents the loss of GAD67 mRNA, protein levels and GAD67+ cells in the CA1 region of the hippocampus. In particular, $\alpha$-MSH protects the loss
of the somatostatin (SST) expressing subtype of GAD67+ inhibitory interneurons. SST is reduced in the CSF and brain of AD patients and SST levels are correlated with cognitive function. By preserving hippocampal GAD67+ cells, especially the SST+ subtype, α-MSH improved spatial memory in TgCRND8 mice and prevented changes in anxiety independent of altering Aβ peptide load in the brain. α-MSH modulated the excitatory/inhibitory balance in the brain by restoring GABAergic inhibition and as a result, improved cognition in TgCRND8 mice.
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List of Abbreviations

ACh  acetylcholine
AChE  acetylcholinesterase
ACTH  adrenocorticotrophin
AD  Alzheimer’s disease
AMPA  glutamatergic $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP  Amyloid precursor protein
APOE  apolipoprotein E
Arc  activity-regulated cytoskeleton-associated protein
Aβ  beta-amyloid peptide
BDNF  brain-derived neurotrophic factor
cAMP  cyclic adenosine monophosphate
CB  calbindin
CCK  cholecystokinin
ChAT  acetylcholine transferase
CLIP  corticotrophin-like intermediate peptide
CR  calretinin
CREB  cyclic adenosine monophosphate response element binding protein
Erk  extracellular signal-regulated kinase
GABA  inhibitory $\gamma$-aminobutyric acid
GAD67  glutamic acid decarboxylase 67
GAPDH  glyceraldehyde 3-phosphate dehydrogenase
IFN  interferon
IL  interleukin
iNOS  inducible nitric oxide synthase
IRAK  IL-1 receptor-associated kinase
LPS  lipopolysaccharide
LTD  long-term depression
LTP  long-term potentiation
MC3R  melanocortin receptor 3
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<th>Full Name</th>
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<tr>
<td>MC4R</td>
<td>melanocortin receptor 4</td>
</tr>
<tr>
<td>MCI</td>
<td>mild cognitive impairment</td>
</tr>
<tr>
<td>MSH</td>
<td>melanocyte stimulating hormone</td>
</tr>
<tr>
<td>NBM</td>
<td>nucleus basalis of magnocellularis</td>
</tr>
<tr>
<td>NFTs</td>
<td>neurofibrillary tangles</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NPY</td>
<td>neuropeptide Y</td>
</tr>
<tr>
<td>NT-3</td>
<td>neurotrphin-3</td>
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<tr>
<td>NT-4/5</td>
<td>neurotrphin-4/5</td>
</tr>
<tr>
<td>NTg</td>
<td>non-Tg littermates</td>
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<tr>
<td>pCREB</td>
<td>phosphorylated CREB</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>POMC</td>
<td>pro-opiomelanocortin</td>
</tr>
<tr>
<td>PS</td>
<td>presenilin</td>
</tr>
<tr>
<td>PV</td>
<td>parvalbumin</td>
</tr>
<tr>
<td>SST</td>
<td>somatostatin</td>
</tr>
<tr>
<td>TLR4</td>
<td>toll-like receptor 4</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Trk</td>
<td>tyrosine kinases</td>
</tr>
<tr>
<td>VIP</td>
<td>vasoactive intestinal polypeptide</td>
</tr>
<tr>
<td>YWAHZ</td>
<td>tyrosine 3-monooxygenase</td>
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Chapter 1
Introduction

1.1 Alzheimer’s Disease Prevalence and Economic Burden

Alzheimer’s disease (AD) is the most common form of dementia with symptoms of memory loss, confusion with time or space, changes in mood and personality and difficulty completing familiar tasks (Thies and Bleiler, 2013). In the US, AD is the 6th leading cause of death. Between 2000 to 2010, the proportion of deaths from heart diseases, stroke and prostate cancer have decreased by 8-23% in contrast to an increase of 68% from AD (Thies and Bleiler, 2013). This devastating disease also creates a societal burden costing an estimated $604 billion USD worldwide in 2010 (Wimo et al., 2013). In Canada, 747,000 Canadians were living with AD or related dementia in 2011 but by 2031, an estimated 1.4 million Canadian are expected to be suffering from AD (Alzheimer’s Society of Canada, 2013). As a result, the economic cost of dementia in Canada is estimated to rise from $33 billion in 2011 to $293 billion in 2040 (Alzheimer’s Society of Canada, 2013). The number of people suffering from AD is increasing worldwide and the cost of care for AD patients is also escalating, leading to an unprecedented economic burden.

1.2 Familial and Sporadic Alzheimer’s Disease

Since AD was first described by Alois Alzheimer in 1906, decades of scientific research have characterized brain pathology in AD patients. Pathologically, AD is characterized by the accumulation of beta-amyloid peptide (Aβ), neurofibrillary tangles of hyperphosphorylated tau (NFTs) as well as synaptic injury and neuronal degeneration possibly due to loss of neurotrophic support in the brain (Selkoe, 1991). These pathological changes result in the impairment of cognitive function in AD. However, the order of pathological events and the trigger for the development of AD still remains largely unknown in sporadic cases. Less than 1% of AD cases are familial and early onset, caused by genetic mutations in the amyloid precursor protein (APP), presenilin 1 (PS1) or presenilin 2 (PS2) genes (Blennow et al., 2006). These genetic mutations
increase the production of Aβ and promote Aβ aggregation within the brain (Hardy and Selkoe, 2002). The majority of AD cases are sporadic and late onset. The two main risk factors in sporadic AD are ageing and apolipoprotein E (APOE) ε4 allele (Blennow et al., 2006; Liu et al., 2013). APOE is a cholesterol carrier with three polymorphic alleles, ε2, ε3 and ε4 (Liu et al., 2013). The allelic frequently of ε3 of 77.9% is the highest in humans with the ε4 frequency being 13.7% (Farrer et al., 1997). However, in the AD population, the allelic frequency of ε4 is as high as 40% (Farrer et al., 1997). APOE ε4 allele is strongly associated with Aβ deposition in senile plaques and cerebral amyloid angiopathy (Zubenko et al., 1994; Liu et al., 2013). In both familial and sporadic AD, genetic mutations or risk factors cause an imbalance in the production and/or clearance of Aβ, which result in the accumulation and aggregation of Aβ in the brain (Hardy and Selkoe, 2002; Liu et al., 2013).

1.3 Beta-amyloid Pathology

The amyloid hypothesis proposes that the accumulation of Aβ is the primary driving force in AD pathogenesis and that neurofibrillary tangles containing hyperphosphorylated tau result from the imbalance of Aβ production and clearance (Hardy and Selkoe, 2002). APP is a membrane-spanning polypeptide that may undergo cleavage by β-secretase followed by γ-secretase to produce Aβ that are 40 (Aβ40) or 42 (Aβ42) amino acids in length (Sisodia and St George-Hyslop, 2002). Aβ aggregates in the brain in a nucleation-dependent process whereby Aβ42 act as seeds to form toxic ordered β-sheet structures (Jarrett et al., 1993; Simmons et al., 1994). Once the nucleus is formed, multiple sites are available for Aβ monomer attachment, which leads to further aggregation to form various sizes of oligomers, protofibrils, fibrils and Aβ plaques (Jarrett and Lansbury, 1993; Glabe, 2005; Figure 1.1). Many toxic intermediates are formed as Aβ aggregation occurs. From human AD brains and brains of transgenic mouse models of AD, many toxic Aβ conformations have been isolated (Gong et al., 2003; Lesné et al., 2006; Townsend et al., 2006; Kayed and Lasagna-Reeves, 2013). The expression of a 56kDa Aβ peptide, Aβ*56, is inversely correlated with spatial memory in Tg2576 mice overexpressing APPswc mutation (Lesné et al., 2006). Aβ-derived diffusible ligands are non-fibrillar small globular oligomers that disrupts long-term potentiation (LTP) and cause hippocampal neuronal
death (Klein et al., 2001). In addition, low molecular weight soluble Aβ oligomers have been shown to inhibit LTP, enhance long-term depression (LTD) and decrease dendritic spine density in the hippocampus (Townsend et al., 2006; Selkoe, 2008). Neuronal synaptic morphology can be altered by Aβ through effects on LTP and LTD as inhibition of LTP limits dendritic spine enlargement and enhanced LTD causes dendritic spine shrinkage (Matsuzaki et al., 2004; Zhou et al., 2004; Koffie et al., 2011). Synaptic loss is the greatest in the vicinity of Aβ plaques (Tsai et al., 2004). Furthermore, Aβ oligomers can bind to the cell surface and synapses to cause functional disruption of many receptors leading to altered calcium homeostasis, decreased LTP, disturbance in autophagy, mitochondrial dysfunction and cell death (Kayed and Lasagna-Reeves, 2013). The amyloid hypothesis proposes that Aβ oligomerization and deposition causes progressive synaptic and neuritic injury (Hardy and Selkoe, 2002).

Figure 1.1. Aβ aggregation pathway. Aβ aggregation starts with Aβ monomers in a nucleation-dependent process to form Aβ oligomers of various sizes. These aggregates then elongate into longer and wider protofibrils, which undergo lateral aggregation to form fibres that deposit in Aβ plaques.
1.4 Hyperphosphorylation of Tau and Neurofibrillary Tangles

In AD, axonal and synaptic loss is more prevalent and precedes neuronal loss (Scheff et al., 2006). Axonal and synaptic degeneration correlate better with cognitive decline than neuronal cell death (Palop et al., 2006). In addition to Aβ oligomerization and deposition, synaptic injury can also be caused by neurofibrillary tangles of hyperphosphorylated tau.

Tau is a microtubule-associated protein that promotes the assembly and stability of microtubules in mature neurons. Tau is found in axons and other somatodendritic compartments such as synapses, rough endoplasmic reticulum and the Golgi apparatus (Iqbal et al., 2010; Pooler et al., 2014). The activity of tau is regulated by its degree of phosphorylation using multiple kinases and phosphatases. In the healthy brain, tau exists in a soluble state whereas in AD, tau is found in soluble, oligomeric and fibrillized forms, which impairs axonal transport and synaptic function (Iqbal et al., 2010). In AD, tau is abnormally hyperphosphorylated and aggregates to form intraneuronal neurofibrillary tangles of paired helical and/or straight filaments (Grundke-Iqbal et al., 1986; Iqbal et al., 2005). Neurons containing these neurofibrillary tangles are found to express reduced levels of the pre-synaptic marker synaptophysin compared to tangle free neurons (Callahan et al., 1999). Similar to Aβ oligomers, soluble oligomeric tau also induces neurotoxicity (Kopeikina et al., 2012). In the absence of neurofibrillary tangles, soluble oligomeric tau induced synaptotoxicity in a mouse model of conditional tauopathy and soluble tau oligomers were correlated with memory impairment (Berger et al., 2007). Both pathological aggregation of hyperphosphorylated tau and Aβ cause synaptic and neuritic injury. To exacerbate the problem of synaptotoxicity and neurotoxicity caused by Aβ and tau, there is also loss of neurotrophic support in AD that would otherwise promote synaptogenesis and neuronal survival in a healthy brain.

1.5 Loss of Neurotrophic Support

Neurotrophins and the transcription factor cyclic AMP response element binding protein (CREB) promote synaptogenesis and neuronal survival, therefore, the loss of this neurotrophic support could exacerbate synaptic and neuronal injury caused by Aβ and tau. CREB is a highly
conserved nuclear transcription factor regulating genes involved in neuronal survival, long term synaptic plasticity and memory (Lopez de Armentia et al., 2007; Benito and Barco, 2010). CREB phosphorylated at ser133 activates genes involved in multiple signaling pathways with an upstream CRE element (Shaywitz and Greenberg, 1999). In AD patients, phosphorylated CREB (pCREB) levels are significantly lower in the hippocampus than controls while total CREB levels are unaltered (Yamamoto-Sasaki et al., 1999). Gene expression profiling of the CA1 region of the hippocampus revealed dysregulation of the CREB signaling pathway in AD patients (Satoh et al., 2009). CREB is also a downstream mediator of neurotrophin induced neuronal survival signaling (Finkbeiner, 2000). The phosphorylated transcription factor CREB participates in signaling pathways involved in neuronal survival, synaptic plasticity and memory but pCREB expression is reduced in the hippocampus of the AD brain (Yamamoto-Sasaki et al., 1999).

Neurotrophins regulate neuronal development, survival, function and plasticity (Huang and Reichardt, 2001). There are four mammalian neurotrophins including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5). Neurotrophins activate two classes of receptors, the tyrosine kinases (Trk) and p75NTR, a member of the tumor necrosis factor receptor family (Huang and Reichardt, 2001). NGF signals through TrKA, BDNF and NT-4/5 signal through TrKB while NT-3 signals through TrKC. All four neurotrophins also bind the low affinity receptor p75NTR (Wong, 2013). Studies on neurotrophins in AD reveal changes in NGF and BDNF expression (Wong, 2013). NGF mRNA and protein levels in AD patients are reported to be either significantly increased or remain unaltered throughout the brain including the hippocampus and the cortex, CSF and serum (Allen et al., 1991; Jette et al., 1994; Murase et al., 1993; Scott et al., 1995; Narisawa-Saito et al., 1996). ProNGF protein levels are increased in both the hippocampus and the cortex of AD patients (Counts et al., 2005; Mufson et al., 2012). However, NGF protein levels are significantly decreased in the nucleus basalis of Meynert (NBM) where cholinergic neurons reside, resulting in decreased neurotrophic support for cholinergic neurons (Scott et al., 1995). There are many cholinergic deficits in AD and drugs targeting the cholinergic system have been used for AD treatment (see section 1.6.1 for details on the cholinergic system in AD). In addition to altered NGF levels in the AD brain, changes to BDNF expression also contributes to loss of neurotrophic support.
BDNF is highly expressed and more widely distributed in the brain than NGF. BDNF mRNA levels are significantly decreased in the hippocampus of the AD patients (Phillips et al., 1991; Connor et al., 1997). Similar to mRNA levels, there is also a decrease in the intensity of the BDNF expression and the number of BDNF-immunoreactive cells in the hippocampus and temporal cortex in AD (Connor et al., 1997). BDNF mRNA expression is regulated by nine different promoters to produce multiple transcripts (Zheng et al., 2012). In AD, mRNA levels of BDNF transcripts I, II and III are down-regulated in the parietal cortex (Garzon et al., 2002). BDNF receptor TrkB immunoreactivity is also significantly reduced in the hippocampus and temporal and frontal cortex in the AD brain (Allen et al., 1999; Wong, 2013). Neurotrophic support from NGF is reduced for cholinergic neurons in the NBM that innervate the cortex and loss of neurotrophic support from BDNF is also observed in the hippocampus and cortex in AD patients. Decreased neurotrophin expression in these brain regions that are important for learning and memory would provide less support for neuronal survival, synaptogenesis and lead to cognitive dysfunction, especially when the brain is under insult from Aβ and tau induced toxicity.

1.6 Dysfunction of Neurotransmitter Systems

1.6.1 Cholinergic System

Synaptic and neuronal degeneration effect many neurotransmitter systems in the brain. Much research has focused on the cholinergic system and preserving cholinergic function has been a major target for AD therapeutics. In the 1970s, postmortem studies of AD brains reported reduction in the activity of the acetylcholine (ACh) synthesizing enzyme acetylcholine transferase (ChAT) and ACh degrading enzyme acetylcholinesterase (AChE) in the hippocampus and cortex; these cholinergic deficits were correlated with Aβ plaque count and with cognitive deficits in AD patients (Davies and Maloney, 1976; Perry et al., 1978). In agreement with decreased ChAT and AChE activity, the number of ChAT-positive cells were drastically reduced in the medial basal forebrain of AD patients (McGeer et al., 1984). Impairment in cholinergic innervation occurs in mild cognitive impairment (MCI) and mild AD, worsening as the disease progresses. In MCI and mild AD, there is a decrease in NGF receptors TrKA and p75NTR in
ChAT-positive cholinergic cells, suggesting a reduction in neurotrophic support for cholinergic cells (Counts et al., 2005). Furthermore, BDNF is also reduced in MCI, further depriving neurons of trophic support (Mufson et al., 2007). As AD progresses, cholinergic cell loss and a decrease in ChAT activity is observed (Counts et al., 2005).

Based on these studies reporting cholinergic deficits and correlation with cognitive deficits in AD patients, cholinesterase inhibitors were used to enhance cholinergic function (Lanctôt et al., 2003; Birks, 2006). Cholinesterase inhibitors block the activity of AChE and/or butyrylcholinesterase, enzymes that hydrolyze ACh, to reduce the degradation of ACh thus prolonging stimulation of postsynaptic receptors. Of all cholinesterase inhibitors tested in clinical trials, donepezil, rivastigmine and galantamine are best tolerated with the least adverse events (Lanctôt et al., 2003).

Donepezil is an AChE inhibitor that is selective for the central nervous system with minimal peripheral activity. Treatment with donepezil significantly improved cognitive function in AD patients (Rogers and Friedhoff, 1996). With donepezil treatment, 11% of AD patients showed cognitive decline compared to 20% on placebo (Rogers and Friedhoff, 1996). Rivastigmine inhibits both AChE and butyrylcholinesterase to prolong ACh activity and also improved cognitive function in AD patients (Rösler et al., 1999). Similarly, treatment with galantamine, another AChE inhibitor, showed cognitive improvement in AD patients compared to placebo treated patients (Wilcock et al., 2000). These three cholinesterase inhibitors are approved for treating mild to moderate AD, however, the treatment effect size is small and only a subset of AD patients show cognitive improvement (Rogers and Friedhoff, 1996; Rösler et al., 1999; Wilcock et al., 2000; Birks, 2006).

Since cholinesterase inhibitors were introduced in 1997, more recent studies reported contradictory results regarding the cholinergic system in MCI, mild and moderate AD cases compared to age-matched controls (Davis et al., 1999; DeKosky et al., 2002). ChAT and AChE activities were reduced only in severe AD and significant correlations of cholinergic deficits with neuritic plaques and with NFTs became insignificant if severe AD cases were excluded from the analyses (Davis et al., 1999; DeKosky et al., 2002). Similarly, the number of ChAT and vesicular ACh transporter immunoreactive cholinergic neurons in MCI and in mild AD are not changed.
compared to controls (Gilmor et al., 1999). Treatment of AD using cholinesterase inhibitors to extend the function of the cholinergic system demonstrates individual variation in response and effect size, which might be explained by the variable pathology reported in post-mortem examinations (Davies and Maloney, 1976; Perry et al., 1978; McGeer et al., 1984; Davis et al., 1999; Gilmor et al., 1999; DeKosky et al., 2002). Studies reporting cholinergic deficits are consistent only in severe AD and cholinesterase inhibitors targeting the cholinergic system provide limited symptomatic relief.

1.6.2 Glutamatergic System

In addition to the cholinergic system, dysfunction of the glutamatergic system leading to excitotoxicity in the brain also contributes to the pathogenesis of AD. Aβ induces dysregulation of the excitatory neuronal network activity via overstimulation of the glutamatergic system (Palop et al., 2007). Regulation of the glutamatergic system to maintain homeostasis in the brain for optimal physiological function is crucial for synaptic plasticity and learning (Parsons et al., 2007). Disturbances in the glutamatergic system have been reported in AD, including elevated levels of glutamate, the principle excitatory neurotransmitter in the brain, and sensitization of the glutamate receptors (Greenamyre and Young, 1989; Butterfield and Pocernich, 2003; Wenk et al., 2006). It has been shown that elevated glutamate levels are due to Aβ stimulated glutamate release from microglia as well as impaired extraneuronal glutamate removal through inhibition of glutamate transporter and decreased activity of glutamine synthetase (Hensley et al., 1995; Noda et al., 1999; Lauderback et al., 2001). In addition to inducing elevated glutamate levels, Aβ also stimulates the glutamatergic system via N-methyl-D-aspartate (NMDA) glutamate receptors by increasing NMDA receptor sensitivity (Wu et al., 1995). Moreover, it has been proposed that Aβ activation of microglia can indirectly cause elevated glutamate levels and increased NMDA receptor sensitivity by inducing oxidative stress and inflammation (Wenk et al., 2006). Overactivation of NMDA receptors by glutamate can inhibit LTP and learning, lead to neuronal calcium overload and eventual cell death (Choi, 1992; Parsons et al., 2007; Danysz and Parsons, 2012). As a result, tonically elevated glutamate levels and increased sensitivity of NMDA receptors lead to excitotoxicity in the AD brain (Danysz and Parsons, 2012).
Based on evidence of glutamatergic dysfunction in AD, memantine, an NMDA receptor antagonist was used in pre-clinical and clinical studies for the treatment of AD (Parsons et al., 2007). Clinical data on patients suffering from moderate to severe AD showed that treatment with memantine offered improvement over placebo in measures of cognitive and daily function (Reisberg et al., 2003). Since 2003, memantine has been a Food and Drug Administration approved drug to treat moderate to severe AD by reducing glutamate induced excitotoxicity (Parsons et al., 2007).

1.6.3 Imbalance between Excitatory and Inhibitory Inputs

Based on Aβ-induced dysfunction of the glutamatergic system, it has been proposed that aberrant excitatory network activity in AD and the compensatory inhibitory responses impair learning and memory to result in cognitive decline (Palop and Mucke, 2010). Pathologically elevated Aβ elicit changes in excitatory and inhibitory synaptic activity, which may cause disturbances in cortical and hippocampal networks, potentially leading to epilepsy (Palop et al., 2007; Figure 1.2).
Figure 1.2. Aβ-induced aberrant network activity. Elevated Aβ levels in the brain alters excitatory and inhibitory synaptic activity, resulting in disturbances in cortical and hippocampal networks.

Epileptic activity is observed in both AD patients and transgenic mouse models. In sporadic AD, patients have 6-10 fold increased risk of developing seizures compared to healthy age-matched controls (Pandis and Scarmeas, 2012). Patients suffering from early onset familial AD experience increased incidence of seizures and epilepsy compared to sporadic AD (Pandis and Scarmeas, 2012). Mutations in PS1 and PS2 genes increase occurrence of seizures by over 30% in AD patients and APP duplication increases the risk to 57% (Cabrejo et al., 2006; Larner and Doran, 2006; Jayadev et al., 2010). The majority of seizures in AD patients are generalized convulsive seizures but the incidence of partial non-convulsive seizures could be underestimated (Pandis and Scarmeas, 2012). Transgenic mouse models of AD carrying familial mutations also display increased seizure activity, which has allowed investigation into disease mechanisms leading to epilepsy (Jolas et al., 2002; Del Vecchio et al., 2004; Palop et al., 2007; Minkeviciene et al., 2009).

In APP transgenic J20 mice, harbouring Swedish and Indiana APP mutations under the control of human platelet-derived growth factor β polypeptide promoter, in vivo electroencephalogram
recordings demonstrated hyperexcitability in the hippocampus and cortex as well as intermittent non-convulsive seizures (Palop et al., 2007). In TgCRND8 mouse model of AD carrying the Swedish and Indiana APP mutations under the control of hamster prion promoter, seizures were observed in a fraction of the transgenic mice (Chishti et al., 2001). Both pre-Aβ plaque and post-Aβ plaque deposition, TgCRND8 mice exhibit lower seizure threshold compared to non-Tg littermates (NTg) when pentylenetetrazole was used to induce seizures (Jolas et al., 2002; Del Vecchio et al., 2004). TgCRND8 mice also experience more severe pentylenetetrazole-induced seizures than NTgs (Del Vecchio et al., 2004). In addition, TgCRND8 mice show increased LTP in the CA1 region of the hippocampus. Hippocampal LTP reflects the complex interaction between excitatory and inhibitory inputs, therefore, the increase in the ratio of excitatory postsynaptic current to inhibitory postsynaptic current observed in TgCRND8 mice indicate an increased ratio of glutamatergic/γ-aminobutyric acid (GABA)-ergic function may help to explain the increased LTP (Jolas et al., 2002). In another AD transgenic mouse model carrying APP Swedish mutation and PS1 mutation controlled by the mouse prion protein promoter, 65% of transgenic mice exhibited electrographic seizures during 3 weeks of electroencephalogram recordings (Minkeviciene et al., 2009). In contrast, no wild-type control mice had seizures. In a subset of these transgenic mice, seizure phenotype was associated with altered expression of inhibitory neuropeptides calbindin (CB) and neuropeptide Y (NPY) (Minkeviciene et al., 2009). Studies from transgenic mouse models show that a disturbance in the excitatory/inhibitory balance in the brain is associated with altered LTP and increased seizure activity.

1.6.4 GABAergic System

Emerging evidence suggests that dysfunction of the inhibitory GABAergic system may be key in the pathogenesis of network dysfunction in AD. In a mouse model of AD, it was demonstrated that decreased inhibition instead of increased excitation contributed to neuronal hyperactivity leading to increased risk of seizures (Busche et al., 2008). The AD mouse model harbouring APP_{Swe}/PS1 mutations controlled by the Thy-1 promoter exhibits hyperactive neurons in close vicinity to Aβ plaques (Busche et al., 2008). These hyperactive neurons display a higher frequency of spontaneous Ca^{2+} transients and fire more frequently in a correlated manner, which may increase the risk of seizure (Busche et al., 2008). However, the hyperactivity of these
neurons is not due to dysfunctional glutamatergic system because glutamate induced Ca\textsuperscript{2+} transients were not different in the hyperactive neurons compared to normal neurons. In contrast, examination of the GABAergic system showed that diazepam, which increases the opening of GABA\textsubscript{A} receptor channels, reduced activity of hyperactive neurons and gabazine, a GABA\textsubscript{A} receptor antagonist, increased the frequency of Ca\textsuperscript{2+} transients (Busche et al., 2008). Taken together, decreased synaptic inhibition rather than increased excitation underlies the hyperactivity observed in these neurons (Busche et al., 2008).

More recent studies on transgenic mouse models of AD reported increasing support for GABAergic deficits in AD pathogenesis and the role of the GABAergic system in learning and memory. To study cognitive deficits in AD, many mouse models have been generated with a growing realization that dysfunction of GABAergic interneurons contribute to the pathophysiology of disease progression (Loreth et al., 2012; Krantic et al., 2012; Ramos et al., 2006; Perez-Cruz et al., 2011). These studies also show that dysfunction of the GABAergic system arises prior to glutamatergic and cholinergic deficits. The deficits reported in the different mouse models demonstrate a loss of GABAergic interneurons but the sub-type, as classified by neuronal neuropeptide and calcium binding protein expression, varies depending on transgene(s) expression (Loreth et al., 2012; Krantic et al., 2012; Ramos et al., 2006; Perez-Cruz et al., 2011). Glutamic acid decarboxylate (GAD) converts glutamic acid to GABA and its two isoforms GAD67 and GAD65 are predominantly localized in GABAergic cell bodies and axon terminals, respectively (Esclapez et al., 1994). Due to the localization within GABAergic cell bodies, GAD67 expression is used to label GABAergic interneurons in the brain. The neuropeptides expressed by GABAergic interneurons include cholecystokinin (CCK), SST, vasoactive intestinal polypeptide (VIP) and NPY (Freund and Buzsáki, 1998). The calcium binding proteins expressed by GABAergic interneurons are parvalbumin (PV), calbindin and calretinin (CR) (Freund and Buzsáki, 1998). These markers of GABAergic interneuron subtypes help to identify specific GABAergic deficits in mouse models of AD. The hippocampal distribution of cells expressing these neuropeptides and calcium binding proteins is illustrated in Figure 1.3.
Figure 1.3. Hippocampal distribution of interneurons expressing various neuropeptides and calcium binding proteins (modified reprint from Freud TF and Buzsaki G (1998) Interneurons of the hippocampus. Hippocampus 6:347-470, with permission from John Wiley and Sons).
In both mouse models of AD and rat models of ageing, GABAergic deficits have been observed (summarized in Table 1.1). In Tau/PS2/APP\textsubscript{Swe} transgenic mouse model of AD, the earliest GABAergic loss was observed in NPY mRNA levels in the hippocampus at 6 months of age (Loreth et al., 2012). Stereological analysis confirmed NPY+ cell loss in the hippocampus at 12 months of age in the dentate gyrus, hilus and CA1-3 regions. Subsequently, GAD67+, PV+ and CR+ cells were also significantly decreased in transgenic mice compared to NTg controls at 18 months of age (Loreth et al., 2012). Even though inhibitory GABAergic deficits were observed as early as 6 months of age, excitatory glutamatergic deficits were not observed in the hippocampus until 18 months of age marked by a decrease in mRNA levels of NMDA receptor subunit 1 and metabotropic glutamate receptor 1 (Loreth et al., 2012). These results suggest that the inhibitory GABAergic system is compromised prior to the excitatory glutamatergic system in this AD model. In the septo-hippocampal pathway, GABAergic loss of PV+ cells in the septum was evident in the Tg mice by 12 months of age whereas the number of ChAT+ cholinergic cells in the septum and AChE+ cholinergic cell fibres in the hippocampus remain comparable between Tgs and NTgs up to 18 months of age (Loreth et al., 2012). In this mouse model, GABAergic dysfunction precedes glutamatergic and cholinergic dysfunction as a result of A\(\beta\) and tau pathology.

In TgCRND8 mouse model of AD, the number of GAD67+ GABAergic interneurons in the hippocampus remained comparable between Tg and NTg mice at 2 and 4 months of age but is significantly decreased in Tg mice at 6 months of age as A\(\beta\) pathology worsens (Krantic et al., 2012). The decrease in GAD67+ interneurons was restricted to the CA1-3 region with comparable interneuron numbers in the dentate gyrus. In primary hippocampal culture, exposure to A\(\beta\)42 selectively caused a decrease in GAD67+ neuron numbers but not the number of total neurons. As A\(\beta\) accumulates in the brain, GABAergic interneurons are targeted in TgCRND8 mice before the glutamatergic dysfunction as suggested by unaltered vesicular glutamate transporter 1 expression in the CA1-3 or dentate gyrus at 6 months of age (Krantic et al., 2012). In a similar model, Tg2576 mice, harbouring APP Swedish mutation under the control of hamster prion protein promoter, the number of SST immunoreactive GABAergic interneuron in the CA1 region of the hippocampus was significantly decreased compared to NTg mice but not within the dentate gyrus (Perez-Cruz et al., 2011). Similar to Tau/PS2/APP transgenic mice,
these APP overexpressing mouse models of AD exhibit GABAergic dysfunction, which precedes glutamatergic dysfunction.

In another mouse model of AD, PS1xAPP<sub>Swe/Lon</sub> mice under the control of HMGCoA-reductase and Thy-1 promoters, GABAergic deficits are also observed prior to glutamatergic deficits. In the hippocampus, SST mRNA levels were decreased starting at 4 months of age and decreases in NPY mRNA levels were observed at 6 months of age (Ramos et al., 2006). Similar to mRNA levels, SST+ cells in the CA1, CA3 and dentate gyrus regions of the hippocampus were also decreased. SST and NPY represent the earliest GABAergic neuropeptide loss as PV, CB, VIP and CCK mRNA levels were not altered at 6 months of age. GABA<sub>A</sub> receptor subunits α1, α2 and α5 were also not significantly different along with glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors GluR1, GluR2, GluR2 and GluR4 (Ramos et al., 2006). In addition to the receptors, the number of excitatory glutamatergic pyramidal neurons in the hippocampus were not altered in PS1xAPP mice (Ramos et al., 2006). Also in the hippocampus, cholinergic muscarinic receptors M1, M2, M3 and M4 as well as nicotinic receptors nα7 and nα4 remain the same between Tg and NTg mice. ChAT mRNA expression in the basal forebrain was also not different in PS1xAPP mice (Ramos et al., 2006). The early decrease in SST and NPY expression was significantly correlated with the amount of Aβ peptide in the hippocampus where higher Aβ levels corresponds to lower SST or NPY levels (Ramos et al., 2006). In these transgenic mouse models of AD, GABAergic deficits appear early in response to Aβ and/or tau pathology in the brain.

The two greatest risk factors for sporadic AD are ApoE4 expression and aging, rodent models of both demonstrate that GABAergic neuronal loss is linked to cognitive deficits (Andrew-Zwilling et al., 2010; Leung et al., 2012; Stanley et al., 2012; Spiegel et al., 2013). ApoE4 is a genetic risk factor in AD that induces learning and memory deficits primarily in female mice (Raber et al., 1998). In aging and ApoE knock-in mouse models, aged ApoE4 knock-in female mice exhibit decreased GAD67+ and SST+ cells in the hilus of the hippocampus; the reduced GABAergic expression in the hilus is correlated with spatial memory (Andrews-Zwilling et al., 2010). However, aging male mice exhibit intact spatial memory with increasing expression of GABAergic interneurons in the hilus of the hippocampus regardless of ApoE4 expression (Leung et al., 2012). In aged rats, animals with reduced numbers of GAD67+ cells and SST+
cells in the hilus of the hippocampus showed impairment in spatial memory while littermates without spatial deficits had no GABAergic neuronal deficits (Spiegel et al., 2013). In outbred rats, GAD67+ and SST+ interneurons are preferentially lost in the stratum oriens of the hippocampus with age (Stanley et al., 2012). The SST+ cell loss leads to cognitive dysfunction and seizures as a result of decreased inhibitory control over entorhinal input to CA1 pyramidal cells (Stanley et al., 2012). Similar to transgenic mouse models of AD, in animal models of ApoE4 and aging that exhibit cognitive impairment, GABAergic neuronal expression is reduced in the hippocampus.
<table>
<thead>
<tr>
<th>Rodent Model</th>
<th>Age</th>
<th>GABAergic Deficits in hippocampus</th>
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<tbody>
<tr>
<td>Tau/PS2/APP&lt;sub&gt;Swe&lt;/sub&gt; mice</td>
<td>6 month</td>
<td>↓ NPY mRNA</td>
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<td>(Loreth et al., 2012)</td>
<td>12 month</td>
<td>↓ NPY+ cells in dentate gyrus, hilus and CA1-3</td>
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<td></td>
<td>18 month</td>
<td>↓ GAD67+, NPY+, CR+ cells in dentate gyrus, hilus and CA1-3</td>
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<td></td>
<td></td>
<td>↓ PV+ cells in dentate gyrus and hilus</td>
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<tr>
<td>TgCRND8 APP&lt;sub&gt;Swe&lt;/sub&gt;/Ind mice</td>
<td>6 month</td>
<td>↓ GAD67+ cells in CA1-3</td>
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<td>(Krantic et al., 2012)</td>
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<tr>
<td>Tg2576 APP&lt;sub&gt;Swe&lt;/sub&gt; mice</td>
<td>5.5 month</td>
<td>↓ SST+ cells in CA1</td>
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<tr>
<td>(Perez-Cruz et al., 2011)</td>
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<tr>
<td>PS1/APP&lt;sub&gt;Swe&lt;/sub&gt;/Lon mice</td>
<td>4 month</td>
<td>↓ SST mRNA</td>
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<tr>
<td>(Ramos et al., 2006)</td>
<td>6 month</td>
<td>↓ SST and NPY mRNA</td>
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<td></td>
<td></td>
<td>↓ SST+ cells in CA1, CA3 and dentate gyrus</td>
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<tr>
<td>ApoE KI female mice</td>
<td>12 month</td>
<td>↓ GAD67+, SST+ cells in hilus</td>
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<td>(Andrews-Zwilling et al., 2012)</td>
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<tr>
<td>Aged male Long-Evans rats</td>
<td>24-26 month</td>
<td>↓ GAD67+, SST+ cells in hilus</td>
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<tr>
<td>(Spiegel et al., 2013)</td>
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<tr>
<td>Aged Fisher 344 Brown Norway F1 hybrid rats</td>
<td>26-30 month</td>
<td>↓ GAD67+, SST+ cells in CA1</td>
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*Table 1.1.* Summary of GABAergic deficits in mouse models of AD and rat models of ageing.
1.6.5 Somatostatinergic System

The neuropeptide SST is decreased in the hippocampus of rodent models of AD and ageing as previously discussed (Andrew-Zwilling et al., 2012; Perez-Cruz et al., 2011; Ramos et al., 2006; Spiegel et al., 2013; Stanley et al., 2012). In AD patients, the somatostatinergic system is consistently affected, displaying reduced levels of the neuropeptide SST in the brain and CSF (Sunderland et al., 1987; Davis et al., 1988; Molchan et al., 1991; Molchan, 1993). SST immunoreactivity shows widespread reduction in the cortex and the hippocampus of post-mortem AD brains and is correlated with cognitive dysfunction (Davies et al., 1980; Davies and Terry, 1981; Beal et al., 1986). SST is highly concentrated in the hippocampus and the cortex and is expressed by a subtype of GABAergic interneurons (Schettini, 1991). Density of neurons expressing somatostatin mRNA and hippocampal neuronal expression of somatostatin mRNA are also reduced in AD (Dournaud et al., 1994). SST expressing cells in the hippocampus provide inhibitory innervation to local principal cells but also exhibit long-range projections to the medial septum, striatum and medial enthorinal cortex (Katona et al., 1999; Melzer et al., 2012). These inhibitory cells are proposed to contribute to the highly synchronized theta activity underlying spatial and temporal coding, which is important for spatial memory (Katona et al., 1999; Melzer et al., 2012). Taken together, these studies demonstrate a reduction in the SST pathways in AD that may contribute to memory, cognitive and emotional changes (Epelbaum et al., 2009).

1.7 α-Melanocyte Stimulating Hormone and Alzheimer’s Disease

1.7.1 α-MSH peptide expression is reduced in Alzheimer’s Disease

α-Melanocyte stimulating hormone (α-MSH) is one cleavage product of the pituitary hormone pro-opiomelanocortin (POMC). POMC is produced in the brain with high expression in the hypothalamus and lower expression in the hippocampus and cortex (Smith and Funder, 1988; Wikberg et al., 2000). POMC is processed to make many functional cleavage products in a tissue-dependent manner (Bicknell, 2008). Figure 1.4 illustrates POMC processing in the brain. Products of POMC processing in the brain include the opiates, lipotropin and β-endorphin and the melanocortins, β-MSH, γ-MSH and adrenocorticotrophin (ACTH) (Bicknell, 2008). ACTH
is further cleaved to produce α-MSH and corticotrophin-like intermediate peptide (CLIP) (Bicknell 2008). The β-endorphin produced is mostly N-acetylated, which prevents it from acting on opiate receptors (Smyth and Zakarian, 1980; Deakin et al., 1980). Lipotropin levels are not altered in AD (Sulkava et al., 1985). CLIP stimulates insulin release from beta cells and regulates sleep (Marshall et al., 1984; Chastrette et al., 1990). Of the melanocortins, α-MSH shows high affinity binding to central nervous system melanocortin receptors (Getting, 2006). In addition, α-MSH has been shown to be down-regulated in AD (Arai et al., 1986; Costa et al., 2011; Rainero et al., 1988). In the AD brain α-MSH immunoreactivity is significantly decreased compared to healthy brains (Arai et al., 1986). CSF levels of α-MSH were also reduced in late onset AD (>65 years of age) (Rainero et al., 1988). Furthermore, a more recent study showed a positive correlation between CSF α-MSH auto-antibodies and Mini-Mental State Examination scores in AD patients and a negative correlation with age (Costa et al., 2011). These studies demonstrate altered α-MSH peptide expression in AD patients, which may play a role in cognitive decline.

![Figure 1.4. POMC processing in the brain. Products of POMC processing in the brains include the opiates: lipotropin, β-endorphin and the melanocortins: ACTH, α-MSH, β-MSH and γ-MSH. POMC, Pro-opiomelanocortin; ACTH, adrenocorticotrophin; MSH, melanocyte-stimulating hormone; CLIP, corticotrophin-like intermediate peptide.](image-url)
1.7.2 Melanocortin Receptors in the Central Nervous System

There are two main melanocortin receptors in the brain, melanocortin receptor 3 (MC3R) (Gantz et al., 1993a) and melanocortin receptor 4 (MC4R) (Gantz et al., 1993b). \(\alpha\)-MSH binds to both G-protein coupled receptors with high affinity. MC3Rs are present in the hypothalamus, thalamus, cortex and hippocampus of the brain. Within the hippocampus, MC3R is expressed in the CA1-3 regions, especially in pyramidal cells, and in the dentate gyrus (Gantz et al., 1993a; Roselli-rehfuss 1993). MC4Rs are found in virtually all brain regions and they are more widespread in the brain than MC3Rs (Liu et al., 2003; Mountjoy 2010; Gantz et al., 1993b). MC4Rs exhibit high expression particularly in the CA1 region of the hippocampus, mostly in the pyramidal layer, and moderate expression in the dentate gyrus (Liu et al., 2003; Gantz et al., 1993b). \(\alpha\)-MSH has many functions in the brain such as inducing grooming, stretching, yawning and appetite suppression (reviewed in Bertolini et al., 2009). More importantly, \(\alpha\)-MSH improves memory and exhibits many anti-inflammatory and neuroprotective effects that may be beneficial in treating AD (Bertolini et al., 2009; Catania 2008).

1.7.3 Neuroinflammation is attenuated by \(\alpha\)-MSH

In the AD brain, neuroinflammation is observed when microglia morphology changes from ramified resting state to amoeboid active state and the number of reactive astrocytes increases (Glass et al., 2010). These microglia and astrocytes release an array of inflammatory mediators such as cyclooxygenase-2, cytokines tumor necrosis factor (TNF)-\(\alpha\), interleukin (IL)-1\(\beta\) and IL-6, nitric oxide, prostaglandins and chemokines (Akiyama et al., 2000; Cartier et al., 2005). \(\alpha\)-MSH has many anti-inflammatory effects in the brain. Using lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, and the cytokine interferon (IFN)-\(\gamma\) to induce inflammation \textit{in vitro}, \(\alpha\)-MSH application decreased the expression of inflammatory mediators in cell culture systems. In rat hypothalamic primary culture, \(\alpha\)-MSH decreased the expression of the cytokines TNF-\(\alpha\) and IL-1\(\beta\), which are possibly regulated via increasing the expression of CRE/CREB complexes downstream of \(\alpha\)-MSH’s G-protein coupled receptors (Caruso et al., 2010). In astrocyte primary culture, LPS and IFN-\(\gamma\) incubation elevated the expression of inducible nitric oxide synthase (iNOS), increased nitric oxide production,
stimulated cyclooxygenase-2 expression to induce elevated levels of the inflammatory mediator prostaglandin E2 (Caruso et al., 2007). LPS and IFN-γ incubation also reduced cell viability by increasing pro-apoptotic Bax and decreasing anti-apoptotic Bcl-2 protein expression to shift the Bax/Bcl2 ratio in the direction that activates apoptosis effector caspase, caspase-3 (Caruso et al., 2007). α-MSH treatment decreased inflammation by reducing the expression of iNOS and nitric oxide production, attenuated prostaglandin E2 release and cyclooxygenase-2 and shifted the Bax/Bcl-2 ratio to prevent apoptosis (Caruso et al., 2007). In macrophages, α-MSH treatment reduced the expression of LPS stimulated nitric oxide, as well as, the cytokines IFN-γ and IL-12p70 (Taylor 2005). Similar to experiments in cell cultures, α-MSH alleviated inflammation in vivo. In a mouse model of cerebral ischemia, induced by middle cerebral artery occlusion followed by reperfusion, an inflammatory response with elevated TNF-α and IL-1β expression was observed (Huang and Tatro 2002). Systemic intraperitoneal (i.p.) administration of α-MSH reduced both TNF-α and IL-1β expression in the cortex (Huang and Tatro 2002). In addition, α-MSH treatment dose-dependently decreased LPS induced nitric oxide expression in the mouse brain (Muceniece et al., 2004). In global models of inflammation, α-MSH treatment reduces inflammatory mediators in in vitro and in vivo experimental systems.

1.7.4 α-MSH Inhibits NF-κB Activation

The anti-inflammatory actions of α-MSH may be exerted via inhibition of nuclear transcription factor NF-κB activation (Manna and Aggarwal, 1998; Ichiyama et al., 1999a; 1999b; Sarkar et al., 2003; Taylor, 2005). Activation of NF-κB can be a downstream consequence of the toll-like receptor 4 (TLR4) signaling pathway or stimulation by other inflammatory agents such as TNF-α, okadaic acid, hydrogen peroxide and ceramide (Siebenlist et al., 1994; Pålsson-McDermott and O'Neill, 2004). Inactive NF-κB complex in the cytoplasm consists of p50, p65 and IκBα (Siebenlist et al., 1994). Once activated, IκBα undergoes phosphorylation and proteolytic degradation, unmask the p65 nuclear localization signal, then NF-κB translocates to the nucleus where it regulates the expression of cytokines (Siebenlist et al., 1994).
α-MSH can either directly inhibit NF-κB activation or target the TLR4 pathway to indirectly inhibit NF-κB (Figure 1.5). α-MSH has been shown to directly act on the NF-κB complex to prevent activation. In human monocyte cells, pre-incubation with α-MSH decreased TNF stimulated NF-κB activation in a dose dependent manner (Manna and Aggarwal, 1998). α-MSH is a potent inhibitor of NF-κB activation stimulated by many inflammatory agents such as LPS, okadaic acid and ceramide and the inhibition extents beyond monocytes to other cell types such as epithelial, glioma and lymphoid cells (Manna and Aggarwal, 1998; Ichiyama et al., 1999b). α-MSH inhibits NF-κB activation by preventing the degradation of IκBα both in vitro in cultured cells and in vivo in brains of mice stimulated with LPS (Manna and Aggarwal, 1998; Ichiyama et al., 1999a; 1999b). In addition, α-MSH inhibits the translocation of the NF-κB subunit p65 to the nucleus (Manna and Aggarwal, 1998). α-MSH receptors in the brain MC3R and MC4R are both G-protein coupled receptors that signal via downstream mediators cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA)(Gantz et al., 1993a; 1993b). α-MSH inhibition of NF-κB was found to be cAMP and PKA dependent as inhibitors of cAMP synthesizing enzyme adenylate cyclase and PKA prevented inhibition of NF-κB by α-MSH (Manna and Aggarwal, 1998). Inhibition of the nuclear transcription factor NF-κB is one mechanism whereby α-MSH reduces inflammation in the brain.

α-MSH may also act on the TLR4 signaling pathway to indirectly inhibit NF-κB. CD14, a coreceptor of TLR4 has been shown to bind Aβ42, especially to fibrillar Aβ42 (Landreth and Reed-Geaghan, 2009). Even though CD14 binds to LPS with higher affinity than with fibrillar Aβ42, CD14-Aβ42 interaction is believed to induce chronic inflammation in the AD brain (Landreth and Reed-Geaghan, 2009). In LPS stimulated macrophage cell culture, α-MSH was shown to down-regulate CD14 (Sarkar et al., 2003). Further downstream of the TLR4 signaling pathway, α-MSH suppressed tyrosine phosphorylation of IL-1 receptor-associated kinase (IRAK)-1 and prevented the activation of IRAK-1 by promoting the binding of IRAK-M to IRAK-1 (Taylor, 2005). Early interference in the TLR4 signaling pathway prevents activation of the downstream NF-κB. By inhibiting the activation of NF-κB, α-MSH may in turn reduce the expression of inflammatory cytokines regulated by NF-κB.
Figure 1.5. Anti-inflammatory effects of α-MSH. α-MSH reduces inflammation by inhibiting NF-κB activation directly and indirectly through TLR4 signaling pathway. α-MSH down-regulated the TLR4 co-receptor CD14, prevents IRAK-1 activation and inhibits NF-κB activation. TLR4, toll-like receptor 4; IRAK, IL-1 receptor-associated kinase; MyD88, myeloid differentiation primary response gene 88.
1.7.5 Neurotrophic and Neurogenic effects of α-MSH

In addition to the anti-inflammatory actions, α-MSH has many neuroprotective effects (Figure 1.6). The neuroprotective effects of α-MSH is mediated through G-protein coupled receptors MC3R and MC4R in the brain (Sarkar et al., 2002; Xu et al., 2003; Giuliani et al., 2011; Caruso et al., 2012). BDNF is important for neuronal development, cell survival and neurogenesis (Binder and Scharfman, 2004). More importantly, BDNF is involved in activity-dependent synaptic plasticity and participates in hippocampal-dependent learning and memory formation, however, BDNF levels are reduced in the AD brain (Tyler et al., 2002; Binder and Scharfman, 2004; Zuccato and Cattaneo, 2009). α-MSH administration increases BDNF expression via activating MC3/4Rs in the rodent brain (Xu et al., 2003; Caruso et al., 2012). In primary cultures of rat astrocytes, α-MSH induced the expression of cAMP and increased downstream BDNF mRNA and protein levels (Caruso et al., 2012). Similarly, in the ventralmedial nucleus of the mouse hypothalamus, a potent MC3/4R agonist also increased BDNF expression (Xu et al., 2003). Blocking α-MSH G-protein coupled receptors and inhibiting downstream signaling abolishes the effect of α-MSH on BDNF expression (Caruso et al., 2012). Inhibition of MC4R using antagonist HS024 reduced downstream cAMP levels. Inhibitors of adenylate cyclase and PKA further downstream of MC4R activation prevented the increase in BDNF (Caruso et al., 2012). In addition to inducing BDNF expression, α-MSH has been shown to up-regulate pCREB in the rat hypothalamus (Sarkar et al., 2002). CREB is involved in many processes of the nervous system such as neuronal survival, neuroprotection, axonal growth and regeneration, synaptic plasticity and memory formation (Alberini, 2009). α-MSH confers neuroprotection by up-regulating BDNF and pCREB expression via receptor activation.
Figure 1.6. Neuroprotective effects of α-MSH. Through MC3/4 receptor activation, α-MSH up-regulates cAMP expression, phosphorylation of CREB and as a result, increases BDNF mRNA levels. α-MSH, α-melanocyte stimulating hormone; MC, melanocortin receptor; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; CREB, cAMP response element binding protein; CRE, cAMP response element.
In addition to increased neurotrophic support, α-MSH also induces neurogenesis. [Nle4, D-phe7]-α-MSH is a potent long-lasting synthetic analogue of α-MSH. When NDP-α-MSH was administered i.p. after cerebral ischemia in Mongolian gerbils, it decreased DNA fragmentation and induced neurogenesis. These effects were abolished by the MC4R antagonist HS024 (Giuliani et al., 2011). NDP-α-MSH treatment increased the expression of Wnt-3A and the early neuronal marker doublecortin in the hippocampus (Giuliani et al., 2011). Wnt-3 regulates adult hippocampal neurogenesis such that overexpression of Wnt-3 increases neurogenesis (Lie et al., 2005). As a result, the number of newborn neurons, BrdU+/NeuN+ cells, in the subgranular zone in the dentate gyrus of the hippocampus is significantly increased in NDP-α-MSH treated animals compared to animals in both the sham group and the ischemic group. To determine whether the newborn cells are functional, the intermediate early gene Zif268, which is expressed in activated neurons upon stimulation by behavioural testing, was used to label active neurons. There was an increase in proliferating cells that also express the intermediate early gene Zif268 post behavior testing, indicating the functional nature of these newborn cells (Giuliani et al., 2011). By activating MC3/4 receptors in the brain, α-MSH induces neuroprotective effects by increasing BDNF expression and CREB activation and increases neurogenesis.

### 1.7.6 α-MSH is Neuroprotective Against Insults to the Brain

α-MSH increases neuronal survival after traumatic insults to the brain. In a rat transient cerebral ischemia model, severe degeneration of hippocampal CA1 pyramidal cells was rescued after i.p. injections of α-MSH (Forslin Aronsson et al., 2006). Ischemia resulted in 47% pyramidal cell loss. However, after α-MSH treatment, the number of viable pyramidal cells increased from 53% to 89%, compared to 100% in sham control (Forslin Aronsson et al., 2006). Kainic acid excitotoxicity induces neurodegeneration in rats as shown by a significant reduction in the number of viable pyramidal cells in the CA1 region of the hippocampus (Forslin Aronsson et al., 2007). Systemic i.p. injection of α-MSH after kainic acid treatment increased the number of viable pyramidal cells in the CA1 region to that of saline injected control animals (Forslin Aronsson et al., 2007). In rats subjected to nigro-striatal hemi-transection, sensorimotor deficits in coordinated limb use and in limb reflexes developed (Zanoli et al., 1988). NDP-α-MSH administered intracerebroventricularly (i.c.v.) after transection improved sensorimotor deficits by
modulating striatal dopamine receptors (Zanoli et al., 1988). Cholinergic deficits are observed in both AD patients and AD mouse models. $\alpha$-MSH administered intraventricularly was shown to increase ACh turnover rate in the rat hippocampus, possibly by acting on melanocortin receptors in the hippocampus (Wood et al., 1979). Surgical transection of projections originating from either the cingulum, entorhinal cortex or septum to the hippocampus does not affect $\alpha$-MSH induced increase in ACh turnover rate (Wood et al., 1979). Overall, these studies show that the neuroprotective and anti-inflammatory actions of $\alpha$-MSH help to improve pathology in the brain.

### 1.7.7 Therapeutic Potential of $\alpha$-MSH

Increased neuronal survival and decreased neuroinflammation could help to ameliorate pathology in the AD brain and improve cognitive function. $\alpha$-MSH suppresses inflammation (Huang and Tatro, 2002; Taylor, 2005; Caruso et al., 2010), protects cells from apoptosis (Caruso et al., 2007) and induces CREB phosphorylation resulting in increased BDNF expression to promote cell survival (Sarkar et al., 2002; Xu et al., 2003; Caruso et al., 2012). At the same time, $\alpha$-MSH promotes a neurogenic niche to induce cell proliferation in the subgranular zone of the hippocampal dentate gyrus (Giuliani et al., 2011). There is chronic neuroinflammation and multiple neurotransmitter alterations in AD (Akiyama et al., 2000; Chen et al., 2011). Deficits are evident in the cholinergic system, the excitatory glutaminergic and inhibitory GABAergic systems including altered expression of many inhibitory neuropeptides (Beal et al., 1987). Although $\alpha$-MSH has many anti-inflammatory and neuroprotective effects in the brain, its potential role in preventing and/or repairing the deficits in these multiple affected neurotransmitter systems in AD has not been elucidated. To date, $\alpha$-MSH treatment has been shown to improve spatial memory in 3xTg AD mouse model overexpressing PS1/APP$_{\text{swe}}$/Tau transgenes (Giuliani et al., 2013). Prophylactic treatment with NDP-$\alpha$-MSH decreased cerebral A\text{B} load in the 3xTg mice, reduced oxidative stress, cytokine levels and apoptosis (Giuliani et al., 2013). These biochemical changes elicited by NDP-$\alpha$-MSH resulted in improved spatial memory as demonstrated by reduced escape latency in the Morris water maze in the 3xTg mice (Giuliani et al., 2013). Transient cerebral ischemia by carotid artery occlusion in Mongolian gerbils produced spatial memory deficits in the Morris water maze test measured by decreased escape latency to the hidden platform. NDP-$\alpha$-MSH administration also rescued spatial memory
deficits in this ischemia model by increasing the number of viable neurons in the hippocampus (Giuliani et al., 2006a; 2006b; 2007; 2011). The therapeutic potential of α-MSH remains to be determined in AD mouse models with well established Aβ and/or tau pathology and cognitive deficits, in a treatment paradigm more translatable to AD patients. α-MSH could mediate neuroprotection and decrease neuroinflammation in the brain, fostering a more suitable environment for the recovery of widespread synaptic dysfunction and deficits in multiple neurotransmitter systems to improve cognitive function in AD.

1.8 TgCRND8 Mouse Model of Alzheimer’s Disease

As previously mentioned, the TgCRND8 mouse model of AD expresses the human amyloid precursor protein 695 containing the Swedish and Indiana mutations under the control of the hamster prion promoter (Chishti et al., 2001). The Swedish mutation favors β-secretase cleavage of APP and the Indiana mutation increases Aβ42/Aβ40 ratio (Mucke et al., 2000). TgCRND8 is a rapid-onset transgenic model of AD such that Aβ plaques can be detected at 3 months of age (Chishti et al., 2001). In AD patients, Aβ deposition first appears in the frontal, parietal, temporal and occipital cortex followed by entorhinal cortex and hippocampus, then ultimately subcortical regions (Thal et al., 2002). In TgCRND8 mice, brain regional accumulation of Aβ is similar to AD patients where the cortex and hippocampus display high Aβ levels and plaque load and less Aβ deposition is observed in subcortical regions (Ma et al., 2011). TgCRND8 mice exhibit cognitive deficits as early as 2 months of age, which progresses with Aβ accumulation (Chishti et al., 2001, Francis et al., 2012a, Hyde et al., 2005). To study the therapeutic potential of α-MSH in AD, I used the transgenic mouse model, TgCRND8.

1.8.1 Behaviour Deficits in TgCRND8 Mice

TgCRND8 mice experience significantly decreased survival rate and lower body weight compared to NTgs (Chishti et al., 2001; Touma et al., 2004). Heath assessment at 7 weeks of age indicated that Tgs and NTgs are comparable in general health status, sensory functions, reflexes and motor abilities but Tgs are more hyperactive than NTg mice (Touma et al., 2004; Hyde et al.,
2005). Behaviourally, TgCRND8 mice exhibit impairment in novel object recognition at 8 weeks of age prior to Aβ plaque formation but Tg mice at this age were not impaired in spatial reference memory of the Morris water maze test (Francis et al., 2012a). Spatial memory impairment in TgCRND8 mice was apparent starting at 11 weeks of age in the reference version of the Morris water maze (Janus et al., 2000; Chishti et al., 2001). At 13 weeks of age, the open field test and elevated plus-maze test revealed no difference in exploratory and anxiety behaviour between Tg and NTg mice (Touma et al., 2004). At 20 weeks of age, TgCRND8 mice in enriched housing conditions display increased anxiety compared to NTg mice as measured by increased entries into the open arm of the elevated plus-maze (Görtz et al., 2008). With advanced plaque pathology at 6-8 months of age, deficits in hippocampal-dependent novel object recognition, spatial reference memory and working memory is observed in TgCRND8 mice compared to NTg s (Janus et al., 2000; Lovasic et al., 2005; McLaurin et al., 2006a; Francis et al., 2012a). In addition, TgCRND8 mice showed impairment in hippocampal-dependent contextual fear conditioning and trace cued fear conditioning tests (Greco et al., 2010). At approximately one year of age, TgCRND8 mice developed weaker taste aversion to saccharine paired with nausea compared to NTg mice in the hippocampal-independent taste aversion test (Janus et al., 2004). TgCRND8 mice did not show any differences in exploration of the novel arm in the Y-maze test either at a time of early Aβ plaque deposition (16-22 weeks) or at advanced plaque pathology (42-46 weeks) (Hyde et al., 2005). TgCRND8 mice exhibit many cognitive deficits in hippocampal-dependent memory tasks, which makes it a suitable model to determine the potential effects of α-MSH on memory.

1.8.2 Neuroinflammation in TgCRND8 Mice

TgCRND8 mice exhibit region-specific accumulation of Aβ where high Aβ levels and Aβ plaques are observed in regions affected by AD, the hippocampus and cortex (Ma et al., 2011). As Aβ plaques first appear at 3 months of age, microgliosis and astrogliosis is observed in the cortex and hippocampus, which becomes more widespread with age (Dudal et al., 2004). Aβ plaques in TgCRND8 mice are associated with focal inflammatory response of activated microglia that are CD11b and Iba-1 immunoreactive as well as high expression of GFAP immunoreactive astrocytes (Chishti et al., 2001; Dudal et al., 2004; McLaurin et al., 2006).
Chronic inflammation in AD is mediated by activated microglia and astrocytes that induce the expression of pro-inflammatory mediators such as cytokines, chemokines and nitric oxide, which contributes to neuronal dysfunction and cell death (Akiyama et al., 2000; Rubio-Perez and Morillas-Ruiz, 2012). At 7 months of age, as Aβ accumulates in the brain, significantly elevated IL-1β cytokine levels were detected in the cortex and the hippocampus (Ma et al., 2011). An increase in Aβ associated glial expression of TNF-α and iNOS in TgCRND8 mice at 7 months of age has also been reported (Luccarini et al., 2012). The elevated expression of cytokines may be a result of increased levels of the upstream transcription factor NF-kB (Luccarini et al., 2012). The expression of iNOS, a marker of nitric oxide production, is found in neurons and astrocytes in the TgCRND8 brain, especially in regions of high Aβ plaque load such as the hippocampus and cortex (Bellucci et al., 2006; Luccarini et al., 2012). By 9 months of age, there is a significant increase in the chemokine CXCL1 levels in the hippocampus (Ma et al., 2011). In addition to increased neuroinflammation, expression of the pro-apoptotic protein Bax is increased whereas levels of the anti-apoptotic protein bcl-2 is decreased (Luccarini et al., 2012). A high Bax/bcl-2 ratio could result in a higher rate of apoptosis in the hippocampus and the cortex. Inflammation is evident in the TgCRND8 brain and activated glia as well as inflammatory mediators coincide temporally and spatially with Aβ deposition.

1.8.3 Neuronal Dysfunction in TgCRND8 Mice

In this APP overexpressing mouse model, Aβ accumulation and aggregation results in neuronal dysfunction. At 5 months of age, TgCRND8 mice exhibit reduced hippocampal neurogenesis in the subgranular zone of the dentate gyrus. The number of newborn NeuN+ neurons are decreased in TgCRND8 mice compared to NTg mice (Herring et al., 2009). At this age, protein levels of the pre-synaptic marker synaptophysin are also significantly decreased in Tg mice (Herring et al., 2009). These results suggest decreased neurogenesis and synaptogenesis in TgCRND8 mice with moderate Aβ plaque deposition.

In 7 month-old TgCRND8 mice, the number and size of ChAT+ cholinergic cell bodies in the nucleus basalis magnocellularis (NBM) is significantly decreased compared to NTgs (Bellucci et al., 2006). Cholinergic neurons in the NBM project to the cortex. As a functional measure of the
NBM cholinergic neurons, ACh release in the prefrontal cortex was evaluated. Extracellular basal ACh levels in the TgCRND8 mice was significantly reduced compared to NTgs (Bellucci et al., 2006). In addition to cholinergic neuronal dysfunction, cholinergic muscarinic 2 receptor immunoreactivity was reduced in the motor cortex of 7 month-old TgCRND8 mice (Bellucci et al., 2006). At approximately 7.5-9 months of age, TgCRND8 mice display a similar percentage of α-internexin+ dystrophic neurites as observed in AD patients (Woodhouse et al., 2009). However, TgCRND8 mice do not exhibit any classical hyperphosphorylated-tau dystrophic neurites found in AD patients (Woodhouse et al., 2009). In aged TgCRND8 mice with plaque levels comparable to end-stage AD in humans, this mouse model exhibits cholinergic deficits and dystrophic neurites.

Possibly due to the synaptic and neuronal deficits, the function of neurons is impaired in TgCRND8 mice. Activity-regulated cytoskeleton-associated protein (Arc) is an intermediate early gene that is required for memory consolidation (Plath et al., 2006; Shepherd and Bear, 2011). Arc expression is induced by spatial exploration of novel environments and it is used as a measure of neuronal activation (Guzowski et al., 1999). In TgCRND8 mice, basal protein levels of Arc are decreased in the hippocampus of TgCRND8 mice compared to NTgs at 5 months of age (Herring et al., 2009). The number of Arc+ cells in the CA1 region of the dorsal hippocampus was significantly decreased compared to NTgs after Morris water maze testing of spatial memory (Yiu et al., 2011). These results demonstrate that neuronal activation in TgCRND8 mice is impaired.

Beside Aβ-induced neurotoxicity, neuronal dysfunction may also be partly explained by the loss of neurotrophic support. In TgCRND8 mice of approximately 3 months of age, neuronal expression of pCREB in the CA1 region of the dorsal hippocampus is significantly decreased compared to NTgs (Yiu et al., 2011). TgCRND8 mice show reduced pCREB expression whether taken from the home cage, after exposure to a novel environment or after behavioural stimulation (Yiu et al., 2011). However pCREB expression is not different between Tg and NTg mice in the CA3 or the dentate gyrus of the dorsal hippocampus. Total CREB levels are comparable between Tg and NTg mice in the hippocampus (Yiu et al., 2011). The reduced expression of pCREB in the TgCRND8 brain contributes to the loss of neurotrophic support to promote neuronal survival and synaptogenesis.
In addition to decreased pCREB levels, expression of the neurotrophin BDNF is also altered in TgCRND8 mice. At different ages and stages of disease progression, total BDNF mRNA levels fluctuate in the hippocampus and cortex of the TgCRND8 mouse brain. In the hippocampus, BDNF mRNA was significantly decreased at 8 weeks of age in TgCRND8 mouse compared to NTgs (Francis et al., 2012a). However, by 9-10 weeks of age, BDNF mRNA levels are not different between TgCRND8 and NTgs (Francis et al., 2012a). At 16 weeks, 8 months and 12 months of age, BDNF levels are significantly decreased in TgCRND8s (Francis et al., 2012a; 2012b). Total BDNF levels in the cortex are comparable between TgCRND8 mice and NTgs at 6 weeks of age but are significantly decreased in TgCRND8 mice compared to NTgs at 9 weeks of age (Francis et al., 2012a). At 11.4 months of age, TgCRND8 show significantly decreased cortical mRNA levels of total BDNF compared to NTg mice. Since multiple promoters control the expression of BDNF, analysis of different transcripts in TgCRND8 revealed a significant decrease of transcripts III and IV mRNA levels (Peng et al., 2009). In addition to reduced pCREB expression, the decrease in BDNF mRNA levels in the brain further deprives neurons of neurotrophic support, contributing to dysfunctional synapses and neurons.

Using Aβ as a driving force to induce AD-like neuropathology and cognitive dysfunction in TgCRND8 mice, this mouse model exhibits synaptic/neuronal deficits, an imbalance of excitatory/inhibitory inputs due to GABAergic deficits and loss of neurotrophic support. α-MSH has been shown to be neuroprotective and improve memory in stroke models. This thesis work focuses on the neuroprotective effect of α-MSH, particularly on the GABAergic neuronal system, to determine whether protecting GABAergic interneurons would restore physiological network activity in the brain and ultimately improve cognitive function.
Chapter 2
Rationale, Hypothesis and Objectives

2.1 Rationale

Recent studies on AD indicate that disruption of the balance between excitatory glutamatergic input and inhibitory GABAergic input in the hippocampus contribute to cognitive deficits. Both mouse models of AD and rat models of ageing exhibit GABAergic cell loss, including subtypes of GABAergic interneurons that express the neuropeptides SST and NPY. α-MSH has many neuroprotective effects such as up-regulating CREB phosphorylation and BDNF expression. CREB and BDNF are involved in synaptic plasticity, axonal growth and regeneration, neuronal survival, learning and memory formation. α-MSH has been shown to promote neuronal survival and improve memory against insults to the brain. However, α-MSH levels are reduced in the brain and CSF of AD patients. Therefore, I investigated whether chronic α-MSH treatment would have a neuroprotective effect in AD mouse models and promote the survival of GABAergic interneurons in the hippocampus to improve memory. As a model of AD, TgCRND8 mice with known GABAergic deficits and cognitive dysfunction were utilized.

2.2 Hypothesis

α-MSH confers neuroprotection to ameliorate cognitive deficits in TgCRND8 mice

2.3 Objectives

1. To determine the effect of α-MSH on hippocampal-dependent tasks such as the Y-maze, fear conditioning and nesting in TgCRND8 mice as well as any treatment-dependent effects in anxiety and locomotion.

2. To determine the effect of α-MSH on Aβ load in TgCRND8 mouse brains, including insoluble and soluble levels of Aβ40 and Aβ42 and Aβ plaque load.

3. To elucidate the neuroprotective mechanism of α-MSH action.
Chapter 3
Methods

Portions of this section have been previously published in:


3.1 Animals

APP transgenic mouse model of Alzheimer’s disease, TgCRND8 mice, harbouring the human Swedish (KM670.671NL) and Indiana (V717F) APP mutations were used in this study. Using the Syrian hamster prion promoter, transgene expression targets neurons in the central nervous system and astrocytes to a much lesser extent. Mice were maintained on an outbred C3H/C57BL6 background and were kept on a 12 hour light/dark cycle with food and water ad libitum. All experiments were performed in accordance with Canadian Council for Animal Care and University of Toronto guidelines.

3.2 Drug Treatment

TgCRND8 and NTg mice were evenly divided per litter and by gender into α-MSH treated or saline vehicle groups. α-MSH, supplied as a trifluoroacetate salt (Bachem, H-1075) was dissolved in sterile saline at a concentration of 0.2mg/ml. Mice received daily i.p. injections of 0.5mg/kg of α-MSH or saline with injection volumes ranging between 50-120µl depending on body weight. Drug treatment was initiated when mice were 20 weeks of age for 28 days and were sacrificed at 24 weeks of age (Figure 3.1). Animals were anesthetized with pentobarbital and exsanguinated by transcardial perfusion with PBS + heplean. Brain tissues were either dissected on ice and flash frozen in dry ice for biochemical analyses or animals were transcardially fix-perfused with Zamboni fixative (2% paraformaldehyde, 15% picric acid, 50% 0.2M sodium phosphate buffer pH7.4, 22.5% distilled water), sectioned using a freezing microtome at 40 µm thickness and stored at -20°C in cryoprotectant (20-25% glycerin, 30% ethylene glycol and 0.1M PO₄).
Figure 3.1. Drug treatment paradigm. All animals were treated for 4 weeks between 20 and 24 weeks of age via *i.p.* injections of either α-MSH or vehicle. The Y-maze test and the open field test were performed before commencing treatment and at the end of treatment. Fear conditioning test, nesting and novel environment exploration were performed at the end of the treatment period. Animals were sacrificed at 24 weeks and tissues were harvested.

3.3 Behavioural Tests

All animals were handled for 3 days prior to behavioural testing and all mice were acclimatized to the testing room 5 min prior to each test session. Testing apparatus were cleaned with 70% ethanol before testing each mouse. The Open Field Test followed by the Y-Maze Test were performed on three separate groups of animals 2-3 days prior to drug administration and on the last 2-3 days of drug administration before animal sacrifice (Figure 3.1). Using a fourth group of animals, nesting behaviour analysis followed by Fear Conditioning Test were performed during the last 2 days of drug administration. On the day of animal sacrifice, novel environment exploration was performed one hour prior to sacrifice.
3.3.1 Open Field Test

Up to 3 mice were placed in 3 separate rat cages (25cmW x 47cmW x 19cmH) simultaneously to freely explore for 10 min. Exploratory behaviour was recorded using Logitech webcam Pro9000 and analyzed using Viewpoint VideoTrack version 3.0. Parameters analyzed in the open field test include percentage of time spent and distance travelled in the centre of the field as well as total distance travelled in the open field, duration of inactivity and number of rotations (circling behaviour), n = 12-23 per treatment group.

3.3.2 Y-Maze Test

The novel arm of the Y-maze was blocked and the mouse was allowed to freely explore the two remaining arms for 10 min. The mouse was then taken back to the colony room for 90 min. After the 90 min inter-trial interval, the novel arm was opened and the mouse was allowed to freely explore all three arms for 5 min. In this forced-trial version of the Y-maze, mouse exploratory behaviour was recorded using Logitech webcam and novel arm exploration and spontaneous alternation was analyzed using Viewpoint VideoTrack version 3.0. n = 13-21 per treatment group.

Exploratory activity in the novel arm (# of entries, duration and distance traveled) was calculated using the formula:

\[
\% \text{ novel arm} = \left( \frac{\text{novel arm}}{\text{novel arm} + \text{arm 2} + \text{arm 3}} \right) \times 100
\]

Spontaneous alternation was calculated using the formula:

\[
\% \text{ alternation} = \left( \frac{\text{number of alternations}}{\text{total number of arms entries} - 2} \right) \times 100
\]

3.3.3 Nest Building

Singly housed mice with food and water *ad libitum* were given bedding and a 5x5cm cotton Nestlet in their cage for nesting (n = 5-8 per treatment group). Nest building progress was
assessed at 1h, 3hrs and 6hrs after commercially available cotton Nestlets were provided. Pictures were taken at each time point and the nest was rated on a scale of 1-5 according to protocol developed by Deacon (Deacon, 2006) by a blind observer.

3.3.4 Fear Conditioning

Each mouse was habituated in the testing room for 5 min prior to each training or testing session and testing chambers were cleaned with 70% EtOH before each use. Two Habitest mouse test cages with a grid shock floor (Coulbourn Instruments) from Centre for Biological Timing and Cognition Department of Psychology, University of Toronto, were used as testing chambers. Animal behaviour was recorded whenever the mouse was in the chamber for analysis using ANYmaze.

Day 1 – training
One mouse at a time was placed in a vanilla scented chamber and freely explored the chamber for 2 min. Then a tone cue was presented for 30s. Three seconds after the end of tone, a foot shock of 0.5mA was given for 2s. The 3s time gap between the tone and shock forces the mouse to associate the tone with the shock, thus this trace cued fear conditioning test paradigm would involve both the hippocampus and amygdala brain regions to successfully complete the task. Five tone-shock pairings were administered with inter-trial gap periods of different times 100s, 90s, 120s and 110s to eliminate anticipation.

Day 2 – testing
Contextual conditioning testing was performed at the same time of the day as on the training day for every mouse. Each animal was placed for 5min in the same vanilla scented chamber as was performed on training day. Mouse activity was recorded using Logitech webcam and the ANYmaze software was used to analyze freezing and mobility behaviours.

Trace cued fear conditioning testing was performed 1h post contextual testing for every mouse. In a separate lemon scented chamber with altered walls, each animal was placed in the chamber for 3min to habituate, then the animal is presented with the same tone cue as in training for 3min continuously. Mouse activity was recorded using Logitech webcam and the ANYmaze software was used to analyze freezing and mobility behaviours.
3.3.5 Novel Environment Exploration

Using the Tru Scan rat arena (60cm x 60cm, Coulbourn Instruments) from the Centre for Biological Timing and Cognition, Department of Psychology, University of Toronto, with spatial cues set up on the outside of transparent arena walls, each mouse was allowed to explore for 5min. Following the protocol by Guzowski et al., 1999, the arena was divided into 9 square sections. During the 5min exploration, each mouse was randomly moved to the centre of one of the 9 squares every 10s, at least 3 times per square, to ensure that all mice sampled the entire environment. All mice were sacrificed and fix-perfused with Zamboni fixative one hour post novel environment exploration for detection of intermediate early gene expression in activated neurons.

3.4 Aβ ELISA

Aβ concentrations were examined in the hippocampus and cortex of TgCRND8 mice administered either α-MSH or vehicle. All tissues were homogenized in sucrose buffer (20 mM Tris pH 7.4, 0.25 M sucrose, 1 mM EDTA, and 1 mM EGTA). Total Aβ was extracted using 70% formic acid and sonicated on ice for 2 x 5s on power setting 3. Soluble Aβ was extracted using 0.4% diethylamine and 100 mM NaCl. All samples were centrifuged at 100,000 x g for 1h at 4°C followed by neutralization of the supernatant. The total Aβ fraction was neutralized with 1 M Tris and 0.5 M Na₂HPO₄ (19x supernatant volume) and the soluble fraction was neutralized with 0.5 M Tris (pH 6.8) (0.1x supernatant volume). Using commercially available sandwich ELISAs (Invitrogen), concentrations of formic acid extracted total Aβ42 and Aβ40 and diethylamine extracted soluble Aβ42 and Aβ40 were determined against a standard concentration curve and values were normalized to protein concentration in the sucrose buffer. Insoluble Aβ concentrations were determined by subtracting soluble Aβ levels from total Aβ levels. Standards and samples were run in triplicate.
3.5 Immunohistochemistry

Brain sections 40 µm thick from 5-11 animals per treatment group were used for immunohistochemistry. For each antibody, 4-6 sections per animal from the same Bregma point were stained and quantification was done on both hippocampi per section. All sections were treated with 0.3%-0.6% H₂O₂ and blocked with 7.5% donkey serum in PBS+0.3% triton X-100. Aβ plaque staining using 6F/3D antibody required an additional antigen retrieval step using 70% formic acid for 5 min. Primary and secondary antibodies were diluted in 7.5% donkey serum in PBS+0.3% triton X-100. Primary antibodies used were mouse anti-human 6F/3D IgG1κ (1:400, Dako), rabbit anti-Arc IgG2a (1:50, Santa Cruz), mouse anti-GAD67 IgG2a (1:1000, Millipore), rat anti-SST IgG2b (1:400, Millipore) and goat anti-ChAT IgGs (1:400, Millipore). Secondary antibodies used were biotinylated horse anti-mouse IgG (H+L) (1:400, Vector), biotinylated goat anti-rabbit IgG (H+L) (1:400, Vector), biotinylated rabbit mouse adsorbed anti-rat IgG (H+L) (1:100, Vector) and biotinylated donkey anti-goat IgG (H+L) (1:200, Vector). Vector ABC kit was used to amplify antigen signal and the staining was visualized with 3, 3′-diaminobenzidine and nickel chloride (Vector). Images of all sections were taken using Leica DMI3000 inverted microscope and quantified using ImageJ. Quantification of Aβ plaques, Arc and ChAT staining were done using appropriate threshold and particle size settings on ImageJ and GAD67+ and SST+ cell quantification were done manually by a blind observer using the cell counter plugin in ImageJ.

3.6 Immunofluorescence

Brain sections of 40 µm thick were blocked with 7.5% donkey serum in PBS+0.3% triton X-100 and incubated in primary and secondary antibodies diluted in 7.5% donkey serum in PBS+0.3% triton. Dilutions for Millipore primary antibodies mouse anti-GAD67 IgG2a and rat anti-SST IgG2b were 1:400 and 1:200, respectively. Alexa Fluor conjugated secondary antibodies goat anti-mouse IgG (H+L) conjugated to Alexa Fluor 488 and goat anti-rat IgG (H+L) conjugated to Alexa Fluor 594 were used for antigen visualization. Representative images were taken with Leica TSC SP5 confocal microscope.
3.7 Immunoblotting

All hippocampal tissues (n = 6-7 per treatment group) were homogenized in RIPA buffer (150mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris pH 8.0 and protease inhibitors) with the exception of lysis buffer used for CREB western blots, which consisted of 50mM Tris, 0.25M sucrose, 25mM KCL, 5mM MgCl₂, protease and phosphatase inhibitors (Yiu et al., 2011). Sample protein concentration was determined using BCA kit from Pierce. All samples were denatured and reduced by adding β-mercaptoethanol to the sample buffer and boiling samples for 5 min. Samples were then separated on a 4-12% Bis-tris gel and transferred using iBlot (Life Technologies) onto nitrocellulose membranes. Membranes were blocked with 5% milk or 1% bovine serum albumin in TBST (0.02% Tween 20). Primary and secondary antibodies were diluted in 5% milk or 1% bovine serum albumin in TBST and membranes were developed with enhanced chemiluminescence (GE Healthcare). Primary antibodies used were mouse anti-GAD67 IgG2a (1:7500, Millipore), rabbit anti-GAD65 IgGs (1:1000, Millipore), rabbit anti-pCREB(ser133) IgG (1:1000, Millipore), mouse anti-CREB IgG1 (1:2000, Millipore), mouse anti-synaptophysin IgG1 (1:5000, BD Biosciences), rabbit anti-dynamin 1 IgGs (1:1000, Affinity BioReagents), mouse anti-PSD95 IgG1κ (1:2000, Affinity BioReagents) rabbit anti-bcl-2 IgG (1:1000, Abcam), mouse anti-gephryin IgG1κ (1:2000, Millipore). Secondary antibodies used were goat anti-mouse IgG (H+L) (1:5000, Thermo Sci) and goat anti-rabbit IgGs (1:2000, Santa Cruz). Optical intensity of target proteins and their respective protein loading control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were measured using ImageJ.

3.8 Quantitative Real-time PCR

Hippocampal RNA was isolated using Aurum Total RNA Fatty and Fibrous Tissue Pack from Bio-Rad (n = 6-7 per treatment group). Isolated RNA was DNase treated to remove genomic DNA contaminants. The purity and integrity of the RNA were verified using absorbance wavelength 260/280 and 260/230 ratios and by running RNA samples on an agarose gel. cDNA from the RNA was made using the Bio-Rad kit iScript Reverse Transcription Supermix for RT-qPCR. Primers for BDNF, β-actin (Francis et al., 2012a; 2012b) and BDNF transcripts I, IV and
VI (Peng et al., 2009) were previously published in the literature and designed by Dr. Margaret Fahnestock’s laboratory. All other primer pairs were designed using Beacon Designer and only primer pairs with efficiencies 90-110% and no secondary products as verified by dissociation curves were used. Real-time PCR was run on the Bio-Rad CFX384 Touch real-time PCR detection system using 12µL reaction volume per sample including forward and reverse primers, the SsoAdvanced SYBR Green Supermix and sample cDNA from 10ng of RNA was diluted in 5µL volume. Data was analyzed with CFX Manager as ratios of target gene/β-actin. A reliable housekeeping gene is critical for data normalization in qPCR. Of all the housekeeping genes tested, β-actin, TATA, GAPDH, S18 and tyrosine 3-monooxygenase (YWAHZ), β-actin expression was the most consistent across genotype and α-MSH treatment using n = 7 per group. Therefore, β-actin was chosen as the housekeeping gene. Table 3.1 lists the primer pairs used in this study. All unknown samples, no-RT, and no-template controls were run in triplicate. The thermal profile used for all measurements are as follows: 2 min at 95°C, 40 cycles of 95°C for 5s and 60°C for 10s.
<table>
<thead>
<tr>
<th>Primers</th>
<th>Forward 5’ to 3’</th>
<th>Reverse 5’ to 3’</th>
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</thead>
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<tr>
<td>BDNF</td>
<td>GCGGCGAGATAAAAAGACTGC</td>
<td>CTTATGAATCGCCAGCCAAT</td>
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<td>BDNF transcript I</td>
<td>AGTCTCCAGGACAGCAAAAGC</td>
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<td>BDNF transcript IV</td>
<td>AGAGGCAGCTGCCTTGATGTT</td>
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<tr>
<td>BDNF transcript VI</td>
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<tr>
<td>NGF</td>
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<td>GCAAGTCAGCCTCTTCTT</td>
</tr>
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<td>TrkA</td>
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<td>TTCACATCAGCCAGAAC</td>
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<tr>
<td>GABA&lt;sub&gt;4&lt;/sub&gt; receptor subunit α1</td>
<td>AGTGGTTGTCGAGAAGATG</td>
<td>AGTGGAAGTGAGTCGTCATAAC</td>
</tr>
<tr>
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<td>GACAACACTCACATG</td>
<td>ATAGCAGACAGCAATGAAC</td>
</tr>
<tr>
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<tr>
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<tr>
<td>VIP</td>
<td>CAGAAGCAAGCCTCAGTT</td>
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</tr>
<tr>
<td>β-actin</td>
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</tr>
<tr>
<td>YWAHZ</td>
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</tr>
<tr>
<td>GAPDH</td>
<td>AAGAAGGTTGGTAAGCAGGCACTC</td>
<td>CGAAGGTTGGAAAGAGTGGGAGTT</td>
</tr>
</tbody>
</table>

**Table 3.1.** Primer pairs used in quantitative real-time PCR.
3.9 Statistical Analyses

For the Y-maze and open field tests, measurements were taken pre-treatment and post-treatment. The change between pre- and post-treatment was calculated for each mouse and the resulting data sets were analyzed using two-way ANOVAs with treatment and genotype as ‘between-subject’ factors and significance set at $P < 0.05$. In the nest building behaviour test, data were analyzed using repeated measures ANOVA with significance set at $P < 0.05$. Behavioral data from either pre-treatment or post-treatment alone and data from quantitative real-time PCR, western blot and immunohistochemistry were analyzed using one-way ANOVAs with Fisher’s LSD post hoc test. Aβ ELISA and Aβ plaque data were analyzed using Student’s t-test. All statistical analyses were done using IBM SPSS Statistics 20.
Portions of this section have been previously published in:


α-MSH is a neuropeptide derived from the precursor pituitary hormone POMC. In AD patients, α-MSH levels are down-regulated in the brain and CSF (Arai et al., 1986; Rainero et al., 1988). α-MSH autoantibody levels are also correlated with cognitive function (Costa et al., 2011). α-MSH exhibits many neuroprotective effects via activation of its CNS G-protein coupled receptors that could be beneficial in treating AD (Sarkar et al., 2002; Xu et al., 2003; Giuliani et al., 2011; Caruso et al., 2012). Both CREB phosphorylation and BDNF expression are involved in neuronal survival, synaptic plasticity and memory formation (Tyler et al., 2002; Binder and Scharfman, 2004; Alberini, 2009; Zuccato and Cattaneo, 2009). It has been shown that α-MSH treatment increases both CREB phosphorylation and BDNF expression (Sarkar et al., 2002; Xu et al., 2003; Caruso et al., 2012). On the cellular level, insults to the brain such as transient cerebral ischemia and drug induced excitotoxicity resulted in a decrease in the number of neurons (Forslin Aronsson et al., 2006; 2007). α-MSH treatment increased the number of viable neurons in these experimental paradigms (Forslin Aronsson et al., 2006; 2007). Moreover, NDP-α-MSH, a potent synthetic analogue of α-MSH, improved sensorimotor and spatial memory deficits in animal models of brain lesion and in one transgenic mouse model of AD (Zanoli et al., 1988; Giuliani et al., 2006a; 2013). A triple transgenic mouse model of AD (PS1/APP/tau) was used to determine the efficacy of NDP-α-MSH in a prophylactic paradigm where treatment was initiated prior to Aβ and tau pathology (Giuliani et al., 2013). NDP-α-MSH treatment decreased Aβ deposits, tau phosphorylation and reduced oxidative stress, cytokine levels and expression of apoptosis effector protein caspase-3 (Giuliani et al., 2013). However, neurotrophic effects of α-MSH on neuronal survival in relation to memory were not examined. In this study, α-MSH was used instead of the potent NDP-α-MSH in order to decrease the risk of receptor desensitization.
and the occurrence of adverse drug reaction in a chronic treatment paradigm. To determine the neuroprotective potential of α-MSH in treating AD, the effect of α-MSH on behavioural deficits in TgCRND8 mice was first assessed, followed by examination of the neuroprotective effects of α-MSH.

4.1 α-MSH Preserves Spatial Memory in TgCRND8 Mice

To determine whether α-MSH treatment preserves cognitive function in the TgCRND8 mice, TgCRND8 and NTg mice were randomly divided into four sex-balanced groups where each genotype was administered either α-MSH or vehicle, n = 10-23 per group. Aβ plaques appear as early as 3 months of age in the TgCRND8 mouse model (Chishti et al., 2001) and cognitive deficits develop starting at 8 weeks of age (Chishti et al., 2001; Francis et al., 2010; Hyde et al., 2005; Janus et al., 2004). α-MSH or vehicle treatment was initiated therapeutically at 20 weeks of age, when Aβ pathology and behavioural deficits were well established in TgCRND8 mice, and continued for 4 weeks until 24 weeks of age. This therapeutic paradigm was used in an attempt to closely parallel translation to human AD patients. The Y-maze spontaneous alternation test and the reference memory version of the Morris water maze test show comparable sensitivity in detecting spatial memory deficits (Stewart et al., 2011). TgCRND8 mice show deficits in the reference memory version of the Morris water maze at 11 weeks of age (Chishti et al., 2001) whereas my results show a significant decline in the Y-maze spontaneous alternation test between 20 and 24 weeks of age, the treatment period in this study (Figure 4.1A). Therefore, the Y-maze test was chosen to assess the effectiveness of α-MSH in preserving spatial memory in TgCRND8 mice. The spontaneous alternation analysis of the Y-Maze utilizes the mouse’s natural tendency to visit less recently explored arm so that a high percentage alternation score relies on the ability to recall which arms were last visited (Sharma et al., 2010; Lalonde 2002). The forced-trial Y-maze spontaneous alternation paradigm was used instead of the free-trial version to increase the alternation rate and to diminish any spatial bias of tendency to make left turns or right turns (Lalonde 2002). TgCRND8 mice and NTg were tested in the Y-Maze both pre-treatment and post-treatment to assess mouse-specific responses.
TgCRND8 mice exhibit a decrease in spontaneous alternation at 24 weeks of age whereas α-MSH treatment improved the spatial memory deficit. Two-way ANOVA analysis of the change in percentage alternation between pre- and post-treatment showed a significant interaction between α-MSH treatment and genotype ($F_{(1, 61)} = 4.62, P = 0.036$; Figure 4.1A). α-MSH treatment significantly improved percentage alternation in TgCRND8 mice ($P < 0.001$) with no effect on NTg mice. At 20 weeks of age, prior to treatment, TgCRND8 mice did not show a significant decrease in percentage alternation suggesting intact spatial memory in the Y-maze task (Figure 4.1B). However by 24 weeks of age, TgCRND8 vehicle treated mice showed a significant decrease in percentage alternation indicating deficits in spatial memory (Figure 4.1C). Four weeks of α-MSH treatment preserved spatial memory in TgCRND8 mice (Figure 4.1A and C).

To determine whether α-MSH affects exploratory behavior, novel arm exploration in the Y-maze was used to test preference for novelty and place discrimination by exploration of the arm not available during habituation (Dellu et al., 2000). In this test, the change between pre-treatment and post-treatment showed no significant main effect of genotype or drug treatment as demonstrated in the amount of time spent in the novel arm ($F_{(1,61)} = 0.29, P = 0.595$; Figure 4.2D), the number of novel arm entries ($F_{(1,61)} = 0.001, P = 0.974$) or distance traveled in the novel arm ($F_{(1,61)} = 0.54, P = 0.466$). TgCRND8 mice did not show a deficit in novel arm exploration compared to NTg either at 20 weeks pre-treatment ($P = 0.53$) or at 24 weeks post-treatment ($P = 0.88$; Figure 4.1E and F). These results are consistent with a previous report that TgCRND8 mice did not show changes in percentage time spent in novel arm compared to NTg mice (Hyde et al., 2005).
**Figure 4.1.** Effect of α-MSH on the performance of TgCRND8 mice in the Y-maze test.

Spontaneous alternation analysis of the Y-maze was used to assess the spatial memory of mice. The change in percentage alternation between pre- and post-treatment shows a significant α-MSH treatment x genotype interaction (A, $F_{(1,61)} = 4.62, P = 0.036$). Pre-treatment at 20 weeks of age TgCRND8 mice did not show a significant deficit in spatial memory (B), however, at 24 weeks of age TgCRND8 vehicle treated mice showed a significant decrease in percentage alternation (C). α-MSH treatment improved spatial memory in TgCRND8 mice ($P < 0.001$) with no effect on NTg mice ($P = 0.786; A$ and C). Novel arm exploration analysis of the Y-maze did not reveal a significant interaction between α-MSH treatment and genotype in the amount of time spent in the novel arm (D, $F_{(1,61)} = 0.29, P = 0.595$). TgCRND8 mice did not show a deficit in novel arm exploration either at 20 weeks or 24 weeks of age (E, F). NTg ctrl = NTg vehicle treated, NTg α-MSH = NTg α-MSH treated, Tg ctrl = Tg vehicle treated, Tg α-MSH = Tg α-MSH treated. Data represent mean ± SEM, $n = 10-23$ per group, $* P < 0.05$. 
4.2 $\alpha$-MSH Maintains Normal Anxiety Levels in TgCRND8 Mice

Decreased anxiety has been previously shown in the TgCRND8 mice in comparison to NTgs after housing in an enriched environment using the elevated plus maze test (Gortz et al., 2008). In agreement, Tg2576 mice that overexpress APP$_{Swe}$ also exhibit less anxiety with increasing Aβ load (Ognibene et al., 2005). In the open field test, exploration of the centre of the field versus the periphery of the field was analyzed as a measure of anxiety because anxious mice avoid open spaces and prefer the protection of walls (Prut et al., 2003). Two-way ANOVA analysis of centre field activity between pre-treatment and post-treatment demonstrated a significant drug and genotype interaction in the amount of the time spent ($n = 10-23$ per group; $F_{(1,68)} = 6.24, P = 0.015$; Figure 4.2A) and the distance traveled in the centre ($F_{(1,68)} = 5.89, P = 0.018$). As previously shown, vehicle treated TgCRND8 mice became less anxious with age compared to NTgs with significant main genotype effects of time spent in the centre and distance traveled within the centre ($P = 0.035$ and $P = 0.029$ respectively; Figure 4.2B and C). $\alpha$-MSH treatment prevented the change in anxiety in TgCRND8 mice using the amount of time spent ($P = 0.008$) and distance traveled in the centre ($P = 0.002$) as outcome measures. Our results demonstrate that $\alpha$-MSH treatment maintained normal anxiety levels in the TgCRND8 mice, which was not statistically different from NTg mice (Figure 4.2A to C).

4.3 Locomotion is not Affected by $\alpha$-MSH

To investigate whether the change in anxiety is confounded by changes in locomotion, we further examined parameters of the open field test. TgCRND8 mice showed a significant increase in locomotion compared to NTgs as assessed by the total distance traveled, duration of inactivity (movement of < 1cm/s) and number of rotations (circling behavior). Two-way ANOVA analysis of changes in all three measurements between pre- and post-treatment showed significant genotype effect where TgCRND8 mice are more hyperactive compared to NTgs ($P < 0.001$). There was no significant drug and genotype interaction in total distance ($F_{(1,68)} = 0.02, P = 0.887$; Figure 4.2D), inactive duration ($F_{(1,68)} = 0.91, P = 0.344$) and rotation ($F_{(1,68)} = 1.52, P = 0.222$). Regardless of age and $\alpha$-MSH treatment, TgCRND8 mice are more hyperactive than NTgs and suggest that locomotion did not affect measures of anxiety (Figure 4.2D to F).
Figure 4.2. Performance of TgCRND8 mice in the open field test as a function of α-MSH treatment. In the open field test, there is a significant interaction between α-MSH treatment and genotype ($F_{(1,68)} = 6.24, P = 0.015$; A). TgCRND8 vehicle treated mice showed a significant decrease in anxiety from 20 to 24 weeks of age and α-MSH treatment prevented this change ($P = 0.008$; B and C). Locomotion of mice was also measured in the open field test. There is no significant interaction between α-MSH treatment and genotype in the total distance traveled ($F_{(1,68)} = 0.02, P = 0.887$; D). TgCRND8 mice, both vehicle and α-MSH treated are more hyperactive compared to NTg mice ($P < 0.001$; E and F). NTg ctrl = NTg vehicle treated, NTg α-MSH = NTg α-MSH treated, Tg ctrl = Tg vehicle treated, Tg α-MSH = Tg α-MSH treated. Data represent mean ± SEM, n = 10-23 per group, * $P < 0.05$. 
4.4 Contextual or Trace Cued Fear Conditioning is not Affected by $\alpha$-MSH

Contextual fear conditioning involves presenting the mouse with the unconditioned stimulus of a foot shock in a new environment. Learning the context of the unconditioned stimulus in this fear conditioning paradigm requires input from the hippocampus and the amygdala (Bucafasco et al., 2009). In the trace cued fear conditioning paradigm, a conditioned stimulus of a tone cue is presented to the mouse followed by a short time interval before the unconditioned stimulus of the foot shock is presented. Learning of the tone-shock association is dependent on the hippocampus (Bangasser et al., 2006; Bucafasco et al., 2009). To assess the hippocampal function of TgCRND8 mice and the effect of $\alpha$-MSH treatment, freezing behaviour was determined as an outcome measure in both fear conditioning paradigms. Freezing behaviour was quantified by the amount of time spent freezing as a percentage of the total duration of the test. Data was analyzed using repeated measures ANOVA to determine any differences between treatment groups over repeated trials or over a time-course. During training, TgCRND8 showed significantly less freezing in repeated trials compared to NTgs with a genotype effect of $P = 0.002$ (Figure 4.3A). In contextual conditioning and trace conditioning, TgCRND8 also showed significantly less freezing than NTg mice with genotype effects $P = 0.003$ and $P = 0.004$ respectively (Figure 4.3B and C). $\alpha$-MSH does not have a significantly effect on the freezing behaviour of TgCRND8 mice (Fig 4.3A to C). However, TgCRND8 mice respond to foot shock and the tone cue by increased running or jumping in the testing chamber with very limited freezing behaviour whereas NTg mice respond by freezing. When the mobility of mice was measured using distance traveled, although not significant, $\alpha$-MSH treated TgCRND8 mice traveled more distance compared to TgCRND8 vehicle mice in the contextual fear conditioning test ($P = 0.103$; Figure 4.3D). In conclusion, fear conditioning may not be a suitable behavioural test for TgCRND8 mice using the traditional measure of freezing as a response to conditioned fear. Therefore the results from this experiment may not truly represent hippocampal function of the mice and drug effects of $\alpha$-MSH.
Figure 4.3. α-MSH does not affect performance of TgCRND8 mice in the fear conditioning test. Performance of mice in learning, contextual fear conditioning and trace cued fear conditioning is represented by the % time freezing during the test. Total distance traveled in the contextual conditioning test illustrates the difference in response between the Tg and NTg mice. Data represent mean ± SEM, n = 5-8 per group, * p<0.05.
4.5 Nest Building Behaviour is not Affected by $\alpha$-MSH

To determine whether $\alpha$-MSH has effects on activities of daily living, nest building behavior was examined. Nesting building is used as a behavioural test to assess hippocampal function (Deacon, 2006). Both cytotoxic hippocampal lesion and transient ischemia that results in death of hippocampal CA1 pyramidal neurons lead to impaired nest building (Deacon et al., 2002; Antonawich et al., 1997). In transgenic mouse models of AD, both Tg2576 and 3xTg mice show nest building deficits, reflecting hippocampal dysfunction as a result of Aβ and/or tau accumulation (Torres-Lista 2013, Wesson et al., 2011). Nest building behaviour is assessed by scoring the nest quality of each singly housed mouse as a function of time. In this study, nest quality of TgCRND8 and NTg mice were scored at 1h, 3h and 6h after nesting material was provided as exploratory experiments showed the greatest difference in nest quality between 0-6h in TgCRND8 and NTg mice (Figure 4.4A). Nests were scored on a 1-5 scale according to the protocol developed by Deacon and colleagues where a score of 1 indicates no nest and intact nesting material and a score of 5 indicates a perfect nest (Deacon, 2006; Figure 4.4B and C). Similar to Tg2576 and 3xTg mouse models of AD, TgCRND8 mice show a statistically significant genotype deficit in nest building compared to NTgs ($P = 0.006$, Figure 4.4A). However, $\alpha$-MSH treatment does not improve nesting in TgCRND8 mice (Figure 4.4A).
Figure 4.4. Assessment of nest building. Quality of nests was assessed on a scale of 1-5 at 1h, 3h and 6h after cotton Nestlets were provided. NTg mice show significantly better nest building than TgCRND8 mice with or without α-MSH treatment (p = 0.006). Data represent mean ± SEM, n = 5-8 per group, * p<0.05.
4.6 Cognitive Improvement is Independent of Aβ Plaque Load

To determine if a reduction in Aβ load is a mechanism of action underlying behavioral benefits of α-MSH treatment, Aβ40 and Aβ42 peptide concentrations were examined in the hippocampus and cortex of TgCRND8 brains (n = 5 mice per group) using commercially available Aβ sandwich ELISA kits. No significant differences in soluble or insoluble Aβ40/Aβ42 were observed between vehicle and α-MSH treated TgCRND8 mice (Figure 4.5A to D). It has been previously reported that 1-fluoro-1-deoxy-scyllo-inositol treatment does not change Aβ concentration within the brain but decreases plaque burden (Hawkes et al., 2012), therefore Aβ plaque load was also examined (n = 6 mice per group). Quantification of Aβ plaques also showed no statistical difference between TgCRND8 vehicle and α-MSH treated mice either by the number of plaques ($P = 0.72$; Figure 4.5E, G and H) or percentage area covered by plaques in the hippocampus ($P = 0.66$; Figure 4.5F, G and H). Since α-MSH did not change Aβ load in the TgCRND8 brain, preservation of behavior elicited by α-MSH treatment is independent of Aβ.
Figure 4.5. Aβ accumulation as a function of α-MSH treatment. α-MSH had no effect on insoluble or soluble levels of Aβ40 (A, B) or Aβ42 (C, D) in the hippocampus and the cortex. The number of Aβ plaques ($P = 0.72$; E) or area covered by plaques ($P = 0.66$; F) in the hippocampus are also not affected by α-MSH. Representative photomicrographs of Aβ plaque staining in vehicle and α-MSH TgCRND8 treated mice are shown in G and H respectively. Data represent mean ± SEM, n = 5-6 per group. Scale bar = 300µm.
4.7 CREB Phosphorylation is not Affected by α-MSH

To further elucidate the underlying mechanism leading to behavioral deficits in TgCRND8 mice and rescue by α-MSH, neuroprotective effects of α-MSH via CREB phosphorylation was examined. Studies in the literature implicate CREB phosphorylation in neuroprotection, synaptic plasticity and memory formation (Benito and Barco, 2010). In addition, α-MSH induced CREB phosphorylation and BDNF expression in vivo in the hypothalamus (Sarkar et al., 2002; Xu et al., 2003) and α-MSH administration increased BDNF expression in vitro in primary astrocyte cultures (Caruso et al., 2012). It has been reported that without altering Aβ levels or Aβ plaque load in hippocampal slices of TgCRND8 mice, induction of CREB phosphorylation rescued LTP and spatial memory in TgCRND8 (Vitolo et al., 2002; Yiu et al., 2011). Therefore, I examined the effect of α-MSH on CREB phosphorylation in the hippocampus of TgCRND8 and NTg mice as a possible mechanism underlying behavioural benefits of α-MSH treatment.

In TgCRND8 and NTg mice treated with α-MSH or vehicle for 28 days, I determined the levels of pCREB in the hippocampus by western blotting using an antibody that specifically detects CREB phosphorylation at Ser133. CREB phosphorylated at ser133 activates genes involved in multiple signaling pathways with an upstream CRE element (Shaywitz and Greenberg, 1999). All mice were sacrificed between 15 to 24 hours after the last injection of either α-MSH or vehicle. There were no significant differences in the pCREB levels between TgCRND8 and NTg mice (P = 0.073), nor did α-MSH have an effect on CREB phosphorylation (P = 0.991, n = 4 per group; Figure 4.6A). Since CREB phosphorylation is a transient event, the pCREB levels observed could be basal levels that are not dependent on α-MSH administration. To rule out this possibility, I performed an exploratory time-course analysis of pCREB levels post acute α-MSH i.p. injection to determine the optimal time point to examine CREB phosphorylation. Based on the study by Wright and Wilson, 1983, i.p. injection of α-MSH in rats yielded a peak plasma concentration at 16.2 minutes. Therefore using NTg mice, mice were sacrificed at time 0 without α-MSH injections and at times 20 min, 45 min and 90 min post α-MSH injections. pCREB showed a slight up-regulation at 20 min and 45 min with n = 3 per group (Figure 4.6B). I chose 30 min, between the two time points of peak pCREB expression, as the time point to conduct my experiment on the effect of α-MSH administration in TgCRND8 mice. There were
no significant differences in pCREB levels between TgCRND8 and NTg mice \((P = 0.77)\) or any differences with \(\alpha\)-MSH treatment \((P = 0.83, n = 6\) per group; Figure 4.6C). Total CREB (tCREB) levels were also uniform among the four groups. In addition, analysis of pCREB to tCREB ratio did not reveal any genotype \((P = 0.46)\) or drug treatment effect \((P = 0.61, n = 6\) per group; Figure 4.6D). \(\alpha\)-MSH treatment does not elicit changes in CREB phosphorylation, therefore, induction of pCREB is not an underlying mechanism of \(\alpha\)-MSH-induced behavioural effects.

![Graphs showing CREB phosphorylation](image)

**Figure 4.6.** Effect of \(\alpha\)-MSH treatment on CREB phosphorylation in the hippocampus. TgCRND8 mice do not show a deficit in pCREB. \(\alpha\)-MSH does not induce CREB phosphorylation in either chronic treatment or acute treatment. Data represent mean ± SEM, \(n = 3-4\) per group for pCREB chronic treatment and pCREB time course, \(n = 6\) for pCREB 30min post \(\alpha\)-MSH injection normalized to either GAPDH or tCREB.
4.8 Expression of Neurotrophic Factors is not Altered by α-MSH

Neurotrophic factors are important for neuronal survival, neurite outgrowth, synaptogenesis and synaptic plasticity. α-MSH increased BDNF expression \textit{in vitro} in primary astrocyte cultures (Caruso et al., 2012); it also induced BDNF expression \textit{in vivo} in the hypothalamus (Sarkar et al., 2002; Xu et al., 2003). Therefore, BDNF and NGF mRNA levels in the hippocampus were examined using quantitative real-time PCR. Both neurotrophin levels in TgCRND8 mice were not different from NTg mice (BDNF, \(P = 0.95\); NGF, \(P = 0.10\)). α-MSH treatment did not have an effect on BDNF (\(P = 0.93\)) and NGF (\(P = 0.66\)) mRNA levels (Figure 4.7A and B). mRNA levels of BDNF receptors in the hippocampus, TrkB and p75\(^{NTR}\) also did not change with genotype (TrkB, \(P = 0.25\); p75\(^{NTR}\), \(P = 0.40\)) or drug treatment (TrkB, \(P = 0.93\); p75\(^{NTR}\), \(P = 0.14\); Figure 4.7C and D). NGF signals via TrkA and p75\(^{NTR}\) receptors. TrkA mRNA levels were too low in hippocampus and could not be detected.

BDNF expression is regulated by different promoters resulting in multiple transcripts. Transcripts I, III, IV and VI are down regulated in the cortex of AD patients (Garzon et al., 2002). These transcripts are highly homologous between humans and mice except transcript III, which only exhibits 62% homology (Aid 2007). Therefore, I examined BDNF transcripts I, IV and VI. At 24 weeks of age, transcripts I and IV levels are not different between TgCRND8 and NTg mice (transcript I, \(P = 0.34\); transcript IV, \(P = 0.30\)) and α-MSH treatment had no effect (transcript I, \(P = 0.77\); transcript IV, \(P = 0.63\); Figure 4.7E and F). Transcript VI levels are significantly reduced in TgCRND8 mice compared to NTgs (vehicle \(P < 0.001\), α-MSH \(P = 0.003\)). α-MSH treatment did not rescue transcript VI levels (\(P = 0.86\); Figure 4.7G). α-MSH treatment does not alter BDNF and NGF mRNA levels in the hippocampus, nor does it alter receptor levels of these neurotrophic factors TrkA, TrkB and p75NTR. Increasing neurotrophin levels or enhancing signaling through up-regulation of neurotrophin receptors does not help to explain improved spatial memory and normalized anxiety levels elicited by α-MSH.
Figure 4.7. α-MSH treatment does not alter the mRNA expression of neurotrophins and neurotrophin receptors in the hippocampus. TgCRND8 mice do not show a deficit in BDNF and NGF mRNA levels or their receptors TrKB and p75NTR. BDNF transcript VI mRNA is decreased in TgCRND8 mice. However, α-MSH does not alter neurotrophins or receptor mRNA levels in either Tg or NTg mice. Data represent mean ± SEM, n = 6-7 per group.
4.9 Expression of Synaptic Markers is not Altered by α-MSH

CREB phosphorylation, BDNF and NGF all play a role in synaptogenesis both in the developing brain and in the mature adult brain. I showed that in the hippocampus of 24 month-old TgCRND8 mice, pCREB protein levels and total BDNF and NGF mRNA levels are comparable to that of the NTg mice. Given the role of pCREB, BDNF and NGF in synaptogenesis and the unaltered mRNA levels in whole hippocampal analyses, it is not surprising that the global hippocampal expression of synaptic markers is not altered in the TgCRND8 mice. Protein levels of the pre-synaptic markers synaptophysin and dynamin-1 and the post-synaptic marker PSD95 are not different between TgCRND8 and NTg mice (synaptophysin, $P = 0.77$; dynamin-1, $P = 0.16$; PSD95, $P = 0.32$). α-MSH treatment also had no effect on levels of these synaptic markers (synaptophysin, $P = 0.84$; dynamin-1, $P = 0.87$; PSD95, $P = 0.98$; Figure 4.8A to D).

Figure 4.8. Hippocampal synaptic markers are not altered by α-MSH treatment. TgCRND8 mice do not show a deficit in the protein expression of pre-synaptic markers synaptophysin and dynamin-1 or in the post-synaptic marker PSD95. α-MSH also does not alter levels of synaptic markers in either Tg or NTg mice. Data represent mean ± SEM, n = 4 per group.
4.10 Neuronal Numbers in the Pyramidal Layer in the CA1 of the Hippocampus is not altered by α-MSH

Neuronal loss is a hallmark in AD pathogenesis. To determine whether there is neuronal loss in the hippocampus, the neuronal marker NeuN was used to label the nuclei of post-mitotic neurons (Mullen et al., 1992). The NeuN antibody recognizes most neuronal cell types in all regions of the mouse brain except the Purkinje cells of the cerebellum, mitral cells of the olfactory bulb and photoreceptor cells of the retina (Mullen et al., 1992). Qualitative observation of NeuN immunohistochemical staining showed comparable number of NeuN+ cells in the hippocampus of TgCRND8 and NTg mice, however, the number of NeuN+ cells in the pyramidal layer of the CA1 region appeared more varied. Therefore I quantified the number of NeuN+ cells in the pyramidal layer of the CA1 region. Exploratory NeuN+ cell quantification of 2 hippocampi per mouse and 6 mice per treatment group showed no genotype ($P = 0.25$) or drug effect ($P = 0.64$; Figure 4.9A, C to F).

Cell death can occur through apoptosis or necrosis. Caspase 3 is an effector of apoptosis, however, no active caspase 3 was detected in the hippocampus by western blot in both TgCRND8 and NTg mice whether treated with α-MSH or vehicle. In addition, protein levels of the anti-apoptotic factor bcl-2 in the hippocampus was highly variable between animals and did not show any significant differences due to genotype ($P = 0.47$) or drug treatment ($P = 0.58$; Figure 4.9B). Global analysis of the hippocampus showed unaltered levels of apoptosis-associated markers caspase 3 and bcl-2. Although whole hippocampal analysis could mask changes in small sub-populations of neurons, unaltered levels of apoptosis effector protein caspase 3 in the hippocampus is consistent with the observation that the number of NeuN+ cells did not change with either genotype or α-MSH treatment.
Figure 4.9. Effect of α-MSH treatment on neuronal numbers. The number of NeuN+ pyramidal cells in the CA1 is not significantly different between TgCRND8 and NTg mice (A, C and E). α-MSH does not affect the numbers NeuN+ pyramidal cells in either Tg or NTg mice (A, C to F). Anti-apoptotic factor bcl-2 protein levels do not change with genotype or α-MSH treatment (B). Data represent mean ± SEM, n = 6 per group.

4.11 α-MSH does not Improve Intermediate Early Gene Expression Post Stimulation

Even though the number of NeuN+ mature neurons in the hippocampus is not altered by genotype or α-MSH treatment, the functional activity of existing neurons could be impaired. Arc is an intermediate early gene that upon behavioural stimulation such as exploration of a novel environment or a maze, transient neuronal expression is up-regulated in the hippocampus (Guzowski et al., 1999; Yiu et al., 2011). To determine whether improper activation of neurons post behavioural stimulation is the underlying cause for behavioural changes in vehicle control or α-MSH treated TgCRND8 mice, I examined Arc expression in the hippocampus. Using the Arc activation protocol initially developed by Guzowski and colleagues where novel environment exploration is used to stimulate Arc expression, TgCRND8 mice showed a
significant decrease in Arc+ neurons in the granule cell layer of the dentate gyrus compared to NTgs \((P = 0.01; \text{Figure 4.10A, C to E})\). \(\alpha\)-MSH had no effect on the expression of Arc in TgCRND8 mice \((P = 0.93, \text{Figure 4.10A, C to E})\). In the pyramidal layer of the CA1, there was no difference in Arc+ neurons between NTg, Tg vehicle and Tg \(\alpha\)-MSH treatment (Figure 4.10B). Up-regulation of Arc in the granule cells of the dentate gyrus is impaired in TgCRND8 mice upon behavioural stimulation and \(\alpha\)-MSH had no effect on Arc expression in the hippocampus.

**Figure 4.10.** Neuronal activation as a function of \(\alpha\)-MSH treatment. In the hippocampus, the number of Arc+ cells one-hour post novel environment stimulation is decreased by more than 50% in TgCRND8 mice compared to NTg mice in the granule cell layer of the dentate gyrus (A, C and D, \(P = 0.01\)). \(\alpha\)-MSH treatment does not rescue neuronal activation deficit (A, C to E, \(P = 0.93\)). In the pyramidal layer of the CA1, there was no difference in Arc+ neurons between NTg ctrl, Tg ctrl and Tg \(\alpha\)-MSH (B). Data represent mean ± SEM, \(n = 5-7\) per group, \(* P < 0.05\). Scale bar = 50\(\mu\)m.
4.12 \( \alpha \)-MSH Rescues GABAergic Neuronal Deficits in TgCRND8 mice

The majority of hippocampal neurons are excitatory and approximately 10% are inhibitory GABAergic interneurons (Freund and Buzsáki, 1998). Since interneurons only account for a small portion of neurons in the hippocampus, a general NeuN count could mask differences in interneurons. GABAergic interneurons modulate the activity of excitatory neurons, therefore, disruption of the GABAergic system interferes with the synchrony of neuronal networks in the hippocampus and leads to cognitive impairment (Katona et al, 1999; Palop et al., 2007; Melzer et al., 2012). GAD converts glutamic acid to GABA and its two isoforms, GAD67 and GAD65, are predominantly localized in GABAergic cell bodies and axon terminals, respectively (Esclapez et al., 1994). TgCRND8 mice exhibit GABAergic deficits that are attenuated by \( \alpha \)-MSH treatment. One-way ANOVA analyses of GABAergic markers revealed that GAD67 mRNA levels are significantly decreased in 24 week-old TgCRND8 vehicle treated mice compared to NTgs (vehicle \( P = 0.044 \), \( \alpha \)-MSH \( P = 0.007 \)) whereas \( \alpha \)-MSH maintained GAD67 mRNA levels in TgCRND8 mice to that of NTg mice (Figure 4.11A). GAD67 protein levels in TgCRND8 vehicle treated mice are decreased compared to NTgs (vehicle \( P = 0.001 \), \( \alpha \)-MSH \( P = 0.016 \)). \( \alpha \)-MSH treated TgCRND8 mice expressed protein levels comparable to NTg mice (Figure 4.11B). However, GAD65 protein levels are not changed with genotype (\( P = 0.45 \)) or \( \alpha \)-MSH treatment (\( P = 0.51 \), \( n = 6 \)-7 mice per group; Figure 4.11C).

Similar to mRNA and protein levels, the number of GAD67+ cells in the CA1 region of the hippocampus are decreased in TgCRND8 vehicle treated mice compared to NTg mice (vehicle \( P = 0.018 \), \( \alpha \)-MSH \( P = 0.001 \)). \( \alpha \)-MSH treatment prevented the loss of GAD67+ cells in TgCRND8 mice (\( P = 0.016 \)) and had no effect on GAD67+ cells in NTg mice (\( n = 6 \) mice per group; Figure 4.12A, D to G). In the CA3 region and hilus of the hippocampus, the number of GAD67+ cells is not altered by genotype (CA3, \( P = 0.48 \); hilus, \( P = 0.64 \)) or \( \alpha \)-MSH treatment (CA3, \( P = 0.22 \); hilus, \( P = 0.90 \)) at 24 weeks of age (\( n = 6 \)-7 mice per group; Figure 4.12B and C). These results suggest that preservation of GABAergic neuronal function in the CA1 region of the hippocampus may contribute to improved behavior post \( \alpha \)-MSH treatment.
Figure 4.11. GABAergic marker expression in the hippocampus. GAD67 mRNA (A) and protein levels (B) are significantly decreased in TgCRND8 mice compared to NTg mice, $P = 0.044$ and $P = 0.001$ respectively. GAD65 protein levels are not changed with genotype or α-MSH treatment (C). α-MSH treatment rescues the GAD67 deficits in TgCRND8 mice in both mRNA ($P = 0.008$) and protein levels ($P = 0.009$). Data represent mean ± SEM, $n = 6-7$ per group, * $P < 0.05$. 
Figure 4.12. α-MSH treatment preserves GABAergic GAD67+ interneurons in the hippocampus. GAD67+ cells in the CA1 region of the hippocampus are decreased in TgCRND8 mice ($P = 0.018$; A), α-MSH treatment rescues the number the GAD67+ cells ($P = 0.016$; A).

Panels D-G shows representative photomicrographs of the GAD67+ GABAergic interneurons in the CA1 region of the hippocampus in NTg vehicle (D), NTg α-MSH treated (E), Tg vehicle (F) and Tg α-MSH treated (G) mice. Neither genotype nor α-MSH treatment changed the number of GAD67+ cells in the CA3 region (B) or the hilus (C) of the hippocampus. Data represent mean ± SEM, $n = 6-7$ per group, * $P < 0.05$. Scale bar = 100µm.
4.13 GABA<sub>A</sub> Receptor Expression is not Affected by α-MSH

Since α-MSH preserved the number of GABAergic neurons in the hippocampus of the TgCRND8 mice, the GABAergic system was further examined to determine the effect of α-MSH on GABA receptors. There are two main classes of GABA receptors, GABA<sub>A</sub> and GABA<sub>B</sub>. GABA<sub>A</sub> receptors regulate fast inhibitory neurotransmission whereas GABA<sub>B</sub> receptors mediate slow inhibitory responses. Drugs that target the GABAergic system such as benzodiazepines and barbiturates act on GABA<sub>A</sub> receptors. GABA<sub>A</sub> receptors are also implicated in disease states such as epilepsy and Alzheimer’s disease (Rissman and Mobley, 2011). Therefore, alterations in GABA<sub>A</sub> receptors in the TgCRND8 mouse model and the effect of α-MSH treatment on GABA<sub>A</sub> receptors were the focus of these experiments.

GABA<sub>A</sub> receptors are composed of multiple subunits with the most common type in the brain being a pentamer with combinations of 2α<sub>x</sub>, 2β<sub>x</sub> and 1γ<sub>x</sub> subunits (Whiting, 2003). Subunits α-1 and α-5 are consistently decreased in the hippocampus of AD patients (Rissman et al., 2007). GABA<sub>A</sub> receptors have either intra-synaptic or extra-synaptic localization for phasic and tonic inhibition respectively. Subunit α-1 is present as part of intra-synaptic receptors, α-5 can be either intra-synaptic or extra-synaptic whereas δ subunit is extra-synaptic (Hines et al., 2012). Analysis of mRNA levels of the α-1 subunit did not reveal any difference between Tg and NTg mice (P = 0.34) nor with α-MSH treatment (P = 0.78; Figure 4.13A). Consistent with this finding, gephyrin, a scaffolding protein that is associated with synaptic GABA<sub>A</sub> receptors, also exhibited similar protein levels in Tgs and NTgs (P = 0.99) with and without α-MSH treatment (P = 0.77; Figure 4.13B). On the other hand, subunit α-5 and δ mRNA levels are significantly reduced in the TgCRND8 mice compared to NTg mice (P = 0.015 and P < 0.001 respectively), suggesting a reduction in tonic inhibition. α-MSH did not rescue α-5 or δ receptor subunit levels (P = 0.79 and P = 0.62 respectively; Figure 4.13C and D). Although there are changes in GABA<sub>A</sub> subunit mRNA levels, suggesting a remodeling of GABA<sub>A</sub> receptor composition in the hippocampus of TgCRND8 mice, α-MSH treatment did not affect expression of GABA<sub>A</sub> subunits. α-MSH preserved the number of GABAergic GAD67+ neurons without altering the subunit composition of GABA<sub>A</sub> receptors.
**Figure 4.13.** Expression of GABA<sub>A</sub> receptor subunits is not altered by α-MSH treatment.

GABA<sub>A</sub> intra-synaptic receptor subunit α1 mRNA levels as well as the intra-synaptic receptor scaffolding protein gephyrin levels are not changed with genotype or α-MSH treatment. mRNA levels of GABA<sub>A</sub> receptor subunits α-5 and δ are significantly decreased in TgCRND8 mice compared to NTg mice but α-MSH treatment does not improve these subunit levels. Data represent mean ± SEM, n = 5-7 per group.
4.14 GABAergic Deficits Precede Cholinergic Deficits

My results demonstrate spatial memory and anxiety deficits in 24 week-old TgCRND8 mice with a coexisting loss of GABAergic interneurons and altered levels of GABA_A receptor subunits in the hippocampus. In order to determine whether GABAergic changes are the primary contributor to cognitive dysfunction at this age, and in light of the cholinergic neuronal loss in the nucleus basalis-cortical pathway previously reported in this model (Bellucci et al., 2006), I examined the cholinergic neurons in the septum in TgCRND8 mice. In the septo-hippocampal pathway, at 24 weeks of age, the number of ChAT+ cells and the area per ChAT+ cell in the septum were not decreased in the TgCRND8 mice compared to NTg mice (NTg vehicle $P = 0.38$, NTg $\alpha$-MSH $P = 0.88$; Figure 4.14A to F). $\alpha$-MSH treatment had no effect on either the number or area of ChAT+ cells in TgCRND8 ($P = 0.57$ number, $P = 0.95$ area) or NTg mice ($P = 0.46$ number, $P = 0.77$ area; Figure 4.14A to F). GABAergic GAD67+ cell loss in the hippocampus precedes cholinergic ChAT+ cell loss in the septum of TgCRND8 mice. $\alpha$-MSH preserved GAD67+ cell numbers but it did not alter GABA_A receptor subunit levels in the hippocampus or ChAT+ cell numbers in the septum. Therefore, preservation of GAD67+ GABAergic neuronal numbers by $\alpha$-MSH correlates with improved cognitive function in the TgCRND8 mouse model.
**Figure 4.14.** ChAT+ cholinergic phenotype in the septum. TgCRND8 mice do not show a decrease in the number or area of ChAT+ cells in the medial septum ($P = 0.38$ and $P = 0.58$ respectively; A and B). α-MSH treatment does not alter the numbers or area of ChAT+ cells in either TgCRND8 ($P = 0.57$ number, $P = 0.95$ area) or NTg mice ($P = 0.46$ number, $P = 0.77$ area; A and B). Panel C-F show representative photomicrographs of the ChAT+ cholinergic cells in the medial septum in NTg vehicle (C), NTg α-MSH treated (D), Tg vehicle (E) and Tg α-MSH treated (F) mice. Data represent mean ± SEM, n = 6 per group. Scale bar = 100µm.
4.15 $\alpha$-MSH Protects Loss of Somatostatin Immunoreactive Neurons in the Hippocampus

To further characterize a potential link between $\alpha$-MSH mediated improvement in behavior and preservation of GABAergic neurons, sub-populations of GABAergic neurons according to their neuropeptide expression were examined (n = 6-7 mice per group). In the hippocampus, cells containing SST, NPY, CCK and VIP are almost exclusively GABAergic neurons (Freund and Buzaki 1998). Investigation of neuropeptide subtype expression of GABAergic neuronal subtypes has not been previously reported in the TgCRND8 mouse at any age. One-way ANOVA analyses revealed a significant decrease of both NPY ($P = 0.01$) and SST ($P = 0.038$) mRNA levels in TgCRND8 mice compared to NTg mice (Figure 4.15A and B). The mRNA levels of neuropeptides CCK and VIP (Figure 4.15C and D) are not affected by either genotype (CCK, $P = 0.36$; VIP, $P = 0.11$) or $\alpha$-MSH treatment (CCK, $P = 0.43$; VIP, $P = 0.18$). $\alpha$-MSH treatment did not prevent NPY mRNA loss in TgCRND8 mice ($P = 0.667$; Figure 4.15A). In contrast, SST mRNA levels in the hippocampus of $\alpha$-MSH treated TgCRND8 mice were significantly greater than vehicle treated TgCRND8 mice ($P = 0.011$), and not different from vehicle or $\alpha$-MSH treated NTg mice (Figure 4.15B).
Figure 4.15. Effect of α-MSH treatment on GABAergic neuronal subtypes in the hippocampus. GABAergic neurons can be divided into subtypes according to the neuropeptide expressed. In the hippocampus, mRNA expression of SST and NPY is significantly decreased in TgCRND8 mice ($P = 0.038$ and $P = 0.01$ respectively, A and B) but not CCK (C) or VIP (D). α-MSH treatment rescued SST mRNA levels (B) but does not affect NPY, CCK or VIP expression (B to D). Data represent mean ± SEM, $n = 6-7$ per group, * $P < 0.05$. 
SST+ cells in the CA1 region of the hippocampus are mostly localized in the stratum oriens. The number of SST+ cells in the stratum oriens of the CA1 was significantly decreased in TgCRND8 mice compared to NTgs (vehicle \( P = 0.038 \), \( \alpha \)-MSH \( P = 0.018 \)). \( \alpha \)-MSH treatment prevented the loss of SST+ cells \( (P = 0.011; \) Figure 4.16A, D to G). In the CA3 region and hilus of the hippocampus, the number of SST+ cells is not affected by genotype (CA3, \( P = 0.28 \); hilus, \( P = 0.95 \)) or \( \alpha \)-MSH treatment (CA3, \( P = 0.46 \); hilus, \( P = 0.32 \); \( n = 6-11 \) per group; Figure 4.16B and C). Confocal images in Figure 4.16H show the co-localization of GAD67+ cells and SST+ cells in the stratum oriens of the CA1. Sub-population analysis of GABAergic neurons demonstrated that GAD67+ cell loss in the CA1 region of the hippocampus is in part due to the loss of the SST+ sub-population. \( \alpha \)-MSH treatment maintained hippocampal SST mRNA levels and the number of CA1 SST+ cells in TgCRND8 mice to levels comparable to NTg mice. In conclusion, preservation of the specific SST-expressing subtype of GABAergic neurons may underlie improved cognitive function in \( \alpha \)-MSH treated TgCRND8 mice as summarized in Figure 4.17.
Figure 4.16. α-MSH treatment preserves SST+ cells in the hippocampus. SST+ cells in the stratum oriens of the CA1 is significantly decreased in TgCRND8 and rescued by α-MSH treatment (A). Panels D-G show representative photomicrographs of the SST+ cells in the CA1 region of the hippocampus in NTg vehicle (D), NTg α-MSH treated (E), Tg vehicle (F) and Tg α-MSH treated (G) mice. Neither genotype nor α-MSH treatment altered the number of SST+ cells in the CA3 region (B) or the hilus (C) of the hippocampus. SST+ cells co-localize with GAD67+ GABAergic interneurons in the CA1 stratum oriens of the hippocampus (H). Data represent mean ± SEM, n = 6-11 per group, * P < 0.05. Scale bar for SST IHC = 100µm, scale bar for GAD67/SST co-localization = 25µm.
**Figure 4.17.** Overall effect of $\alpha$-MSH treatment in TgCRND8 mouse model of AD. $\alpha$-MSH improves spatial memory and prevents changes in anxiety in TgCRND8 mice via neuroprotection of GABAergic GAD67+ interneurons, particularly SST-expressing interneurons.
Portions of this section have been previously published in:


TgCRND8 mice develop spatial memory deficits and decreased anxiety between 20 and 24 weeks of age as measured by percentage alternation in the Y-Maze test and exploration of the centre field in an open field test, respectively. At this stage of Aβ accumulation, TgCRND8 mice exhibit concomitant loss of hippocampal GAD67 mRNA and protein expression as well as GAD67+ GABAergic interneurons in the CA1 region. The number of GAD67+ interneurons in the CA3 and hilus of the hippocampus are not statistically different between TgCRND8 and NTg mice. These results are in agreement with recent reports of decreased GAD67+ interneurons in the CA1 region of triple transgenic mice (TauPS2APP) (Loreth et al., 2012) and CA1-CA3 region of TgCRND8 (Krantic et al., 2012). In 24 week-old TgCRND8 mice, the decrease of GAD67+ cells in the CA1-CA3 regions previously observed by Krantic and colleagues may be accounted for by the decrease in the CA1 region because my results demonstrate no changes in the CA3 region (Krantic et al., 2012).

Furthermore, I examined the subtypes of GABAergic interneurons and showed for the first time that in TgCRND8 mice, the loss of GAD67+ GABAergic interneurons is of the SST and NPY expressing subtypes. SST+ cells in the stratum oriens of the CA1 region of the hippocampus is significantly decreased in TgCRND8 compared to NTg mice. GABAergic cell loss in the CA3 or the hilus of the hippocampus was not detected, however, cell loss with more advanced disease in these mice cannot be ruled out. These results are consistent with previous reports that showed decreased tonic inhibition leading to increased long term potentiation in the CA1 region (Jolas et al., 2002) and increased seizure threshold and severity in TgCRND8 mice (Del Vecchio et al., 2004). Our results are also consistent with the age-dependent deficits in multiple cognitive systems in the TgCRND8 mice (Hyde et al., 2005b; Hanna et al., 2009).
GABAergic interneuron loss has been previously reported in mouse models of AD (Ramos et al., 2006; Perez-Cruz et al., 2011; Krantic et al., 2012; Loreth et al., 2012). Similar to our results, Tg2576 mice overexpressing human APP<sub>Swe</sub> demonstrate a loss of hippocampal SST+ cells in the CA1 region but not the dentate gyrus (Perez-Cruz et al., 2011). Krantic and colleagues also did not detect a loss of GAD67+ cells in the dentate gyrus of TgCRND8 mice (Krantic et al., 2012). In APPxPS1 mice, a loss of SST and NPY GABAergic neurons was detected in all regions of the hippocampus (Ramos et al., 2006). This group demonstrated that PS1 mice did not have a loss of GABAergic neurons and suggested that the GABAergic neuronal loss could be attributed to Aβ. In contrast, J20 mice that overexpress APP<sub>Swe</sub> showed decreased GABAergic neurons of the PV and CR subtypes in the septo-hippocampal pathway. The loss of PV neurons resulted in network dysfunction and increased spontaneous epileptiform activity (Rubio et al., 2012; Verret et al., 2012). In TauPS2APP mice, the hyperactive phenotype and spatial memory deficits were linked to loss of NPY+, PV+ and CR+ GAD67+ cells in the dentate gyrus without a loss of SST+ or cholinergic cells (Loreth et al., 2012). The difference in subtype loss between the AD mouse models may be linked to genetic background, expression of other transgenes, gender differences and level of Aβ expression.

The two greatest risk factors for sporadic AD are ApoE4 expression and aging, rodent models of both demonstrate that GABAergic neuronal loss is linked to cognitive deficits (Andrews-Zwilling et al., 2010; Leung et al., 2012; Stanley et al., 2012; Spiegel et al., 2013). ApoE4 is a genetic risk factor in AD that induces learning and memory deficits primarily in female mice (Raber et al., 1998). Aged ApoE4 knock-in female mice exhibit decreased GAD67+ and SST+ cells in the hilus of the hippocampus, which is correlated with spatial memory (Andrews-Zwilling et al., 2010). The toxic effect of the ApoE4(Δ272-299) fragment in female mice results in decreased SST+ and NPY+ GAD67+ cells in the hilus that is dependent on tau expression (Andrews-Zwilling et al., 2010). These data are in agreement with APP x Tau<sup>−/−</sup> mice, which demonstrated that cognitive loss in the TgAPP mouse was dependent on the expression of tau (Roberson et al., 2007). In contrast, aging male ApoE4 knock-in mice exhibit intact spatial memory with increasing expression of GABAergic interneurons in the hilus of the hippocampus (Leung et al., 2012). In aged F344 rats, animals with reduced numbers of GAD67+ SST+ cells in the hilus of the hippocampus showed impairment in spatial memory, while littermates without spatial deficits had no GABAergic neuronal deficits (Spiegel et al., 2013). In outbred rats,
GAD67+ and SST+ interneurons are preferentially lost in the stratum oriens with age (Stanley et al., 2012). The SST+ cell loss leads to cognitive dysfunction and seizures as a result of decreased inhibitory control over entorhinal input to CA1 pyramidal cells (Stanley et al., 2012). These combined studies demonstrate the important link between GABAergic neuronal and cognitive function, more specifically the SST expressing interneurons.

Collectively, these studies use various transgenic mouse models of AD harbouring familial mutations or rodent models of ageing or ApoE expression to recapitulate risk factors of AD in order to study the GABAergic system in disease models. Results from these studies all demonstrate a loss of GABAergic interneuronal markers and the loss of neuropeptide expression in GABAergic interneurons of the hippocampus are predominantly the SST and/or NPY (Ramos et al., 2006; Andrews-Zwillling et al., 2010; Perez-Cruz et al., 2011; Leung et al., 2012; Loreth et al., 2012; Stanley et al., 2012; Spiegel et al., 2013).

NPY levels are decreased in the CSF of AD patients but no correlation was found between NPY CSF levels and the degree of cognitive impairment or with age of disease onset (Alom et al., 1990; Nilsson et al., 2001). NPY knockout mice show behavioral changes of altered anxiety in both the open field test and the elevated plus-maze test and changes in locomotion in the open field test (Karl et al., 2008). However, NPY knockout mice exhibit no cognitive changes in the hippocampal-dependent passive avoidance task of learning and memory (Karl et al., 2008). In contrast, SST levels are consistently decreased in the brain and CSF of AD patients and decreased SST levels are correlated with cognitive dysfunction (Davies et al., 1980; Davies and Terry, 1981; Beal et al., 1986; Sunderland et al., 1987; Tamminga et al., 1987; Davis et al., 1988; Molchan et al., 1991; Molchan, 1993; Dournaud et al., 1994). Animal studies show that i.c.v. administration of SST improves cognitive functions and alters anxiety (Bollók et al., 1983; Vécsei et al., 1983a; 1983b; 1984; Engin et al., 2008). My results show that in TgCRND8 mice, both NPY and SST mRNA levels are decreased in the hippocampus and SST+ cell number in the stratum oriens of the CA1 is decreased. In line with behavioral effects of NPY and SST in the literature, TgCRND8 mice exhibited a cognitive deficit in the Y-maze test of spatial memory, altered anxiety and locomotion in the open field test.
In addition to showing GABAergic deficits, my results demonstrate that in the septo-hippocampal pathway, GABAergic GAD67+ and SST+ interneuron loss in the hippocampus precedes cholinergic ChAT+ cell loss in the septum. At 24 weeks of age when TgCRND8 mice exhibit GABAergic deficits, the ChAT+ cholinergic cell number in the medial septum in TgCRND8 mice was not significantly different from NTg mice. Also, TgCRND8 mice showed no changes in the number of NeuN+ cells in the CA1 pyramidal cell layer of the hippocampus. These results illustrate that the loss of GABAergic GAD67+ cells in the hippocampus precedes the loss of ChAT+ cholinergic cells in the septum as well as NeuN+ neurons in the pyramidal cell layer of the hippocampus, which are predominantly excitatory pyramidal neurons.

In agreement with my results, the triple transgenic mouse model harboring tau/PS2/APP mutations showed a decrease in GAD67+ cells in the hippocampus, specifically NPY+ cells in the CA1-CA3 and NPY, PV and CR positive cells in the dentate gyrus of the hippocampus (Loreth 2012). At this stage of disease progression, AChE immunoreactive fibres in the hippocampus and ChAT+ cells in the septum remained unchanged (Loreth 2012). In addition, PS1xAPP transgenic mouse model exhibits GABAergic deficits in the hippocampus as demonstrated by decreased SST and NPY mRNA levels and SST+ cells (Ramos et al., 2006). These GABAergic changes preceded changes in markers of the glutamatergic and cholinergic systems in addition to unaltered numbers of NeuN+ pyramidal neurons in the CA1 region of the hippocampus (Ramos et al., 2006). At 24 weeks of age, TgCRND8 mice only exhibit GAD67+ GABAergic cell loss. Since the majority of hippocampal neurons are excitatory and only approximately 10% are inhibitory GABAergic interneurons (Freund and Buzsáki, 1998), active caspase-3 indicative of apoptosis in the hippocampus of TgCRND8 mice could not be detected; the anti-apoptotic factor bcl-2 levels are also not different between Tgs and NTgs. The GABAergic dysfunction is an early pathological event prior to widespread apoptosis through activation of caspase-3 or the loss of NeuN+ pyramidal neurons and ChAT+ cholinergic neurons.

In addition to the loss of GAD67+ GABAergic neurons in the hippocampus, my results show for the first time that in the TgCRND8 mice, there are alterations in GABA_A receptors. TgCRND8 mice show decreased mRNA levels of the subunits α5 and δ in the hippocampus compared to NTg mice. GABA_A receptors are chloride channels that regulate fast inhibitory transmission in the brain and are made up of mostly hetero-oligomeric pentamers consisting of subunits ranging
from \(\alpha_1-6, \beta_1-3, \gamma_1-3, \delta, \theta, \pi\) and \(\rho_1-3\) (Olsen and Sieghart, 2008). The \(\alpha_5\) subunit is particularly abundant in the CA1 and CA3 regions of the hippocampus and it is present at both synaptic and extrasynaptic GABA\(\text{A}\) receptors (Sur et al., 1998; Serwanski et al., 2006). Consistent with the down-regulation of \(\alpha_5\) mRNA levels in the hippocampus of TgCRND8 mice, in AD patients, \(\alpha_5\) mRNA and protein levels are decreased in the hippocampus (Rissman et al., 2007). In contrast, no studies to date have reported \(\delta\) subunit levels in AD patients or in AD transgenic mouse models in the brain. The \(\delta\) subunit is present in extrasynaptic GABA\(\text{A}\) receptors and it has been implicated in neurogenesis as well as modulation of dentate gyrus-dependent behavior such as recognition memory and contextual discrimination (Belelli et al., 2009; Whissell et al., 2013). My novel finding that the GABA\(\text{A}\) receptor subunit \(\delta\) is down-regulated in the APP transgenic mouse model TgCRND8 identifies a potential target for improving neurogenesis and memory in AD.

GABA\(\text{A}\) receptor subunit \(\alpha_1\) mRNA levels are not changed in TgCRND8 mice compared to NTgs. Consistent with the synaptic expression of the \(\alpha_1\) subunit, the synaptic receptor scaffolding protein gephyrin also showed comparable protein levels between TgCRND8 and NTg mice. Although the GABAergic system is thought to be relatively preserved in AD compared to the glutamatergic and the cholinergic system, remodeling of the GABA\(\text{A}\) receptors is observed (Rissman et al., 2007; Luchetti et al., 2011; Rissman and Mobley, 2011). The subunit composition of GABA\(\text{A}\) receptors is changed in the AD brain, particularly in the hippocampus (Rissman et al., 2007; Luchetti et al., 2011; Rissman and Mobley, 2011). Studies consistently report a significant decrease in both the mRNA and protein expression of \(\alpha_1\) and \(\alpha_5\) subunits of the GABA\(\text{A}\) receptors (Rissman et al., 2007). Furthermore, functionally, the altered GABA\(\text{A}\) receptors from AD patients show decreased GABA current amplitude, less sensitivity to GABA and faster rate of desensitization (Limon et al., 2012). TgCRND8 mice at 24 weeks of age exhibit GABAergic deficits in the hippocampus including decreased number of GAD67+ cells, in particular the SST- and NPY- expressing subtypes as well as altered GABA\(\text{A}\) receptor subunit levels.

Treatment of TgCRND8 mice with \(\alpha\)-MSH for 4 weeks preserved GAD67+ GABAergic interneurons and prevented the loss of SST-expressing subtype of interneurons in the
hippocampus but not the NPY-expressing subtype. NPY knockout mice show behavioral changes in anxiety and locomotion but not cognition (Karl et al., 2008). In contrast, decreased SST levels is correlated with cognitive dysfunction and increased SST levels improves cognitive functions and alters anxiety (Bollók et al., 1983; Vécsei et al., 1983a; 1983b; 1984; Tamminga et al., 1987; Engin et al., 2008). Therefore, protection of the somatostatinergic system in AD models could improve cognition and anxiety. The GABAergic preservation of GAD67+ and SST+ cells by α-MSH does not extend to GABA\(_A\) receptors. α-MSH does not increase the α-5 and δ subunit mRNA levels in TgCRND8 mice. α-MSH targets the early pathology of the GABAergic system by preventing the loss of GAD67+ interneurons and the SST+ subtype. Taken together, protection of the somatostatinergic system by α-MSH and not remodeling of the GABA\(_A\) receptors could help to explain behavioural improvements in TgCRND8 mice.

In TgCRND8 mice, α-MSH targets the early pathology of the GABAergic system by preventing the loss of GAD67+ interneurons of the SST+ subtype. However, α-MSH treatment did not alter Aβ levels in the hippocampus and cortex of TgCRND8 mice, either soluble and insoluble Aβ40 and Aβ42 concentration or Aβ plaques. α-MSH treatment was initiated therapeutically after the onset of Aβ pathology in TgCRND8 in an attempt to create a treatment paradigm translatable to human AD patients. In the TgCRND8 mouse model where considerable Aβ40 and Aβ42 levels are present in the brain at 10 weeks of age and Aβ plaques appear by 3 months of age, I initiated treatment starting at 20 weeks of age when Aβ pathology is well established and continued treatment for 4 weeks. In contrast, prophylactic treatment with a long-lasting potent synthetic analogue of α-MSH, NDP-α-MSH, prior to the onset of Aβ pathology for an extended period of 18 weeks reduced Aβ deposits and Aβ42 levels in the brain of 3xTg-AD mice (Giuliani et al., 2013). The 3xTg-AD mice harbor APP/PS1/tau mutations with Aβ accumulation starting intraneuronally at 16 weeks of age, Aβ plaques appear at 24 weeks of age and extensive tau immunoreactivity at 52 weeks of age (Oddo et al., 2003). Prophylactic treatment using i.p. injections of NDP-α-MSH began in male mice at 12 weeks of age for 18 weeks until 30 weeks of age. NDP-α-MSH treatment improved performance in the Morris water maze. NDP-α-MSH treatment also decreased hyperphosphorylation of tau, inflammation, oxidative stress and apoptosis (Giuliani et al., 2013). There are a number of differences between these two AD models and experimental paradigms utilized. The TgCRND8 model is an aggressive mouse...
over-expressing 5 copies of APP, while 3xTg mouse model expresses a single copy of APP and thus will have substantially reduced Aβ levels. The nature of the treatment paradigm, therapeutic vs. prophylactic may also have contributed to the different outcomes, as passive immunization strategies have shown prophylactic benefit but not therapeutic (Janus et al., 2000; Morgan et al., 2000; Das et al., 2001). An increasing number of studies have shown cognitive improvement after different treatments without altering Aβ levels (Janus et al., 2000; Yiu et al., 2011; Francis et al., 2012b). In my study, independent of changes in Aβ load in the hippocampus and the cortex, α-MSH treatment improved cognition by preserving the GABAergic system. This finding also reveals the GABAergic system and more specifically the somatostatinergic system as a novel target for drug intervention to improve cognitive function in AD.

α-MSH does not rescue all pathology examined in the TgCRND8 mice in this study. Either a longer treatment period may be required or a combination therapy involving compounds that target more than one aspect of the multifactorial nature of AD. Functionally, the numbers of Arc+ cells in the granule layer of the dentate gyrus of the hippocampus post novel environment exploration is decreased by more than 50% in the TgCRND8 mice compared to NTg mice. α-MSH treatment did not rescue Arc+ cell numbers in the dentate gyrus. The impairment in neuronal activation could help to explain nesting deficits in TgCRND8 mice. Hippocampal lesions lead to nesting deficits (Deacon et al., 2002), therefore, if the number of functional neurons are decreased, nesting ability may be affected. In addition, many transgenic mouse models of AD exhibit nesting deficits as a result of transgene(s) expression producing Aβ pathology and removal of Aβ pathology using immunization improved nesting behaviour (Wesson and Wilson, 2011; Min et al., 2013; Morales-Corraliza et al., 2013; Torres-Lista and Giménez-Llort, 2013). Since α-MSH did not rescue the number of Arc+ neurons in the dentate gyrus or decrease the Aβ load in the hippocampus, it may help to explain the lack of effect of α-MSH on the nesting deficit in TgCRND8 mice.

In the literature, α-MSH has been reported to exhibit many neuroprotective functions such as increasing CREB phosphorylation and BDNF levels, which may contribute to the increased viability of neurons and improvement in spatial memory after α-MSH treatment (Sarkar et al., 2002; Forslin Aronsson et al., 2006; Giuliani et al., 2006a; 2006b; Forslin Aronsson et al., 2007;
Based on these studies, neuroprotective mechanisms elicited by \( \alpha \)-MSH were examined. However, in the hippocampus, TgCRND8 mice at 24 weeks of age were not deficient in CREB phosphorylation (Ser 133), mRNA levels of neurotrophic factors BDNF and NGF or their receptors TrKB and p75NTR.

In young TgCRND8 mice when A\( \beta \) plaques first appear in the hippocampus, the number of pCREB+ cells in CA1 of the hippocampus and pCREB protein levels in the dorsal hippocampus were decreased in the TgCRND8 mice compared to NTgs (Yiu et al., 2011). While total CREB protein levels remained the same in TgCRND8 and NTg mice, both basal levels of pCREB expression in TgCRND8 mice taken from home cage and stimulated pCREB expression with novel context or water maze behavioural tasks were reduced (Yiu et al., 2011). However, in 24 week-old TgCRND8 mice where A\( \beta \) plaque pathology is well established, my results show no difference in hippocampal pCREB protein levels in TgCRND8 mice from home cage compared to NTg mice. The same western blot protocol with the same homogenization buffer and antibody were utilized in these two studies. Sucrose and RIPA homogenization buffers were also tested with slight adjustments to the protocol but no differences in Tg and NTg pCREB protein levels were found. CREB phosphorylation upon \( \alpha \)-MSH administration after a pre-determined optimal time point was also not up-regulated in either TgCRND8 mice or NTgs. Furthermore, \( \alpha \)-MSH treatment did not affect total CREB protein levels. The discrepancy in pCREB levels could be due to the different genetic background of the mice, 129S6/SvEvTac x C57BL/6NTac in Yiu’s experiment and C57BL6/C3H in my experiment. Studies have reported that the same mutation on mice of different genetic backgrounds could result in different phenotypes (Threadgill et al., 1997; Linder, 2001). The difference in A\( \beta \) pathology, early vs. well-established, could also contribute to the difference in pCREB levels. Elevated levels of A\( \beta \)40 induced pCREB (ser133) expression via extracellular signal-regulated kinase (Erk)1/2-dependent pathway in rat pheochromocytoma PC12 cells (Sato et al., 1997). Fibrillar A\( \beta \) has also been shown to activate Erk 1/2 in microglia and result in the downstream event of CREB phosphorylation at Ser133 (McDonald et al., 1998). The decreased pCREB expression observed in young TgCRND8 mice when A\( \beta \) plaques first appear may be offset by highly elevated A\( \beta \)40 levels and A\( \beta \) fibrils in 24 week-old TgCRND8 mice with well-established A\( \beta \) pathology. In conclusion, CREB
phosphorylation is not different between Tg and NTg mice at 24 weeks of age and it is not a mechanism by which α-MSH improves behaviour in TgCRND8 mice.

Next I examined the effect of α-MSH on BDNF mRNA levels. In the literature, total BDNF mRNA levels in the TgCRND8 fluctuate depending on age/Aβ pathology (Peng et al., 2009; Francis et al., 2012a; 2012b). In the hippocampus of TgCRND8 mice, BDNF mRNA levels are decreased at 6 weeks of age, increased at 9-10 weeks of age, decreased at 16 weeks, 8 months and 1 year (Peng et al., 2009; Francis et al., 2012a; 2012b). Similarly in human AD patients, serum BDNF levels are significantly decreased in MCI, increased or not changed in mild/moderate AD, and both serum and brain BDNF levels are decreased in late AD (Laske et al., 2005; Yasutake et al., 2006; Yu et al., 2008; O'Bryant et al., 2009). In both TgCRND8 mouse model of AD and AD patients, BDNF levels fluctuate depending on disease severity. At 24 weeks of age in TgCRND8 representing approximately moderate AD, hippocampal BDNF mRNA levels have not been previously determined.

Using the same primer pairs to determine BDNF mRNA levels as previous TgCRND8 mouse studies (Peng et al., 2009; Francis et al., 2012a; 2012b), my results show that BDNF levels are not altered in the TgCRND8 mice at 24 weeks compared to NTg mice. α-MSH treatment does not have an effect on either genotype. BDNF expression is controlled by nine different promoters, resulting in multiple transcripts that are translated into the same BDNF polypeptide (Aid et al., 2007; Zheng et al., 2012). To examine regulation of BDNF transcripts, I determined mRNA levels of transcripts I, IV and VI because the expression of these transcripts is down-regulated in AD patients and they are also highly homologous between humans and mice. Of the three transcripts, transcripts I and IV levels are not different between TgCRND8 and NTg mice whereas transcript VI levels are reduced in TgCRND8 mice. It has been previously reported that in 11.4 month-old TgCRND8 mice with Aβ pathology more severe than end-stage AD in humans, total BDNF mRNA levels was decreased as well as transcript IV levels but not transcripts I and VI (Peng et al., 2009). The considerable difference in Aβ pathology of TgCRND8 at 24 weeks (6 month) vs. 11.4 month may contribute to the differences in BDNF transcript levels. α-MSH treatment at 24 weeks had no effect on transcripts I, IV and VI mRNA levels in either TgCRND8 or NTg mice. CREB phosphorylation at Ser 133 activates CREB
mediated transcription. pCREB binds to the promoter regions of BDNF exons I and IV and induces the transcription of transcripts I and IV (Shieh et al., 1998; Tao et al., 1998; Tabuchi et al., 2002). Since pCREB levels are not altered in TgCRND8 mice or with α-MSH treatment at 24 weeks of age, it may help to explain the unaltered BDNF transcript I and IV mRNA levels in the hippocampus either with genotype or with α-MSH treatment.

In human patients with cases ranging from MCI to severe AD, NGF levels in the hippocampus are maintained at the same levels as age-matched healthy individuals; some studies found increased NGF levels but mostly in patients with severe AD (Allen et al., 1991; Murase et al., 1993; Scott et al., 1995; Narisawa-Saito et al., 1996). In agreement with human studies, at 24 weeks of age where moderate levels of Aβ plaques are present in the brain, TgCRND8 mice did not show changes in NGF mRNA levels compared to NTg mice. α-MSH also did not alter NGF levels either in Tg or NTg mice. NGF receptor TrkA mRNA levels are too low to detect in the hippocampus but the BDNF receptor TrkB and the low affinity receptor for both trophic factors p75NTR are comparable between Tgs and NTgs. Neurotrophic support from both BDNF and NGF are maintained in the hippocampus of TgCRND8 mice and α-MSH does not affect either neurotrophin or receptor levels.

Given the role of BDNF and NGF as neuronal survival factors and regulators of synaptic plasticity and synaptogenesis (Lessmann et al., 2003), it is not surprising that protein levels of hippocampal synaptic markers synaptophysin, dynamin-1 and PSD95 are also not changed in the TgCRND8 mice compared to NTGs. However, synaptophysin immunoreactive cell bodies and boutons were previously examined in the CA1 region of the hippocampus in 24 weeks old TgCRND8 mice and a decrease in synaptophysin immunoreactivity was observed (McLaurin et al., 2006). This discrepancy could be due to the sub-regional CA1 analysis of synaptophysin instead of the whole hippocampus. Examining a larger region could mask changes in a smaller sub-region. Likewise in the J20 mice, synaptophysin protein levels of the whole brain determined by western blot showed no differences between Tg and NTg mice. However, synaptophysin immunoreactivity was decreased in the hippocampus in J20 mice of the same age (Shankar et al., 2009). In conclusion, the effect size of the sub-regional decrease of synaptophysin in CA1 is too small to be reflected in the more global analysis of the whole
hippocampus. Similar to synaptophysin, protein levels of dynamin-1 and PSD95 were also not altered in TgCRND8 compared to NTg mice and α-MSH had no effect in either Tg or NTg mice.

In contrast, a significant decrease in GAD67 protein levels in the whole hippocampus was observed in 24 week-old TgCRND8 mice even though a previous study showed that GAD67+ cells are decreased in the CA1-CA3 of the hippocampus but not the dentate gyrus (Krantic et al., 2012). In addition, GAD67 mRNA levels in the whole hippocampus are also decreased. The detection of GAD67 loss at mRNA, protein and cellular levels either in the whole hippocampus or the CA1 sub-region suggest that the loss of the GABAergic marker GAD67 is more global and of greater magnitude than neurotrophic factors and synaptic markers. Furthermore, a sub-population of GAD67+ interneurons, SST+ interneurons, is also decreased in the CA1 region of the hippocampus. The loss of interneurons expressing GAD67 and SST is a more overt phenotype than deficits in synaptic markers.

Changes in brain SST levels have been shown to affect cognitive functions. Protection of the somatostatinergic system by α-MSH may explain the behavioural improvements observed in TgCRND8 mice. In early studies, i.c.v. administration of somatostatin improved performance in active avoidance tests (Bollók et al., 1983; Vécsei et al., 1983a; 1983b; 1984). In active avoidance tests, rats were trained to respond to a conditioned stimulus to avoid the unconditioned stimulus of foot shock. Avoidance behaviour weakens over time in saline treated rats but SST administration inhibited the extinction of avoidance behaviour. In addition, SST rescued electroconvulsive shock induced deficit in avoidance latency of rats (Bollók et al., 1983; Vécsei et al., 1983a; 1983b; 1984). In contrast, cysteamine administration (SST depletion) caused memory deficits in passive avoidance as well as Morris water maze (Bakhit and Swerdlow, 1986; Schettini et al., 1988; DeNoble et al., 1989; Matsuoka et al., 1995). In passive avoidance tests, rats are trained to remain in brightly illuminated space instead of the preferred dark space in order to avoid a foot shock administered when the rat enters the dark space. Cysteamine depletes SST in the brain and causes memory retention deficits in both the active and passive avoidance tests as well as in the Morris water maze test of spatial memory (Bakhit and Swerdlow, 1986; Schettini et al., 1988; DeNoble et al., 1989; Matsuoka et al., 1995). SST rescued the memory retention deficit in the passive avoidance test caused by a variety of factors including cysteamine, cholinergic dysfunction through scopolamine (muscarinic receptor
antagonist) administration or lesion of the NBM where cholinergic neurons reside (Matsuoka et al., 1994). Increased SST levels in the brain improve cognitive function.

SST+ interneurons in the stratum oriens of the CA1 help to induce increased inhibition onto CA1 pyramidal neurons, which is necessary in trace eyeblink conditioning task, illustrating the importance of SST+ interneurons in maintaining the inhibitory-excitatory balance (McKay et al., 2013). SST+ cells in the hippocampus form long-range projections to both the medial septum and the medial entorhinal cortex. These projections are found to target local interneurons and possibly mediate synchronized theta oscillations between the hippocampus and entorhinal cortex (Melzer et al., 2012). Theta oscillation is critical for temporal and spatial coding as well as memory encoding and retrieval (Buzsáki, 2002). Melzer and colleagues proposed that local interneuron modulation by long-range SST+ GABAergic neurons could contribute to the mechanism underlying spatial memory (Melzer et al., 2012). In addition, i.c.v. administration of SST suppresses the frequency of hippocampal theta oscillation, which is a common mechanism of action of anxiolytic drugs (Engin et al., 2008). SST induced an anxiolytic effect in rats as measured by spending significantly more time in the open arms of the elevated-plus maze (Engin et al., 2008). Studies in the literature help to establish the function of SST+ interneurons in behavioural phenotypes. α-MSH rescued the SST deficit in TgCRND8 mice. According to previous research, preserving SST in the hippocampus of TgCRND8 mice may have led to the improvement in spatial memory and altered anxiety observed.

Overall my results demonstrate that in the septo-hippocampal pathway, GABAergic deficits precede cholinergic deficits. α-MSH targets the early GABAergic deficit and prevents the loss of SST+ cells to improve spatial memory and prevent changes in anxiety independent of Aβ levels. My results present a proof of concept that cognition can be improved by preserving the GABAergic system, in particular SST+ interneurons. In addition, α-MSH emerges as a peptide that is neuroprotective against degeneration of the GABAergic system.
Chapter 6
Conclusions and Future Directions

6.1 Overall Conclusions

The AD mouse model TgCRND8 exhibits spatial memory decline and altered anxiety between 20-24 weeks of age. Concomitant with the behavioural changes, GABAergic deficits were observed at 24 weeks of age. The GABAergic marker GAD67 mRNA and protein expression are reduced in the hippocampus and the number of GABAergic GAD67+ interneurons are reduced in the CA1 region of the hippocampus but not the CA3 or hilus. mRNA levels of neuropeptides expressed by GABAergic interneurons NPY and SST are also reduced in the hippocampus. In particular, SST+ interneurons were significantly decreased in the CA1 region of the hippocampus but not the CA3 or hilus. In the septo-hippocampal pathway, GABAergic deficits are observed prior to cholinergic deficits, suggesting that GABAergic loss may underlie behavior deficits. α-MSH treatment of TgCRND8 mice between 20-24 weeks of age preserves GAD67 mRNA and protein expression in the hippocampus and prevents the loss of GAD67+ interneurons as well as the SST expressing subtype in the CA1 region of the hippocampus. Without decreasing Aβ load in the brain, α-MSH improves spatial memory in TgCRND8 mice and prevents alterations in anxiety. α-MSH modulated the excitatory/inhibitory balance in the brain by restoring GABAergic inhibition and improved cognitive function in TgCRND8 mice.

6.2 Future Directions

In this thesis, the neuroprotective properties of α-MSH were examined in 24 week-old TgCRND8 mice. At this stage of Aβ pathology, GABAergic deficits of decreased numbers of GAD67+ interneurons and reduced expression of neuropeptides NPY and SST were observed in the hippocampus. α-MSH treatment preserved the SST+ GABAergic interneurons in the CA1 region of the hippocampus. In addition to neuroprotective properties, studies in the literature have shown that α-MSH also promotes neurogenesis and reduces inflammation. TgCRND8 mice exhibit both reduced neurogenesis and neuroinflammation, therefore, it is a suitable mouse model for examining the effect of α-MSH on neurogenesis and neuroinflammation (Chishti et al., 2001; Dudal et al., 2004; Herring et al., 2009; Ma et al., 2011).
The effect of α-MSH on neurogenesis in AD has not been previously determined. GABA and its activation of GABA_A receptors play an important role in adult neurogenesis of the subgranular zone (SGZ) in the dentate gyrus of the hippocampus. GABA helps to regulate cell proliferation, migration, differentiation and synaptic integration of newborn neurons (Ge et al., 2007). In examining the GABAergic system in TgCRND8 mice, I found that both NPY and GABA_A receptor subunit δ mRNA levels are reduced in the hippocampus, which may lead to decreased hippocampal neurogenesis. NPY has been shown to modulate adult neurogenesis and exogenous administration of NPY increases cell proliferation in the SGZ of the dentate gyrus by activating NPY receptor Y1 (Decressac et al., 2011; Malva et al., 2012). GABA_A receptor subunit δ also plays a role in adult neurogenesis as mice lacking the subunit δ gene showed disrupted cell migration, maturation and dendritic development of newborn neurons (Whissell et al., 2013). α-MSH has been shown to promote neurogenesis in Mongolian gerbils by increasing cell proliferation in the SGZ after transient cerebral ischemia (Giuliani et al., 2011). However, in TgCRND8 mice, α-MSH treatment did not have an effect on reduced mRNA levels of NPY or GABA_A receptors in the hippocampus. It remains to be investigated whether α-MSH treatment promotes neurogenesis in TgCRND8 mice. Future experiments are required to determine if α-MSH treatment increases cell proliferation and differentiation in the SGZ of TgCRND8 mice. α-MSH may act through an alternative mechanism of increasing Wnt signaling to increase neurogenesis because administration of NDP-α-MSH analogue increased Wnt-3A expression to induce neurogenesis in Mongolian gerbils (Giuliani et al., 2011). Wnt signaling has been found to be the principal regulator of neurogenesis (Lie et al., 2005; Wu and Hen, 2013). α-MSH may promote neurogenesis in TgCRND8 mice via increased Wnt signaling instead of increasing NPY and GABA_A receptor subunit δ expression.

In addition to increasing neurogenesis, α-MSH reduces inflammation in the brain. α-MSH administration decreases expression of pro-inflammatory cytokines upon LPS stimulation (Huang and Tatro, 2002; Taylor, 2005; Caruso et al., 2007). Anti-inflammatory actions of α-MSH may be exerted via direct and indirect inhibition of NF-κB as discussed in the Introduction (section 1.7.3). To determine whether α-MSH suppresses inflammation in the TgCRND8 brain, activation of the immune cells astrocytes and microglia could be examined. Furthermore, a
multi-panel cytokine analysis could be performed to establish a cytokine expression profile for examining the inflammatory status in the brain. If α-MSH reduces inflammation, the mechanism of action could be inhibiting TLR4 downstream signaling by down-regulating CD14 expression, decreased IRAK-1 phosphorylation and inhibiting NF-κB activation. The anti-inflammatory action of α-MSH in the TgCRND8 brain remains to be investigated.

α-MSH has many effects on the brain. I have shown that α-MSH treatment protects GABAergic cell loss in an AD mouse model, in particular SST+ cells, to improve cognitive function. AD is a multifactorial disease with many pathological hallmarks. α-MSH treatment did not decrease Aβ concentration or Aβ plaque load in the TgCRND8 brain. In order to target more pathological features of AD to help ameliorate symptoms, combination therapy may be more effective. Our lab has previously shown that scyllo-inositol treatment inhibited Aβ aggregation into high-molecular-weight oligomers and decreased Aβ plaque load in the brain of TgCRND8 mice (McLaurin et al., 2006). As a result, neuroinflammation was attenuated by decreased activation of astrocytes and microglia and synaptic pathology was improved as the number of synaptophysin immunoreactive cell bodies and bouton was increased in the CA1 region of the hippocampus in treated TgCRND8 mice (McLaurin et al., 2006). Combination treatment with α-MSH and scyllo-inositol may confer neuroprotection while attenuating the toxic effects of Aβ to help reduce inflammation, promote neuronal survival and synaptogenesis.

α-MSH has been shown to induce neurogenesis by increasing the expression of Wnt-3A (Giuliani et al., 2011). However, NPY and the GABA_A receptor subunit δ have also been shown to regulate neurogenesis (Decressac et al., 2011; Malva et al., 2012; Whissell et al., 2012). My results show that both NPY and GABA_A receptor subunit δ mRNA levels are down-regulated in TgCRND8 mice. Since TgCRND8 mice exhibit decreased neurogenesis (Herring et al., 2009), combination therapy of α-MSH with exogenous administration of NPY and/or selective agonist for GABA_A receptor subunit δ (THIP), may increase the neurogenic potential of α-MSH by targeting GABAergic deficits not improved by α-MSH treatment. AD exhibits many pathological features that combination therapy may be required to target multiple aspects of the disease.
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