

ABSTRACT

Title of Document: CIRCUIT ANALYSIS OF SYNAPTIC
DYSFUNCTIONS IN THE CA3 AREA OF
BACE1 KNOCKOUT MICE

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Beta-amyloid precursor protein cleaving enzyme 1 (BACE1), a major neuronal β -secretase critical for the formation of β -amyloid ($A\beta$) peptide, is thought to be one of the key therapeutic targets that can prevent the progression of Alzheimer's disease (AD). Although complete ablation of *BACE1* gene prevents $A\beta$ formation, I found that at the mossy fiber projections in CA3 area of hippocampus, where BACE1 is highly expressed in normal brain, BACE1 knockout (KO) mice display reduced presynaptic function, as measured by an increase in paired-pulse facilitation ratio, and abolished mossy fiber LTP, which is very likely due to presynaptic Ca^{2+} signaling abnormality.

In order to determine the function of BACE1 in an intact CA3 circuit, whole-cell recordings were performed from pyramidal cells and inhibitory interneurons in the CA3 area that receive mossy fiber inputs. My analyses revealed a decrease in presynaptic release at mossy fiber synapses onto CA3 pyramidal cells of BACE1 KO

mice as determined by a significantly reduction in the frequency of miniature excitatory postsynaptic currents (mEPSCs) and enhanced paired-pulse facilitation ratio. In contrast, BACE1 KO mice do not exhibit significant dysfunction at mossy fiber input on CA3 inhibitory interneurons. However, presynaptic function at inhibitory input on CA3 pyramidal neurons is impaired in BACE1 KOs, as seen from a reduction in paired-pulse depression of inhibitory postsynaptic responses and a significant decrease in the frequency of miniature inhibitory postsynaptic currents (mIPSCs).

Finally, to restore the deficits caused by BACE1 inhibition, I demonstrated that brief application of nicotine can improve presynaptic release and recover mossy fiber LTP in BACE1 KOs by activating $\alpha 7$ -nAChRs, which recruits Ca^{2+} induced Ca^{2+} release to rescue the abnormal presynaptic Ca^{2+} signaling.

In summary, my studies suggest that BACE1 may play a critical role in regulating presynaptic function, especially activity-dependent strengthening of presynaptic release. The presynaptic dysfunction seen in BACE1 KOs is likely specified by the postsynaptic target, the CA3 pyramidal neurons, independent of the type of inputs. And nicotine or $\alpha 7$ -nAChR agonists may be a potential pharmacological means to circumvent the synaptic dysfunctions caused by BACE1 inhibition.

CIRCUIT ANALYSIS OF SYNAPTIC DYSFUNCTIONS IN THE CA3 AREA OF
BACE1 KNOCKOUT MICE

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Dedication

To my parents, Yujiao and Jizhong

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First of all, I would like to thank my advisor Dr. Hey-Kyoung Lee, who opened my mind in the world of science and guided me through step by step during these years. She is a great scientist and also incredible mentor, whenever I have difficulties or confusions about my research, she is always there, helps me, supports me and encourages me. I am very grateful to her and really enjoy working with her.

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Chapter 1: Introduction

Section 1 Alzheimer's disease and synaptic alteration

Subsection 1 What is Alzheimer's disease?

First described by German psychiatrist and neuropathologist Alois Alzheimer in 1906, Alzheimer's disease (AD) has been recognized as one of the most common forms of senile dementia around the world. AD is clinically characterized by a global decline of cognitive function including memory loss (Citron, 2004a). Although it has been studied for 100 years, there is still no effective disease-modifying treatment currently available for AD. It has been found that extracellular amyloid plaques consisting predominantly of amyloid beta 42 ($A\beta_{42}$), and intraneuronal tangles consisting of an aggregated form of the neuronal protein tau, are the two pathological hallmarks of AD (Citron, 2004a). Current theories implicate the production of $A\beta$ as a key molecular event (Hardy and Selkoe, 2002), that initiates synaptic dysfunction, which may be the basis for memory loss in early stages of the disease (Walsh and Selkoe, 2004a; Shankar and Walsh, 2009), ultimately, disease progression leads to severe neurodegeneration and memory loss. $A\beta$ is produced from sequential proteolytic cleavage of amyloid precursor protein (APP) by two endoproteolytic enzymes, β - and γ -secretase (Fig. 1.1). Consequently, inhibiting the activity of these enzymes has surfaced as one of the major disease-modifying approaches for AD (Citron, 2004a). Recently, it has been found that γ -secretase cleaves other important substrates necessary for normal cell development and function, such as Notch

(Sisodia and St George-Hyslop, 2002; Selkoe and Kopan, 2003). Therefore, inhibiting β -secretase is now receiving renewed attention (Vassar, 2002; Citron, 2004a, b). In order to develop effective therapeutics, a detailed molecular and cellular understanding of the role of β -secretase in synaptic function is necessary. In addition, accumulating evidence suggests that the initial pathology of AD is a result of synaptic dysfunction (Walsh and Selkoe, 2004b; Shankar and Walsh, 2009). Therefore, in my thesis, I focused on the consequences of inhibiting BACE1, identified as the major neuronal β -secretase (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999), at synaptic function and plasticity level, I tried to identify the synapses affected by losing BACE1 activity and the underlying mechanisms of the deficits in CA3 area of hippocampus, where the expression of BACE1 is most prominent in normal brain (Laird et al., 2005; Zhao et al., 2007). These findings will aid in the development of effective therapeutics that can overcome the negative effects of long-term BACE1 inhibition needed for AD treatment. First of all, I will briefly introduce the topic and current understanding of synaptic plasticity in the following two sections, which are relevant for the later discussions.

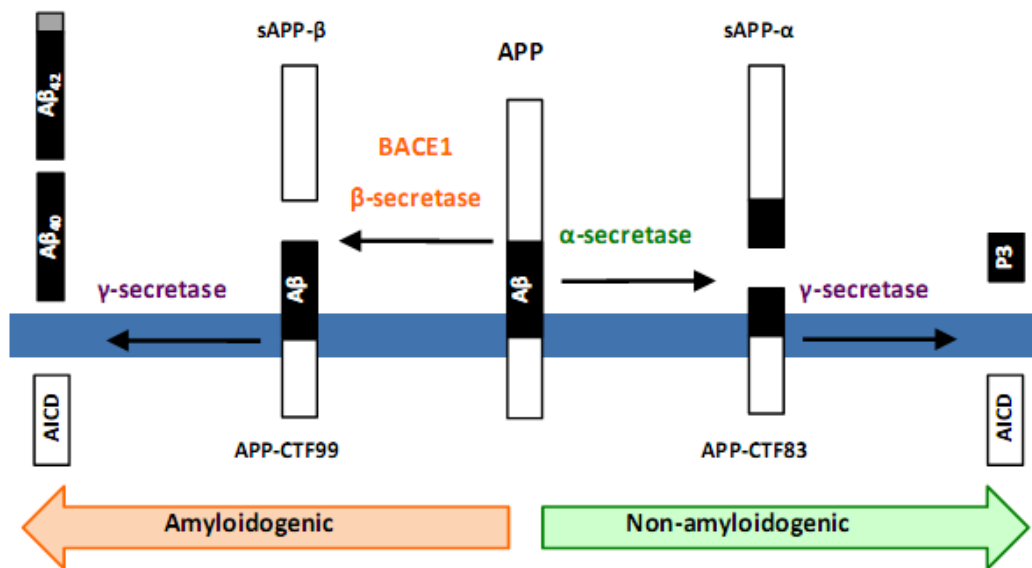


Figure 1.1. A diagram of amyloid precursor protein (APP) processing pathways.

The transmembrane protein APP (membrane indicated in blue) can be cleaved by two pathways, the non-amyloidogenic α -secretase pathway and the amyloidogenic β -secretase pathway. In the non-amyloidogenic pathway, α -secretase cleaves in the middle of the β -amyloid ($A\beta$) region (black) to release soluble APP-fragment sAPP- α . The APP C-terminal fragment 83 (APP-CTF83) is then cleaved by γ -secretase to release APP intracellular domain (AICD) and P3 fragment. In the amyloidogenic pathway, β -secretase cleaves APP and produces soluble fragment sAPP- β . The APP-CTF99 is then cleaved by γ -secretase to form $A\beta_{40}$, $A\beta_{42}$ and AICD.

Subsection 2 Synaptic plasticity and memory formation

It is widely believed that long-term changes in the strength of synaptic transmission underlie the formation of memories. Hebb is often recognized as the first person to crystallize this idea by proposing that coincident activity of pre- and postsynaptic neurons strengthens synaptic connections (Hebb, 1949). It was subsequently recognized that uncorrelated activity between two neurons should

decrease the strength of synaptic transmission between them (Stent, 1973). It has been demonstrated experimentally that high frequency stimulation, which can lead to correlated activity in pre- and postsynaptic cells, can indeed strengthen synapses (Bliss and Lomo, 1973). On the other hand, a prolonged low frequency stimulation of afferents, which would lead to presynaptic activation in the absence of correlated postsynaptic activity, produces a long-term decrease in synaptic transmission (Dudek and Bear, 1992; Mulkey and Malenka, 1992). The strengthening of synaptic connections is termed long-term potentiation (LTP), while the weakening of synaptic transmission is called long-term depression (LTD). Since their initial discovery, both LTP and LTD have been found to occur in a diverse set of synapses across many different brain areas (reviewed in (Malenka and Bear, 2004)). These long lasting forms of synaptic plasticity share similar mechanisms of induction, expression, and maintenance with those of long-term consolidation of several forms of memory (Lisman, 1989; Bailey et al., 1996; Bear, 1996; Martin et al., 2000; Paulsen and Sejnowski, 2000; Bliss et al., 2003; Lynch, 2004; Barco et al., 2006; Morris, 2006). Moreover, long-term alterations in synaptic transmission, similar to characteristics of LTP and LTD, have been observed *in vivo* during various learning paradigms (Rioult-Pedotti et al., 1998; Rodrigues et al., 2004; Schafe et al., 2005; Stefan et al., 2006; Whitlock et al., 2006), which further suggests that LTP and LTD may be cellular substrates for memory formation.

While LTP and LTD are effective models for mediating synapse-specific changes required for memory formation, theoretical considerations indicate that maintaining the stability of the nervous system requires additional homeostatic

plasticity mechanisms that operate at a slower time scale (hours to days) (Bienenstock et al., 1982; Bear et al., 1987; Abraham and Bear, 1996; Turrigiano et al., 1998; Turrigiano and Nelson, 2004). For example, without homeostatic regulation, the increase in postsynaptic activity after LTP might result in a vicious cycle of potentiation that not only degrades the capacity of neural circuits to store specific information, but could also culminate in a run-away excitation of the neural network. There are several mechanisms of homeostasis that can stabilize the nervous system: adjusting excitatory synaptic transmission postsynaptically (Bienenstock et al., 1982; Bear et al., 1987; Turrigiano et al., 1998; Abbott and Nelson, 2000; Turrigiano and Nelson, 2004), modulating the excitability of neurons (Desai et al., 1999; Aizenman et al., 2003; Maffei et al., 2004), changing inhibitory circuits (Kilman et al., 2002; Morales et al., 2002; Maffei et al., 2004; Maffei et al., 2006), and altering presynaptic function (Burrone et al., 2002; Thiagarajan et al., 2002; Thiagarajan et al., 2005). While most studies of synaptic plasticity related to memory formation focus on LTP and LTD, it is prudent to understand that alterations in homeostatic plasticity can also affect learning and memory.

Subsection 3 Molecular mechanisms of synaptic plasticity

While LTP and LTD have been observed in many different brain areas, the majority of knowledge about their molecular mechanisms comes from studies in the hippocampus. This is partly because the hippocampus is an area of the brain that is critically involved in the formation of long-term memories (reviewed in (Lynch, 2004)). In addition, the hippocampus is one of the areas highly susceptible to amyloid

pathology in most AD brains (reviewed in (Walsh and Selkoe, 2004b)). Therefore, I will briefly review the mechanisms of synaptic plasticity in the hippocampus.

In the hippocampus, two major forms of LTP and LTD are observed: one that is dependent on NMDA receptor (NMDAR) activation and another that is independent of NMDARs (Nicoll and Malenka, 1995; Lynch, 2004). The most widely studied forms of LTP and LTD are those dependent on NMDARs in the CA1 region; hence, their mechanisms have been fairly well characterized. NMDARs, due to activity-dependent relief of their Mg^{2+} block (Malenka and Nicoll, 1999), act as coincident detectors for pre- and postsynaptic activity. In addition, activation of NMDARs allows influx of Ca^{2+} (Connor et al., 1999; Yuste et al., 1999; Kovalchuk et al., 2000), which can act as a second messenger to activate various downstream effectors in the postsynaptic neuron. It is thought that both the magnitude and temporal pattern of Ca^{2+} increase determines the expression of either LTP or LTD, by differentially regulating the activity of protein kinases and phosphatases (Lisman, 1989). One of the key downstream events of LTP and LTD is regulation of synaptic AMPA receptors (AMPA receptors) (for review see (Malinow and Malenka, 2002; Lee, 2006)). AMPARs are the major mediators of fast excitatory synaptic transmission in the central nervous system (CNS), therefore their function directly dictates synaptic strength. Several studies demonstrated that LTP increases synaptic content of AMPARs, predominantly by an activity-dependent insertion of receptors containing the GluA1 subunit (GluR1) (Shi et al., 1999; Hayashi et al., 2000; Shi et al., 2001). This requires concomitant activation of Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) and phosphorylation of the AMPAR subunit GluA1 at serine 818 (S818)

(Boehm et al., 2006) and serine 845 (S845) (Esteban et al., 2003) or serine 831 (S831) (Esteban et al., 2003; Lee et al., 2010). GluA1-S818 is a protein kinase C (PKC) phosphorylation site (Boehm et al., 2006), while GluA1-S845 is a protein kinase A (PKA) phosphorylation site (Roche et al., 1996), and GluA1-S831 can be phosphorylated by both PKC (Roche et al., 1996) and CaMKII (Barria et al., 1997; Mammen et al., 1997). Many studies confirm that CaMKII, PKC, and PKA are involved in NMDAR-dependent LTP (reviewed in (Lee, 2006)). Consistent with a dominant role for GluA1 in mediating synaptic potentiation, GluA1 knockout mice (Zamanillo et al., 1999), as well as mice lacking specific phosphorylation sites on GluA1 (Lee et al., 2003), display LTP deficits. On the other hand, NMDAR-dependent LTD is associated with an activity-dependent removal of synaptic AMPARs (Carroll et al., 2001). This process depends on endocytosis of GluA2-containing receptors (Luthi et al., 1999; Daw et al., 2000; Lin et al., 2000; Man et al., 2000; Osten et al., 2000; Kim et al., 2001a; Lee et al., 2004), but also requires dephosphorylation at GluA1-S845 (Lee et al., 1998; Lee et al., 2000a; Lee et al., 2003).

While regulation of synaptic AMPARs, through synaptic targeting and phosphorylation, is involved in the initial expression of LTP and LTD, maintenance of these forms of plasticity involve additional mechanisms. Collectively, data from many studies report that blocking new protein synthesis inhibits the late phase of long-term synaptic plasticity (Krug et al., 1984; Stanton and Sarvey, 1984; Frey et al., 1988; Huber et al., 2000; Manahan-Vaughan et al., 2000; Kelleher et al., 2004). This parallels the requirement for new protein synthesis in the formation of long-term

memory in intact animals (Flexner et al., 1963; Davis and Squire, 1984) (see review(Sutton and Schuman, 2006)). Transcriptional activation is also necessary for the maintenance of some forms of long-term synaptic plasticity (Nguyen et al., 1994). So far, it is known that multiple transcription factors are activated immediately after induction of LTP. Increased transcription of several immediate early genes (IEG) is especially important (Tischmeyer and Grimm, 1999) since they enhance new protein synthesis (Lynch, 2004; Barco et al., 2006). Interestingly, some, if not all, of these transcriptional regulators are also required for long-term memory formation. Disruption of cAMP Response Element-Binding Protein (CREB) levels, a Ca^{2+} -dependent transcription factor, in either the hippocampus or the amygdala has been found to impair specific long-term memory but not initial acquisition or short-term memory formation (Guzowski and McGaugh, 1997; Lamprecht et al., 1997; Silva et al., 1998). Inhibiting the expression of Arc/Arg 3.1 (activity-regulated cytoskeletal protein/activity regulated gene 3.1), one of the immediate early genes (IEGs), in the hippocampus also impairs long-term memory consolidation (Guzowski et al., 2000).

Subsection 4 Exogenous A β application alters synaptic function

Molecular understanding of AD came from studying familial AD (FAD)-linked mutations which have been found in genes encoding APP and presenilin 1 and 2 (PS1 and 2) in AD patients. These mutations are linked to elevated A β production (Scheuner et al., 1996; Lendon et al., 1997). This is because many FAD-linked mutations make APP a more favorable substrate for the amyloidogenic cleavage pathway leading to increased A β production. Since FAD patients often harbor multiple mutations, many of the AD mouse models carry several FAD mutations.

However, depending on the combination of the mutations and their variants, distinct phenotypes are observed across age and brain regions studied (for an extensive recent review on electrophysiological studies of various AD transgenic (Tg) mouse models see (Marchetti and Marie, 2011)).

Although different AD mouse models show deficits in synaptic function, it cannot be taken for granted that these deficits are caused directly by the enhanced production of A β peptides (especially the A β ₄₂, which is the major component of extracellular senile plaques). In order to directly test the role of A β in altering synaptic function, many studies have been done to characterize synaptic properties and synaptic plasticity following exogenous application of various A β peptides.

In vitro studies done in either the medial perforant path to dentate granule cells or the Schaffer collateral inputs to CA1 neurons, reported that application of subneurotoxic concentrations of A β peptides (i.e. A β ₄₂, A β ₄₀, or A β ₂₅₋₃₅) inhibits LTP induction without affecting basal synaptic transmission (Chen et al., 2000; Chen et al., 2002; Zhao et al., 2004). A similar result was found in an *in vivo* study, where naturally secreted A β collected from cells expressing mutated APP (V717F mutation in APP₇₅₁) was injected into the CA1 region of hippocampus which prevented stable LTP maintenance (Walsh et al., 2002). This study further showed that soluble A β oligomers are responsible for blocking LTP, not monomeric A β , or A β fibrils (Walsh et al., 2002). In addition, *in vivo* injection of A β peptides (i.e. A β ₄₂ or the C-terminal of APP containing the A β fragment) is reported to facilitate LTD and LTP reversal (called depotentiation) in the CA1 region (Kim et al., 2001b). A majority of studies suggest that while fibrillar A β accumulation is found in senile plaques that are a

hallmark of AD, it is the soluble A β oligomers that disturb synaptic function and lead to neurodegeneration in AD (Walsh et al., 2002; Tanzi, 2005).

How is A β affecting long-term synaptic plasticity? Soluble A β oligomers in AD brains have been found to bind to neuronal surfaces (Gong et al., 2003), specifically to a subset of synapses where they colocalize with a postsynaptic density marker PSD95 (Lacor et al., 2004), suggesting that A β may regulate postsynaptic function directly. One candidate target of A β is NMDARs. It was found that synthetic A β_{40} peptides can selectively augment NMDAR current, without affecting AMPAR current, in the dentate gyrus of acute hippocampal slices (Wu et al., 1995). Consistent with this, APP_{Ind} (V717F mutation) Tg mice show an enhancement in the ratio of NMDAR-to-AMPA-mediated synaptic transmission in the CA1 region (Hsia et al., 1999). However, contradictory results are reported from later studies. A recent study showed that both application of synthetic A β_{42} peptides and naturally secreted A β , from APP_{Swe} (K670N/M671L mutation) Tg mice, promote endocytosis of surface NMDARs and hence depresses NMDAR current in wildtype cultured cortical neurons (Snyder et al., 2005). Moreover, they also found reduced surface expression of NMDARs in cultured cortical neurons from APP_{Swe} Tg mice (Snyder et al., 2005). Other studies found down-regulation of surface AMPARs in neurons overexpressing either wildtype or APP_{Swe}, or when wildtype neurons were treated with exogenous A β_{42} peptides (Almeida et al., 2005; Hsieh et al., 2006). The mechanisms involved are not only endocytosis of synaptic AMPARs via mechanisms shared by LTD (Hsieh et al., 2006), but also a reduction of basal levels of S845 phosphorylation by activating the calcium-dependent phosphatase, calcineurin, as well as interrupting

extrasynaptic delivery of AMPARs (Minano-Molina et al., 2011). Contradictory results on the effects of A β on AMPAR and NMDAR regulation may be due to several variables. First, there is evidence that A β_{40} and A β_{42} peptides may have distinct functions in AD pathology. For example, a majority of FADs caused by PS1 mutations have reduced A β_{1-40} peptides and therefore an increase in the A β_{42} /A β_{40} ratio (Borchelt et al., 1996; Thinakaran and Sisodia, 2006). Second, there are differences in experimental preparations. Both Wu et al. (Wu et al., 1995) and Hsia et al. (Hsia et al., 1999) were working with acute adult hippocampal slices, while Snyder et al. (Snyder et al., 2005), Almeida et al. (Almeida et al., 2005), Hsieh et al. (Hsieh et al., 2006) and Minano-Molina et al. (Minano-Molina et al., 2011) were using either cultured neurons from embryonic mice or organotypic hippocampal slice cultures prepared from early postnatal mice. Third, the presence or absence of APP itself may have also affected the results. Indeed there is evidence that uncleaved full-length APP may promote synapse formation and enhance excitatory synaptic function (see (Hoe et al., 2012) for a recent review).

In any case, A β mediated alterations in NMDAR function suggests that A β will affect downstream Ca²⁺-dependent signaling pathways. Calcineurin, a Ca²⁺-activated protein phosphatase, may be one of the downstream signaling molecules affected by A β , since it is required for the inhibition of perforant pathway LTP (Chen et al., 2002), endocytosis of surface AMPARs (Hsieh et al., 2006), as well as dephosphorylation of GluA1-S845 (Minano-Molina et al., 2011). In addition to activating calcineurin, A β prevents the activation of CaMKII, a Ca²⁺-dependent protein kinase necessary for LTP, and decreases the synaptic cluster of CaMKII,

which correlates with a reduction in the phosphorylation of GluA1-S831, surface expression of GluA1, and AMPAR mediated EPSCs (Zhao et al., 2004; Gu et al., 2009). Together, these data are consistent with the idea that A β oligomers impair LTP and facilitate LTD (Lee et al., 2000a; Knobloch et al., 2007; Li et al., 2009).

A β has also been found to modify regulation of gene expression. A β peptides have been found to alter CREB signaling, which causes synaptic dysfunction and memory deficits (reviewed in (Saura and Valero, 2011)). In addition, treating cultured hippocampal neurons with soluble A β oligomers induces rapid expression of the IEG Arc/Arg 3.1 (Lacor et al., 2004), which is implicated in synaptic plasticity (Guzowski et al., 2000; Steward and Worley, 2001; Shepherd et al., 2006). Because overexpression of Arc/Arg 3.1 causes learning dysfunctions (Guzowski, 2002), possibly via reducing surface expression of GluA1-containing AMPARs (Shepherd et al., 2006), this would suggest that A β oligomer-induced Arc/Arg3.1 expression may in fact interfere with synaptic plasticity. However, this study is seemingly at odds with the results of Echeverria and colleagues, which reported a strong inhibition of BDNF-induced increase in Arc expression in cultured cortical neurons treated with A β oligomers (Echeverria et al., 2007). Similarly, there is also a report that synaptic plasticity related genes, including Arc/Arg3.1, are reduced in transgenic mice expressing FAD-linked mutations in APP and PS1 (Dickey et al., 2003). The apparent differences in Arc expression caused by A β could be due to different experimental systems, or that A β may modulate Arc expression via distinct pathways which may depend on the effective concentration of A β oligomers.

Besides influencing postsynaptic function, A β is also implicated in presynaptic modifications. A recent study reported that 8 nM A β_{42} globulomer (a highly stable globular oligomeric A β) could directly inhibit presynaptic P/Q type Ca $^{2+}$ channels and decrease vesicle release (Nimmrich et al., 2008). Moreover, application of synthetic A β to cultured hippocampal neurons causes a down-regulation of dynamin, a protein critical for synaptic vesicle endocytosis, and interrupts synaptic vesicle recycling (Kelly et al., 2005; Kelly and Ferreira, 2007). This result is consistent with the observed reduction in dynamin levels in human AD brains (Yao et al., 2003). These findings may explain the observation that A β_{42} globulomer causes a decrease in basal synaptic transmission at the Schaffer collateral to CA1 synapses in hippocampal slice culture (Nimmrich et al., 2010). Recently, Kelly et al. reported that the reduction in dynamin is dependent on Ca $^{2+}$ influx through activated NMDARs as well as activation of a calcium-activated intracellular cysteine protease calpain (Kelly et al., 2005; Kelly and Ferreira, 2006). These results not only suggest that there may be retrograde signaling from postsynaptic to presynaptic terminals, but also establish an interesting relationship between A β , NMDARs and calpain. It has been found that A β_{42} peptides can activate calpain-mediated cleavage of p35 to p25 (Lee et al., 2000b), which then upregulates mRNA and protein expression of β -secretase (BACE1) (Wen et al., 2008; Liang et al., 2010), a critical enzyme for A β formation (discussed in the following sections). This indicates that there is a positive feedback between A β production and calpain activation. The observation that calpain inhibitors can fully recover deficits in basal synaptic transmission caused by A β globulomer application in hippocampal slice culture, to the level that is comparable to using an

NMDAR antagonist (Nimmrich et al., 2010), suggests that A β induced activation of NMDARs and calpain may share a common pathway. It is likely that A β induces Ca²⁺ influx through NMDARs and activates intracellular calpain, which further promotes p25/cdk5 dependent transcription of downstream genes, including BACE1 (Wen et al., 2008).

In addition to NMDARs, recent studies suggest that the α 7-nicotinic acetylcholine receptor (α 7-nAChR), a Ca²⁺-permeable homopentameric ion channel highly expressed in the hippocampus and cerebral cortex (Seguela et al., 1993), is another potential target of A β . High affinity binding between A β ₄₂ peptides and α 7-nAChRs (Wang et al., 2000b; Wang et al., 2000a) either inhibit (Guan et al., 2001; Liu et al., 2001a; Pettit et al., 2001; Chen et al., 2006) or activate α 7-nAChR signaling (Dineley et al., 2001). It is possible that A β ₄₂ peptides may facilitate α 7-nAChRs at low concentrations, but may inhibit α 7-nAChRs when the burden of A β increases (Dineley et al., 2001; Dougherty et al., 2003). This concentration-dependent dual role of A β ₄₂ peptides is also suggested from a study showing that picomolar concentrations of synthetic A β ₄₂ peptides facilitate, but nanomolar concentrations abolish, hippocampal LTP and learning via their interaction with α 7-nAChRs (Puzzo et al., 2008).

Moreover, the double-edged sword effect of A β is also reflected by its ability to regulate reactive oxygen species (ROS). ROS have been found to have normal physiological roles in maintaining normal synaptic plasticity. However, high levels of ROS have been found in both AD animal models and human patients, leading to oxidative damage related to AD pathology (reviewed in (Massaad and Klann, 2011)).

Recently, Ma and colleagues found that exogenous treatment of A β ₄₂ (500 nM) increased mitochondria superoxide, which they reported is a cause of synaptic dysfunction induced by A β . In particular, decreasing the level of mitochondrial superoxide reversed A β -induced CA1 LTP impairments (Ma et al., 2011). Given the normal physiological role of A β and ROS at low levels, this finding suggests that ROS imbalance, caused by A β toxicity, may lead to synaptic dysfunction in AD. Also, it implies that A β levels exceeding the normal range may initiate the abnormalities in synaptic function (Fig. 1.2).

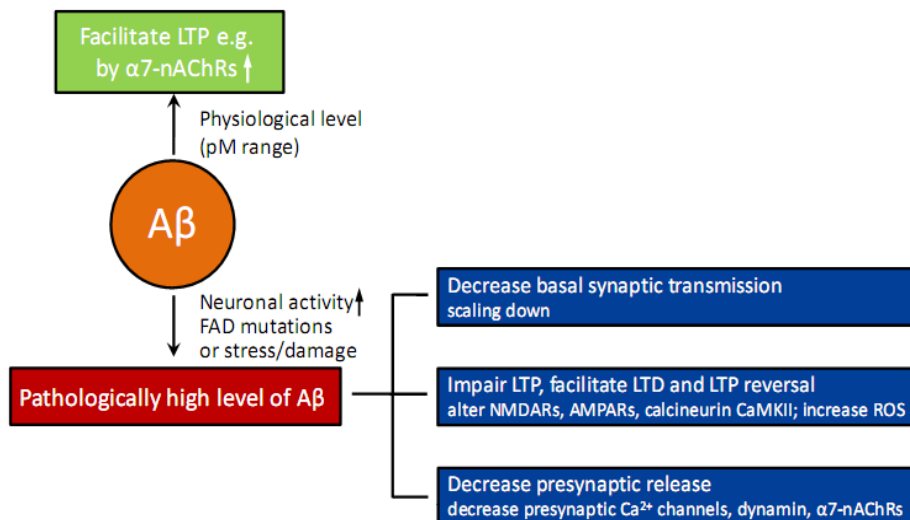


Figure 1.2. A β is double-edged sword on synaptic function.

At normal physiological levels (picomolar range), A β peptides have positive effects on synaptic function: they can facilitate learning and LTP in CA1 by activating α 7-nAChRs. However, under pathological conditions, such as increased neuronal activity, stress or the presence of familial Alzheimer’s disease (FAD) mutations, the increase in A β peptide concentration produces pathological effects, including decreased basal synaptic transmission, decreased presynaptic release, LTP impairment and LTD facilitation. Therefore, maintaining the concentration of A β peptides within a normal physiological level is essential, which should be the goal for developing effective treatments for Alzheimer’s disease.

In summary, pathologically high levels of A β can disturb ROS balance and interfere with both pre- and postsynaptic function, presumably by affecting NMDARs, presynaptic P/Q Ca²⁺ channels, and/or α 7-nAChRs; thereby interrupting subsequent Ca²⁺ signaling leading to altered synaptic function.

Subsection 5 Neuronal activity can regulate APP processing and A β levels

Data from both transgenic mice and exogenous A β application studies suggest that alterations in A β levels change neuronal activity and synaptic function. It is now evident that neuronal activity itself can also regulate APP processing leading to alterations in A β production. In 1993, a study reported that electrical stimulation not only increases neurotransmitter release in rat hippocampal slices, but also enhances the release of APP cleavage products (Nitsch et al., 1993). In agreement with this finding, ten years later, Kamenetz and colleagues (Kamenetz et al., 2003) found that neuronal activity can bidirectionally control A β levels in organotypic hippocampal slice cultures from APP_{Swe} Tg mice. Blocking neuronal activity in this preparation by tetrodotoxin (TTX) treatment reduced A β levels, while increasing neuronal activity with picrotoxin (PTX) enhanced A β secretion (Kamenetz et al., 2003). The experimental paradigm used by Kamenetz et al. to manipulate neuronal activity is reported to produce homeostatic synaptic plasticity termed “synaptic scaling” (Turrigiano et al., 1998), which globally up- or down-regulates all excitatory synaptic synapses following prolonged decrease or increase, respectively, in neuronal activity (Turrigiano and Nelson, 2004). This suggests that A β may play a role in regulating homeostasis of excitatory synapses in normal brains. In addition, the cellular mechanism responsible for regulating APP processing and A β production in response

to neuronal activity, is possibly through enhancing the accessibility of APP to γ -secretase cleavage (Kamenetz et al., 2003) and/or depressing γ -secretase function (Lesne et al., 2005). It has recently been shown that PS1, the catalytic subunit of the γ -secretase complex, is necessary to scale up excitatory synapses following reduced network activity and that PS1 knockout mice show deficits in synaptic scaling (Pratt et al., 2011). Moreover, Wu and colleagues have reported that the IEG Arc is required for activity-dependent increases of A β production (Wu et al., 2011). They found that Arc directly binds the N terminus of PS1 and plays an important role in trafficking γ -secretase to early endosomes where APP is processed through β -secretase pathway to produce A β . In addition, Arc contributes to A β levels and plaque load in APP_{swe};PS1 Δ E9 AD mice, and Arc expression is elevated in medial frontal cortex of AD patients (Wu et al., 2011). These results provide cellular mechanism of A β generation coupled to neuronal activity, and may explain why people who suffer from hypoxia, which usually causes an abnormal enhancement in neuronal activity (Talos et al., 2006), have a higher risk for developing AD (Desmond et al., 2002).

Consistent with the idea that A β induces homeostatic adaptation to increase in activity, *in vivo* studies have also shown that either electrical stimulation or endogenous whisker activity proportionally regulate interstitial fluid (ISF) A β levels in Tg2576 mice, which overexpress human APP carrying the Swedish (K670N/M671L) mutation (Cirrito et al., 2005; Cirrito et al., 2008; Bero et al., 2011). However, there are also contradictory results. Tampellini et al. have shown that synaptic activity decreases intracellular A β in primary neuronal culture, as well as in the barrel cortex of 4 month old Tg19959 mice, which overexpress human APP

carrying the Swedish (K670N/M671L) and Indiana (V717F) mutations (Li et al., 2004a), likely by enhancing A β degradation (Tampellini et al., 2009). Zhang et al. have reported that prolonged olfaction deprivation facilitates amyloid plaque deposition in the olfactory bulb and piriform cortex of 7-24 month old Tg2576 mice (Zhang et al., 2010). These contradictions may be due to age, region, and paradigm differences. Another possibility is that normal neuronal activity regulates A β levels by balancing A β release and degradation, and that either hyperactivity or hypoactivity may break this balance leading to A β accumulation.

Subsection 6 Physiological roles of APP and A β

Proteolytic processing of APP not only produces A β peptides, but also other products. Some functions of these products have been identified (reviewed in (Pearson and Peers, 2006)). For example, the cytoplasmic tail of APP, APP intracellular domain (AICD), is shown to participate in transcriptional regulation (Cao and Sudhof, 2001). To evaluate other physiological roles of APP, mice lacking APP were generated. APP knockouts show enhanced excitatory synaptic activity and neurite growth (Priller et al., 2006), which is consistent with the finding that APP-deficient mice are more susceptible to glutamate-induced toxicity (Steinbach et al., 1998). Similar to APP, A β peptides also have normal physiological functions. Normal physiological levels (picomolar range) of A β peptides regulate synaptic function by facilitating learning and LTP in CA1 (Puzzo et al., 2008). Additionally, preventing A β production by adding β - or γ -secretase inhibitors in cultured neurons causes cell death, which can be rescued by applying synthetic A β peptides to culture medium (Plant et al., 2003). The resulting cell death is likely caused by excitotoxicity, as A β

has been identified as a negative feedback regulator of excitatory synaptic transmission (Kamenetz et al., 2003).

Collectively, these data suggest that proteolytic processing of APP and the presence of a physiological dose of A β may be required for maintaining proper neuronal activity and brain function. While the therapeutic benefits of targeting APP processing and A β production are still attractive, it should be noted that AD pathology is most likely triggered only when A β levels exceed the normal range, and that the physiological processing of APP and A β production may be important in maintaining normal brain functions. Therefore, partial inhibition, but not complete blockade, of A β production might be a useful approach for AD therapeutics. A recent study supports this view. Immunizing APP_{Ind} Tg mice against A β , which lowered A β levels, decreased senile plaque formation, and rescued loss of neuronal integrity seen previously in aged mice (Buttini et al., 2005).

Subsection 7 Role of BACE1 in synaptic function

As mentioned in Subsection 1, A β peptides are generated from sequential cleavage of APP by β - and γ -secretase (Fig. 1.1). In the brain, beta-site APP cleaving enzyme (BACE1), a transmembrane aspartic protease, has been found to be the major neuronal β -secretase (Vassar et al., 1999; Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001). Mice lacking the BACE1 gene show no β -secretase activity and essentially no A β (A β ₄₀ and A β ₄₂) production in the brain compared to wildtype littermates. Initial characterization of BACE1 knockouts (BACE1 KOs) showed that they are viable and fertile, with no gross differences in behavior and development (Cai et al., 2001; Luo et al., 2001; Roberds et al., 2001; Ohno et al., 2004). Furthermore,

knocking out the *BACE1* gene in mouse models of AD was able to rescue hippocampus-dependent memory deficits (Ohno et al., 2004; Laird et al., 2005; Ohno et al., 2006) and ameliorate impaired hippocampal cholinergic regulation of neuronal excitability (Ohno et al., 2004). These findings were quite encouraging and suggested that BACE1 may be a good therapeutic target for treating AD (Citron, 2002; Vassar, 2002; Citron, 2004b).

However, recent studies have found that BACE1 has normal physiological functions in synaptic transmission and plasticity in CA1 region of hippocampus. Laird et al. found that BACE1 KO mice display deficits in both synaptic transmission and plasticity at the hippocampal Schaffer-Collateral to CA1 synapses (Laird et al., 2005). While BACE1 KO mice display normal AMPAR- and NMDAR-mediated synaptic transmission, these synapses show a larger paired-pulse facilitation (PPF) ratio compared to wildtype littermates when tested with paired-pulse stimuli at a 50ms interstimulus interval (Laird et al., 2005). Changes in PPF ratio are linked to alterations in presynaptic function (Manabe et al., 1993). Therefore, the increase in PPF ratio observed in BACE1 KO mice, indicates a reduction in presynaptic function, which is consistent with the high expression of BACE1 in presynaptic terminals (Laird et al., 2005). In addition to reflecting presynaptic changes, recent data suggest that alterations in PPF ratio can also be caused by postsynaptic modifications, such as by varying the subunit composition of AMPARs (Rozov et al., 1998). Therefore, it is possible that knocking out of *BACE1* may also affect postsynaptic AMPAR function. Besides alterations in the PPF ratio, BACE1 KO mice also showed a larger depression (reversal of LTD) induced by high frequency theta burst stimulation (TBS)

at the Schaffer collateral inputs to CA1 (Laird et al., 2005). In contrast, the same TBS protocol induced-LTP remained unchanged (Laird et al., 2005). As LTP and de-depression have separate underlying mechanisms (Lee et al., 2000a), these data suggest BACE1 may play a regulatory role in the de-depression pathway, while not affecting the mechanisms that lead to LTP. Laird and colleagues also found evidence that the enhanced de-depression is due to larger summation of responses during TBS, specifically following LTD induction. Enhanced summation of synaptic responses during the induction of de-depression despite normal basal synaptic transmission suggests that BACE1 may play a specific role in activity-dependent high frequency information transfer across synapses. Also, the abnormal increase in the magnitude of de-depression reflects that LTD expression may be easily disrupted when knocking out *BACE1*, which could interfere with memory formation and storage. Consistent with this interpretation, detailed behavioral studies on BACE1 KO mice reported problems in both cognitive and emotional memory tests (Harrison et al., 2003; Laird et al., 2005; Ma et al., 2007). Although the majority of studies characterizing synaptic function of BACE1 KOs have been performed in the CA1 region of hippocampus (Ohno et al., 2004; Laird et al., 2005; Ma et al., 2007), the expression of BACE1 is most prominent in the CA3 area of hippocampus (Laird et al., 2005; Zhao et al., 2007). Therefore, it is essential to investigate the synaptic alteration caused by BACE1 inhibition in CA3 area, which is the main focus of my thesis.

Since synaptic deficits are seen in the CA1 region of hippocampus in BACE1 KO mice, it indicates that BACE1 may play a general role in regulating synaptic function. Whether synaptic deficits in BACE1 KO mice are solely due to a lack of

APP processing is unclear. From the view that A β suppresses synaptic transmission (discussed in Subsection 4), it seems counterintuitive that abolishing A β production, as in BACE1 KO mice, would reduce presynaptic function. However, considering the fact that physiological concentration of A β (pM range) facilitates synaptic plasticity (Puzzo et al., 2008), the absence of A β could produce synaptic deficits. An alternative possibility is that the synaptic dysfunction of BACE1 KO mice may arise from abnormal processing of substrates other than APP (Fig. 1.3).

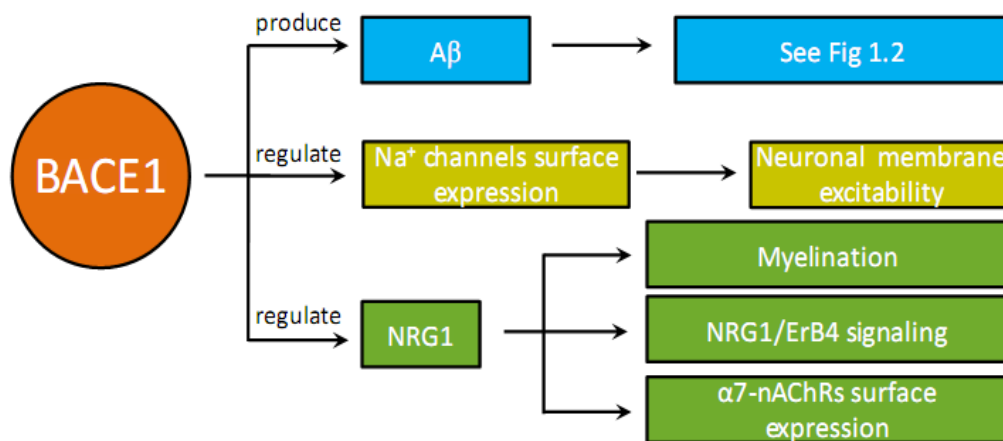


Figure 1.3. The roles of BACE1 in synaptic function.

Besides cleaving APP to produce A β peptides, BACE1 has been found to have other substrates. It can process the β 2 subunit of voltage-gated sodium (Na⁺) channel, which can regulate Na⁺ channel surface expression and in turn modulate neuronal excitability. In addition, BACE1 can cleave NRG1 which plays a crucial role in myelination and NRG1/ErB4 signaling. Recently, it has been showed that NRG1 can regulate the cell surface expression of α 7-nAChRs, which can also affect synaptic transmission.

It has been shown that the auxiliary $\beta 2$ subunit of the voltage-gated sodium channel (Na_v1), is a substrate of BACE1 (Wong et al., 2005; Kim et al., 2007). The $\beta 2$ subunit of the Na_v1 channel is important for plasma membrane expression of functional Na^+ channels, which is critical for generating action potentials. Among the ten different types of Na_v1 channels, $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$ and $\text{Na}_v1.6$ are expressed mainly in the central nervous system (CNS) (Lai and Jan, 2006). By cleaving the $\beta 2$ subunit, BACE1 regulates the surface expression of these types of Na_v1 channels. In transgenic mice over-expressing BACE1, there is an increase in $\text{Na}_v1.1$ α -subunit mRNA and protein levels, but a decrease in the surface expression of functional $\text{Na}_v1.1$ channels due to cleavage of the $\beta 2$ subunits (Kim et al., 2007; Kim and Kovacs, 2011). The interpretation is that the full-length $\beta 2$ subunit promotes surface expression of $\text{Na}_v1.1$ channels, but the $\beta 2$ -intracellular domain (ICD), which is produced by a sequential cleavage by BACE1 and γ -secretase, increases transcription of the $\text{Na}_v1.1$ α -subunit gene. Consistent with this, BACE1 KO mice display a decrease in $\text{Na}_v1.1$ α -subunit mRNA and protein (Kim et al., 2011). However, there is a compensatory increase in the surface expression of $\text{Na}_v1.2$ in the BACE1 KO mice, which correlates with the hyperexcitability and seizure phenotypes seen in BACE1 KOs (Hu et al., 2010). These results suggest that the ability of BACE1 to regulate the Na_v1 family of Na^+ channels is rather complex, but suggests a role for BACE1 in regulating neuronal excitability.

Another candidate substrate for BACE1 is neuregulin-1 (NRG1), which is an axonal signaling molecule critical for regulating myelination (Lemke, 2006). Willem and colleagues found that BACE1 KO mice show hypomyelination in the peripheral

nerves (Willem et al., 2006), while another study detected loss of myelination in the central nerves (Hu et al., 2006). Both of these studies showed an accumulation of unprocessed NRG1 and a reduction in its cleavage products, suggesting that NRG1 is a potential substrate for BACE1 cleavage and that this process is important for myelination of axons (Hu et al., 2006; Willem et al., 2006). Recently, it has been shown that the absence of NRG1 processing in BACE1 KO mice decreased postsynaptic function of ErbB4, a receptor for NRG1 (Savonenko et al., 2008). NRG1/ErbB4 signaling has been suggested to regulate synaptic function and plasticity, mainly via regulation of postsynaptic glutamate receptors (Huang et al., 2000; Gu et al., 2005b; Li et al., 2007). Additionally, abnormal processing of NRG1 may also affect presynaptic release by regulating the expression of $\alpha 7$ -nAChRs (Liu et al., 2001b; Zhong et al., 2008), which allows Ca^{2+} influx (Seguela et al., 1993). Indeed, presynaptic nAChRs can increase glutamate release (McGehee et al., 1995; Gray et al., 1996; Maggi et al., 2003a), likely via the $\alpha 7$ containing nAChRs (Le Magueresse et al., 2006). These results suggest that the abnormal NRG1 cleavage caused by BACE1 inhibition can alter synaptic function both pre- and postsynaptically.

Accumulating data on the biological roles of BACE1 suggest caution for using BACE1 inhibitors as a treatment for AD. More and more studies indicate that complete inhibition of BACE1 activity is deleterious for neuronal function. In order to improve the development of effective therapeutics that target this enzyme, we need to seek potential ways to overcome the synaptic dysfunction associated with blocking BACE1.

Section 2 Synaptic plasticity at mossy fiber synapses

As one of the three important pathways in hippocampus, the mossy fiber pathway is composed of dentate gyrus granule cell axon projections, the so called mossy fibers on dendrites of pyramidal cells within the stratum lucidum of CA3 area and the filopodial extensions from mossy fiber boutons, which contact inhibitory interneurons forming feedforward inhibition in local CA3 circuitry (Chicurel and Harris, 1992; Acsady et al., 1998) (Fig. 1.4). Although they are originated from common mossy fiber input, synapses on CA3 pyramidal cells and those on CA3 interneurons are not only anatomically different, but also functionally specialized.

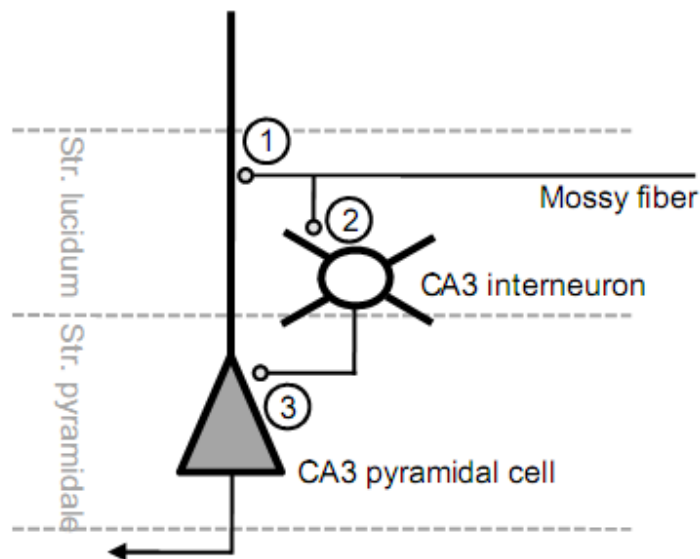


Figure 1.4. A sketch of hippocampal CA3 circuitry.

Mossy fibers (MF) form glutamatergic excitatory synapses onto CA3 pyramidal cells (PCs) (1) as well as onto inhibitory interneurons (INTs) (2) in the stratum lucidum; inhibitory neurons project GABAergic synapses onto CA3 PCs (3). This configuration is feedforward inhibition, in which PCs and INTs are excited simultaneously by common afferents (MF inputs), with INTs in turn inhibiting PCs with a short delay relative to the monosynaptic excitation.

Subsection 1 Mossy fiber at CA3 pyramidal cell synapses

i) Short-term plasticity

The axons of the dentate gyrus granule cells innervate their pyramidal neuron targets via giant mossy fiber boutons which have multiple active zones (Chicurel and Harris, 1992; Henze et al., 2000). The mossy fiber synapse onto CA3 pyramidal cells is distinct from most other synapses in the CNS due to its large paired-pulse facilitation (PPF) and uniquely robust frequency facilitation (Salin et al., 1996), which are presynaptic forms of short-term plasticity. The large PPF and robust frequency facilitation are consistent with a low probability of neurotransmitter release at these synapses (Jonas et al., 1993; Lawrence et al., 2004).

CA3 pyramidal cells not only receive mossy fiber inputs, they also receive inputs from other CA3 pyramidal cells, forming associational-commissural synapses. Associational inputs are the projections from other CA3 pyramidal cells on the same side of the brain, commissural inputs are CA3 - CA3 connections between the two hemispheres. Unlike mossy fiber synapses onto CA3 pyramidal cells, associational-commissural synapses show very little facilitation (Salin et al., 1996; Dobrunz and Stevens, 1999), suggesting a stronger presynaptic release.

ii) Long-term potentiation

In striking contrast to LTP in CA1 region of hippocampus discussed in Section 1, high frequency stimulation induced mossy fiber LTP is a presynaptic form of LTP, which is NMDAR independent (Harris and Cotman, 1986). As early as 1994, Weisskopf and colleagues reported that the mechanism of mossy fiber LTP is mediated by presynaptic cAMP pathway (Weisskopf et al., 1994), which is initiated

by Ca^{2+} influx in response to high frequency stimulation at mossy fiber terminals. The increased Ca^{2+} activates calmodulin, which in turn stimulates adenylyl cyclase, therefore increasing cAMP level and activating cAMP dependent protein kinase A (PKA). PKA phosphorylation of specific synaptic vesicle proteins facilitates presynaptic vesicle release, which results in a long-lasting increase in presynaptic release. Recent studies have indicated several PKA phosphorylation targets that are indispensable for mossy fiber LTP. The synaptic vesicle protein Rab3A is required for mossy fiber LTP as shown by an absence of mossy fiber LTP in mice lacking Rab3A (Castillo et al., 1997). In addition, Lonart et al. identified that RIM1 α , an active zone protein that binds to Rab3A, is a PKA substrate that is necessary to presynaptic LTP (Lonart et al., 2003). Another candidate of PKA phosphorylation is Rabphilin, an effector protein of Rab3A, PKA phosphorylation of Rabphilin is specifically detected in CA3 region in response to LTP induction compared to CA1 (Lonart and Sudhof, 1998).

Although LTP at mossy fiber terminals on CA3 pyramidal cell synapses is widely accepted as a presynaptic form of LTP, recently it has been found that NMDAR dependent LTP can also be induced by relative weak stimulation at these synapses. Kwon and colleagues found that this form of LTP is not due to an increase in presynaptic release probability. In contrast to the “classical” mossy fiber LTP, the induction of NMDAR dependent mossy fiber LTP requires not only NMDAR, but also mGluR5 activation, which stimulates postsynaptic PKC pathway, and further induces postsynaptic Ca^{2+} release from internal Ca^{2+} store (Kwon and Castillo, 2008b). Identification of NMDAR mediated mossy fiber LTP has profound

significance. Given the slow kinetics of NMDAR-mediated synaptic responses, NMDAR mediated mossy fiber LTP could modify the temporal nature of synaptic integration in CA3 pyramidal cells. Moreover, it has been reported that selective removal of NMDAR NR1 subunit from CA3 pyramidal cells by genetic manipulation has an important impact on memory acquisition (Nakazawa et al., 2003), associative memory recall, and pattern completion (Nakazawa et al., 2002), contextual learning (Cravens et al., 2006), and trace conditioning learning (Kishimoto et al., 2006). Before NMDAR mediated mossy fiber LTP was discovered, it is commonly assumed that NMDARs at associational - commissural synapses mediate this effect on learning and memory, but with Kwon and colleagues' finding, it suggests that NMDARs at mossy fiber CA3 pyramidal neuron synapses may also contribute.

iii) Long-term depression

Metabotropic glutamate receptor 2 (mGluR2), one of Group-II mGluRs, is found primarily at the presynaptic terminals of mossy fiber-CA3 pyramidal cell synapses (Yokoi et al., 1996). These receptors are thought to suppress transmission in response to excess glutamate release (Scanziani et al., 1997), and also play an essential role in inducing LTD (Kobayashi et al., 1996; Yokoi et al., 1996). The mechanistic studies of this form of LTD have found that the G proteins coupled with mGluR2 are Gi proteins, which contain an inhibitory α -type subunit (Pin and Duvoisin, 1995; Nicholls et al., 2006). Activation of mGluR2 activates Gi proteins, which results in a decrease in adenylyl cyclase activity followed by a decrease in PKA activity (Tzounopoulos et al., 1998), which leads to reduction of neurotransmitter release. Therefore, this LTD is a reversal of the presynaptic

processes involved in mossy fiber LTP. Since mGluR2 is exclusively expressed at mossy fiber terminals but not at associational – commissural synapses, detecting the existing of mGluR2 can be used to distinguish mossy fiber inputs, therefore, (2S,s'R,3'R)-2-(2',3'-dicarboxycyclopropyl) glycine (DCG-IV), a mGluR2 agonist, is commonly applied to suppress synaptic transmission originated from mossy fiber terminals (Toth and McBain, 1998; Toth et al., 2000; Torborg et al., 2010).

Besides mGluR2 mediated LTD, there is another form of LTD existing at these synapses. In young animals, it has been reported that high frequency stimulation (Domenici et al., 1998) or prolonged depolarization (5 min) in the absence of stimulation (Lei et al., 2003) induces LTD that is independent of NMDARs and mGluRs, but is dependent on a rise in postsynaptic Ca^{2+} . This LTD coexists with, and is mechanistically distinct from, the mGluR-dependent LTD discussed above. However, there is disagreement as to whether this form of LTD is expressed presynaptically (Domenici et al., 1998) or postsynaptically (Lei et al., 2003).

Subsection 2 Mossy fiber at CA3 interneuron synapses

i) Short-term plasticity

Unlike pyramidal cells which receive inputs from large mossy fiber boutons, interneurons within the stratum lucidum are innervated by small en passant or filopodial mossy fiber synapses (Acsady et al., 1998). Not only is there structure differences, the subunit composition of postsynaptic AMPARs at these two kinds of synapses are also different. Whereas mossy fiber onto CA3 pyramidal neuron synapses express only calcium-impermeable (CI) AMPARs, which contain the GluA2 (or GluR2) subunit (Toth et al., 2000); mossy fiber to CA3 interneuron synapses

contain both calcium-impermeable (CI) and GluA2-lacking calcium-permeable (CP) AMPARs (Toth and McBain, 1998). In addition, pronounced short-term facilitation is particularly prominent for the large mossy fiber boutons; mossy fiber terminals targeting interneurons show much less frequency facilitation or can even undergo depression, which is not due to the subunit composition of postsynaptic AMPARs (Toth et al., 2000). However, CP-AMPA synapses show a voltage-dependent facilitation because of relief from blockade by polyamine spermine at these GluA2-lacking AMPARs (Toth et al., 2000).

ii) Long-term plasticity

High frequency stimulation, which induces NMDAR independent LTP at mossy fiber to CA3 pyramidal cell synapses, generates NMDAR dependent LTD at interneuron CI-AMPA synapses (Lei and McBain, 2002), and NMDAR independent LTD at CP-AMPA synapses on interneurons (Toth et al., 2000). The induction of both forms of LTD required elevation of postsynaptic Ca^{2+} through NMDAR activation in the case of the former, and CP-AMPA activation for the latter.

Although early studies failed to show LTP at mossy fiber synapses onto interneurons, more recent studies indicate that this form of plasticity can be induced. Alle et al. found that high frequency stimulation evokes LTP at mossy fiber synapses to dentate basket cells. This LTP is attenuated by high doses of BAPTA in the basket cell. Compared to mossy fiber to pyramidal cell LTP, LTP at the mossy fiber to dentate basket cell synapses is also expressed presynaptically but is abolished by PKC inhibition (Alle et al., 2001). Moreover, Pelkey et al. have discovered a presynaptic

form of LTP at mossy fiber on stratum lucidum interneurons expressing CP-AMPARs at synapses. In the presence of group III mGluR agonist L-AP4, a brief tetanus induces LTP. Surprisingly, this form of LTP actually requires the internalization of mGluR7, but not mGluR activity blockade, because a tetanus with mGluR7 antagonist application does not induce LTP (Pelkey et al., 2005).

The differences of synaptic transmission and plasticity at the two types of mossy fiber innervated synapses suggest that the regulation of synaptic function at these sites is specialized depending on the nature of the postsynaptic target cell.

The following three chapters, which include two papers I have published in *Journal of Neuroscience* and one paper under preparation, are the result sections of my thesis. In Chapter 2 and 3, I used field potential recording as well as whole-cell patch-clamp recording to examine the synaptic function of all three synapses within CA3 circuit (see Fig. 1.4), and specifically located the synaptic dysfunction of this circuit in BACE1 KOs. Furthermore, in Chapter 4, I report that nicotine and alpha-7 nicotinic acetylcholine receptor activators can rescue the presynaptic deficits seen in BACE1 KOs.

Chapter 2: Beta-amyloid precursor protein cleavage enzyme 1 (BACE1) knockouts display deficits in activity-dependent potentiation of synaptic transmission at mossy fiber to CA3 synapses in the hippocampus

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My contribution: All of the experiments reported in this study.

Section 1 Introduction

Alzheimer's disease (AD) is the most prevalent form of senile dementia. Current treatment of AD remains limited, and there is no effective disease-modifying treatment as of yet (Citron, 2004b). It is widely believed that AD is initiated as a synaptic dysfunction, which correlates with the loss of memory function in the early stages of the disease (Selkoe, 2002). A current hypothesis states that over-production of amyloid-beta ($A\beta$) peptide initiates the pathogenesis of AD (Hardy and Selkoe, 2002; Citron, 2004b; Walsh and Selkoe, 2007). $A\beta$ is produced from the sequential cleavage of amyloid precursor proteins (APPs) by β - and γ -secretases, which are one of the major disease-modifying targets to treat AD (Citron, 2004b). However, it became apparent that γ -secretase processes other critical substrates essential for normal cell development and function, such as Notch (Sisodia and St George-Hyslop, 2002; Selkoe and Kopan, 2003). Therefore, inhibiting β -secretase is now receiving renewed attention (Vassar, 2002; Citron, 2004a, b). The amount and activity of β -secretase is elevated in sporadic AD brains (Yang et al., 2003; Li et al., 2004b; Zhao et al., 2007), further suggesting that effective methods to reduce its activity may be beneficial to a large population of AD patients.

A transmembrane aspartic protease, called BACE1 (beta-site APP cleavage enzyme 1), was identified as the major neuronal β -secretase (Hussain et al., 1999; Sinha et al., 1999; Vassar et al., 1999; Yan et al., 1999). BACE1 knockout mice were generated to determine the functional consequences of chronically inhibiting the activity of β -secretase. Initial characterization of the BACE1 knockouts suggested that there are no gross anatomical or functional abnormalities (Luo et al., 2001; Luo et al., 2003). Moreover, knocking out BACE1 in APP transgenic lines, which normally develop A β plaques and behavioral deficits, essentially alleviated the AD symptoms (Luo et al., 2003; Ohno et al., 2004; Laird et al., 2005). However, recent studies, including our own, showed that BACE1 knockouts display specific dysfunctions in synaptic transmission and plasticity (Ohno et al., 2004; Laird et al., 2005), as well as behavioral deficits (Harrison et al., 2003; Laird et al., 2005; Savonenko et al., 2008). While all of the studies characterizing synaptic function of BACE1 knockouts so far have been carried out in the CA1 region of the hippocampus (Ohno et al., 2004; Laird et al., 2005; Ma et al., 2007), the expression of BACE1 is most prominent in the mossy fiber terminals that synapse onto CA3 pyramidal neurons (Laird et al., 2005; Zhao et al., 2007). Therefore, we examined synaptic function and plasticity of the BACE1 knockouts at the mossy fiber synapses.

Section 2 Methods and Materials

Subsection 1 Animals

All mice used (BACE1 $+/+$ and $-/-$) were derived from heterozygous breeders ($+/-$) as described previously (Laird et al., 2005). The Institutional Animal Care and

Use Committees of both University of Maryland at College Park and Johns Hopkins University approved all procedures involving animals.

Subsection 2 Acute hippocampus slices preparation for electrophysiology

Hippocampal slices were prepared from adult (3-6 months old) male BACE1 knock-out or wild-type mice as described previously (Laird et al., 2005). Briefly, under deep anesthesia by isoflurane, mice were killed by decapitation, and their brains were removed quickly and transferred to the ice-cold dissection buffer containing the following (in mM): 212.7 sucrose, 2.6 KCl, 1.23 NaH₂PO₄, 26 NaHCO₃, 10 dextrose, 3 MgCl₂, and 1 CaCl₂ (bubbled with a mixture of 5% CO₂ and 95% O₂). A block of hippocampus was removed and sectioned into 400 μm-thick slices using a vibratome. The slices were recovered for 1 h at room temperature in artificial CSF (ACSF) (in mM): 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 dextrose, 1.5 MgCl₂, and 2.5 CaCl₂ (bubbled with a mixture of 5% CO₂ and 95% O₂).

Subsection 3 Electrophysiological Recordings

Recordings were done in a submersion-type recording chamber perfused with ACSF (saturated with 5% CO₂/95% O₂; 29.5°C–30.5°C, 2 ml/min). Synaptic responses were evoked through bipolar stimulating electrodes (double-barreled borosilicate glass capillaries [Sutter Instruments, Novato, CA]) placed in the dentate granule cell layer to activate the mossy fibers with pulse durations of 0.2 ms (baseline stimulation at 0.067 Hz), and recorded extracellularly in the stratum lucidum of CA3. Both the stimulating and recording electrodes were filled with ACSF. To induce long-term potentiation (LTP), three trains of 100 Hz (1 sec) stimuli were given at 20 sec

intervals. Long-term depression (LTD) was induced by a paired-pulse 1Hz protocol (interstimulus interval (ISI) = 50 ms, 15min). For measurement of paired-pulse facilitation (PPF), ISIs of 25, 50, 100, 200, 400, 1000, and 2000 ms were used. In some experiments, extracellular Ca^{2+} concentration was increased to 5.0 mM for 10 minutes before delivering HFS (Castillo et al., 2002). To activate cAMP production, 50 μM forskolin (Sigma-Aldrich, St. Louis, MO) was applied for 5 minutes. All experiments were done in the presence of 100 μM D,L-2-amino-5-phosphonovaleric acid (D,L-APV) (Sigma-Aldrich, St. Louis, MO) to block NMDA receptors. At the end of each experiment, 1 μM (2S,s'R,3'R)-2-(2',3'-dicarboxycyclopropyl) glycine (DCG-IV) (Tocris Bioscience, Ellisville, MI) was added, and blockade $\geq 80\%$ were taken to be mossy fiber inputs.

Subsection 4 Analysis of Electrophysiology data

The evoked extracellular field potential were digitized and stored in the computer by the Igor software (WaveMetrics). Using a custom-made Igor program, initial slope (for measurement of AMPA receptor responses) of population field potential was measured as the strength of synaptic transmission, and data are expressed as mean \pm standard error of mean. For statistic, Fisher's PLSD post hoc test was used to compare the PPF ratio. For the LTP and LTD data, the measurements at the end of each recording (60 min after baseline for LTP and LTD) were compared using student's t-test.

Section 3 Results

Subsection 1 Reduction in presynaptic function at mossy fiber synapses in BACE1 knockouts

We previously observed that mossy fiber terminals are enriched in BACE1 protein compared to other hippocampal subfields (Laird et al., 2005). Therefore, we hypothesized that BACE1 knockouts may exhibit alterations in synaptic transmission and plasticity at this particular set of synapses. We first measured presynaptic function by comparing paired-pulse facilitation (PPF) ratio at various interstimulus intervals (ISIs). We found a significant interaction between the genotype and ISIs (Two-factor ANOVA, genotype * ISI: $F(6, 203) = 2.586$, $P < 0.02$), particularly BACE1 KOs displayed larger PPF ratios at shorter ISIs (25 ms ISI: WT = 3.4 ± 0.57 ; KO = 6.1 ± 0.79 ; 50 ms ISI: WT = 3.6 ± 0.65 , $n = 14$; KO = 5.7 ± 0.77 , $n = 17$; Fisher's PLSD post hoc test: $P < 0.002$ for 25 and 50 ms ISI between WT and KO; Fig. 2.1A). The increase in PPF ratio suggests a reduction in presynaptic release.

Pyramidal cells in the CA3 region receive inputs not only from mossy fibers but also from CA3 pyramidal cell collaterals. Therefore, it was important to confirm in that the recorded field potentials originated from granule cell activation. Group II metabotropic glutamate receptors (mGluRs) are expressed exclusively on mossy fiber terminals but not on CA3 collaterals (Nicoll and Schmitz, 2005). DCG-IV, a group II mGluRs agonist, can therefore be used to identify synaptic transmission at mossy fiber to CA3 synapses. Bath application of 1 μ M DCG-IV at the end of the recording produced a comparable reduction in basal synaptic transmission in both knockouts

and wildtypes (WT: $12 \pm 4\%$ of baseline at 20 min DCG-IV, $n = 14$; KO: $11 \pm 2\%$, $n = 17$; Fig. 2.1B).

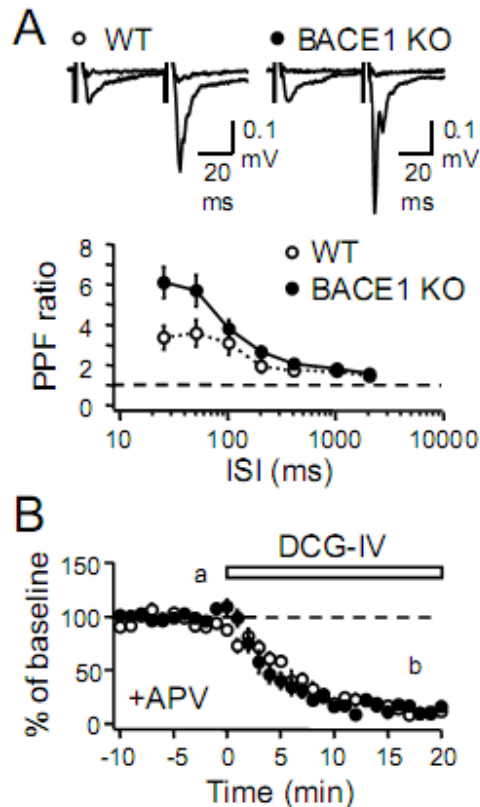


Figure 2.1. BACE1 knockouts display a reduction in presynaptic function at the mossy fiber synapses.

A. Larger PPF ratio in BACE1 knockouts. The difference between wildtype (WT: white circles) and knockouts (KO: black circles) are significant at 25 and 50 msec ISIs.

B. Application of Group II mGluR agonist (1 μ M DCG-IV) reduces mossy fiber synaptic transmission in wildtype (white circles) and knockouts (black circles). Top: Representative field potential (FP) traces following paired-pulse stimulation at 50 msec ISI before and after DCG-IV application in wildtype and knockout.

Subsection 2 Activity-dependent synaptic strengthening at mossy fiber synapses is abolished in BACE1 knockouts

Next we examined whether knocking out BACE1 affects synaptic plasticity at the mossy fiber synapses. We first compared LTP induced by high frequency

stimulation (HFS: 3 x 100 Hz, 1 sec). BACE1 knockouts showed a larger initial potentiation, suggesting an enhanced facilitation during HFS, however, the responses relaxed back to baseline by 1 hour (WT: $149 \pm 10\%$ of baseline at 1 hour post-HFS, $n = 13$ slices/6 mice; KO: $96 \pm 7\%$, $n = 16$ slices/7 mice; t-test: $P < 0.01$; Fig. 2.2A). Consistent with a presynaptic locus of expression, LTP in wildtypes was accompanied by a decrease in PPF ratio measured at 50 ms ISI (baseline = 3.1 ± 0.5 , 1 hour post-HFS = 2.6 ± 0.4 , $n = 13$ slices/6 mice, paired t-test: $P < 0.03$), but knockouts displayed no change in PPF ratio (baseline = 5.9 ± 1.0 , 1 hour post-HFS = 6.1 ± 1.2 , $n = 16$ slices/7 mice, paired t-test: $P = 0.26$).

To test LTD, we used a paired-pulse 1 Hz protocol (PP-1 Hz, 15 min), because a standard 1 Hz (15 min) protocol (Kobayashi et al., 1996) failed to produce LTD in the wildtypes at the ages used for our study (data not shown). LTD induced by the PP-1 Hz was slightly larger in BACE1 knockouts (WT: $75 \pm 4.3\%$ of baseline at 1 hour post-onset of PP-1 Hz, $n = 7$ slices/5 mice; KO: $62 \pm 3.8\%$ of baseline, $n = 6$ slices/4 mice; t-test: $P < 0.04$; Fig. 2.2B). This form of LTD did not significantly change PPF ratio either in wildtypes or knockouts (WT: baseline = 3.8 ± 1.0 , 1 hour post-PP 1 Hz = 3.2 ± 0.9 , $n = 6$ slices/4 mice, paired t-test: $P = 0.16$; KO: baseline = 7.1 ± 1.5 , 1 hour post-PP 1Hz = 5.5 ± 1.1 , $n = 6$ slices/4 mice, paired t-test: $P = 0.10$). Unlike in wildtypes, HFS failed to reverse LTD in the knockouts (WT: $195 \pm 28.0\%$ of renormalized baseline at 1 hour post-HFS, $n = 6$ slices/4 mice; KO: $100 \pm 5.4\%$, $n = 6$ slices/4 mice; t-test: $P < 0.02$; Fig. 2.2B).

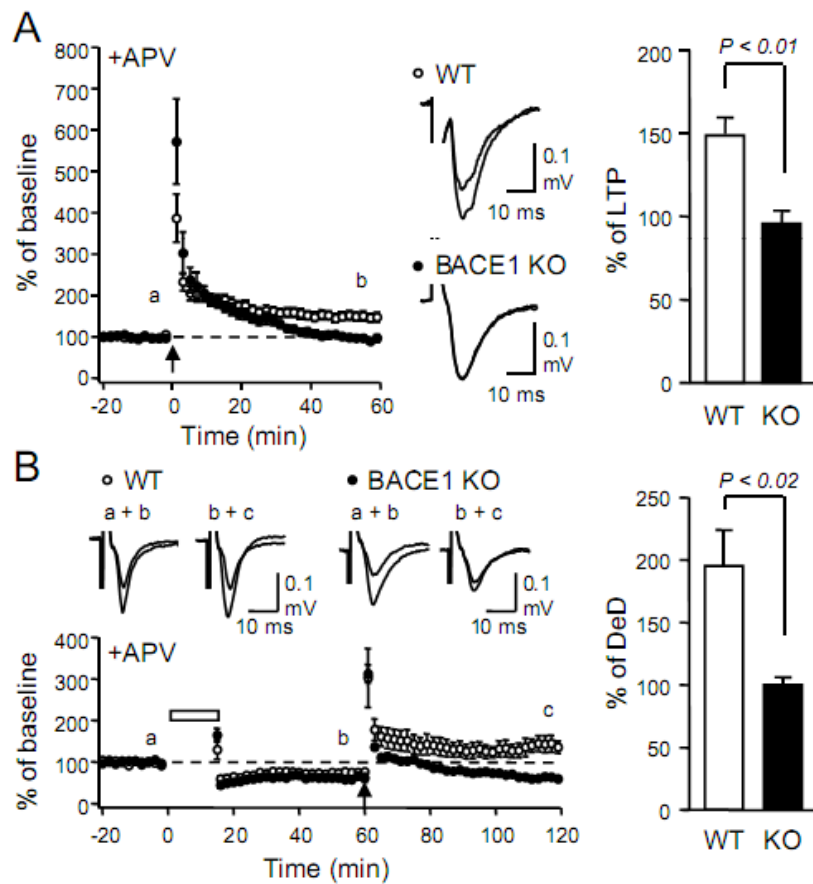


Figure 2.2. Absence of activity-dependent potentiation at mossy fiber synapses in BACE1 knockouts.

A. Mossy fiber LTP is absent in BACE1 knockouts. Left: Summary graph plotting changes in normalized field potential against time. The arrow depicts when HFS (100 Hz, 1 sec x 3) was delivered. Note that knockouts (black circles) showed larger post-tetanic potentiation, but no LTP when compared to wildtypes (white circles). Middle: Superimposed representative FP traces taken from wildtype and knockouts at times indicated in the left panel. Right: Average magnitude of LTP at 1-hour post-HFS (last 10 min averages) between wildtype and knockouts.

B. BACE1 knockouts show a slightly larger LTD, but no dedepression. Left bottom: Summary graph of the averages. The bar and arrow indicate delivery of PP-1 Hz (15 min), and HFS (100 Hz, 1 sec x 3), respectively. Left top: Representative FP traces taken at times indicated in the summary graph. Right: Average magnitude of dedepression measured 1-hour post-HFS (averages of last 10 min) between wildtype and knockouts. The responses were re-normalized to the 10 min preceding HFS.

Subsection 3 Rescue of mossy fiber LTP in BACE1 knockouts by increasing extracellular Ca²⁺ concentration

Mossy fiber LTP is triggered by a rise in presynaptic Ca²⁺ (Castillo et al., 1994), and a further recruitment of cAMP-dependent signaling mechanisms (Nicoll and Schmitz, 2005). Therefore, we investigated whether the lack of mossy fiber LTP in BACE1 knockouts is due to abnormal regulation of presynaptic Ca²⁺ or signaling downstream. We found that transiently increasing the concentration of extracellular Ca²⁺ (from 2.5 mM to 5 mM) during HFS recovered mossy fiber LTP in BACE1 knockouts ($146.7 \pm 8.5\%$ of baseline at 1 hour post-HFS, $n = 6$ slices/4 mice; paired t-test: $P < 0.01$; Fig. 2.3). Furthermore, LTP was accompanied by a decrease in PPF ratio measured at 50 ms ISI (baseline = 3.8 ± 1.0 , 1 hour post-HFS = 2.2 ± 0.4 , $n = 6$ slices/4 mice, paired t-test: $P < 0.05$) consistent with a presynaptic expression. This suggests that the induction of mossy fiber LTP is defective in the BACE1 knockouts

To further confirm whether signaling downstream of the Ca²⁺ influx is intact in BACE1 knockouts, we directly activated cAMP signaling by a brief application of an adenylyl cyclase activator forskolin. This caused a dramatic enhancement of synaptic transmission in both wildtypes and knockouts to similar magnitudes (WT: $622.5 \pm 57.8\%$ of baseline at 1 hour post-forskolin, $n = 7$ slices/5 mice; KO: $741.8 \pm 110.1\%$, $n = 7$ slices/4 mice; t-test: $P = 0.36$; Fig. 2.3). This was accompanied by a significant decrease in PPF ratio in both genotypes (WT: baseline = 3.2 ± 0.34 , 1 hour post-forskolin = 1.5 ± 0.15 , $n = 7$ slices/5 mice, paired t-test: $P < 0.01$; KO: baseline = 4.9 ± 0.63 , 1 hour post-forskolin = 1.7 ± 0.16 , $n = 7$ slices/4 mice, paired t-test: $P < 0.01$), consistent with a presynaptic mechanism of potentiation. This demonstrates

that the presynaptic deficits seen in BACE1 knockouts are upstream of cAMP signaling.

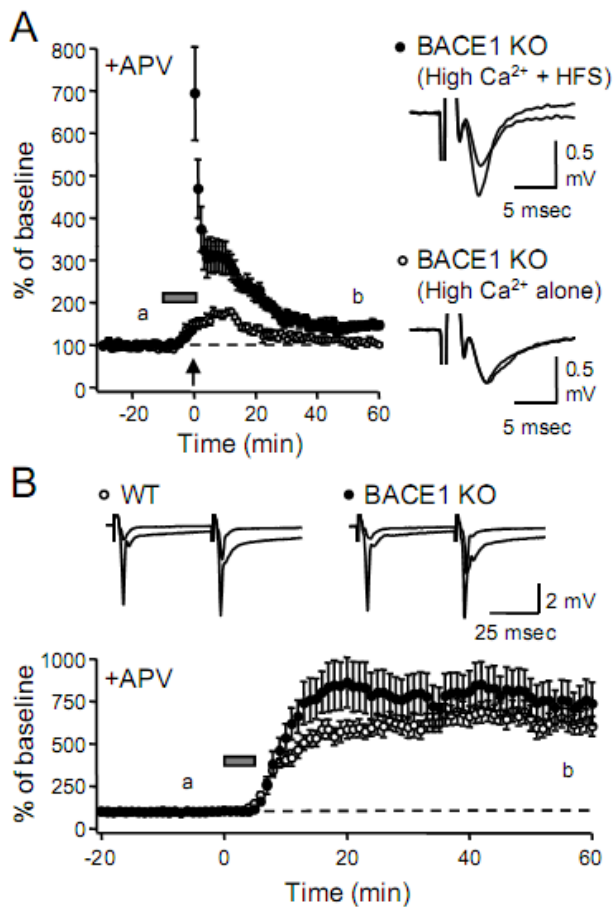


Figure 2.3. BACE1 knockouts express mossy fiber LTP under high extracellular Ca^{2+} , and produce normal forskolin-induced potentiation.

A. Transient elevation of external Ca^{2+} concentration (5 mM Ca^{2+} , 10 min; gray bar) rescued mossy fiber LTP in knockouts.

Right: Representative traces taken at times indicated.

B. Transient application of forskolin (50 μM , 5 min; gray bar) potentiated mossy fiber synaptic transmission in wildtypes (white circles) and knocks (black circles) to the same magnitude. Top: Representative traces taken at times indicated.

Section 4 Discussion

We found that BACE1 knockouts display severe deficits in presynaptic function at mossy fiber synapses in CA3: a reduction in presynaptic release and an absence of mossy fiber LTP. In addition, BACE1 knockouts exhibited a slightly larger LTD, which could not be reversed. These results suggest that BACE1 function is critical for normal synaptic transmission and plasticity, especially activity-dependent potentiation, at these synapses. We further found that the specific deficit in mossy fiber LTP in BACE1 knockouts can be rescued by increasing extracellular Ca^{2+} concentration. Since a direct activation of cAMP production was not impaired in the BACE1 knockouts, our data suggests that the presynaptic dysfunction is likely at the level of presynaptic Ca^{2+} regulation.

Previous studies suggest that BACE1 is highly localized to presynaptic terminals, especially at the mossy fiber boutons in the CA3 (Laird et al., 2005; Zhao et al., 2007). This localization is consistent with our observation of a deficit in presynaptic function and plasticity at this synapse. Taken together with our previous results from the CA1 also showing an increase in PPF ratio (Laird et al., 2005), these results indicate that BACE1 may play a general role in regulating presynaptic function under physiological conditions. However, whether presynaptic deficits in BACE1 knockouts are directly due to lacking APP processing is unclear. Previous studies suggest that generation of excess $\text{A}\beta$ depresses excitatory synaptic transmission (Kamenetz et al., 2003; Hsieh et al., 2006; Ting et al., 2007) mainly by postsynaptic removal of AMPA receptors and loss of synapses (Hsieh et al., 2006; Priller et al., 2006; Ting et al., 2007). These results would predict that lacking $\text{A}\beta$

production, as in BACE1 knockouts, would cause a postsynaptic increases in AMPA receptor function, not a decrease in presynaptic function as observed in our studies. Our previous work in CA1, where BACE1 knockouts showed normal basal synaptic transmission despite the apparent reduction in presynaptic release (Laird et al., 2005), implies that there may be a compensatory increase in postsynaptic function.

Another possibility is that the presynaptic effects of BACE1 knockout may be from abnormal processing of substrates other than APP. It is now known that BACE1 can also cleave APP-like proteins (APLPs) (Li and Sudhof, 2004), β subunits of voltage-gated Na^+ channel (Wong et al., 2005; Kim et al., 2007), and neuregulin-1 (NRG1) (Hu et al., 2006; Willem et al., 2006). Regulation of the latter two substrates is particularly interesting. The $\beta 2$ subunit of Na^+ channel is critical for plasma membrane expression of functional Na^+ channels (Schmidt and Catterall, 1986), which are essential for action potential generation. However, over-expressing BACE1 actually decreases the density of functional Na^+ channels (Kim et al., 2007), hence it cannot directly account for the observed reduction in presynaptic release in the BACE1 knockouts. Potential regulation of NRG1 by BACE1 was discovered from observations that BACE1 knockouts display a hypomyelination phenotype with a correlated accumulation of full-length NRG1 and a significant loss of NRG1 cleavage products (Hu et al., 2006; Willem et al., 2006). Recently, we demonstrated that the lack of NRG1 processing in BACE1 knockouts reduces postsynaptic function of ErbB4, a receptor for NRG1 (Savonenko et al., 2008). NRG1/ErbB4 signaling has been suggested to regulate synaptic function and plasticity, mainly via regulation of postsynaptic glutamate receptors (Huang et al., 2000; Gu et al., 2005a; Li et al., 2007).

Nevertheless, abnormal processing of NRG1 may also affect presynaptic release by regulating the expression of nicotinic acetylcholine receptor (nAChR) subunit $\alpha 7$ (Liu et al., 2001b), which allows Ca^{2+} influx (Seguela et al., 1993). Indeed, presynaptic nAChRs can increase glutamate release (McGehee et al., 1995; Gray et al., 1996; Maggi et al., 2003b), likely via the $\alpha 7$ containing nAChRs (Le Magueresse et al., 2006). These results suggest that lacking NRG1 cleavage, as in BACE1 knockouts, would reduce presynaptic release. Whether this is the case for mossy fiber synapses is unclear (Vogt and Regehr, 2001).

Our results indicate that a complete inhibition of BACE1 activity is deleterious for neuronal function, especially at the mossy fiber synapses in CA3 compared to Schaffer collateral inputs in CA1. This suggests that mossy fiber dysfunction may have had a larger impact on the behavioral phenotypes seen in the BACE1 knockouts (Harrison et al., 2003; Laird et al., 2005; Savonenko et al., 2008). We demonstrate that signaling downstream of presynaptic Ca^{2+} influx is intact in BACE1 knockouts. Therefore, we were able to restore mossy fiber LTP in the BACE1 knockouts by simply increasing extracellular Ca^{2+} concentration during LTP induction. This has significant clinical implications, because it suggests that means to enhance presynaptic Ca^{2+} will circumvent synaptic deficits, and perhaps alleviate the behavioral phenotypes, associated with inhibiting BACE1 activity.

Chapter 3: Synaptic dysfunctions in the CA3 circuit of BACE1 knockout mice

This manuscript is in preparation.

Putative authors: Hui Wang, Philip C. Wong, and Hey-Kyoung Lee

My contribution: All of the experiments performed in this study.

Section 1 Introduction

Beta-amyloid precursor protein cleaving enzyme 1 (BACE1), a major neuronal β -secretase critical for the formation of β -amyloid (A β) peptide, is thought to be one of the key therapeutic targets that can prevent the progression of Alzheimer's disease (AD) (Vassar, 2002; Citron, 2004b, a; Vassar et al., 2009). However, many recent studies, including my own in Chapter 2, have shown that although BACE1 knockouts (KOs) lack A β peptides (Cai et al., 2001) and show no gross anatomical or functional abnormalities (Luo et al., 2001; Luo et al., 2003), they display specific synaptic dysfunctions in CA1 and CA3 regions of the hippocampus (Laird et al., 2005; Wang et al., 2008). In particular, we investigated synaptic function and plasticity of the BACE1 knockouts using extracellular field recordings, and found that BACE1 KOs showed presynaptic dysfunctions especially at the mossy fiber to CA3 synapses, which is one of the major loci of BACE1 expression in the brain (Laird et al., 2005), we also pinpointed the presynaptic dysfunction of BACE1 KOs to the level of presynaptic Ca²⁺ signaling (Wang et al., 2008).

The CA3 pyramidal neurons not only receive excitatory inputs from mossy fibers, but also get feedforward inhibition formed by the interneurons (INTs) within stratum lucidum of CA3, which are activated by mossy fibers (Lawrence and McBain,

2003). How blocking BACE1 influences excitatory and/or inhibitory synapses in this feedforward inhibitory circuit is unknown. Because many of the neuronal functions depend on a critical balance between excitatory and inhibitory circuits, understanding the impact of BACE1 inhibition on each synapse-type present in an intact circuitry is critical. Furthermore, by providing understanding of how a lack of BACE1 activity affects specific synapses, it will aid in the development of effective methods to overcome the synaptic deficits and potentially benefit the therapeutics of AD. Therefore, in this chapter, we expanded the analysis to a circuit level in the CA3 area of hippocampus by performing whole-cell recording from pyramidal cells (PCs) and inhibitory interneurons (INTs) in the CA3 area that receive mossy fiber inputs to examine the mossy fiber (MF) synapses onto CA3 PCs and INTs, as well as inhibitory synapses from INTs to CA3 PCs, to specifically locate the synapses affected by losing BACE1 activity (see Fig. 1.4 in Chapter 1).

Section 2 Methods and Materials

Subsection 1 Animals

All mice used (BACE1 $+/+$ and $-/-$) were derived from heterozygous breeders ($+/-$) as described previously (Laird et al., 2005). The Institutional Animal Care and Use Committees of both University of Maryland and Johns Hopkins University approved all procedures involving animals.

Subsection 2 Acute hippocampus slices preparation for electrophysiology

Acute hippocampal slices were prepared from 16 - 25 d-old BACE1 KO or WT mice as described previously (Laird et al., 2005; Wang et al., 2008). Briefly, each mouse was euthanized by decapitation following overdose of isoflurane. Hippocampi were rapidly removed and sectioned into either 300- μm (for whole cell recording) or 400- μm (for field potential recording) slices on a vibratome (Vibratome 3000 series, Ted Pella Inc.) using ice-cold dissection buffer (212.7 mM sucrose, 2.6 mM KCl, 1.23 mM NaH_2PO_4 , 26 mM NaHCO_3 , 10 mM dextrose, 3 mM MgCl_2 , and 1 mM CaCl_2) saturated with 5% $\text{CO}_2/95\% \text{O}_2$. The slices were transferred to a holding chamber containing artificial cerebrospinal fluid (ACSF: 124 mM NaCl, 5 mM KCl, 1.25 mM NaH_2PO_4 , 26 mM NaHCO_3 , 10 mM dextrose, 1.5 mM MgCl_2 , and 2.5 mM CaCl_2 , saturated with a mixture of 5% $\text{CO}_2/95\% \text{O}_2$) and recovered for at least 1 h at room temperature before being used for all experiments.

Subsection 3 Field Potential Recording from mossy fiber pathway in CA3 area

Recordings were done in a submersion-type chamber perfused with ACSF (29.5°C–30.5°C, 2 ml/min). Synaptic responses were evoked through glass bipolar stimulating electrodes placed in the dentate granule cell layer to activate MFs with pulse duration of 0.2 ms (at 0.067 Hz), and recorded extracellularly in the stratum lucidum of CA3. Paired-pulse facilitation (PPF) was measured at 25, 50, 100, 200, 400, 1000, and 2000 ms interstimulus intervals (ISIs). To induce mLTP, three trains of 100 Hz (1 sec) stimuli were given at 20 sec intervals. All experiments were done in the presence of 100 μM D,L-2-amino-5-phosphonovaleric acid (D,L-APV) (Sigma-

Aldrich) to isolate the presynaptic NMDAR-independent mLTP (Nicoll and Schmitz, 2005). At the end of each experiment, 1 μ M (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl) glycine (DCG-IV) (Tocris Bioscience) was added, and blockade \geq 80% were taken to be MF inputs. Field potential slopes were measured, and data are expressed as mean \pm standard error of mean.

Subsection 4 Whole-cell recording of evoked AMPAR-mediated excitatory postsynaptic currents (EPSCs)

The slices were visualized by an upright microscope (Nikon E600FN) equipped with infra-red oblique illumination. CA3 PCs and INTs located within the stratum lucidum of CA3 were initially identified on the basis of somata shape and position within the CA3 subfield, and patched by a whole-cell patch pipette (tip resistance, 3-5 M Ω) filled with the following (in mM): 120 CH₃O₃SCs, 5 MgCl₂, 8 NaCl, 1 EGTA, 10 HEPES, 1 QX-314, 0.5 Na₃GTP, and 2 MgATP (pH 7.2-7.3, 280-290 mOsm) in presence of 20 μ M bicuculline, and 100 μ M D,L-APV in the ACSF to isolate AMPAR-mediated EPSCs. Biocytin (Sigma-Aldrich, 1mg/ml) was routinely added to the recording electrode solution to allow post hoc morphological processing of the recorded cells. Recordings were made at 30°C at a holding potential of -70 mV. Additionally, 150 nM CNQX (Tocris Bioscience) was added to prevent polysynaptic responses for evoked EPSC in CA3 PCs. For evoked EPSC in INTs, CNQX was not used, as INTs were reported to show less recurrent excitation (Toth et al., 2000). A double-barrel glass electrode filled with ACSF as stimulation electrode was placed in the dentate granule cell layer to activate the MF inputs. Minimum-intensity stimulation (15–25 μ A intensity) was used to induce monosynaptic response. DCG-

IV was applied at the end of the recordings to verify that the currents were elicited by MF inputs. 20-30 waveforms were used to produce the average evoked EPSCs, and the amplitude was calculated by Igor Pro software (WaveMetrics). PPF was measured at 50 ms ISI. Only the cells and recording conditions that met the following criteria were studied: no obvious multiple EPSCs or polysynaptic waveforms, $R_{\text{input}} \geq 125 \text{ M}\Omega$ and $R_{\text{series}} \leq 25 \text{ M}\Omega$, and R_{input} or R_{series} changed less than 15% during the course of the experiment.

Subsection 5 Whole-cell recording of miniature AMPAR-mediated EPSCs (mEPSCs)

To record AMPAR-mediated mEPSCs, the same intracellular solution as evoked EPSCs was used in the recording pipette, 1 μM TTX (Tocris Bioscience), 20 μM bicuculline (Enzo Life Science) and 100 μM DL-APV were added to the ACSF (29.5°C–30.5°C, 2 ml/min). mEPSCs were recorded at a holding potential of -70mV in CA3 PCs or INTs using an Axopatch 700B amplifier (Molecular Devices), digitized at 10 kHz by a data acquisition board (National Instruments), and acquired using a custom-made Igor Pro software (WaveMetrics). Acquired mEPSCs were analyzed using the Mini Analysis program (Synaptosoft). The threshold for detecting mEPSCs was set at three times the root mean square (RMS) noise. mEPSCs with > 3 ms rise time (measured between 10 and 90% of amplitude) were excluded from analysis. Average mEPSC amplitude and frequency were calculated and compared between two genotypes using unpaired Student's t test as noted in text. Only the cells with $R_{\text{input}} > 200 \text{ M}\Omega$ and $R_{\text{series}} < 25 \text{ M}\Omega$ were studied, cells were discarded if R_{input} or R_{series} changed > 15%.

Subsection 6 Whole-cell recording of evoked GABA_AR-mediated inhibitory postsynaptic currents (IPSCs)

To record evoked GABA_AR currents, 10 μM NBQX (Sigma-Aldrich) and 100 μM DL-APV were added to isolate GABA_AR component. Evoked IPSCs were recorded at 30°C at a holding potential of -70 mV in CA3 PCs with recording pipette filled with (in mM): 140 CsCl, 0.2 CaCl₂, 8 NaCl, 2 EGTA, 10 HEPES, 0.5 Na₃GTP, and 4 MgATP (pH 7.2, 275-285 mOsm). A double-barrel glass electrode filled with ACSF as stimulation electrode was placed in the stratum lucidum of CA3 where cell bodies of INTs are located. Minimum-intensity stimulation (15–30 μA intensity) was used to induce monosynaptic response. 20-30 waveforms were used to generate the average evoked EPSCs, and the amplitude was calculated by Igor Pro software (WaveMetrics). PPF was measured at 50, 100, 400 and 1000ms ISIs. Only cells with no obvious multiple EPSCs or polysynaptic waveforms, $R_{\text{input}} \geq 125\text{M}\Omega$ and $R_{\text{series}} \leq 25\text{M}\Omega$, as well as cells showing less than 15% change in R_{input} or R_{series} during the course of the experiment, were included in the analyses.

Subsection 7 Whole-cell recording of miniature GABA_AR-mediated IPSCs (mIPSCs)

For mIPSCs in CA3 PCs, the recording pipette was filled with (in mM): 140 CsCl, 8 KCl, 10 EGTA, 10 HEPES, and 10 QX-314 (pH 7.3, 275-285 mOsm) in the presence of 1 μM TTX, 100 μM D,L-APV, and 10 μM NBQX in the ACSF to isolate GABA_AR-mediated currents. mIPSCs were recorded at a holding potential of -70mV and analyzed with the Mini Analysis program (Synaptosoft). The threshold for detecting mIPSCs was set at three times the RMS noise. mIPSCs with > 5 ms rise

time (measured between 10 and 90% of amplitude) were excluded from analysis. 350 to 500 consecutive events from each experiment were considered for the determination of mIPSC frequency, but highly superimposed events constituting “bursts” (more than two events, interevent interval < 10 ms) were excluded from the measurement of amplitudes (300 non-burst events from each cell were used for average mIPSC amplitude calculations). The decay time constant was calculated using the average of 150–200 well-isolated events.

Section 3 Results

Subsection 1 Younger BACE1 KO mice show similar synaptic dysfunctions at mossy fiber to CA3 synapses as adults

In the Chapter 2, we found that 3-6 month old BACE1 KO mice display severe deficits in presynaptic function at mossy fiber synapses in CA3 including a reduction in presynaptic release and an absence of mossy fiber LTP. In order to use young BACE1 KO mice for whole-cell recordings, we need to first verify that similar synaptic deficits at CA3 area can be detected at this age. Therefore, we performed extracellular field potential recording at mossy fiber to CA3 pathway of hippocampus in 3-week old BACE1 WT and KO mice. We measured presynaptic function by comparing paired-pulse facilitation (PPF) ratio at various interstimulus intervals (ISIs). Similar to results from older animals we found that young BACE1 KO mice displayed significantly larger PPF ratios at shorter ISIs (25 ms ISI: WT = 3.4 ± 0.24 ; KO = 6.0 ± 0.44 ; 50 ms ISI: WT = 3.5 ± 0.23 , n = 8 slices/4 mice; KO = 5.6 ± 0.49 , n = 8 slices/4 mice; ANOVA: P < 0.001; Fisher’s PLSD post hoc test: P < 0.001 for 25 and

50 ms ISI between WT and KO; data from slices were used for statistical analysis, see Appendix on Page 125; Fig. 3.1A) The increase in PPF ratio suggests a reduction in presynaptic function.

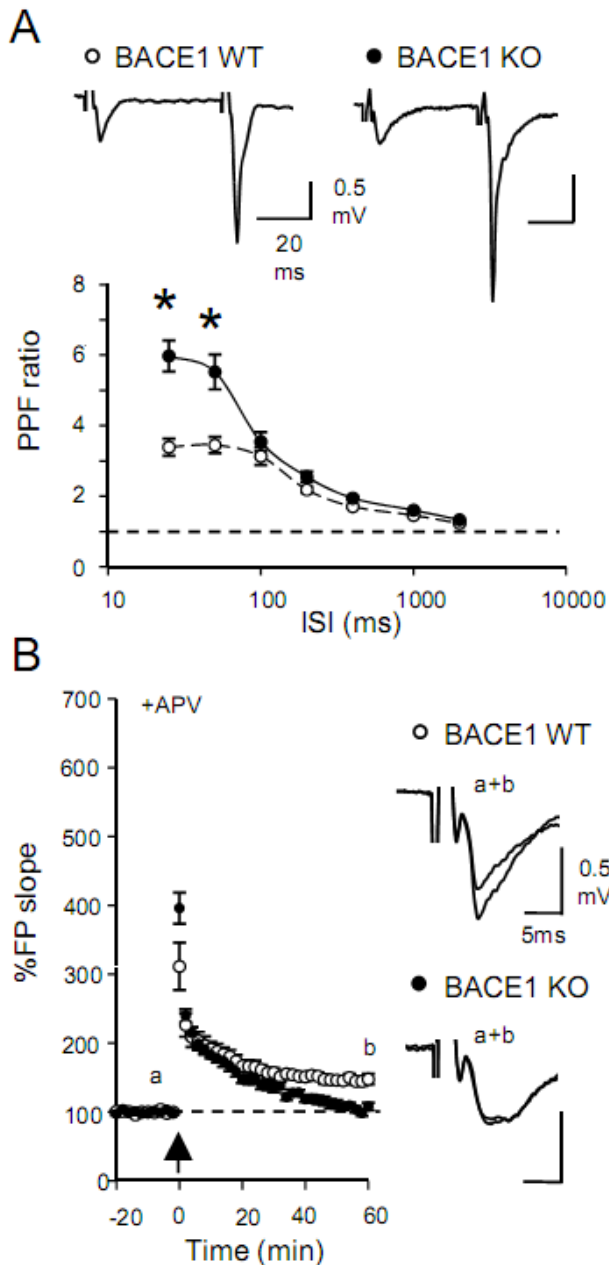


Figure 3.1. Abnormal synaptic function at MF to CA3 synapses in young BACE1 KOs.

A. Young BACE1 KOs (black circles) displayed larger PPF ratio (especially at 25 and 50 msec ISIs) compared to WT (white circles), which indicates a reduction in presynaptic release. Top panel: Representative field potential traces following paired-pulse stimulation at 50 msec ISI. *ANOVA: $P < 0.001$; Fisher's PLSD post hoc test: $P < 0.001$ between the two genotypes.

B. Absence of mossy fiber LTP in young BACE1 KOs. Left: Summary graph plotting changes in normalized field potential against time. The arrow depicts when HFS (100 Hz, 1 sec x 3) was delivered. Note that KOs (black circles) showed no LTP 60 minutes after LTP induction compared to WT (white circles). Right: Superimposed representative field potential traces taken from WT and KO at times indicated in the left panel.

Next we compared mossy fiber LTP induced by high frequency stimulation (HFS: 3 x 100 Hz, 1 sec) from 3-week old BACE1 WT and KO mice. Again similar to our previous data from adults, we found that young BACE1 KOs display a larger initial potentiation, suggesting an enhanced facilitation during HFS, however, the responses returned to baseline by 1 hour (WT: $146 \pm 5\%$ of baseline at 1 hour post-HFS, $n = 8$ slices/4 mice; KO: $106 \pm 4\%$, $n = 8$ slices/4 mice; t-test: $P < 0.001$; Fig. 3.1B). Consistent with a presynaptic locus of expression, LTP in WTs was accompanied by a decrease in PPF ratio measured at 50 ms ISI (baseline = 3.4 ± 0.26 , 1 hour post-HFS = 2.6 ± 0.20 , $n = 8$ slices/4 mice, paired t-test: $P < 0.001$), but KOs displayed no change in PPF ratio (baseline = 5.4 ± 0.54 , 1 hour post-HFS = 5.5 ± 0.56 , $n = 8$ slices/4 mice, paired t-test: $P = 0.54$).

These results suggest that 3-week old BACE1 KOs display both presynaptic release decrease and mossy fiber LTP abolishment at CA3 area of hippocampus similar to adults. Therefore, animal at this age were used in subsequent whole-cell patch-clamp recording experiments.

Subsection 2 Reduced presynaptic function at MF to CA3 PC synapses in BACE1KOs

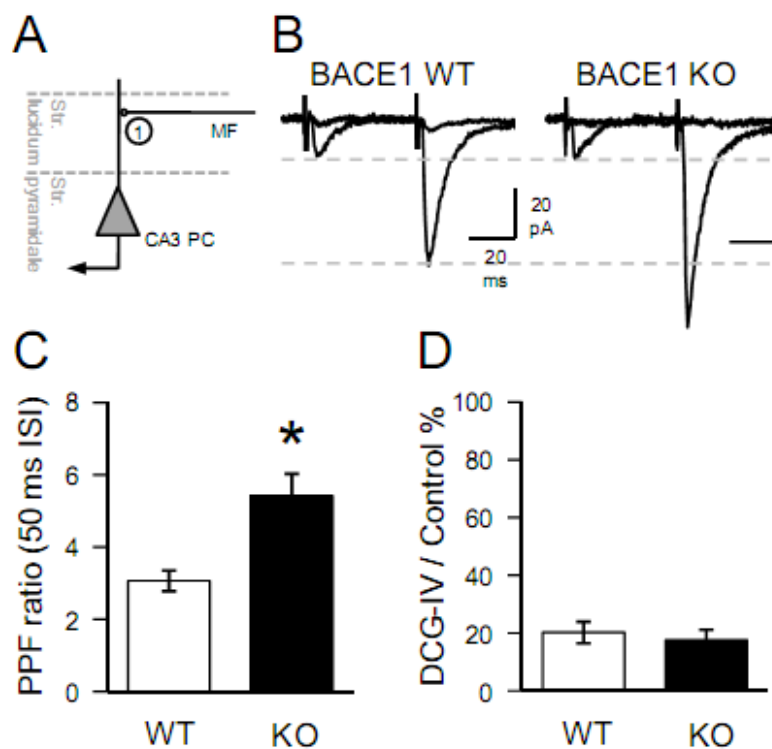
The first set of synapses we examined in the CA3 circuit was the excitatory monosynaptic MF inputs to CA3 PCs. We stimulated dentate gyrus granule cell layer (Langdon et al., 1993) and performed whole-cell patch-clamp recordings from CA3 PCs in CA3 pyramidal cell layer. Monosynaptic AMPAR-mediated EPSCs were isolated pharmacologically by applying APV, which is an antagonist of NMDARs, and bicuculline, which is a GABA_AR antagonist. The latency, rise time and decay

time of the evoked EPSC traces were analyzed, and there were no differences of these properties between the two genotypes (t test: $P > 0.6$ for each property; Table 3.1). We calculated PPF ratio as a measure of presynaptic function. Consistent with our previous extracellular field potential recording findings, BACE1 KO mice displayed dramatic increase in PPF ratio at 50 ms ISIs compared to WT mice (WT = 3.1 ± 0.28 , $n = 11$; KO = 5.4 ± 0.61 , $n = 10$; t test: $P < 0.01$; Fig. 3.2C), suggesting a decrease in presynaptic function. To verify that the responses were elicited from MF inputs, $1 \mu\text{M}$ DCG-IV was bath applied at the end of the recording, which produced a comparable reduction in basal synaptic transmission in both genotypes (WT: $20.3 \pm 3.7\%$ of baseline, $n = 11$; KO: $17.6 \pm 3.6\%$, $n = 10$; Fig. 3.2D).

We next examined miniature EPSCs (mEPSCs) from CA3 PCs and compared the frequency and amplitude of the recorded mEPSCs between the two genotypes. BACE1 KO mice showed a significant reduction in frequency (WT = 1.4 ± 0.3 Hz, $n = 19$; KO = 0.6 ± 0.1 Hz, $n = 19$; t test: $P < 0.01$; Fig. 3.3B), without changes in mEPSC amplitude distribution (the cumulative probability curve of mEPSC amplitude from KO and WT were overlapped, Kolmogorov-Smirnov test: $P > 0.5$; Fig. 3.3C) or the average mEPSC amplitude (WT = 19.4 ± 1.0 pA, $n = 19$; KO = 17.5 ± 1.4 pA, $n = 19$; t test: $P = 0.28$; Fig. 3.3C inset). These results are consistent with PPF ratio changes indicating presynaptic alteration. Together, these findings suggest that there is a decrease in presynaptic function at MF to CA3 PC synapses in BACE1 KO mice.

Table 3.1. ePSC properties

Experiment	Genotype	Num. of cells	Latency (ms)	Rise time (ms)	Decay time (τ , ms)
PCs eEPSCs	WT	n = 11	2.4 \pm 0.1	1.8 \pm 0.1	7.7 \pm 0.5
	KO	n = 10	2.5 \pm 0.1	1.9 \pm 0.2	7.9 \pm 0.6
INTs eEPSCs	WT	n = 8	2.4 \pm 0.1	1.1 \pm 0.1	3.1 \pm 0.3
	KO	n = 5	2.3 \pm 0.2	1.0 \pm 0.1	3.0 \pm 0.2
PCs eIPSCs	WT	n = 11	1.5 \pm 0.1	2.3 \pm 0.2	20.6 \pm 1.9
	KO	n = 12	1.6 \pm 0.1	2.2 \pm 0.2	20.0 \pm 1.1

**Figure 3.2. Reduced presynaptic release at MF to CA3 PC monosynapses in BACE1 KOs.**

A. A sketch showing MF terminals onto PCs monosynapses within CA3 circuit.
B. Representative evoked EPSCs traces from CA3 PCs following paired-pulse stimulation at 50 msec ISI before and after DCG-IV application in WT and KOs.
C. PPF ratio was significantly increased in BACE1 KOs (black bar) at 50 msec ISI compared to WT (white bar). *t test: $P < 0.01$.
D. Application of 1 μ M DCG-IV reduced eEPSCs from CA3 PCs elicited by mossy fiber inputs in both WT (white bar) and KOs (black bar).

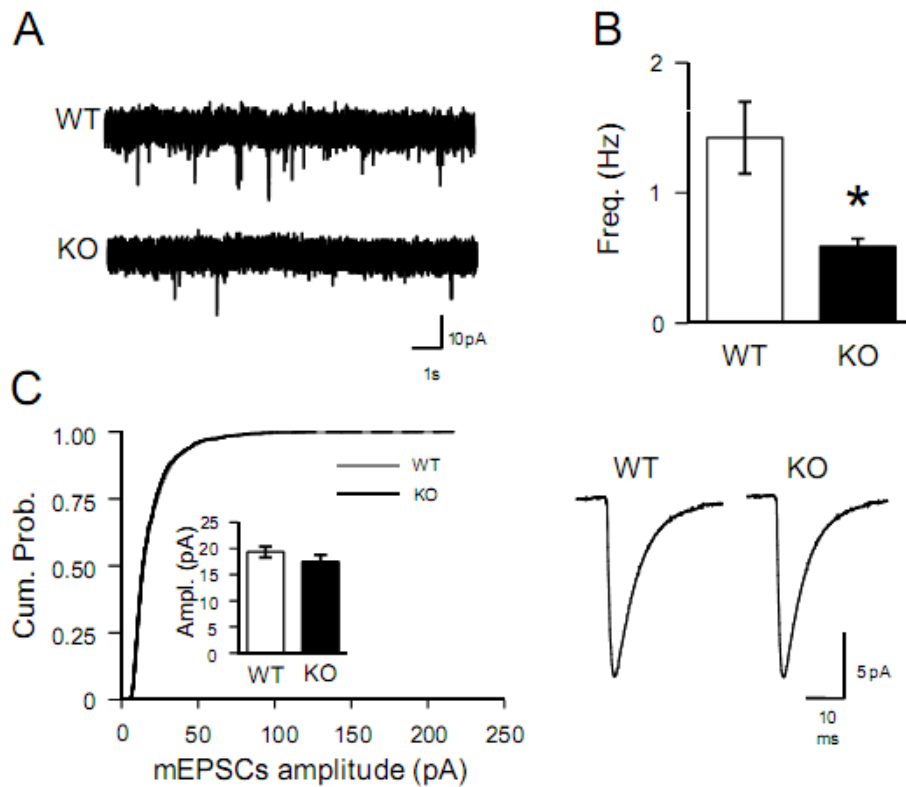


Figure 3.3. Altered mEPSCs from CA3 PCs in BACE1 KOs.

A. Representative miniature EPSCs traces from CA3 PCs in WT and KOs.

B. BACE1 KOs (black bar) showed significantly decreased mEPSCs frequency in CA3 PCs compared to WT (white bar). *t test: $P < 0.01$.

C. Amplitude of mEPSCs in CA3 PCs was not altered in KOs. Left: The cumulative probability curve of KO mEPSC amplitudes (black solid line) superimposed with that of WT (gray solid line) (K-S test, $P > 0.5$). Inset: Average of mEPSC amplitude from CA3 PCs showed no change between the two groups (WT: white bar, KO: black bar). Right: Average mEPSC traces from PCs of the two groups.

Subsection 3 Normal synaptic function at MF to CA3 INT synapses in BACE1KOs

The granule cell axons (MFs) have more than one terminal type including large complex mossy boutons, small en passant terminals, and small filopodial extensions of the mossy fiber boutons. MFs only innervate PCs via the large complex mossy boutons, whereas either small en passant or filopodial terminals preferentially target the stratum lucidum INTs (Chicurel and Harris, 1992; Acsady et al., 1998). To assess whether the MF synapses onto INTs in the stratum lucidum are altered in BACE1 KOs, we isolated excitatory MF inputs on INTs and recorded evoked EPSCs. The latency, rise time and decay time of the evoked EPSC traces were analyzed, and there were no differences of these properties between the two genotypes (t test: $P > 0.5$ for each property; Table 3.1). In addition, there was no change in the PPF ratio between the two genotypes (WT = 1.8 ± 0.29 , $n = 8$; KO = 1.7 ± 0.16 , $n = 5$; t test: $P = 0.84$; Fig. 3.4C) suggesting that presynaptic function is not affected at this synapse. Bath application of 1 μM DCG-IV at the end of the recording resulted in a significant reduction in basal synaptic transmission in WTs and KOs (WT: $13.4 \pm 5.5\%$ of baseline, $n = 8$; KO: $12.7 \pm 6.4\%$, $n = 5$; Fig. 3.4D), which confirmed that we were recording MF mediated synaptic responses.

In addition to the evoked EPSCs, we examined mEPSCs from CA3 INTs and compared the frequency and amplitude between the two genotypes. We did not observe a significant change in the frequency (WT = 4.8 ± 0.7 Hz, $n = 19$; KO = 4.4 ± 0.7 Hz, $n = 14$; t test: $P = 0.69$; Fig. 3.5B) or the amplitude of mEPSCs (the cumulative probability curve of mEPSCs amplitude from KO and WT were

overlapped, Kolmogorov-Smirnov test: $P = 0.2$; Average: WT = 22.5 ± 2.0 pA, $n = 19$; KO = 22.0 ± 2.2 pA, $n = 14$; t test: $P = 0.88$; Fig. 3.5C). These results suggest that there is no change in pre- or postsynaptic function at MF to INT synapses of BACE1 KOs.

These observations indicate that only MF synapses onto CA3 PCs are impaired, while the same afferent inputs projecting onto INTs are normal in BACE1 KOs. This suggests that the effect of BACE1 KO is postsynaptic target specific.

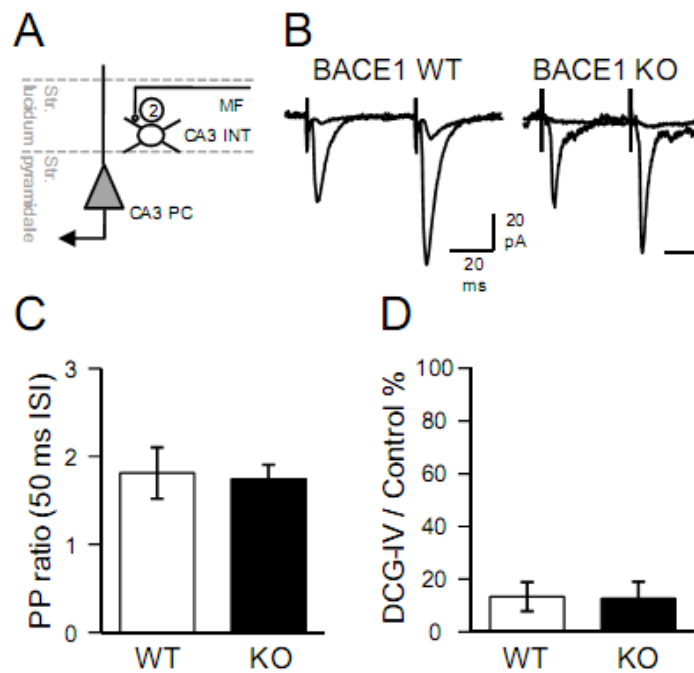


Figure 3.4. No change has been detected at MF onto CA3 INT synapses in BACE1 KOs.

A. A sketch showing the synapses of MF terminals onto inhibitory neurons within stratum lucidum.

B. Representative evoked EPSCs traces from CA3 INTs following paired-pulse stimulation at 50 msec ISI before and after DCG-IV application in WT and KO.

C. PPF ratio was not changed between the two genotypes (WT: white bar, KO: black bar) at 50 msec ISI.

D. Application of 1 μ M DCG-IV reduced eEPSCs from CA3 INTs elicited by mossy fiber inputs in both WT (white bar) and KO (black bar).

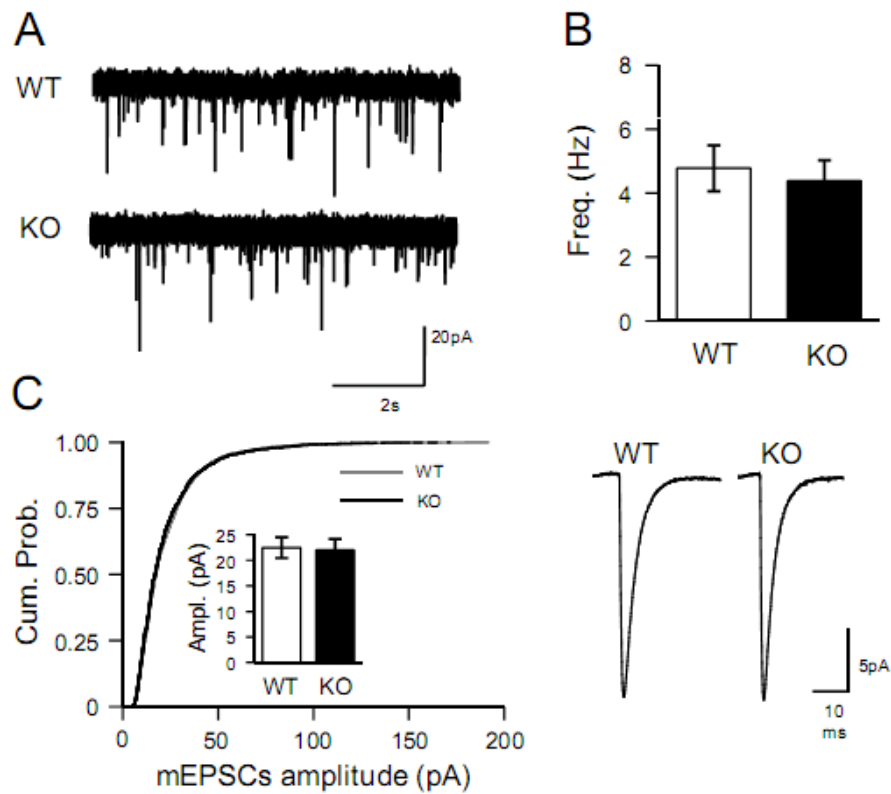


Figure 3.5. No change in mEPSCs from CA3 INTs between BACE1 WT and KOs.

A. Representative miniature EPSC traces from CA3 INTs in WT and KOs.

B. There was no change in frequency of mEPSCs from CA3 INTs (WT: white bar, KO: black bar).

C. Amplitude of mEPSCs in CA3 INTs was not altered in KOs. Left: The cumulative probability curve of KO mEPSC amplitudes (black solid line) superimposed with that of WT (gray solid line) (K-S test, $P = 0.2$). Inset: Average of mEPSC amplitude from CA3 INTs showed no change between the two groups (WT: white bar, KO: black bar). Right: Average mEPSC traces from INTs of the two groups.

Subsection 4 Reduced presynaptic function at CA3 INT to PC inhibitory synapses in BACE1KOs

We have demonstrated that BACE1 can regulate excitatory synaptic transmission, which is postsynaptic target specific. However, whether BACE1 plays a role in modulating inhibitory synapses is unknown. We therefore investigated inhibitory inputs from CA3 INTs onto PCs in BACE1 KOs by isolating GABA_AR-mediated IPSCs within CA3 area using AMPAR antagonist NBQX and NMDAR antagonist APV. Stratum lucidum of CA3 were stimulated where INT cell bodies are located and recordings were made from CA3 PCs in CA3 pyramidal cell layer. We measured evoked IPSCs at a negative holding potential of -70mV using a symmetrical chloride internal solution that reverses IPSCs at 0 mV, and confirmed the isolation of IPSCs by showing a complete and reversible block of current by application of bicuculline (Fig. 3.6B). The latency, rise time and decay time of the evoked IPSC traces were analyzed, and there were no differences of these properties between the two genotypes (t test: $P > 0.4$ for each property; Table 3.1). Consistent with the high release probability at inhibitory synapses (Jiang et al., 2010), BACE1 WT mice showed large paired-pulse depression (PPD) especially at shorter ISIs (PPD ratio: 50 ms ISI = 0.59 ± 0.04 , $n = 11$; Fig. 3.6C). Surprisingly, the PPD was significantly impaired in BACE1 KOs (PPD ratio: 50 ms ISI = 0.92 ± 0.08 , $n = 12$; ANOVA: $P < 0.001$; Fisher's PLSD post hoc test: $P < 0.001$ for 50 ms ISI between WTs and KOs; Fig. 3.6C). The reduction in PPD seen in KOs suggests that there is a decrease in presynaptic function at these synapses. To our knowledge, it is the first evidence that BACE1 regulates inhibitory synaptic transmission.

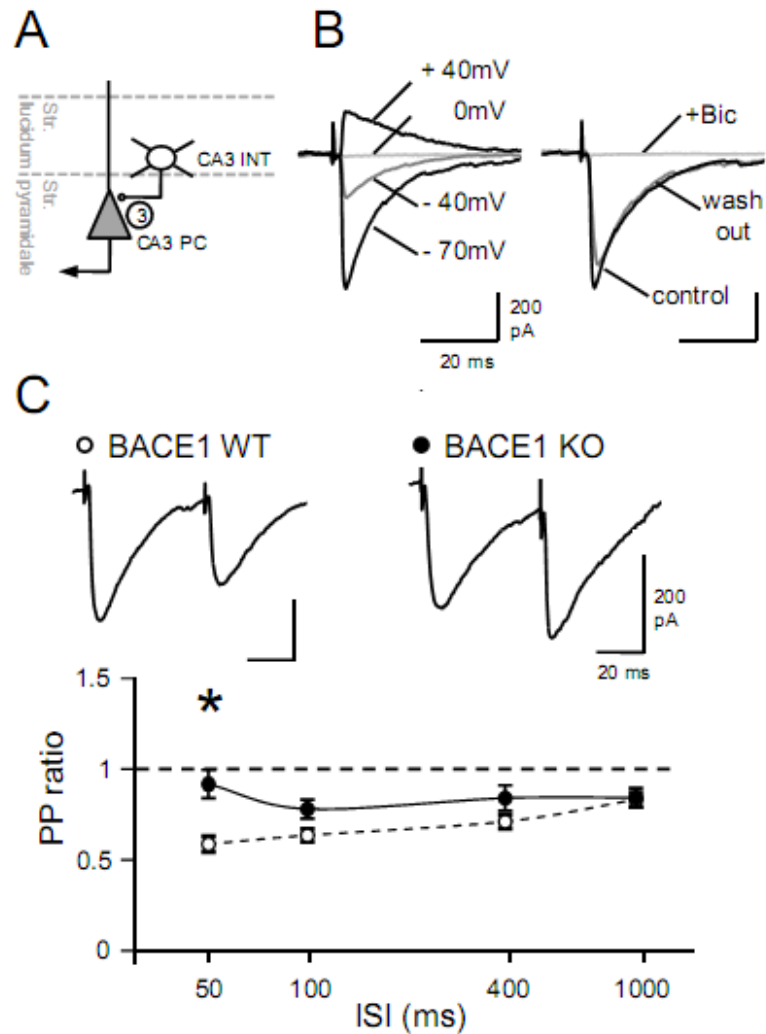


Figure 3.6. Reduced presynaptic function at INT to CA3 PC inhibitory synapses in BACE1 KOs.

A. A diagram showing the inhibitory projections from CA3 INTs to PCs.

B. Left: Verification that the internal solution we used reversed mIPSCs at 0 mV. Each evoked IPSCs trace was recorded under different holding voltage as indicated. Right: The pharmacologically isolated eIPSCs were blocked by addition of 20 μ M bicuculline (+Bic), and the currents were reversible when bicuculline was washed out.

C. Paired-pulse depression was impaired in BACE1 KOs (black circles) especially at 50 msec ISI compared to WTs (white circles). Top panel: Representative evoked IPSCs traces from CA3 PCs following paired-pulse stimulation at 50 msec ISI. *ANOVA: $P < 0.001$; Fisher's PLSD post hoc test: $P < 0.001$ between the two genotypes.

In addition, we compared mIPSCs from CA3 PCs across the two genotypes. Consistent with evoked IPSCs results showing a decrease in presynaptic function, BACE1 KOs displayed a significant reduction in frequency (WT = 18.6 ± 1.3 Hz, n = 19; KO = 13.7 ± 1.3 Hz, n = 17; t test: $P < 0.05$; Fig. 3.7B), without changes in mIPSC amplitude distribution (The cumulative probability curve of mIPSC amplitude from KO and WT were overlapped, Kolmogorov-Smirnov test: $P > 0.7$; Fig. 3.7C) nor averaged mIPSC amplitude (WT = 42.2 ± 3.2 pA, n = 19; KO = 45.7 ± 3.6 pA, n = 17; t test: $P = 0.46$; Fig. 3.7C inset).

These data suggest that in the CA3 circuit, BACE1 inhibition not only reduce presynaptic function at excitatory inputs, but also at inhibitory synapses.

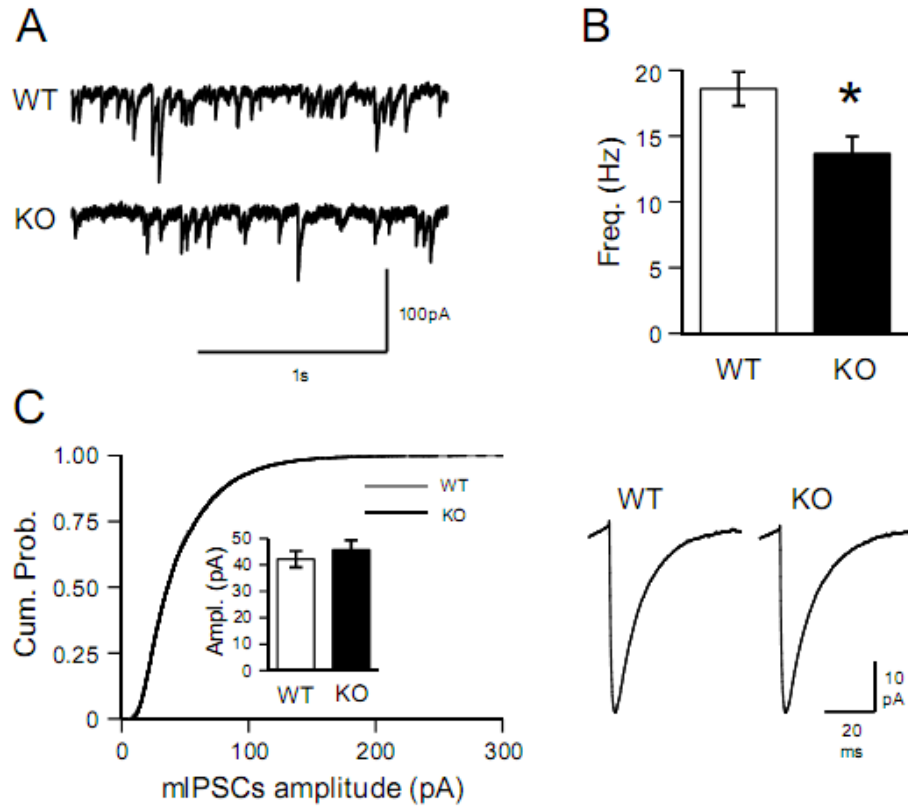


Figure 3.7. Reduced frequency of mIPSCs from CA3 PCs in BACE1 KOs.

A. Representative miniature IPSCs traces from CA3 PCs in WT and KOs.

B. BACE1 KOs (black bar) showed significantly reduced mIPSCs frequency in CA3 PCs compared to WT (white bar). *t test: $P < 0.05$.

C. Amplitude of mIPSCs in CA3 PCs was not altered in KOs. Left: The cumulative probability curve of KO mIPSC amplitudes (black solid line) superimposed with that of WT (gray solid line) (K-S test, $P > 0.7$). Inset: Average of mIPSC amplitude from CA3 PCs showed no change between the two groups (WT: white bar, KO: black bar). Right: Average mIPSC traces from PCs of the two groups.

Section 4 Discussion

This study indicates that within the CA3 circuit of hippocampus, presynaptic function of both excitatory inputs from MF and inhibitory inputs from INTs onto CA3 PCs are reduced in the BACE1 KOs. On the other hand, there was no change at excitatory projections from MF onto INTs. Our data, for the first time, suggest that BACE1 can regulate synaptic transmission of both excitatory and inhibitory component within the same neuronal circuit. The fact that the synapses affected by BACE1 inhibition are both targeted to CA3 PCs reveals that this effect is likely specified by the postsynaptic target independent of the type of inputs.

Our finding that only synapses of MF inputs onto CA3 PCs, but not MF projections onto INTs, were altered by BACE1 inhibition is very intriguing. This further strengthens the idea that even though these two sets of synapses have common inputs, the functional characteristic of these two sets of synapses is quite different. Previous studies have shown that MF synapses onto CA3 PCs display distinct high level of facilitation indicative of low release probability (Nicoll and Schmitz, 2005); while MF synapses to INTs exhibit a higher probability of release and display either facilitation or depression by brief trains of stimulation (Toth et al., 2000). Recently, it has been found that different types of AMPARs are present at the two sets of synapses. MF inputs onto CA3 PC synapses contain only calcium-impermeable (CI) AMPARs (Toth et al., 2000); whereas MF inputs onto CA3 INT synapses contain both calcium-impermeable (CI) and calcium-permeable (CP) AMPARs (Toth and McBain, 1998). Interestingly, high frequency stimulation (100 Hz), which induces LTP at MF to CA3 PC synapses, produce NMDAR dependent LTD at interneuron

CI-AMPA synapses (Lei and McBain, 2002), and NMDAR independent LTD at CP-AMPA synapses on interneurons (Toth et al., 2000). Our results add to these differences and suggest that BACE1 function is more critical for maintaining normal synaptic transmission at the MF to CA3 PC synapses.

Our study, for the first time, provides evidence that inhibiting BACE1 not only affected excitatory inputs, but also impaired feedforward inhibition of the CA3 circuit. When interneurons and principal cells receive the same excitatory input, the inhibitory projections from interneurons onto principal cells form disynaptic feedforward inhibition (Buzsaki, 1984), which inhibits the excitability and tunes the firing pattern of the principal cells. Feedforward inhibition is one of the major components within neuronal circuits in many brain areas, such as hippocampal formation, visual cortex, sensory cortex, etc. (Buzsaki, 1984; Swadlow, 2003; Callaway, 2004), and plays a crucial role in circuit development as well as balancing excitation and inhibition to maintain neural activity (Chittajallu and Isaac, 2010). In the CA3 area of hippocampus, MF terminals innervating PCs are large with multiple release sites; whereas INTs receive small MF branches but large number of synapses. In addition, a single MF targets tens of inhibitory neurons, and each inhibitory interneuron can contact hundreds of CA3 PCs. This anatomy allows the CA3 circuit to form a high frequency filter, which permits high frequency responses to pass but low frequency responses are dampened by feedforward inhibition (Lawrence and McBain, 2003). If inhibitory synaptic transmission is damaged by blocking BACE1 activity, the function of CA3 circuit is certainly impacted in BACE1 KOs.

The evidence that both of the synapses modified by blocking BACE1 activity are synapsing onto CA3 PCs suggests that BACE1 in the postsynaptic PCs may actually play an important role, and may bring about target specific regulation of synaptic transmission. However, it is still unknown how BACE1 at postsynaptic site would modulate presynaptic function. One potential candidate is A β generated by BACE1 processing of APP. A β , which can be secreted into synaptic cleft, may act as a retrograde signal from postsynaptic to presynaptic terminals to modify presynaptic function. In support of this, A β is implicated in presynaptic modifications by affecting presynaptic P/Q type Ca²⁺ channels and critical synaptic vesicle endocytosis protein dynamin (Kelly et al., 2005; Kelly and Ferreira, 2007; Nimmrich et al., 2008). Although from those studies, A β suppresses synaptic transmission, hence it seems counterintuitive that abolishing A β production, as in BACE1 KO mice, would reduce presynaptic function. However, we need to consider the fact that physiological concentration of A β (pM range) facilitates synaptic plasticity (Puzzo et al., 2008), and in this context the absence of A β in BACE1 KO mice is expected to produce synaptic deficits.

Another possibility is that the presynaptic effects of BACE1 KOs may be from abnormal processing of neuregulin-1 (NRG1), a substrate of BACE1 (Hu et al., 2006; Willem et al., 2006), which affects presynaptic release by regulating the surface expression of presynaptic Ca²⁺ permeable α 7-nAChRs (Seguela et al., 1993; Liu et al., 2001b; Zhong et al., 2008). Presynaptic nAChRs can increase glutamate release (McGehee et al., 1995; Gray et al., 1996; Maggi et al., 2003b), likely via the α 7 containing nAChRs (Le Magueresse et al., 2006). Whether activation of α 7-nAChRs

can rescue the presynaptic deficits seen at MF to CA3 pathway in BACE1 KO mice will be discussed in the next chapter.

By isolating excitation and inhibition within the same neuronal circuit, we demonstrated that the absence of BACE1 produces presynaptic dysfunction at both excitatory and inhibitory components on CA3 PCs. Although reduction of presynaptic function at both types of synapses may be a compensation mechanism to keep the balance of excitation and inhibition, these changes likely impact synaptic function in the CA3 area of hippocampus as seen by an abolishment of mossy fiber LTP (Wang et al., 2008). The current study reveals BACE1 function at a circuit level, and may provide useful mechanistic information to circumvent the negative side effects caused by BACE1 inhibition.

Chapter 4: Mossy fiber LTP deficits in BACE1 knockouts can be rescued by activation of $\alpha 7$ nicotinic acetylcholine receptors

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My contribution: Measurement of field potentials recordings and analyses

Section 1 Introduction

Beta-site amyloid precursor protein cleaving enzyme 1 (BACE1), the neuronal β -secretase responsible for producing β -amyloid (A β) peptides, emerged as one of the key therapeutic targets of Alzheimer's disease (AD) (Vassar, 2002; Citron, 2004b, a; Vassar et al., 2009). Although complete ablation of the BACE1 gene prevents A β formation (Cai et al., 2001), in the previous two chapters, we have shown that BACE1 KOs display severe presynaptic dysfunctions (Wang et al., 2008). The deficits include a reduction in presynaptic release at mossy fiber to CA3 synapses and an absence of mossy fiber long-term potentiation (mfLTP), which are due to abnormal presynaptic Ca²⁺ signaling (Wang et al., 2008). These studies caution the use of BACE1 inhibitors as a practical treatment for AD.

Cholinergic system modulates neurotransmitter release from glutamatergic and GABAergic terminals via the action of nicotinic acetylcholine receptors (nAChRs) (Gray et al., 1996; Radcliffe et al., 1999; Giocomo and Hasselmo, 2005; Jiang and Role, 2008; Bancila et al., 2009). Among them, $\alpha 7$ -nAChR is a Ca²⁺-permeable homopentameric ion channel highly expressed in the hippocampus and cerebral

cortex (Seguela et al., 1993). Several studies have linked $\alpha 7$ -nAChR with neurodegenerative disorders, including AD (Perry et al., 2000). In this chapter, we present data that activating $\alpha 7$ -nAChRs, by nicotine or a specific agonist PNU282987, can restore presynaptic function at mossy fiber to CA3 synapses and mfLTP in BACE1 KOs via recruiting Ca^{2+} -induced Ca^{2+} release (CICR).

Section 2 Methods and Materials

Subsection 1 Animals

All mice used (BACE1 +/+ and -/-) were derived from heterozygous breeders (+/-) as described previously (Laird et al., 2005). The Institutional Animal Care and Use Committees of both University of Maryland and Johns Hopkins University approved all procedures involving animals.

Subsection 2 Acute hippocampus slices preparation for electrophysiology

Hippocampal slices were prepared from adult (3-6 months old) male BACE1 knock-out or wild-type mice as described previously (Laird et al., 2005). Briefly, under deep anesthesia by isoflurane, mice were killed by decapitation, and their brains were removed quickly and transferred to the ice-cold dissection buffer containing the following (in mM): 212.7 sucrose, 2.6 KCl, 1.23 NaH_2PO_4 , 26 NaHCO_3 , 10 dextrose, 3 MgCl_2 , and 1 CaCl_2 (bubbled with a mixture of 5% CO_2 and 95% O_2). A block of hippocampus was removed and sectioned into 400 μm -thick slices using a vibratome. The slices were recovered for 1 h at room temperature in

artificial CSF (ACSF) (in mM): 124 NaCl, 5 KCl, 1.25 NaH₂PO₄, 26 NaHCO₃, 10 dextrose, 1.5 MgCl₂, and 2.5 CaCl₂ (bubbled with a mixture of 5% CO₂ and 95% O₂).

Subsection 3 Electrophysiological Recordings

Recordings were done in a submersion-type recording chamber perfused with ACSF (saturated with 5% CO₂/95% O₂; 29.5°C–30.5°C, 2 ml/min). Synaptic responses were evoked through glass bipolar stimulating electrodes placed in the dentate granule cell layer to activate MFs with pulse duration of 0.2 ms (at 0.067 Hz), and recorded extracellularly in the stratum lucidum of CA3. PPF was measured at 25, 50, 100, 200, 400, 1000, and 2000 ms ISIs. To induce mfLTP, three trains of 100 Hz (1 sec) stimuli were given at 20 sec intervals. We used α 7-nAChR agonists (-)-Nicotine (Sigma-Aldrich) and PNU282987 (Tocris Bioscience), and an antagonist α -bungarotoxin (Tocris Bioscience). To block intracellular Ca²⁺ release, ruthenium red (Tocris Bioscience) or ryanodine (Tocris Bioscience) was applied. All experiments were done in the presence of 100 μ M D,L-2-amino-5-phosphonovaleric acid (D,L-APV) (Sigma-Aldrich) to isolate the presynaptic NMDAR-independent mflTP (Nicoll and Schmitz, 2005). At the end of each experiment, 1 μ M (2S,2'R,3'R)-2-(2',3'-dicarboxycyclopropyl) glycine (DCG-IV) (Tocris Bioscience) was added, and blockade \geq 80% were taken to be MF inputs. Field potential slopes were measured, and data are expressed as mean \pm standard error of mean.

Subsection 4 Immunohistochemistry and confocal imaging

Hippocampal slices (300- μ m-thick) prepared from transgenic mice expressing yellow fluorescence protein (YFP) in a subset of neurons (YFP-2J line, Jackson

Laboratory) were placed in 4% paraformaldehyde overnight (4°C) and transferred to 30% sucrose [in 0.1 M sodium phosphate (SP) buffer] overnight before re-sectioning to 20 µm thickness using a freezing sliding microtome (Leica). The sections were collected in cryoprotectant (20% sucrose, 30% ethylene glycol in pH 7.4 SP buffer) and kept at -20°C. The day before primary antibody incubation, the sections were frozen at -80°C in 30% sucrose (in 0.1 M SP buffer). The following day, sections were defrosted and rinsed in PBS (4×5 min) and permeabilized [2% Triton X-100 (TX-100) in SP buffer, 1 hour]. They were then incubated in -20°C methanol (10 min), rinsed in PBS (4×5 min), and placed in 1% TX-100 blocking solution [10% normal donkey serum (NDS), 4% BSA in PBS] for 1 h at room temperature. Sections were then incubated in 0.2% TX-100 blocking solution with $\alpha 7$ -nAChR antibody (1:500, rabbit polyclonal antibody, Abcam, Cambridge, MA) and synaptophysin antibody (1:20, mouse monoclonal antibody, Millipore, Billerica, MA) for 7 days at 4°C. After washing (PBS, 4×5 min), sections were incubated for 2 h at room temperature in secondary antibodies [Alexa633 goat anti-rabbit IgG (1:500, Molecular Probes, Eugene, OR) and Alexa555 goat anti-mouse IgG (1:200, Molecular Probes, Eugene, OR)] in 1.5% NDS in PBS. Sections were rinsed in PBS, mounted on glass slides, and air-dried. The slides were coverslipped with ProlongTM mounting solution (Molecular Probes, Eugene, OR) and sealed with nail polish. The stained sections were imaged using a Leica SP5X confocal microscope with a 63×oil immersion objective lens. The CA3 subfield of the hippocampus was imaged through the z-axis at 0.5 µm steps with x/y/z resolution of 0.24/0.24/0.50 µm/pixel.

Subsection 5 Steady-state surface biotinylation

Hippocampal slices (400 μm thick) were prepared as described above, and the CA3 region was isolated. After 30 min recovery at room temperature, the isolated CA3 slices were transferred to 30°C for additional 30 min recovery. The slices were then transferred to ice-cold ACSF for 10 min, and subsequently to ice-cold ACSF containing 2 mg/ml biotin (EZ-Link Sulfo-NHS-Biotin, Pierce) saturated with 5% $\text{CO}_2/95\% \text{O}_2$ for 15 min. The slices were then washed in tris-buffered saline (TBS: 50 mM Tris, 0.9% NaCl, pH 7.4) containing 100 mM glycine (5 times, 1 min each) before homogenized in ice-cold 0.2% SDS/1% Triton X-100 IPB (20 mM Na_3PO_4 , 150 mM NaCl, 10 mM EDTA, 10 mM EGTA, 10 mM $\text{Na}_4\text{P}_2\text{O}_7$, 50 mM NaF, and 1 mM Na_3VO_4 , pH 7.4; with 1 μM okadaic acid and 10 KIU/ml aprotinin) by ~30 gentle strokes using glass-teflon tissue homogenizers (Pyrex). The homogenates were centrifuged for 10 min at $13,200 \times g$, 4°C. Protein concentration of the supernatant was measured and normalized to 2 mg/ml. Some of the supernatants were saved as inputs by adding gel sampling buffer and boiled for 5 min. 300 μg of each supernatant was mixed with neutravidin slurry [1:1 in 1% Triton X-100 IPB (TX-IPB)] and rotated overnight at 4°C. The neutravidin beads were isolated by brief centrifugation at $1,000 \times g$. Some of the supernatants were saved by adding gel sample buffer and boiled for 5 min. The neutravidin beads were washed 3 times with 1% TX-IPB, 3 times with 1% TX-IPB containing 500 mM NaCl, followed by 2 washes in 1% TX-IPB. The biotinylated surface proteins were then eluded from the neutravidin beads by boiling in gel sampling buffer for 5 min. The input (I, total homogenate), supernatant (S, intracellular fraction), and biotinylated samples (B,

surface fraction) were run on the same gel, and processed for immunoblot analysis using $\alpha 7$ -nAChR and actin antibodies. The band intensity in the input lanes and biotin lanes, which fell within the linear range, was quantified to calculate the % of total $\alpha 7$ -nAChR on the surface for each sample.

Subsection 6 Immunoblot analysis

SDS-PAGE gels were transferred to polyvinyl difluoride (PVDF) membranes (ImmobilonTM, Millipore). The PVDF membrane blots were blocked for ~1 hr in blocking buffer (1% bovine serum albumin and 0.1% Tween-20 in phosphate buffered saline (PBS), pH 7.4), and subsequently incubated for 1-2 hrs in primary antibodies diluted in blocking buffer. After 5 x 5 min washes in blocking buffer, the blots were incubated for 1 hr in secondary antibodies linked to Cy3 and Cy5. After washes, blots were scanned using Typhoon Trio (GE Health), and signals were quantified using Image Quant TL software (GE Health). The fluorescence intensity values for each band was then normalized to the average value of WT samples on the same blot to obtain the % of average WT values, which were compared across different experimental groups using unpaired Student's t-test. The biotinylation blots were also probed simultaneously with $\alpha 7$ -nAChR and actin antibodies using the ECL plex system. The band intensities of biotinylated samples were normalized to that of the input lanes (total protein) to obtain the level of surface $\alpha 7$ -nAChR as a % of total value.

Section 3 Results

Subsection 1 Nicotine restores presynaptic function at MF to CA3 synapses in BACE1 KOs

We first examined the effect of nicotine on the presynaptic function of MF to CA3 synapses in BACE1 KOs by measuring paired-pulse facilitation (PPF). The results showed that nicotine decreased PPF ratio in a dose dependent manner at 25 and 50 ms ISIs in KOs (n = 7 slices/3 mice; ANOVA: $P < 0.05$; Fig. 4.1A), and 10 μM was the lowest concentration that significantly decreased PPF ratio in both genotypes (KO: control = 4.81 ± 0.16 , nicotine = 4.05 ± 0.22 , n = 15 slices/10 mice, paired t-test: $P < 0.001$; WT: control = 3.77 ± 0.43 , nicotine = 3.50 ± 0.40 , n = 10 slices/9 mice, paired t-test: $P < 0.001$; Fig. 4.1B). We previously showed that BACE1 KOs display a significant increase in PPF ratio at MF synapses indicating a reduction in presynaptic release (Wang et al., 2008). Nicotine at 10 μM concentration decreased the PPF ratio of KOs to a similar level of WTs (t-test: $P = 0.57$) without affecting synaptic transmission in either genotype (KO: $100 \pm 1\%$ of baseline at 20 min post-nicotine, n = 15 slices/10 mice; paired t-test: $P = 0.97$; WT: $99 \pm 1\%$, n = 10 slices/9 mice; paired t-test: $P = 0.54$; Fig. 4.1B). These results suggest that 10 μM nicotine reverses PPF deficits in BACE1 KOs without affecting synaptic strength. Therefore, 10 μM nicotine was used in subsequent experiments.

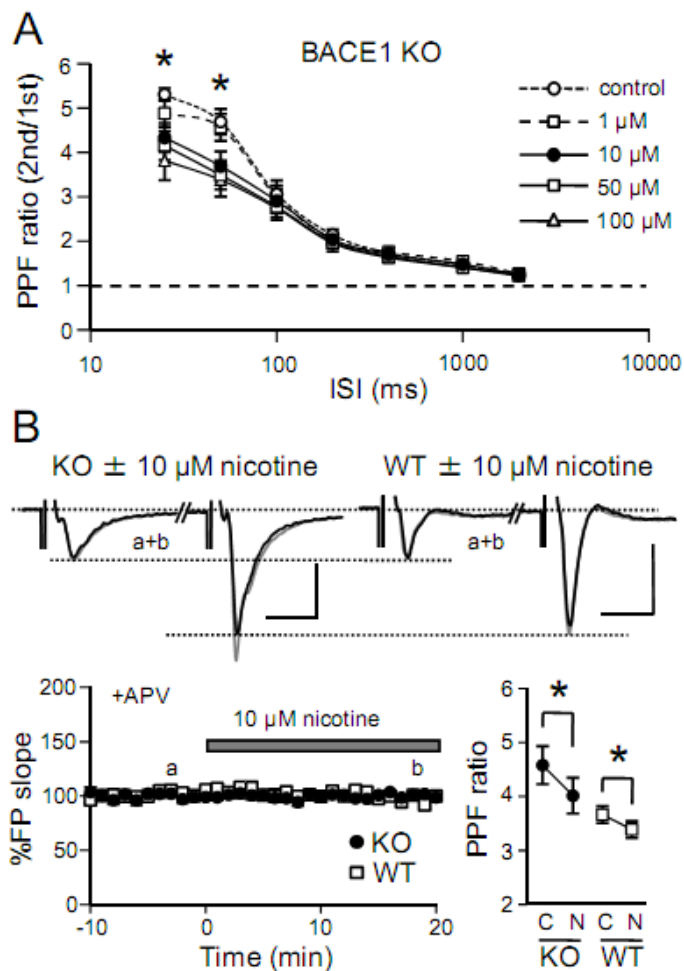


Figure 4.1. Nicotine recovers deficits in PPF at MF synapses in BACE1 KOs.

A. Nicotine reduced PPF ratio in a dose-dependent manner, which was significant at 25 and 50 ms ISIs. *ANOVA, $P < 0.05$; Fisher's PLSD, $P < 0.05$ between control and 10, 50, 100 μM nicotine groups.

B. Nicotine (10 μM) significantly decreased PPF ratio in both genotypes, but did not influence basal synaptic transmission. Top: Representative FP traces of paired-pulse stimulation (50 ms ISI) before (thin traces) and after (thick traces) nicotine. Scale: KO 1 mV, WT 0.5 mV, 10 ms. Bottom left: No change in basal synaptic strength with nicotine (KO, black circles; WT, white circles). Bottom right: Comparison of PPF ratio (50 ms ISI) before (C) and after (N) nicotine application. *paired t-test, $P < 0.001$.

Subsection 2 Nicotine rescues mfLTP in BACE1 KOs without affecting mfLTP in WTs

Consistent with our previous results, KOs lacked mfLTP under control conditions, but 10 μ M nicotine applied during the whole duration of the experiment restored mfLTP (control: $95 \pm 4\%$ at 1 hour post-HFS, $n = 6$ slices/4 mice; nicotine: $133 \pm 7\%$, $n = 8$ slices/7 mice; t-test: $P < 0.001$; Fig 4.2A). Nicotine-induced rescue of mfLTP was accompanied by a significant decrease in PPF ratio (50 ms ISI; baseline: 4.36 ± 0.26 , 1 hour post-HFS: 3.01 ± 0.27 , paired t-test: $P < 0.001$; Fig. 4.2A inset) suggesting presynaptic expression. Interestingly, 10 μ M nicotine did not alter the magnitude of mfLTP in WTs (control: $148 \pm 3\%$ at 1 hour post-HFS, $n = 5$ slices/3 mice; nicotine: $144 \pm 6\%$, $n = 7$ slices/6 mice; t-test: $P = 0.52$; Fig. 4.2B).

To investigate whether nicotine affects the induction mechanisms of mfLTP, we transiently applied nicotine for 10 min before and during the HFS. KOs displayed significant mfLTP, which was similar in magnitude with that evoked in WTs (KO = $147 \pm 2\%$ at 1 hour post-HFS, $n = 8$ slices/5 mice, paired t-test: $P < 0.001$; WT: $157 \pm 8\%$, $n = 8$ slices/5 mice, paired t-test: $P < 0.001$; Fig. 4.2C, D). Furthermore, mfLTP was accompanied by a significant decrease in PPF ratio (50 ms ISI) in both genotypes (WT: baseline = 3.66 ± 0.16 , 1 hour post-HFS = 2.55 ± 0.21 , paired t-test: $P < 0.001$; KO: baseline = 4.59 ± 0.35 , 1 hour post-HFS = 2.95 ± 0.33 , paired t-test: $P < 0.001$; Fig. 4.2C, D insets), consistent with an increase in presynaptic release. These results demonstrate that nicotine specifically rescues the induction mechanisms of mfLTP in BACE1 KOs.

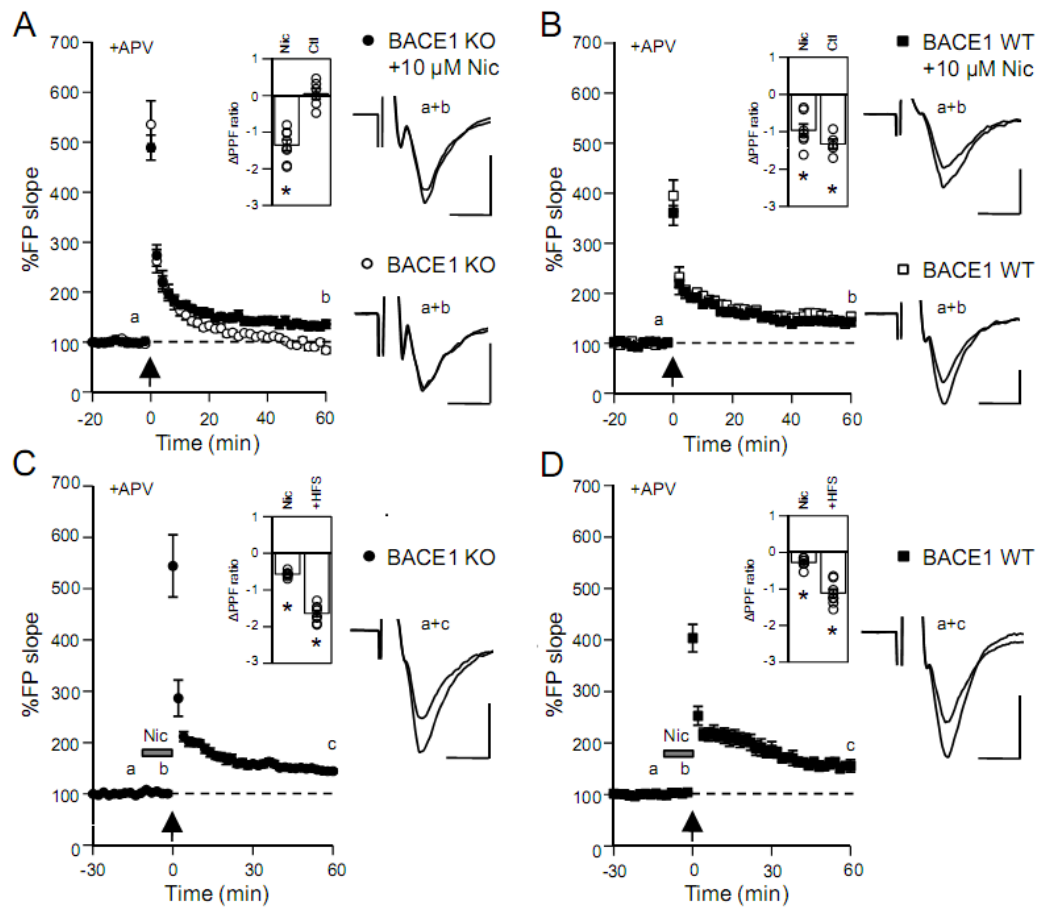


Figure 4.2. Nicotine rescues mfLTP in BACE1 KOs without effects in WTs.
A. KO slices treated with 10 μ M nicotine (black circles) showed significant mfLTP compared to control slices without nicotine (white circles).
B. The magnitude of mfLTP in WT slices treated with 10 μ M nicotine (black squares) was similar to that of control WT slices (white squares).
C. Transient application of nicotine (10 μ M, 10 min; gray bar) before and during HFS rescued mfLTP in KOs (black circles).
D. The same transient nicotine (10 μ M, 10 min; gray bar) application did not influence mfLTP in WT (black squares).
Insets: (A, B) Changes in PPF ratio with HFS [Δ PPF ratio = (PPF ratio at time b) – (PPF ratio time a)] for control (Ctl) and nicotine (Nic); (C, D) Δ PPF ratio with nicotine application [= (PPF ratio at time b) – (PPF ratio at time a)] and with HFS [= (PPF ratio at time c) – (PPF ratio at time a)]. Bars: average \pm sem. Open circles: individual data points. *paired t-test, $P < 0.001$.
 Arrow: HFS (100 Hz, 1s \times 3). Right panels: Superimposed FP traces taken at times indicated in the left panels. Scale: 0.5 mV, 5 ms.

Subsection 3 Nicotine-induced rescue of mfLTP in BACE1 KO is mediated by $\alpha 7$ -nAChRs

We showed that presynaptic dysfunction of MF synapses in BACE1 KO is at the level of Ca^{2+} regulation (Wang et al., 2008). To determine whether nicotine acts via the Ca^{2+} -permeable $\alpha 7$ -nAChRs, we used a specific agonist PNU282987 (Bodnar et al., 2005). A brief application of PNU282987 (500 nM, 10 min) before and during HFS recovered mfLTP in KOs (1 hour post-HFS: $167 \pm 19\%$, $n = 8$ slices/5 mice; paired t-test: $P < 0.05$; Fig 4.3A). Furthermore, PPF ratio decreased significantly after PNU282987 application and further by LTP induction (baseline: 6.29 ± 0.77 , +PNU282987: 5.81 ± 0.76 , 1 hour post-HFS: 4.80 ± 0.69 , Fig. 4.3A inset). PNU282987 alone did not produce changes in synaptic strength (1 hour post-PNU282987: $105 \pm 4\%$, $n = 4$ slices/2 mice; paired t-test: $P = 0.30$; Fig 4.3A).

To further test whether nicotine-induced rescue of mfLTP was mediated by $\alpha 7$ -nAChRs, we applied 100 nM α -bungarotoxin (α BTX), a selective antagonist. α BTX abolished the effect of nicotine on mfLTP (1 hour post-HFS: $105 \pm 4\%$, $n = 10$ slices/6 mice; paired t-test: $P > 0.05$; Fig 4.3B) and PPF ratio (α BTX: 5.22 ± 0.65 , α BTX+nicotine: 5.17 ± 0.65 , 1 hour post-HFS: 5.19 ± 0.73 ; Fig. 4.3B inset) in KOs. Application of α BTX and nicotine in the absence of HFS did not alter synaptic transmission (1 hour-post α BTX+Nic: $100 \pm 1\%$, $n = 4$ slices/2 mice; paired t-test: $P = 0.86$; Fig 4.3B). These results suggest that nicotine-induced rescue of presynaptic deficits in BACE1 KO is mediated by $\alpha 7$ -nAChRs.

Finally, we tested whether $\alpha 7$ -nAChRs are required for mfLTP in WTs. A brief application of α BTX (10 min) before and during HFS failed to block mfLTP in

WTs (1 hour post-HFS: $148 \pm 6\%$, $n = 9$ slices/7 mice; paired t-test: $P < 0.001$; Fig. 4.3C). This indicates that activation of $\alpha 7$ -nAChRs is not necessary for m/LTP induction in WT, hence the rescue of m/LTP in KOs by $\alpha 7$ -nAChR activation is probably via recruitment of an alternative pathway not normally used in WT.

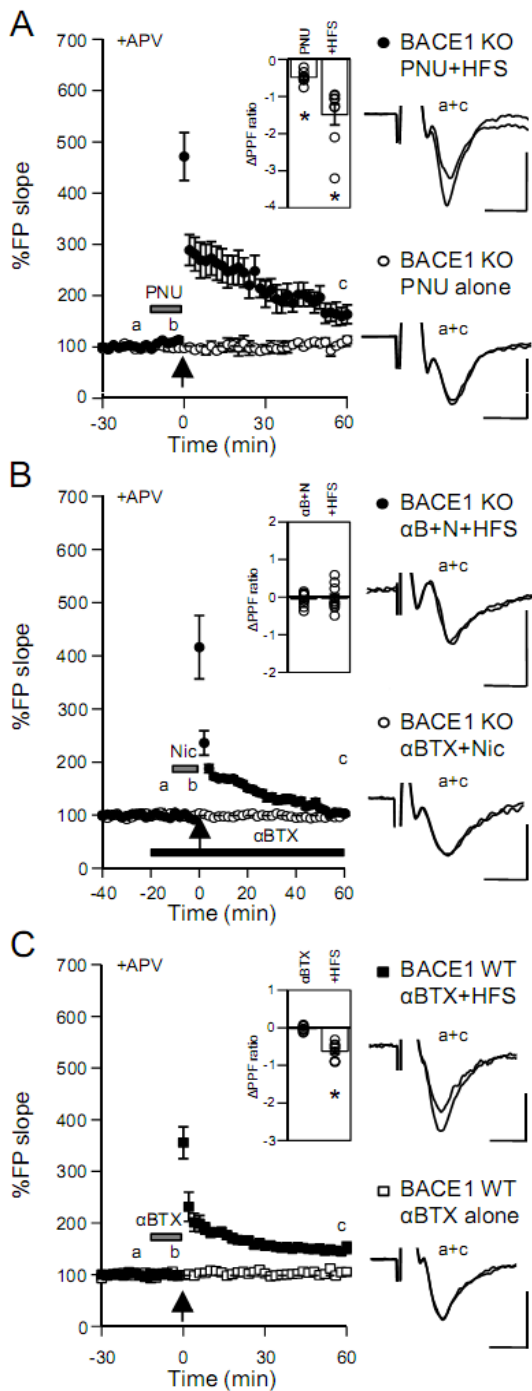


Figure 4.3. Nicotine-induced rescue of m/LTP in BACE1 KO is mediated by $\alpha 7$ -nAChRs.

A. Transient bath application of PNU282987 (PNU: 500 nM, 10 min; gray bar) rescued m/LTP in KOs (black circles). PNU282987 alone did not alter synaptic transmission (white circles). Inset: Δ PPF ratio in KO PNU+HFS experiments. Δ PPF ratio with PNU282987 application [= (PPF at b) – (PPF at a)]; Δ PPF ratio with HFS [= (PPF at c) – (PPF at a)], *paired t-test: $P < 0.01$.

B. Application of α BTX (100 nM, black bar) blocked nicotine-induced rescue of m/LTP in KOs (black circles). Application of α BTX and nicotine without HFS did not influence basal synaptic transmission (white circles). Inset: Δ PPF ratio in KO α BTX+Nic+HFS experiments. Δ PPF ratio with nicotine application in the presence of α BTX [= (PPF at b) – (PPF at a)]; Δ PPF ratio with HFS [= (PPF at c) – (PPF at a)].

C. m/LTP in wildtype is not blocked by α BTX. α BTX alone (100 nM, 10 min; gray bar) did not affect synaptic transmission (white squares). α BTX+HFS: black squares. Inset (for α BTX+HFS experiments): Δ PPF ratio with α BTX [= (PPF at b) – (PPF at a)]; Δ PPF ratio with HFS [= (PPF at c) – (PPF at a)]; *Paired t-test, $P < 0.001$. Right: FP traces. Scale: 0.5 mV, 5 ms.

Subsection 4 Calcium-induced calcium release (CICR) is involved in nicotine-induced rescue of mfLTP in BACE1 KOs

Activation of $\alpha 7$ -nAChRs enhances CICR from ryanodine-sensitive Ca^{2+} stores (Sharma and Vijayaraghavan, 2003; Sharma et al., 2008). To investigate whether CICR is also involved in nicotine-induced rescue of mfLTP in KOs, we used 20 μM ruthenium red (RR) or 100 μM ryanodine (Ryan), which are blockers of ryanodine-sensitive stores. Both drugs completely abolished nicotine-induced recovery of PPF ratio (RR: 5.00 ± 0.69 , RR+Nic: 4.97 ± 0.70 , 1 hour post-HFS: 4.62 ± 0.74 , Fig. 4.4A inset; Ryan: 5.01 ± 0.20 , Ryan+Nic: 5.03 ± 0.23 , 1 hour post-HFS: 4.93 ± 0.25) and mfLTP in KOs (1 hour-post RR+Nic: $91 \pm 5\%$, $n = 9$ slices/5 mice; paired t-test: $P = 0.18$; 1 hour-post Ryan+Nic: $100 \pm 2\%$, $n = 6$ slices/3 mice; paired t-test: $P = 0.66$; Fig 4.4A) without influencing basal synaptic transmission. MfLTP was present in WTs treated with RR (1 hour-post HFS: $124 \pm 5\%$, $n = 9$ slices/5 mice; paired t-test: $P < 0.01$; Fig 4.4B), but was significantly less than that in control WTs (t-test: $P < 0.01$), suggesting that CICRs are only partially involved.

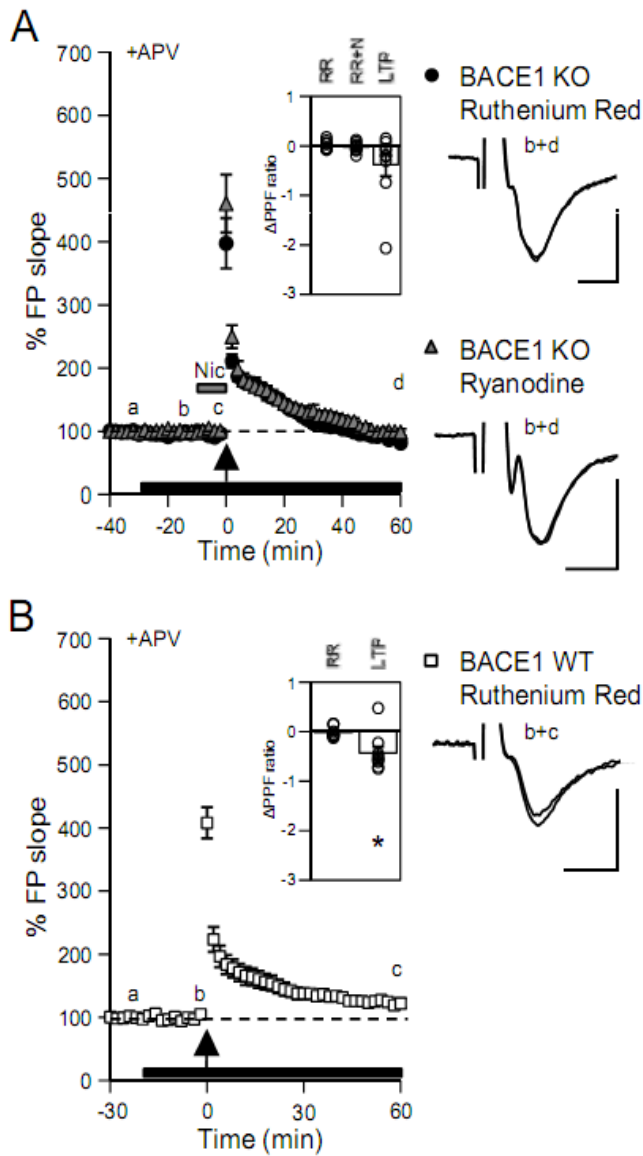


Figure 4.4. Nicotine-induced rescue of mflLTP in BACE1 KOs requires Ca^{2+} induced Ca^{2+} release (CICR).

A. Application (black bar) of ruthenium red (RR, 20 μM) or ryanodine (Ryan, 100 μM) abolished nicotine-induced rescue of mflLTP in KOs (RR: black circles, Ryan: gray triangles). Inset: ΔPPF ratio of RR application [= (PPF at b) - (PPF at a)]; +nicotine [= (PPF at c) - (PPF at b)]; +HFS [= (PPF at d) - (PPF at b)].

B. RR (20 μM ; black bar) reduced mflLTP in WT (white squares). Inset: ΔPPF ratio of RR application [= (PPF at b) - (PPF at a)]; +HFS [= (PPF at c) - (PPF at b)], *paired t-test: $P < 0.01$.

Right: FP traces. Scale: 0.5 mV, 5 ms.

Section 4 Discussion

We found that nicotine restores PPF and LTP at MF to CA3 synapses in BACE1 KOs. The nicotine effect was mimicked by $\alpha 7$ -nAChR specific agonist PNU282987, and blocked by $\alpha 7$ -nAChR antagonist α BTX. We have evidence that nicotine acts via recruiting CICR. These results suggest nicotine and $\alpha 7$ -nAChR agonists as potential pharmacological means to circumvent the presynaptic deficits caused by BACE1 inhibition.

MfLTP is presynaptically expressed requiring an increase in presynaptic Ca^{2+} and a subsequent activation of cAMP-PKA signaling pathway (Nicoll and Schmitz, 2005). We previously demonstrated that presynaptic dysfunction seen in BACE1 KOs is at the level of Ca^{2+} regulation, but the downstream PKA signaling is intact (Wang et al., 2008). These results predict that restoring presynaptic Ca^{2+} signaling should recover mfLTP in BACE1 KOs. Presynaptic $\alpha 7$ -nAChR elevates the intracellular concentration of free Ca^{2+} (Vijayaraghavan et al., 1992) and enhances glutamate release at MF terminals (Sharma and Vijayaraghavan, 2003; Sharma et al., 2008; Bancila et al., 2009). The nicotine-induced rescue of PPF and mfLTP without much effect on basal synaptic transmission is likely via the recruitment of CICR, which is known to preferentially amplify use-dependent release (Shimizu et al., 2008). Short-term presynaptic plasticity, including PPF, does not depend on CICR at MF terminals (Carter et al., 2002). Consistent with this, inhibiting CICRs in WTs did not alter PPF ratio, but reduced mfLTP magnitude, which suggests that HFS recruits CICR. In the case of KOs, it is clear that the CICR triggered by $\alpha 7$ -nAChR activation is needed to rescue mfLTP. Although we cannot rule out the possible involvement of $\alpha 7$ -nAChRs

on interneurons, the detection of $\alpha 7$ -nAChR immunoreactivity in the MF input region (Fig 4.5) provides a substrate for $\alpha 7$ -nAChR agonists to act on MF terminals. This is further corroborated by a recent electron microscopy study, which localized $\alpha 7$ -nAChRs on MF terminals (Bancila et al., 2009). Interestingly, the $\alpha 7$ -nAChRs were present away from the active zone suggesting an indirect regulation of presynaptic release.

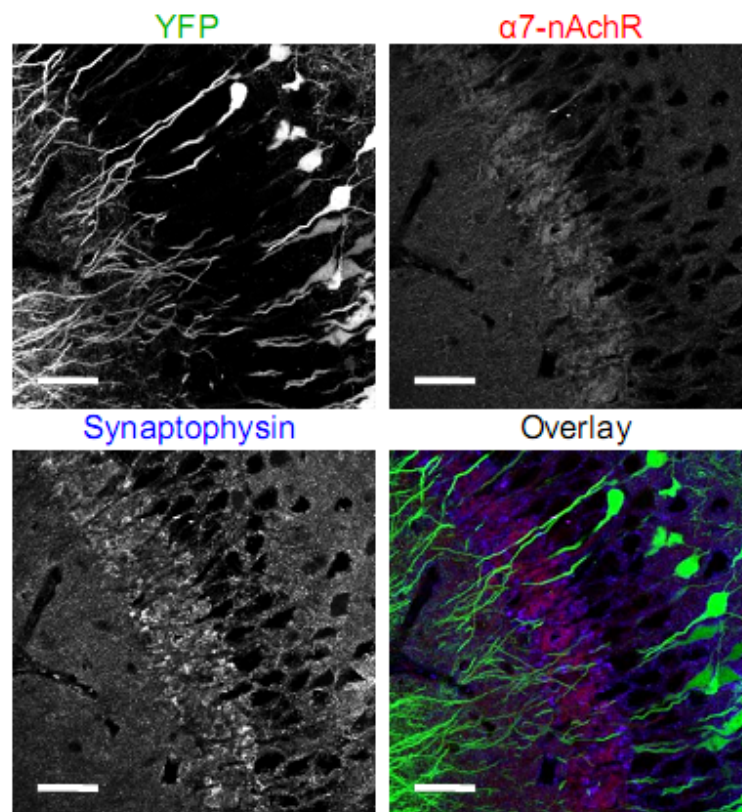


Figure 4.5. Immunohistochemical labeling of $\alpha 7$ -nAChRs in the CA3 region of a YFP-2J mouse.

Top left: YFP signal in a subset of CA3 neurons. Top right: $\alpha 7$ -nAChR immunoreactivity (Alexa633-linked secondary antibody). Note a higher signal in the stratum lucidum. Bottom left: Synaptophysin immunoreactivity (Alexa555-linked secondary antibody). Bottom right: Overlay of YFP (green), $\alpha 7$ -nAChR (red), and synaptophysin (blue). Note purple signal in stratum lucidum, which indicates overlap of $\alpha 7$ -nAChR and synaptophysin immunoreactivity. Scale: 40 μm .

It is known that $\alpha 7$ -nAChRs can rapidly desensitize upon agonist binding in a dose-dependent manner (Peng et al., 1994). Because nicotine-induced rescue of mfLTP was blocked by α BTX, we suspect residual $\alpha 7$ -nAChR activity even with the prolonged application of nicotine used in our study. Interestingly, the increase in glutamate release at MF terminals with $\alpha 7$ -nAChR activation is rather slow and involves presynaptic Ca^{2+} increase via CICR from internal stores (Sharma and Vijayaraghavan, 2003; Sharma et al., 2008). In synaptosomes isolated from the prefrontal cortex, $\alpha 7$ -nAChR agonist-induced glutamate release is dependent on CICR and a downstream activation of extracellular signal-regulated kinase (ERK) signaling (Dickinson et al., 2008). These results suggest that presynaptic signaling of $\alpha 7$ -nAChRs leading to glutamate release may outlast the initial activation of the receptor.

The regulation of $\alpha 7$ -nAChRs has been implicated in the pathology of AD. There are studies reporting high affinity binding between $\text{A}\beta 42$ and $\alpha 7$ -nAChRs (Wang et al., 2000b; Wang et al., 2000a), which either inhibit (Guan et al., 2001; Liu et al., 2001a; Pettit et al., 2001) or activate $\alpha 7$ -nAChR signaling (Dineley et al., 2001). It is possible that $\text{A}\beta 42$ may facilitate $\alpha 7$ -nAChRs at low concentration, but may inhibit nAChRs when the burden of $\text{A}\beta$ peptides increases (Dineley et al., 2001; Dougherty et al., 2003). The concentration-dependent dual role of $\text{A}\beta 42$ is evident in a study showing that picomolar range of $\text{A}\beta 42$ facilitates, but nanomolar range abolishes, LTP in CA1 and learning via its action on $\alpha 7$ -nAChRs (Puzzo et al., 2008). It is unlikely that endogenous $\text{A}\beta 42$ acts in this manner to influence mfLTP, because blocking $\alpha 7$ -nAChRs with α -BTX did not affect mfLTP in WTs. This result indirectly

argues that the lack of mLTP in BACE1 KO mice may not be a strict consequence of lacking A β . Interestingly, BACE1 has been found to regulate neuregulin-1 (NRG1) cleavage (Hu et al., 2006; Willem et al., 2006), and indeed this process is affected in BACE1 KO mice (Savonenko et al., 2008). NRG1 is critically involved in maintaining surface expression of presynaptic α 7-nAChRs (Hancock et al., 2008; Zhong et al., 2008). However, in isolated CA3 slices, we did not see a change in the total or cell surface levels of α 7-nAChRs and NRG1 in the KO mice (Fig 4.6). Furthermore, our ability to rescue mLTP in KO mice with α 7-nAChR agonists suggests sufficient presence of functional α 7-nAChRs.

Several potential methods are being developed to overcome dysfunctions caused by complete BACE1 inhibition, such as partial BACE1 inhibition (Vassar et al., 2009). While our results might reflect a developmental loss of BACE1, they suggest that combining α 7-nAChR agonists with BACE1 inhibitors may be another alternative.

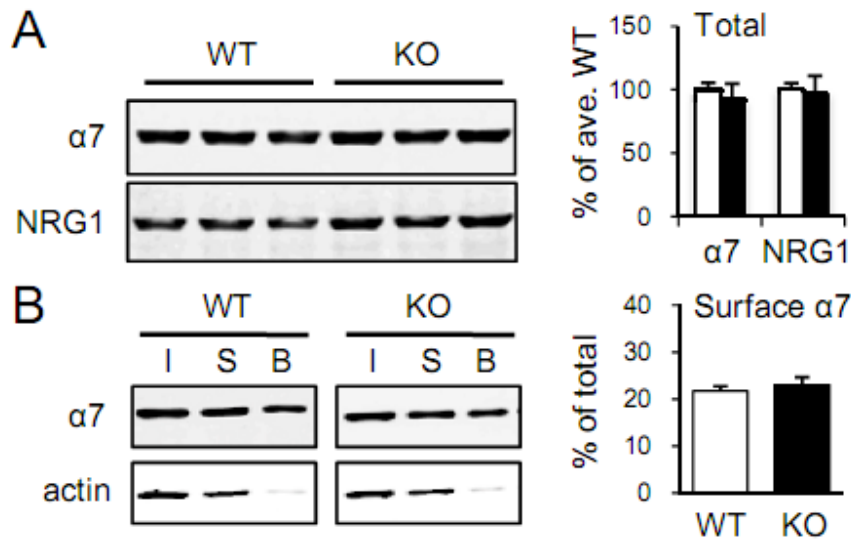


Figure 4.6. Normal expression of $\alpha 7$ -nAChRs in the CA3 region of BACE1 KOs.

A. Comparison of $\alpha 7$ -nAChR and neuregulin-1 (NRG1) levels in the total homogenates of isolated CA3 regions of WT and KOs. Left: An example immunoblots probed with $\alpha 7$ -nAChR or NRG1 antibody. Right: Quantification of the immunoblots for total $\alpha 7$ -nAChR (WT = 100 ± 5.7% of average WT value; KO = 92 ± 12.9% of average WT value; n = 10 mice each group; t-test: P > 0.58) and total NRG1 (WT = 100 ± 5.2% of average WT value; KO = 96 ± 14.6% of average WT value; n = 10 mice each group; t-test: P > 0.82).

B. Comparison of cell surface $\alpha 7$ -nAChR levels in isolated CA3 slices between WT and KO. Cell surface $\alpha 7$ -nAChRs were isolated using steady-state surface biotinylation. The total protein (input, I), the resulting supernatant (S: intracellular protein fraction), and biotin pull-down (B: cell surface fraction) were loaded onto the same gel, transferred to blots, and simultaneously probed with $\alpha 7$ -nAChR and actin antibodies using the ECLplex system (GE Health). The intensity of signals in the input and the biotin lanes was used to calculate the fraction of surface $\alpha 7$ -nAChRs as a % of total $\alpha 7$ -nAChR. Actin signal was used to assess the success of the biotinylation assay. Only the blots showing less than 5% actin signal in the biotin lane was used for quantification. Left: An example immunoblot from WT and KO. Right: Quantification of biotinylated $\alpha 7$ -nAChR signals (Surface $\alpha 7$ -nAChR: WT = 21 ± 1.0% of total, n = 10 mice; KO = 23 ± 1.7% of total, n = 8 mice; t-test: P = 0.51).

Chapter 5: General discussions and Future directions

My thesis focused on the effect of inhibiting BACE1 on synaptic transmission and plasticity at synaptic loci where BACE1 is highly expressed in brain. In addition, I identified pharmacological means to circumvent the synaptic dysfunctions caused by BACE1 inhibition. In this chapter I will summarize the conclusions obtained from my studies, and discuss their significance and future directions.

Section 1 Physiological function of BACE1

From my study, the physiological function of BACE1 in both synaptic transmission and plasticity was characterized for the first time in the CA3 circuit of hippocampus by using BACE1 KO mice. At mossy fiber synapses on CA3 pyramidal neurons, BACE1 KO mice displayed severe deficits in presynaptic function including a reduction in presynaptic function and an absence of mossy fiber LTP, which is normally expressed by a long-term increase in presynaptic release (Weisskopf et al., 1994). Moreover, BACE1 KO mice exhibited a slightly larger mossy fiber LTD, which could not be reversed. These results suggest that BACE1 function is crucial for normal synaptic transmission and activity-dependent presynaptic potentiation at these synapses. I further found evidence that the presynaptic dysfunction in BACE1 KO is likely at the level of presynaptic Ca^{2+} signaling, because the mossy fiber LTP deficit in BACE1 KO mice could be recovered by increasing the extracellular Ca^{2+} concentration. This suggests that the signaling downstream of Ca^{2+} is more or less intact in the BACE1 KO, which was confirmed by the fact that the magnitude of

presynaptic potentiation resulting from direct activation of the cAMP signaling pathway is normal in the BACE1 KO mice. The presynaptic deficits seen in BACE1 KOs may be due to the absence of A β peptides. There is evidence that A β is involved in presynaptic modifications by affecting presynaptic P/Q type Ca²⁺ channels and critical synaptic vesicle endocytosis protein dynamin (Kelly et al., 2005; Kelly and Ferreira, 2007; Nimmrich et al., 2008), which indicate the possible role of A β in regulating presynaptic Ca²⁺ and vesicle release. At first glance of these results, the evidence that A β reduces synaptic transmission, may contradict our findings, because the absence of A β as in BACE1 KOs is expected to increase presynaptic release. However, there is accumulating evidence that A β is a double-edged sword on regulating synaptic function (discussed in Chapter 1), where normal physiological concentration of A β (pM range) facilitates, but higher burden of A β peptides jeopardizes (Puzzo et al., 2008), synaptic function. Therefore, it is not surprising that the absence of A β in BACE1 KO mice may result in presynaptic deficits.

Another possibility is that the presynaptic dysfunction of BACE1 KOs may be because of abnormal processing of neuregulin-1 (NRG1), a substrate of BACE1 (Hu et al., 2006; Willem et al., 2006), which affects presynaptic release by regulating the surface expression of presynaptic Ca²⁺ permeable α 7-nAChRs (Seguela et al., 1993; Liu et al., 2001b; Zhong et al., 2008). Presynaptic nAChRs can increase glutamate release (McGehee et al., 1995; Gray et al., 1996; Maggi et al., 2003b), likely via the α 7 containing nAChRs (Le Magueresse et al., 2006). Indeed, I have demonstrated that activation of α 7-nAChRs by nicotine or α 7-nAChRs agonist can restore presynaptic release as a measure of decrease in PPF ratio, and recover mossy fiber LTP in

BACE1 KO mice. The cellular mechanism of nicotine-induced rescue is dependent on the recruitment of Ca^{2+} induced Ca^{2+} release from intracellular Ca^{2+} stores through ryanodine receptors after $\alpha 7$ -nAChR activation.

A third possibility is the augmented α -secretase activity in the BACE1 KOs. α -secretase and β -secretase have been shown to compete with each other to cleave APP (reviewed in (Turner et al., 2003)), hence in BACE1 KO mice APP tends to be processed through the non-amyloidogenic α -secretase pathway, consistent with the evidence that BACE1 KO mice have higher level of sAPP α , a product of the α -secretase (Luo et al., 2001). sAPP α has been shown to reduce the resting intracellular Ca^{2+} level and regulate cell excitability (Mattson et al., 1993; Furukawa et al., 1996). Therefore, the increased sAPP α level in the BACE1 KOs may downregulate the presynaptic function via these processes.

To examine the effect of BACE1 on an intact neuronal circuit, I expanded the analysis to CA3 circuit containing both excitatory and inhibitory synaptic transmission. I found that not only is the presynaptic release at mossy fiber synapses on CA3 pyramidal cells decreased, the presynaptic function at the inhibitory inputs on CA3 pyramidal neurons is also impaired in the BACE1 KOs. Interestingly, BACE1 KOs do not exhibit significant dysfunction at the mossy fiber input to CA3 inhibitory interneurons. These results suggest that the role of BACE1 in regulating synaptic function is specifically dependent on the nature of the postsynaptic target. My observation that both excitatory and inhibitory inputs to CA3 pyramidal cells are influenced by BACE1 ablation indicates that BACE1 modulates synaptic integration in CA3 pyramidal cells, which not only has impact on the excitability of CA3

pyramidal cells, but also has the potential to control the output of these neurons which relays information signals to CA1 region of hippocampus. The modification of input/output properties of CA3 neurons may be the cellular mechanisms of behavioral deficits seen in BACE1 KOs (Laird et al., 2005).

Section 2 Nicotine and AD

Subsection 1 The role of nicotine in modulating synaptic function and plasticity

Nicotine has been found to regulate synaptic transmission in the brain, especially playing a prominent role in presynaptic modification of neurotransmitter release (for review, see (McGehee and Role, 1996; Wonnacott, 1997)). It has been clearly documented that nicotinic enhances dopaminergic transmission, which may be an important mechanism underlying the addictive properties of nicotine (for review, see (Stolerman and Shoaib, 1991; Dani and Heinemann, 1996)). Recently, nicotinic enhancement of glutamatergic transmission has been observed in several brain areas (McGehee et al., 1995; Alkondon et al., 1996; Guo et al., 1998), including the hippocampus (Gray et al., 1996) and neocortex (Vidal and Changeux, 1993). The mechanism underlying these involves interaction of nicotine with presynaptic nAChRs, especially $\alpha 7$ -nAChRs. $\alpha 7$ -nAChR has a pentameric structure, but differs from other neuronal nAChRs which are comprised of combination of α ($\alpha 2$ - $\alpha 6$) and β ($\beta 2$ - $\beta 4$) subunits, it contains five $\alpha 7$ subunits (Decker et al., 1995; Jones et al., 1999; McGehee, 1999; Hogg et al., 2003). The special property of this receptor is that it is highly Ca^{2+} permeable after activation (Seguela et al., 1993). There is evidence that

$\alpha 7$ -nAChR activation plays an important role in nicotine induced enhancement of glutamate release due to its ability to increase presynaptic Ca^{2+} . Radcliffe et al. found that a brief nicotine application strongly increased presynaptic glutamate release in hippocampal cell culture as measured by a significant increase in mEPSCs frequency and reduction of PPF ratio. This effect was mediated by $\alpha 7$ -nAChRs and required Ca^{2+} influx in presynaptic terminals (Radcliffe and Dani, 1998). At mossy fiber to CA3 pyramidal cell synapses, it has been reported that 20 μM nicotine can increase spontaneous release, which is accompanied by Ca^{2+} increase in response to $\alpha 7$ -nAChR activation in mossy fiber boutons very likely via recruiting Ca^{2+} induced Ca^{2+} release through ryanodine receptors (Sharma et al., 2008). This study as well as others suggests that although $\alpha 7$ nAChRs desensitize rapidly upon exposure to agonist (Castro and Albuquerque, 1993; Peng et al., 1994; Zhang et al., 1994), it may contribute to prolonged enhancement of presynaptic release by allowing increases in Ca^{2+} in the presynaptic terminals via triggering Ca^{2+} release from internal stores (Sharma and Vijayaraghavan, 2003; Dickinson et al., 2008; Sharma et al., 2008). In addition to regulating synaptic transmission, nicotine is also demonstrated to modulate synaptic plasticity. Numerous studies have been done to investigate the effect of nicotine on Schaffer collateral-CA1 LTP and perforant path-dentate gyrus LTP (reviewed in (Kenney and Gould, 2008)). However, whether nicotine can influence mossy fiber-CA3 LTP has not been shown before. I have found that 10 μM nicotine significantly increases glutamate release at mossy fiber synapses on CA3 pyramidal cells in WTs and BACE1 KOs. Furthermore, nicotine, by activating $\alpha 7$ -nAChRs and recruiting Ca^{2+} induced Ca^{2+} release, restores mossy fiber LTP in

BACE1 KOs with no influence on mossy fiber LTP in WT mice. This finding is very promising because we expected to rescue the deficits in BACE1 KOs without introducing new side effects in the system. The fact that nicotine does not affect normal mossy fiber LTP in WT mice is likely because presynaptic Ca^{2+} signaling in response to high frequency stimulation in WT mice is already saturated. And the evidence that inhibiting $\alpha 7$ -nAChRs by α -bungarotoxin during mossy fiber LTP induction does not block LTP in WT mice supports the idea that activation of $\alpha 7$ -nAChRs is not normally required for LTP induction at these synapses. These results suggest that nicotine-induced rescue of mossy fiber LTP in BACE1 KOs utilizes a bypass mechanism, which is not normally recruited in WT mice.

Besides regulating glutamatergic transmission, nicotine has also been found to modulate GABAergic transmission. It has been reported that nicotine can increase inhibitory inputs to the principal cells of local circuits in both CNS and PNS (Radcliffe et al., 1999; Genzen and McGehee, 2005; McGehee, 2007), which involves presynaptic nAChRs activation on interneurons in a Ca^{2+} -dependent manner (Radcliffe et al., 1999; Liu et al., 2007). Interestingly, in the CA1 circuit of hippocampus, activation of interneurons not only directly inhibits pyramidal neuron activity, but also produces inhibition of other GABAergic cells leading to disinhibition of pyramidal cells. Both the inhibition and disinhibition effects are mediated by $\alpha 7$ -nAChRs (Ji and Dani, 2000; Buhler and Dunwiddie, 2002). The dual mechanism allows fine-tuned control of nicotine on inhibition within a neuronal circuit, which in turn modulates the input/output of the information flow. Although my study suggests that nicotine can regulate excitatory synaptic transmission at

mossy fiber synapses in CA3 area, whether nicotine can also regulate inhibitory inputs in the circuit is unknown. Future studies will be necessary to verify whether nicotine has inhibition and/or disinhibition effects on the CA3 circuit, which may control the output from CA3 to CA1 area of hippocampus where many learning and memory formations are associated (Kenney and Gould, 2008). Besides, it is very interesting to know whether nicotine can also rescue the deficits in GABAergic terminals of CA3 interneurons caused by BACE1 inhibition, which will provide full evidence of the mechanism underlying nicotine induced rescue of the synaptic dysfunction, hence will allow better development of therapeutics that can overcome the negative effects of long-term BACE1 inhibition needed for AD treatment.

Subsection 2 Nicotine therapy as a potential treatment for AD

Another characteristic of AD is degeneration of the cholinergic neurons in the basal forebrain, reduction of cholinergic projections, and loss of nicotinic receptors in the cortex and hippocampus (Paterson and Nordberg, 2000; Auld et al., 2002). Therefore, nicotine and nicotinic receptor agonist application could be potential therapy for AD. The emerging evidences suggest that it may be the case. An *in vivo* study showed that nicotine treatment (2 mg/kg/day, 6 weeks) prevents CA1 LTP and LTD deficits in an AD mouse model (Alkadhi et al., 2011). Echeverria et al. found that cotinine, the main metabolite of nicotine, which does not have addictive side effects in humans, significantly decreased A β deposition, inhibited A β ₄₂ aggregation, and more impressively, improved working and reference memories in a transgenic AD mouse model (Echeverria et al., 2011). Although a lot of work still needs to be done, current studies are revealing that nicotine or nicotinic agonists may have

significant effect on AD. Indeed, $\alpha 7$ -nAChR has become a drug target for many cognitive disorders including AD (reviewed in (Wallace and Porter, 2011; Russo et al., 2012)). In my study, I have shown that nicotine or $\alpha 7$ -nAChR agonist can overcome presynaptic dysfunction and restore the abolished mossy fiber LTP due to blocking of BACE1. My results suggest that nicotine or $\alpha 7$ -nAChR agonist application combining with BACE1 inhibition minimizes the side-effect of presynaptic dysfunction, hence may be a better treatment for AD. Future studies will be necessary to examine whether nicotine or $\alpha 7$ -nAChR agonist can rescue the behavioral deficits seen in BACE1 KOs, and more importantly the combination treatment needs to be examined in AD models. Furthermore, studies need to be done to determine the optimal dose and treatment duration of nicotine or $\alpha 7$ -nAChR agonist to avoid addiction during AD treatment.

Section 3 Feasibility of BACE1 inhibition as AD treatment

Inhibition of BACE1, the major neuronal form of β -secretase, has received more and more attention as the treatment of AD. It was initially promising because knocking out *BACE1* in APP transgenic lines, which normally develop A β plaques and behavioral deficits essentially, alleviated the AD symptoms (Luo et al., 2003; Ohno et al., 2004; Laird et al., 2005). However, recent studies, including mine, showed that BACE1 knockouts display specific dysfunctions in synaptic transmission and plasticity (Ohno et al., 2004; Laird et al., 2005; Wang et al., 2008), as well as behavioral deficits (Harrison et al., 2003; Laird et al., 2005; Savonenko et al., 2008). In addition, studies showing that BACE1 has other substrates, besides APP, which

have normal physiological functions, further caution the use of BACE1 inhibition as a practical treatment for AD (Wong et al., 2005; Hu et al., 2006; Willem et al., 2006; Kim et al., 2007). In order to improve the development of effective therapeutics that targets this enzyme, we need to find potential ways to overcome the synaptic deficits due to the absence of BACE1 activity or resort to partial inhibition strategies.

Subsection 1 Partial inhibition or conditional knockdown of BACE1

It has been shown that A β burden is dose-dependent on BACE1 activity. Therefore, partial inhibition of BACE1 may be beneficial for AD treatment. To test this, Kimura and colleagues crossed BACE1 heterozygous (HT) mice with a line of transgenic mice carrying human APP and PS1 with 5 familial AD mutations (5XFAD), and found that partial reduction of BACE1 improved remote and recent memory and restored CA1 LTP (Kimura et al., 2010). In addition, I have tested synaptic function at mossy fiber CA3 synapses using BACE1 HT mice. My preliminary data showed that presynaptic function at these synapses in BACE1 HTs is recovered compared to KOs as a measure of reduced PPF ratios at 25 and 50 ms ISIs (25 ms ISI: HT = 4.3 ± 0.25 ; KO = 6.1 ± 0.79 ; 50 ms ISI: HT = 4.0 ± 0.21 , n = 12; KO = 5.7 ± 0.77 , n = 17; Fisher's PLSD post hoc test: P < 0.01 between HTs and KOs; Fig. 2.1A and 5.1A), although the ratios are not restored completely back to the levels of WTs (25 ms ISI: WT = 3.3 ± 0.27 ; HT = 4.3 ± 0.25 ; 50 ms ISI: WT = 3.4 ± 0.21 , n = 12; HT = 4.0 ± 0.21 , n = 12; Fisher's PLSD post hoc test: P < 0.01 between WTs and HTs; Fig. 5.1A). BACE1 HTs display significant mossy fiber LTP compared to BACE1 KOs (HT: $128 \pm 3\%$ of baseline at 1 hour post-HFS, n = 12 slices/5 mice; KO: $96 \pm 7\%$, n = 16 slices/7 mice; t-test: P < 0.01 between HTs and

KOs; Fig. 2.2A and 5.1B), although the magnitude of the LTP is less than that of WT (WT: $149 \pm 3\%$ of baseline at 1 hour post-HFS, $n = 12$ slices/6 mice; HT: $128 \pm 3\%$, $n = 12$ slices/5 mice; t-test: $P < 0.01$ between WT and HT; Fig. 5.1B). These data suggest that although the presynaptic release is also impaired in BACE1 HTs, the damage is not as severe as that in BACE1 KOs, and mossy fiber LTP is partially expressed in BACE1 HTs. Therefore partial inhibition of BACE1 produces less synaptic dysfunction at mossy fiber synapses to CA3 pyramidal cells compared to completed BACE1 inhibition.

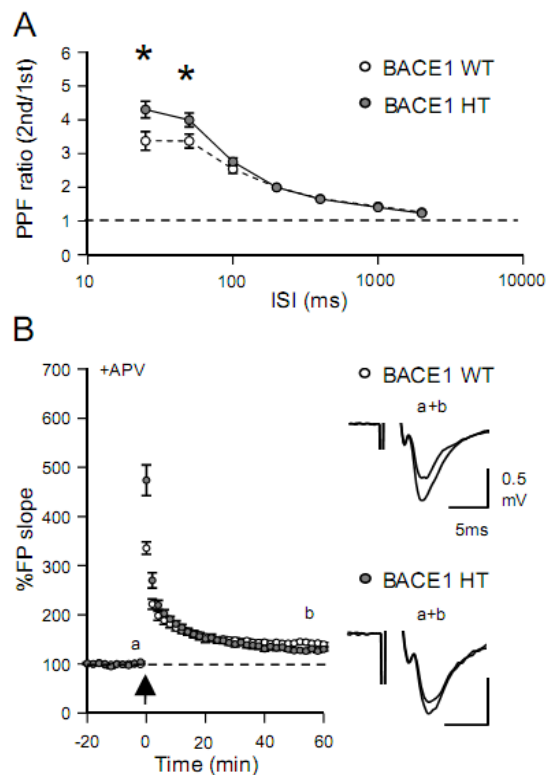


Figure 5.1. BACE1 HTs display a slight reduction in presynaptic function at mossy fiber to CA3 PCs synapses remaining partial mossy fiber LTP.

A. Slightly larger PPF ratio in BACE1 HTs. The difference between WT (white circles) and HTs (grey circles) are significant at 25 and 50 msec ISIs. *ANOVA: $P < 0.01$; Fisher's PLSD post hoc test: $P < 0.01$ between the two genotypes.

B. BACE1 HTs display partial mossy fiber LTP. Left: Summary graph plotting changes in normalized field potential against time. The arrow depicts when HFS (100 Hz, 1 sec x 3) was delivered. Right: Superimposed representative field potential traces taken from WT and HTs at times indicated in the left panel.

Another potential method is knockdown of BACE1. Researchers have successfully suppressed BACE1 activity by using RNA interference (RNAi) *in vitro* (Kao et al., 2004; Dong et al., 2006) and *in vivo* (Laird et al., 2005; Singer et al., 2005). Lentiviral BACE1 siRNA delivered into the hippocampus has been found to effectively reduce A β production, neurodegeneration, and behavioral deficits in APP transgenic mice (Laird et al., 2005; Singer et al., 2005). Characterizing synaptic function in the BACE1 siRNA knockdown models may provide information about acute effects of blocking BACE1 function. In addition, siRNA knockdown of BACE1 in APP transgenic lines will better approximate clinical situations, and hence allow us to better estimate the feasibility of developing an effective treatment of AD by BACE1 inhibition.

Subsection 2 BACE1 inhibitors

Since the identification of BACE1, the development of BACE1 inhibitors has been initiated. However, the progress was slow, probably due to the difficulty of identifying small molecules that can pass through the blood brain barrier and also have high stability and good pharmaceutical properties (Citron, 2004a; Ghosh et al., 2008a). So far, several BACE1 inhibitors have been discovered, among them only CTS-21166 has passed Phase I clinical trials (see review (Ghosh et al., 2008a; Luo and Yan, 2010)). Many BACE1 inhibitors have been shown to decrease soluble A β production, amyloid plaque deposition, as well as improve cognitive function in AD animal models (Hussain et al., 2007; Ghosh et al., 2008b; Fukumoto et al., 2010; Takahashi et al., 2010; Zhu et al., 2010; Chang et al., 2011). Surprisingly, none of them have been tested to determine their ability to improve synaptic dysfunction, the

cellular mechanism that correlates with cognitive decline. A critical question is whether these inhibitors can recover synaptic deficits seen in AD models, or whether they may produce additional defects in the normal brain.

Subsection 3 Transcriptional and miRNA regulation of BACE1 regulation of BACE1

There are several reports of transcriptional regulation of BACE1. Nie et al. have shown that activation of $\alpha 4\beta 2$ nAChR can decrease BACE1 transcription through the ERK1-NF κ B pathway in SH-EP1 cell line expressing human APP695 and human nAChR $\alpha 4$ and $\beta 2$ subunit (Nie et al., 2011); Wen and colleagues reported that overexpression of p25, an activator of cdk5, can increase levels of BACE1 mRNA and protein likely through interactions of signal transducer and activator of transcription (STAT3) with the BACE1 promoter (Wen et al., 2008). In addition, in sporadic AD patients' brains, an increase in BACE1 levels is correlated with a decrease in a subset of microRNAs (miRNA), especially the miR-29a/b-1 miRNA cluster (Hebert et al., 2008). miRNAs can regulate mRNA translation. Therefore, it is possible that an increase in specific miRNA levels can down regulate BACE1 protein expression and decrease A β burden. These findings provide various ways to regulate BACE1 expression.

Subsection 4 Endogenous BACE1 activity modulators

Recently, studies have shown that during sporadic AD or in AD animal models, the activities of certain endogenous molecules are modified, which upregulates BACE1 activity. For example, sphingosine-1-phosphate (S1P),

phosphorylation of the translation initiation factor eIF2 α and calpain activity are increased in AD, which can lead to an increase in BACE1 activity (O'Connor et al., 2008; Liang et al., 2010; Nimmrich et al., 2010; Ill-Raga et al., 2011; Takasugi et al., 2011). On the other hand, conjugated linoleic acid (CLA), acetylcholinesterase inhibitor galantamine (Gal), copper chaperone for superoxide dismutase (CCS), PPAR γ co-activator-1 α (PGC-1 α), trafficking molecule GGA3, as well as Fbx2-E3 ligase activity are decreased in AD, which leads to increased BACE1 protein levels (Tesco et al., 2007; Sarajarvi et al., 2009; Gong et al., 2010; Gray et al., 2010; Kang et al., 2010; Li et al., 2010; Katsouri et al., 2011; Li et al., 2011). So far, only the effect of Fbx2 on synaptic plasticity has been tested. Adenoviral-Fbx2 transfection significantly improves LTP in the CA1 region of the hippocampus of Tg2576 mice without affecting basal synaptic transmission (Gong et al., 2010). Although modulating these molecules or signaling pathways can be potential methods to control BACE1 activity, further studies need to be done to verify whether synaptic function can be improved by manipulating BACE1 modulators.

Subsection 5 Pharmacological means that increase presynaptic Ca²⁺ signaling

From my study, I have found that the presynaptic dysfunction in BACE1 KOs is likely at the level of presynaptic Ca²⁺ signaling, therefore, it is possible that manipulations that enhance presynaptic Ca²⁺ may overcome the synaptic deficits caused by inhibiting BACE1 activity. This opens up possibilities of using pharmacological means to enhance presynaptic Ca²⁺ signal, which in turn can restore synaptic dysfunctions in BACE1 KOs. Exploring these possibilities will allow

development of effective pharmacological means for treating AD by combining BACE1 inhibitors with specific agents that can enhance presynaptic Ca^{2+} signal.

One possible way is modulating presynaptic Ca^{2+} permeable $\alpha 7$ -nAChRs. My study showed that activation of $\alpha 7$ -nAChRs, by nicotine or $\alpha 7$ -nAChRs agonist, can restore PPF ratio and mossy fiber LTP in BACE1 KO mice. The cellular mechanism of nicotine-induced rescue is dependent on the recruitment of Ca^{2+} induced Ca^{2+} release from intracellular Ca^{2+} stores through ryanodine receptors. These results suggest that nicotine or $\alpha 7$ -nAChR agonists may be potential pharmacological means to circumvent the presynaptic dysfunctions caused by BACE1 inhibition.

Another candidate is mGluR2 which acts as inhibitory autoreceptor at mossy fiber terminals to potentially block glutamate release by a mechanism that likely includes inhibition of presynaptic voltage-gated Ca^{2+} channels (Kamiya and Ozawa, 1999; Pelkey et al., 2006). Therefore, it is possible that by using specific antagonist of mGluR2, it would facilitate presynaptic voltage-gated Ca^{2+} channels and enhance presynaptic Ca^{2+} influx. However, recent study has shown that repetitive stimulation can activate mGluR2, which limits the magnitude of frequency facilitation, and antagonist of mGluR2 is reported to enhance frequency facilitation during low frequency stimulation (Kwon and Castillo, 2008a). Therefore, mGluR2 inhibition may not reduce PPF ratio in BACE1 KOs. To verify whether blocking mGluR2 is beneficial to presynaptic function recovery, future study should be done to compare frequency facilitation in BACE1 KOs with and without mGluR2 antagonist, to see whether BACE1 KOs show deficits in frequency facilitation, and whether mGluR2 antagonist overcomes this defect.

A third potential target that is thought to regulate presynaptic release is kainate receptor (KAR), which is another ionotropic glutamate receptor aside from NMDAR and AMPAR. It has been shown that dentate gyrus granule cells strongly express KARs especially in stratum lucidum which is the mossy fiber terminal zone suggesting presynaptic location of KARs (Monaghan and Cotman, 1982; Wisden and Seeburg, 1993; Darstein et al., 2003). Interestingly, low concentrations of kainate (20-100 nM), which activate KARs, have been found to enhance synaptic transmission at hippocampal mossy fiber synapses (Schmitz et al., 2001; Ji and Staubli, 2002; Contractor et al., 2003; Rodriguez-Moreno and Sihra, 2004). This enhancement is mediated, at least partially, by increased release probability (Schmitz et al., 2001; Ji and Staubli, 2002). Additionally, several studies have suggested that presynaptic KARs may facilitate the induction of mossy fiber LTP (Contractor et al., 2001; Lauri et al., 2001; Bortolotto et al., 2003; Schmitz et al., 2003). Although the detailed mechanism of KAR mediated enhancement of glutamate release is not very clear, Kamiya et al. found that increase of presynaptic Ca^{2+} is likely involved (Kamiya et al., 2002). These evidences provide possibility that activation of KARs may rescue the presynaptic deficits seen in BACE1 KO. However, contradictory results also exist. Kwon and colleagues have evidence suggesting that activation of KARs by 50nM kainate does not enhance transmitter release at mossy fiber to CA3 synapses, and short-term plasticity at these synapses is KAR independent (Kwon and Castillo, 2008a). If this is the case, KARs activation may not rescue the presynaptic dysfunction in BACE1 KO; but if future studies show that activation of these receptors rescues phenotypes in BACE1 KO, it would suggest that KAR mediated

rescue of presynaptic deficits involve utilizing signaling pathways which are normally not recruited.

Appendix

Comparison of statistical analysis using data from slices or data from animals

Take paired-pulse facilitation (PPF) ratio data from wild type animals as an example:

Statistics using data from slices

ISI(ms)	Ave. PPF ratio	SD	SEM	CV	Num. of slices
25	3.392429	0.682648	0.241353	0.201227	8
50	3.452732	0.657079	0.232313	0.190307	8
100	3.122592	0.595195	0.210433	0.190609	8
200	2.185701	0.230974	0.081662	0.105675	8
400	1.708131	0.114219	0.040383	0.066868	8
1000	1.460462	0.113193	0.040020	0.077505	8
2000	1.232870	0.070771	0.025021	0.057404	8

Statistics using data from animals

ISI(ms)	Ave. PPF ratio	SD	SEM	CV	Num. of animals
25	3.392429	0.355143	0.177571	0.104687	4
50	3.452732	0.417338	0.208669	0.120872	4
100	3.122592	0.424468	0.212234	0.135935	4
200	2.185701	0.198730	0.099365	0.090923	4
400	1.708131	0.051059	0.025530	0.029892	4
1000	1.460462	0.059047	0.029523	0.040430	4
2000	1.232870	0.037512	0.018756	0.030426	4

The tables above show that the Coefficient of Variation (CV) of data from slices is consistently larger than that from animals, suggesting that the major variability tends to come from the difference among the slices not that across the animals. Therefore, data from slices were used to perform all the statistical analysis.

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