REGULATION OF SYNAPTIC TRANSMISSION BY CYTOPLASMIC ATM

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ABSTRACT OF THE DISSERTATION

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Ataxia-telangiectasia (A-T) is a devastating childhood disorder caused by mutation of the ataxia-telangiectasia mutated gene (ATM) which encodes a member of the PI3 kinase family. In most cells, ATM is localized in the nucleus and is involved in DNA damage repair. In neurons, however, a substantial fraction of ATM is cytoplasmic, and the function of this cytoplasmic pool is poorly understood and not well studied.

Since likely binding partners of ATM include the molecules synapsin-I and VAMP2, we examined the possibility that cytoplasmic ATM is a regulator of synaptic plasticity. FM4-64 dye tracing experiment showed a deficit of spontaneous vesicle releasing in ATM deficient cells. Hippocampal slice recordings revealed that Shaffer collateral long-term potentiation (LTP) in homozygous Atm^{tm1Awb} mice was significantly reduced comparing to wild-type controls. In Atm^{tm1Awb} mutant mice, ATM protein is still

made, but is a novel, catalytically less active, splice mutant product. By comparison in *Atm*^{tm1Bal} mutant mice in which the protein is not made, short-term plasticity such as synaptic fatigue and paired-pulse facilitation were compromised, whereas LTP was less affected.

Binding studies show that cytoplasmic ATM serves as a scaffold that brings together ATR, synapsin-I and VAMP2. This observation suggests that cytoplasmic ATM in the *Atm*^{tm1Awb} mice may be acting to disable the complex, whereas the simple absence of ATM is less disruptive. The next stage of this work will focus on understanding the mechanism of how cytoplasmic ATM is involved in synaptic function.

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Thank you all and always be in light!!!

Sincerely

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INTRODUCTION

Ataxia-Telangiectasia – a genetic neurodegenerative disorder.

Ataxia-Telangiectasia (A-T) is also called Louis-Bar syndrome. It was first brought into medical literature by Syllaba and Henner in 1926 (Syllaba and Henner, 1926), then thirty years later defined and named by Border and Sedgwick in 1958 (Boder and Sedgwick, 1958).

Common facts about Ataxia-Telangiectasia (A-T)

The disorder's name comes from two likely recognized abnormalities: ataxia (lack of muscle control) and telangiectasia (abnormal dilation of capillary vessels that often result in tumors and red skin lesions). However, A-T involves more than just the sum of these two findings. It is a multisystem disorder, and it covers a wide range of symptoms: e.g. speech difficulty, choreoathetosis, oculomotor dysfunction, growth retardation, cancer susceptibility, hypersensitivity to ionizing radiation and sterility. A-T also affects the immune system and increases the risk of leukemia and lymphoma in affected individuals [For reviews see: (Boder, 1985, Chun and Gatti, 2004, Nowak-Wegrzyn et al., 2004)].

A-T is a progressive neurodegenerative disease. Infants with A-T initially often appear very healthy. At around age two, ataxia and nervous system abnormalities become apparent. A toddler becomes clumsy, loses balance easily and lacks muscle control. Speech becomes slurred and more difficult, and the symptoms progressively

worsen. Between ages two and eight, telangiectasias, or tiny, red "spider" veins, often appear on the cheeks and ears and in the eyes. Around age 10-12, children with A-T can no longer control their muscles and become wheel chair bound. Immune system deficiencies become common, and affected individuals are extremely sensitive to radiation. Immune system deficiencies vary between individuals but include lower-than-normal levels of proteins that function as antibodies (immunoglobulin) and white blood cells. The thymus gland, which aids in development of the body's immune system, is either missing or has developed abnormally. Intelligence of A-T patients is usually normal, but growth may be retarded probably due to hormonal deficiencies. Individuals with A-T are also sometimes afflicted with diabetes, prematurely graying hair and difficulty swallowing. As the children grow older, the immune system becomes weaker and less capable of fighting infection. In the later stages, recurrent respiratory infections and blood cancers, such as leukemia or lymphoma, are common. A-T is fatal, individuals with A-T typically die by their twenties with few surviving into thirties.

A-T is an autosomal recessive disorder, and the occurrence is rare with an estimated frequency of between 1/40,000 and 1/100,000 live births. But it is believed that many A-T cases, particularly those who die at a young age, are never properly diagnosed. Therefore, this disease may actually be much more prevalent. According to the A-T Project Foundation, it is estimated that ~1% of the general population in the United States carries defective A-T genes. Carriers of one copy of this gene do not develop A-T, but have a significantly increased risk of cancer (Easton, 1994, Meyn, 1995).

Classical A-T diagnosis relies on recognizing the hallmarks of A-T: progressive ataxia and telangiectasia. However, this may delay the golden opportunity of early

treatment of the disease because these symptoms usually don't appear till later childhood. Another way to diagnosis A-T is based on the cellular phenotype of A-T. Cell lines derived from patients with A-T display characteristic abnormalities, including poor growth, increased serum growth factor requirements, premature senescence, sensitivity to IR, and failure to establish effective cell-cycle arrest after genotoxic insult (Brown et al., 1999). The cellular phenotype diagnosis is certainly more accurate and reliable, however it requires the generation of cell lines from A-T patient, which takes time and effort.

A-T Mutated (ATM) is the cause of A-T

Because of the wide range of symptoms shown by A-T patients, the mechanism of the disease has long been considered elusive (Boder, 1985). Progress towards the identification of the defective gene(s) in A-T was hampered by the suspicion of as many as five possible complementation gene groups existing in early cellular studies (Jaspers and Bootsma, 1982). In 1988, Gatti and colleague first localized the A-T gene(s) to chromosomal region 11q22-23 using genetic linkage analysis of 31 A-T families (Gatti et al., 1988). This initiated extensive positional cloning studies from several laboratories and the search for A-T gene(s) was narrowed to less than 500 kilobases (Lange et al., 1995). But not until 1995 was one single gene identified as the cause (Savitsky et al., 1995). The gene that is defective in A-T was subsequently named ATM (A-T mutated). The ATM gene was shown to occupy 160 kb of genomic DNA, encoding a 13 kb transcript of 66 exons (Uziel et al., 1996).

Basic properties of ATM

ATM gene is conserved from yeast to humans (Zakian, 1995). The protein encoded by this gene is a large protein of 370 KD. ATM contains an N-terminal substrate-binding domain that binds to several known substrates such as p53, Nijmegen Breakage Syndrome-1 (NBS1) and Breast Cancer Susceptibility Protein-1 (BRCA1). This binding domain is a crucial region of ATM because its deletion inactivates the protein. The ATM protein also has a number of other domains, including a FAT domain, an extreme C-terminal FATC domain, a Leu zipper, a Pro-rich region that enables it to bind to ABL kinase, a peroxisomal targeting signal sequence and a Ser/Thr protein kinase domain at the site close to the C terminal end (Shafman et al., 1997). The C-terminal kinase is a member of the phosphoinositide 3-kinase (PI3K)-related protein kinase (PIKK) family, which also includes ATR (Ataxia Telangiectasia- and RAD3 related) protein and DNA-dependent Protein Kinase (DNA-PK). The ATM PI3 kinase recognizes a general consensus motif – a serine or threonine residue followed by a glutamine (the 'SQ/TQ' motif). Positively charged amino acids near the target serine/threonine generally seem to diminish phosphorylation, whereas hydrophobic or negatively charged amino acids enhance it (Kim et al., 1999).

Nuclear ATM responds to DNA damage

At present, the best-known function of ATM is its response to DNA damage. Hypersensitivity to ionizing radiation (X-rays, γ-rays) was reported in A-T patients after radiotherapy for cancer in the 1960s (Gotoff et al., 1967) and in A-T cells in culture in the 1970s (Taylor et al., 1975, Chen et al., 1978). ATM displays increased kinase

activities in response to IR or to the radiomimetic compound neocarzinostatin but not to UV irradiation (Banin et al., 1998, Canman et al., 1998). IR usually produces severe Double-Strand Breaks (DSBs) inside the genome, while UV irradiation causes relatively less significant DNA damage.

Upon detection of DSBs, the MRE11-RAD50-NBS1 (MRN) complex is assembled at the DNA damage site and then ATM protein is recruited to the lesion site by association with the MRN complex. This association is very important for proper DNA Damage Repair (DDR) and disruption of the complex can result in a profound physiological deficit. Disorders have also been reported for hypomorphic mutants in two members of this complex: Nijmegen Breakage Syndrome by NBS-1 mutants and A-T-Like disorder (ATLD) by MRE11 mutants (Carney et al., 1998, Matsuura et al., 1998, Varon et al., 1998, Stewart et al., 1999). Although these two syndromes are distinct from A-T, they share many close clinical symptoms and cellular characteristics (Uziel et al., 2003). MRN complex subsequently acts as an adaptor for ATM's autophosphorylation at its Serine 1981 site in human (S1987 in mouse). This autophosphorylation will dissociate the non-functioning ATM dimer into two active monomers thus activating the PI-3 kinase and allowing ATM to phosphorylate downstream DDR targets such as histone variant H2AX. H2AX is converted into γH2AX by ATM dependent phosphorylation. This phosphorylation change seems to be the initial signal for subsequent accumulation of DNA-damage-response proteins (Bassing et al., 2003, Celeste et al., 2003). A second substrate of ATM, Mediator of DNA-damage Checkpoint protein (MDC1), then binds to γH2AX via its breast cancer susceptibility protein-1 (BRCA1) C-terminal (BRCT)

domain. MDC1 serves as a "master regulator" and initiates the subsequent repair of DNA DSBs (Stucki and Jackson, 2004).

ATM also plays a key role in cell-cycle-checkpoint regulation. Cell-cycle-checkpoints are part of a surveillance system that maintains genomic integrity after damage of DNA, or other cellular macromolecules, by agents such as IR or UV. These checkpoints either result in programmed cell death (apoptosis) to eliminate the damaged cell, or they stall the cell cycle to prevent replication of potentially damaged DNA. The cell cycle can potentially arrest at several stages, but it usually stalls before S phase or during S phase (known as the G1 and S-phase checkpoints, respectively) to prevent the cell from progressing through DNA replication, or before mitosis (the G2 checkpoint) to prevent aberrant segregation of damaged chromosomes. Failure of these mechanisms would be expected to result in genomic instability and cancer predisposition.

Cells from A-T patients showed abnormal cell-cycle arrest (Houldsworth and Lavin, 1980, Painter and Young, 1980, Kastan et al., 1992, Beamish and Lavin, 1994). As the cell cycle arrest at the G1 and S checkpoints are directly involved ATM, none of the proteins that regulate progression from G2 into M in mammalian cells has been identified as an ATM target (Kastan and Lim, 2000). In the G1 checkpoint arrest, tumor suppressor protein p53 is phosphorylated by ATM on serine 15 upon detection of DNA damage. Phosphorylated p53 then activates the production of p21 (also known as WAF1 or CIP1) through its transcription-factor activity. p21 in turn inhibits cyclin E and its partner CDK2 – a complex required for progression of the cell cycle from G1 to S phase (Shieh et al., 1997, Siliciano et al., 1997). A second target of ATM, the checkpoint kinase Chk2, is also phosphorylated in this process. Activated Chk2 then phosphorylates

p53 on serine 20. This phosphorylation event is believed to result in an increase in the level of p53 protein (Chehab et al., 2000, Hirao et al., 2000, Shieh et al., 2000).

ATM is also important in the cell cycle arrest at S-phase checkpoint. Actually one of the first abnormalities to be characterized in ATM-deficient cells was a failure to arrest DNA synthesis after ionizing radiation (Houldsworth and Lavin, 1980, Painter and Young, 1980). The phenomenon was called Radioresistant DNA Synthesis (RDS). The S-phase checkpoint arrest is a distinct process from G1 checkpoint, and does not depend on p53 (Larner et al., 1994, Morgan et al., 1997). Instead, a new substrate p95/NBS1 is involved (Kim et al., 1999). Upon DSBs, activated ATM phosphorylates NBS1 on serine 343. The following signal transduction pathway has not yet been fully revealed. But as a result of this NBS1 phosphorylation, the Replicon – new DNA synthesis site will be inhibited and the cell will be stalled at S-phase (Painter and Young, 1980).

To summarize, upon detection of DNA double strand damage, nuclear ATM is activated and then initiates comprehensive programs of cell cycle arrest and DNA repair that helps ensure the integrity of the genome.

Novel functions of cytoplasmic ATM

ATM's involvement in DNA damage response makes certain symptoms of the disease easy to understand, e.g. cancer predisposition, hypersensitivity to ionizing radiation, immune deficiency and sterility. However, many other neurological symptoms such as ataxia, speech defects and abnormal body movements remain difficult to explain. Consistent with this, ATM mutations in mice interfere with DNA damage but have only

mild neurological symptoms (Barlow et al., 1996, Alterman et al., 2007, Katyal and McKinnon, 2007, Biton et al., 2008), indicating the link between DNA damage and the death of neurons and astrocytes can be broken (Herzog et al., 1998, Gosink et al., 1999, Lee et al., 2000).

Cytoplasmic ATM associates with cytoplasmic vesicles

Another interesting observation about ATM is its significant residence in the cytoplasm. ATM was found to co-fractionate (Lakin et al., 1996) and co-localize (Watters et al., 1997) with cytoplasmic vesicles in A-T cell lines. Parallel work from other groups found that cytoplasmic ATM was associated with peroxisomes and endosomes (Lim et al., 1998, Watters et al., 1999) and was involved in the functions of these organelles. The cytoplasmic pool of ATM does not appear to change in quantity or localization in response to IR (Brown et al., 1997, Watters et al., 1997), so it is unlikely that cytoplasmic ATM is recruited to the nucleus after DNA damage. This suggests the cytoplasmic ATM might be involved in cellular processes other than function in genome surveillance in nucleus.

Cytoplasmic ATM was also found to be bound with vesicle-associated protein β-adaptin both in vitro and in vivo (Lim et al., 1998). β-adaptin is a component of the AP-2 adaptor complex, which interacts with several proteins that are important in membrane trafficking and cell signaling and in endocytosis of clathrin-coated vesicles. Several ATM-related lipid kinases, in particular Vps34 and PI-3K, also play a critical role in stimulating vesicle and protein transport (De Camilli et al., 1996). The consequence of

the interaction between ATM and β-adaptin is still not quite unknown, but it is tempting to speculate that cytoplasmic ATM may act in vesicle transport.

Cytoplasmic ATM also involves in Insulin signaling pathway

Another interesting finding about cytoplasmic ATM function has come from the discovery of its involvement in insulin signaling pathways. In the first report demonstrating a role of ATM in insulin signaling, ATM kinase activity was found to increase 3-fold in response to insulin in rat 3T3-L1 cells that had differentiated into adipocytes (Yang and Kastan, 2000). In addition, insulin leads to phosphorylation of 4E-BP1 (also called PHAS-I), an insulin-responsive cytoplasmic protein, in an ATM-dependent manner. Phosphorylation of 4E-BP1 at Ser111 by ATM promotes initiation of mRNA translation (Yang and Kastan, 2000). More recently, it was discovered that ATM stimulates insulin-induced Akt phosphorylation at Ser473 and mediates the full activation of Akt activity (Viniegra et al., 2005, Halaby et al., 2008). Akt participates in multiple physiological processes, including protein translation, glucose uptake, cell proliferation and cell survival, in response to insulin and many other growth factors. Therefore, these findings open the door for exploring the unknown functions of ATM and could provide explanations for many of the clinical phenotypes of A–T that are difficult to explain by the nuclear localization and functions of ATM.

Neuronal cytoplasmic ATM is associated with synaptic vesicle proteins

Yet more exciting studies come from research of cytoplasmic ATM in neurons. It was previously reported that ATM is predominantly localized in the nucleus of dividing

cells, where in post-mitotic cells such as Purkinje and granular neurons in cerebella ATM had a significant distribution in the cytoplasm (Lakin et al., 1996, Brown et al., 1997, Oka and Takashima, 1998). A recent study has verified that speculation, cytoplasmic ATM was found specifically in neurons but not in other peripheral tissue (Li et al., 2009).

Furthermore, Li et al. identified two novel cytoplasmic ATM binding partners: Synapsin-I and VAMP2. With regard to neural function, these two stand out among hundreds of potential binding targets (Matsuoka et al., 2007). Both Synapsin-I and VAMP2 are well known synaptic vesicle proteins found in pre-synaptic nerve terminals. Synapsins are commonly known as linker proteins that tether synaptic vesicles (SV) to the cytoskeleton in presynaptic terminals, and by doing so stabilize a reserved pool of SVs near terminal plasma membrane and make them ready to be released (Greengard et al., 1994). Synaptobrevin (VAMP2) forms a central part of the SNARE (soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor) complex that mediates synaptic vesicle fusion with the cell membrane during neurotransmitter release (Schiavo et al., 1992). Both proteins regulate synaptic vesicle mobility at the synaptic terminal, indicating that cytoplasmic ATM might also play a role in that process. Indeed FM4-64 dye tracing experiment confirmed that speculation showing a much slowed down spontaneous dye release in ATM deficient cells.

The physiological function of Synapsin and VAMP2

To better understand how cytoplasmic ATM may regulate synaptic transmission, it is important to take a more thorough look at what Synapsin-I and VAMP2 do in neurons. Extensive electrophysiological studies have been performed to examine the role of Synapsin-I and VAMP2 in synaptic transmission. But before presenting the observation and conclusions drawn from electrophysiological experiments performed by other laboratories, it is important to discuss briefly the types of analyses that can be used, how they are interpreted, and what potential mechanism are involved.

One type of analysis examines miniature postsynaptic current, either inhibitory (mIPSCs) or excitatory (mEPSCs), induced by the spontaneous release of single vesicle of neurotransmitter called quanta. Quantal release is stochastic and the total amplitude of the resulting mEPSCs, for example, is an integer of the smallest unit quantal event. Experimentally, a change in the frequency of mPSCs would be reflective of a change in the probability that transmitter will be secreted whereas changes in the amplitude of mEPSCs are thought to result from a change in the sensitivity of the postsynaptic receptor to a given quantum.

A second type of analysis involves stimulus-evoked transmitter release that produces evoked inhibitory (eIPSCs) or excitatory (eEPSCs) postsynaptic currents. This type of response can be elicited by either electrical stimulation nearby a traveling axon or direct injection of current into a presynaptic cell to make the cell fire an action potential. When an action potential reaches the presynaptic terminal, the depolarization causes

voltage-gated Ca2+ channels to open and Ca2+ enters. Ca2+ enables Synaptic Vesicles (SVs) to fuse to the presynaptic membrane and then release neurotransmitter (NT) into the synaptic cleft to produce a postsynaptic current (ePSC). A change in the amplitude of an ePSC could have either a pre- or postsynaptic basis, therefore, a conclusion from this type of experiment requires further analysis of, for example, mPSCs. Conversely, a change in the decay rate of the ePSC would be indicative of a change in postsynaptic receptor sensitivity. A major assumption with these analyses is that the population of postsynaptic receptors remains constant. However, recent evidence suggests that the number of postsynaptic receptors may change in response to activity [For reviews see, (Luscher et al., 2000, Malinow et al., 2000)]. If the number of postsynaptic receptors changes then interpretation of changes in mPSC frequency or amplitude becomes much less straightforward.

A third commonly used method for studying regulation of synaptic transmission is analysis of Long Term Potentiation (LTP). LTP was first discovered by Bliss, Gardner-Medwin and Lomo in 1973 when they induced a long-lasting increase in synaptic efficacy with brief trains of rhythmic high-frequency electrical stimulation of the rabbit hippocampus (Bliss and Gardner-Medwin, 1973, Bliss and Lomo, 1973). The hippocampus is a deep brain structure which is thought to play a role in converting short-term memory into long-term memory in mammals. It is a highly organized structure in that different neuronal cell types are neatly packed into distinctive lamina. The hippocampus is large enough to be cut into 400-micron slices for electrical recording, allowing the circuits within this brain region to remain largely intact thus making hippocampus a good model for studying neurophysiology in vitro. However LTP is not

just confined to hippocampus, it is also reported from amygdala (Chapman et al., 1990, Clugnet and LeDoux, 1990), and neocortex (Racine et al., 1983, Artola et al., 1990, Hirsch and Crepel, 1990, Laroche et al., 1990).

There are many molecules both presynaptic and postsynaptic that are involved in the induction, expression and maintenance of LTP [For reviews see, (Bliss and Collingridge, 1993, Maren and Baudry, 1995)]. But one of the key components is believed to be the postsynaptic N-methyl-D-aspartate (NMDA) receptors. During high-frequency stimulation of excitatory afferents, strong postsynaptic depolarization coupled with presynaptic glutamate release results in the activation of NMDA receptors by releasing the voltage-dependent Mg2+ blockade of their ionic channels (Mayer et al., 1984, Nowak et al., 1984). NMDA activation results in Ca2+ influx into postsynaptic terminals, which triggers a series of enzymatic cascades that lead to a persistent modification of synaptic efficacy. LTP is considered to involve parallel changes in both presynaptic neurotransmitter release (Bekkers and Stevens, 1990, Bliss et al., 1990, Malgaroli and Tsien, 1992)and postsynaptic receptor composition (Ambros-Ingerson et al., 1991, Xiao et al., 1991, Maren et al., 1993). Depending upon the induction protocol, LTP can persist for many hours in vitro, and even days in vivo (Barnes, 1979, Staubli and Lynch, 1987).

Besides LTP, there is another category of synaptic plasticity that is more short-lived, thus termed short-term plasticity (STP). Paired-Pulse Facilitation (PPF) is one type of use-dependent short-term synaptic plasticity (Zucker, 1989). PPF refers to postsynaptic potentials (PSPs) evoked by an impulse are increased when that impulse closely follows a prior impulse. The mechanism underlying PPF is believed to be due to

Ca2+ accumulation in the presynaptic terminal during the paired pulse stimulation leading to enhanced mobilization and release of synaptic vesicles [residual Ca2+ hypothesis, (Katz and Miledi, 1968)]. Ca2+ plays a significant role in transmitting signals at chemical synapses. The amount of neurotransmitter released is correlated with the amount of Ca2+ influx. Therefore, PPF results from a build up of Ca2+ within the presynaptic terminal when action potentials propagate close together in time (Creager et al., 1980). PPF usually lasts for hundreds of milliseconds.

Similar to PPF, but when the number of stimuli is increased, each action potential enhances synaptic strength by 1-15%. Because each enhancement lasts for several seconds, the integrated effect of a train of hundreds of pulses can lead to a many-fold enhancement of synaptic strength (Zucker and Regehr, 2002). This enhancement is termed Post-Tetanic Potentiation (PTP) and usually lasts for 30s to several minutes (Abbott and Regehr, 2004).

Short-term plasticity can also be expressed as Synaptic Fatigue (SF), or short-term synaptic depression. It refers to sustained presynaptic activity (e.g. a train of high frequency stimulation) resulting in a progressive decline in postsynaptic potential amplitude (Zucker, 1989, Zucker and Regehr, 2002). The underlying cause of fatigue on the synapse is believed to be the temporary depletion of synaptic vesicles in the presynaptic terminals. Thus it is generally considered to be primarily a presynaptic phenomenon (Zhang et al., 2005, Simons-Weidenmaier et al., 2006) although this does not always have to be the case.

The physiological function of VAMP2

The fusion of synaptic vesicles (SVs) with the pre-synaptic plasma membrane underlies synaptic transmission at chemical synapses. This fusion event, termed exocytosis, occurs spontaneously at a low and asynchronous rate, whereas an increase in presynaptic Ca2+ mediates a larger and synchronous fusion of SVs with the plasma membrane (Neher and Sakaba, 2008). Central to SV exocytosis is the formation of a protein complex between SNARE proteins present on the plasma membrane and SV membrane (Sollner et al., 1993a, Sollner et al., 1993b). In neurons, Vesicle-Associated Membrane Protein2 (VAMP2; also known as Synaptobrevin 2) functions as the vesicle (or v) SNARE, whereas the plasma membrane proteins SNAP25 and Syntaxin1 act as target membrane (or t) SNAREs. These three SNARE proteins interact to form a trans-SNARE complex which is thought to represent the minimal membrane fusion machinery (Weber et al., 1998). Genetically mutated VAMP2 -/- mice pups are not viable upon birth probably due to an inability to breathe. VAMP2 +/- mice are viable, and express ~50% of the VAMP2 level compared to wild type controls. Moreover, VAMP2 +/- mice also showed up to a 50% reduction in the peak amplitude of both resting and evoked glutamate release in CA1, CA3 and DG of the hippocampus (Matveeva et al., 2012).

High-density primary cultures of hippocampal neurons from both VAMP2 -/- and wild type mice embryos were used in an electrophysiology study to determine VAMP2's modulation on synaptic transmission (Schoch et al., 2001). Even though immunocytochemistry revealed both mutant and control neurons formed a similar dense meshwork of synapses, spontaneous synaptic vesicle fusion and fusion induced by

hypertonic sucrose were decreased ~10-fold in VAMP2-/- neurons. Moreover, fast Ca2+ triggered fusion was decreased more than 100-fold. Thus VAMP2 is crucial in catalyzing SVs fusion reactions and maintaining a proper rate of fusion activities during both spontaneous and evoked synaptic events.

The physiological function of Synapsins

One of the unique characteristics of neuronal nerve terminals is that they can sustain vesicular release at a high rate. Although the detailed mechanisms that account for the distinctive features of neurotransmitter release still not known, it is suggested that synapsins – a group of presynaptic neuron-specific proteins, are involved. Synapsins form one of the most abundant family of phosphoproteins in the brain and it is estimated to account for approximate 9% of the total synaptic vesicle protein (Huttner et al., 1983, De Camilli et al., 1990). Three synapsin genes have been identified in mammals (Sudhof et al., 1989, Kao et al., 2002). In addition, each gene is alternatively spliced to produce related isoforms. The various isoforms possess different combinations of the phosphorylation sites that regulate their binding to SVs and cytoskeletal elements (Greengard et al., 1993, Hilfiker et al., 1999). And they also differ in their ability to bind Ca2+ and ATP (Hosaka and Sudhof, 1998).

Synapsins are commonly expressed in both the central and peripheral nervous system. They localize at nerve terminals, in which they interact with each other, as well as with SVs and Actin. In the classical view, synapsins have been proposed to maintain a reserve pool of vesicles by tethering SVs to each other and to actin. By doing so, it stabilizes the SV membrane and causes SVs to cluster, thus keeping a reserve pool of

SVs away from plasma membrane, while preventing them from the possibility of diffusion and random fusion. Upon repetitive synaptic activity, synapsins control the availability of SVs for release through their phosphorylation-dependent dissociation from SVs and actin. Thus the major function of synapsins has been proposed to be a form of homeostatic regulation of synaptic transmission [For reviews see: (Cesca et al., 2010)].

To better understand the function of synapsins, genetically engineered knockout mice lacking either synapsin I, synapsin II, or both, were subjected to biochemical and electrophysiological studies (Rosahl et al., 1993, Rosahl et al., 1995). All single- and double-knockout mice were viable and fertile and had an apparently normal life expectancy. The mutant mice did not exhibit any obvious behavioral abnormalities, and experienced no major morbidity except mild seizures. The seizures developed only after two months of age. Histological examination showed that brains from mice lacking synapsins had a normal size, form and structure. In cultured hippocampal neurons, neurite outgrowth appeared to be identical between mutant and wild-type neurons. The structures and distributions of synapses were similar between wild-type and double-knockout brains except for a dramatically reduced amount of synaptic vesicles in mutant synapses (~50%).

Electrophysiological studies revealed that brains slices from all single- and double-knockout mice exhibited normal LTP. However, the mutants showed quite different phenotypes in short-term plasticity (Spillane et al., 1995, Silva et al., 1996). Synapsin-I knockout mice showed normal post-tetanic potential (PTP) but increased paired-pulse facilitation (PPF). Interestingly, in synapsin-II knockouts, PPF was normal but the magnitude of PTP was decreased. PTP was even more severely depressed in the

double knockouts, but PPF remained normal in them. When subject to repetitive stimulation tests, wild-type and synapsin-I deficient synapses both showed an initial enhancement of neurotransmitter release followed by a continuous decline. In contrast, synapses lacking synapsin II or both synapsins exhibited a rapid and pronounced activity-dependent depression. More interestingly, when the extracellular Ca2+ concentration was lowered to decrease the probability of transmitter release, wild-type synapses exhibited no significant depression, but synapses lacking both synapsins showed an almost immediate decrease in transmitter release. This suggested that the inability of mutant synapses to adjust to higher release rates occurs after only a few stimuli and results from increased sensitivity susceptibility to surrounding Ca2+ concentration.

Taken together, synapsin knockout studies have shown that synapsins are not required for neurite outgrowth, synaptogenesis or the basic mechanics of synaptic vesicle release, but are essential for acceleration of synaptic vesicle traffic during repetitive stimulation. And failure in synapsin function could result in the defective short-term plasticity of synaptic transmission.

Cytoplasmic ATM may play a role in regulation of synaptic transmission in neurons

As shown by various knockout studies, Synapsins and VAMP2 are both very important presynaptic proteins that regulate docking and release of synaptic vesicles. Consequently, the disruption of either molecule could result in a multi-level changes in synaptic transmission. As indicated by our recent study (Li et al., 2009), ATM in

neuronal cytoplasm is tightly associated with both Synapsin-I and VAMP2, thus prompting us to hypothesize that cytoplasmic ATM inside neurons might also be involved in the regulation of synaptic transmission. The neurological dysfunction seen in A-T patients and the neurodegeneration displayed by A-T patients, could result from this defective synaptic transmission regulation as well as abnormal axonal or synaptic transport.

Compared to the extensive studies of nuclear ATM and its function in DNA damage responses, cytoplasmic ATM has received much less attention (Yang et al., 2011). Although many functions of ATM haven be deduced from its amino acid sequence, corresponding laboratory studies have not yet identified functions for >90% of the remaining sequence. This indicates that ATM could have many, as yet unknown, additional functions (Shiloh and Kastan, 2001). So to better elucidate the functions of ATM, in particular the fraction residing in the cytoplasm, more thorough biochemical and electrophysiological studies are needed.

Currently, the molecular basis of A-T disease is still poorly understood. A more in-depth understanding of the cellular functions of ATM, including its role in the cytoplasm, could greatly assist the understanding of the etiology of the disease and help the development of effective approaches to treat this devastating disorder. Known pharmaceutical agents that specifically target ATM, such as chloroquine and KU55933, can be used in proof-of-concept studies and the further exploration of their potential for treating A-T. Furthermore the knowledge gained from ATM and its relationship with multi-facet symptoms of A-T disease could help us get a better understanding of how the

system coordinates several important cellular pathways under stress, thus shedding light on cancer and many other related diseases.

METHODS

Animals

Our colony of mutant mice was maintained in the animal facilities at Rutgers University. Atm^{tm1Awb} mice were procured originally from The Jackson Laboratory; Atm^{tm1Bal} mice were a gift from Dr. Yang Xu. Generation of mutants was achieved through the mating of heterozygous Atm^{tm1Awb} males and Atm^{tm1Awb} females as well as the mating of heterozygous Atm^{tm1Bal} males and Atm^{tm1Bal} females. Timed pregnancies were established from these matings as needed; the date of appearance of a vaginal plug was considered embryonic day 0.5. Embryos were taken at embryonic day 16.5 (E16.5) for either primary cultures or histology. For LTP, 4-6 week old wild-type or homozygous mutant mice were chosen. All animal procedures were carried out in accordance with Rutgers University IACUC standards. The animal facilities at Rutgers University are fully AAALAC accredited.

Antibodies and chemical regents

Antibodies against ATM [2C(1A1) and 5C2], phospho-Ser1981 ATM, VAMP2, synapsin-I, syntaxin-1, PSD95 and ATR were from Abcam (Cambridge Science Park, UK), GFP antibody was purchased from Santa Cruz (San Diego). Hsp90 and HDAC-1 were purchased from Biovision (Mountain View, CA). Flag-tag antibody was from Sigma-Aldrich (St. Louis, MO) and phospho-(Ser/Thr)-Gln ATM/ATR substrate antibody was from Cell Signalling (Boston, MA). An antibody specific for VAMP2

phosphorylated on T35 was made by ProSci, Incorporated. Secondary antibodies used for immunocytochemistry were all procured from Invitrogen (Eugene, OR). All were fluorescent tagged as follows: Chicken anti-mouse Alexa 488 and Donkey anti-mouse Alexa 594; chicken anti-rabbit Alexa 488 and Donkey anti-rabbit Alexa 594. ATM kinase inhibitor KU-55933 was purchase from Calbiochem (VWR International AB, Stockholm, Sweden).

Cell culture and differentiation

N2a cells were cultured in DMEM medium supplemented with 10% FBS. To differentiate N2a cells, 24 hours after transfection, culture medium was replaced by DMEM with 1mM dbcAMP and 0.25% FBS. Cells were kept in this differentiation medium for 3-7 days before use.

Primary neuronal cultures

Embryonic cortical neurons were isolated by standard procedures. For ATM-deficient cultures, all embryos from a heterozygous mutant intercross were harvested and treated separately. Isolated E16.5 embryonic cerebral cortices were treated with 0.25% trypsin-EDTA and dissociated into single cells by gentle trituration. Cells were suspended in Neurobasal medium supplemented with B27 and 2 mM glutamine, then plated either on coverslips or dishes coated with poly-L-Lysine (0.05 mg/ml) and laminin (5 μg/ml). All cultures were grown for a minimum of 5 days in vitro (DIV) before any experiment.

Preparation of hippocampal brain slices

Mice of either sex were decapitated under isoflurane anesthesia, and the brains removed. Hippocampal slices (400 μm) were prepared using a vibrating slicer (Campden Instruments) and cut in a solution containing (in mM): sucrose (228), glucose (10), NaHCO3 (24), CaCl2 (2), MgSO4 (2), KCl (3), NaH2PO4 (1.25) aerated continuously (95%/5% O2/CO2) at 4 °C. After slicing, brain tissue was transferred to an aerated (95%/5% O2/CO2) holding chamber containing artificial cerebro-spinal fluid (ACSF) solution (in mM): NaCl (128), KCl (3.45), CaCl2 (2.5), MgCl2 (1.3), NaH2PO4 (1.25), NaHCO3 (24), glucose (10), equilibrated with 95%/5% O2/CO2 at room temperature. After at least 1 hour of recovery, slices were transferred as needed to an interface recording chamber where they were maintained at 30 °C and perfused with ACSF at 2ml/min. Each slice was allowed to equilibrate to the new temperature for 20-30 min before any recordings were made.

FM4-64-tracking vesicles recycling

Dissociated cortical neurons cultured in vitro for 14 days or differentiated transfected N2a cells were incubated with 10 μM FM4-64 (Molecular Probes). Dye uptake was induced by exposure to a Tyrode's solution containing 47 mM KCl and 2mM CaCl2 for 90 s at room temperature (Kavalali et al., 1999, Sara et al., 2005). Fluorescent images of FM 4-64 labeled vesicles were captured after 15 min of perfusion with dye-free normal Tyrode's solution. Fluorescent signals were imaged using a Leica 40x water immersion objective using Leica's image tool suite (IP lab 4.0). For live imaging, one frame was captured every 10 seconds over a total analysis period of one hour. To analyze FM4-64 uptake and release, the mean intensity of the fluorescence at a specific region of interest, e.g., a synapse was calculated. A small adjacent region was then selected to

determine the background. The mean intensity of this region was subtracted from the synapse intensity to correct for variability in background FM4-64 levels.

Constructs and plasmids

The GFP-ATM plasmid was kindly provided by Dr. Kum Kum Khanna; the Flag-ATM WT was from Dr.Michael Kastan; the Flag-ATR was provided by Aziz Sancar; Flag-VAMP2 was gift from Dr. Mitsunori Fukuda and VAMP2-GFP was provided by Dr. Bonnie L. Firestein. GFP-ATR was subcloned by inserting GFP into ATR-wt-pcDNA4TO construct (from Dr.Paul Nghiem). Myc-tagged synapsin I and m-Cherry-synapsin I were cloned from mouse brain mRNA (BC022954) by SuperScript III one-step RT-PCR system. The Myc-tag vector and pmCherry-C1 vector were bought from Clontech Laboratories (Mountain View, CA). Site-direct mutation of Flag-VAMP2 or VAMP2-GFP on T35A, S75A and T35AS75A as well as Myc- or mCherry-synapsin I on S656A were performed by QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA).

Immunocytochemistry

Cultured cells were rinsed once with PBS and then fixed in buffered 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at room temperature followed by three rinses with PBS. Primary antibody concentrations used for cell culture were: 1:500 for mouse anti-ATM, rabbit anti-ATR and rabbit anti-1981S, 1:1000 for rabbit anti-VAMP2, rabbit anti-Synapsin I, rabbit anti-ser15(p53) and rabbit anti-T68(Chk2). All secondary antibodies were used at 1:1000. Cells were counterstained with 1 μg/ml DAPI.

For DAB/brightfield staining, 10μm mouse brain frozen sections were soaked in 0.3% hydrogen peroxide in methanol for 20 min to remove endogenous peroxidase

activity, rinsed in Tris-buffered saline (TBS), and pre-treated in a solution of 0.1 M citrate buffer heated to 90-95°C for 10 min. Sections were cooled and rinsed in TBS. Slides were incubated in a blocking solution consisting of 0.1% blocking reagent (Boehringer Mannheim, Mannheim, Germany) and 10% goat serum in PBS at room temperature for 1 hr. After overnight incubation with the primary antibody (4°C), the sections were washed three times in TBS before applying the secondary antibody. The secondary antibody was left on the section for 1 hr at room temperature; afterward, sections were rinsed in TBS. Rinsed sections were then incubated in Vectastain ABC Elite reagent for 1 hr, followed by three successive washes. The sections were then incubated in diaminobenzidine (DAB) as a substrate for visualization of the chromagen, according to the manufacturer's specification (Vector Peroxidase Substrate DAB kit; Vector Laboratories, Burlingame, CA). Antibody concentrations used for immunohistochemistry were mouse anti-ATM 1:500, Rabbit anti-ATR 1:500.

Field potential recording

Brain slices were placed in a temperature controlled recording chamber kept at 30 °C. Extracellular recordings of field EPSPs (fEPSPs) were made with ACSF-filled glass electrodes (5-10 μm tip diameter). Test stimuli (0.1 ms) were delivered with a bipolar platinum/iridium stimulating electrode at 1 min intervals except for specialized protocols that elicited changes in synaptic strength (see below). For recordings of CA1 activation by Schaffer collateral stimulation, recording and stimulating electrodes were both placed in stratum radiatum. Each experiment was begun by obtaining input-output relationships to establish the strength of baseline synaptic transmission. A Grass S8800 stimulator connected to a Grass PSIU6 photoelectric stimulus isolation unit was used to deliver a

series of increasing intensity constant current pulses. Current magnitude was adjusted to elicit responses ranging from just-suprathreshold to near maximal. Following this, stimulus intensity is adjusted to evoke fEPSPs 30-40% of maximum, typically 30-40 μA. To elicit LTP, theta burst stimulation (TBS) was used. A single TBS consists of 12 bursts of 4 100 Hz pulses spaced 200 ms apart. Response magnitude was quantified using the slope of the field potential. Field potential traces during the TBS were saved and the peak amplitude of EPSP versus stimulus number was used to assess synaptic fatigue. The response to paired-pulse stimulation at different interpulse intervals (25, 50, 100, 150, 200 and 300 msec) was used to measure PPF.

Data analysis

For LTP experiments, data were analyzed by measuring the slope of the field potential during the baseline period and the after TBS stimulation. For the PPF study, the slope of the field potential from the two paired stimuli were measured and the ratio (post/pre) was calculated and then plotted in accordance to the interpulse intervals. For the synaptic fatigue study, the peak amplitude of the field potentials from the stimulation response traces in the Theta Burst Stimulation were measured, normalized to the first response, and then plotted. Recordings that did not exhibit a stable baseline or in which the field potential decreased to below baseline level after the 3-hr LTP testing period were not used. Statistical comparisons were made using Student's t-test with $p < 0.05$ taken to indicate a significant difference.

Hindpaw footprint

The back paws of each mouse were dipped into black ink, and then the mouse was

placed at the entry of a dark tunnel (9.2cm * 6.3cm * 35.5cm). The footprints were recorded on a clean sheet of white paper placed in the floor of the tunnel. Stride lengths were determined by measuring the distance between each step on the same side of the body (e.g., the distance between one right footprint and the next right footprint). Average stride length was calculated. The distance of the shortest stride was subtracted from the distance of the longest stride to determine the maximum difference in stride length for each subject.

RESULTS

Chapter 1. Cytoplasmic ATM binds to synaptic vesicle proteins and modulates synaptic vesicle mobility

Preface to the results section.

The work described in this section was done by our main collaborator Dr. Jiali Li from Dr. Karl Herrup's lab. These data are included to show the unique cytoplasmic existence of ATM in the central nerve cells, and its modulation of synaptic vesicle cycling. In our published work (Li et al., 2009) it was clearly showed that in neurons ATM has a unique distribution in both the nucleus and the cytoplasm whereas in other organ or tissue ATM only resides inside nucleus. When western blot analysis was performed with subcellular fractionations of tissue extracts from mouse brain, spleen and thymus (Figure 1A), ATM was detectable in cytoplasm only in brain (lane 1) but not spleen or thymus (lanes 2 & 3), where ATM clearly showed up in the nucleus in all three tissues (lanes 4-6). This result was confirmed by histological staining of the brain slices from cerebellum and cerebral cortex (Figure 1B). This tissue specificity was also preserved in cell cultures (Figure 1C). In both cultured neurons and N2a cells (neuron-like cells), ATM was detectable in the cytoplasm, but not in NIH3T3 or HeLa cells (non-neuronal cells). This distribution pattern was also verified by either immunostaining of native ATM or the transfection of GFP-ATM in all three cell cultures: neurons, N2a and NIH3T3 (Figure 1D).

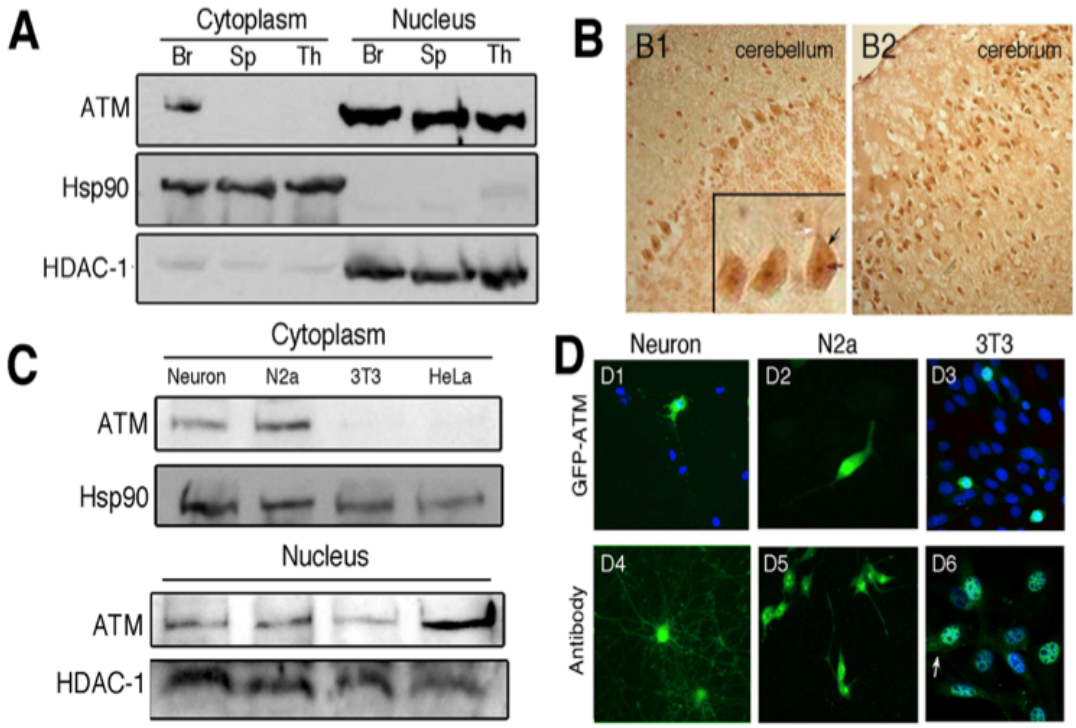

Figure 1. ATM is present in both nucleus and cytoplasm in the CNS. A) Cytoplasmic and nuclear protein extracts from adult mouse brain (Br), spleen (Sp) and thymus (Th) were blotted with ATM antibody. Hsp90 and HDAC-1 were used as cytoplasmic and nuclear markers respectively. B) ATM immunostaining was performed on 10 μm cryostat sections from adult mouse cerebellum (B1) and cortex (B2). The inset illustrates the presence of immunoreactivity in Purkinje cell cytoplasm (black arrow) as well as nucleus (brown arrow) with weak staining in the primary dendrite (white arrow). C) Cytoplasmic extracts from cultured neurons and N2a cells contain ATM; NIH 3T3 and HeLa cell cytoplasm was immunonegative. D) ATM immunostaining (D4 – D6) and GFP-ATM deployment (D1 – D3) is found in the cytoplasm of cultured neurons (D1 and D4) and N2a cells (D2 and D5) but not of 3T3 cells (D3 and D6).
<Figure adapted from (Li et al., 2009)>

ATM binds to Synapsin-I and VAMP2 in cytoplasm

The subsequent set of experiments was designed to determine the potential interacting molecules of cytoplasmic ATM. Co-immunoprecipition revealed two novel cytoplasmic ATM binding partners: Synapsin-I and VAMP2. ATM partially co-localized with VAMP2 in cultured neurons in the immunocytochemical staining experiments (Figure 2A). This association was confirmed by immunoprecipitation with antibody to either ATM or VAMP2 from protein extracts of mouse brains (Figure 2B). We next asked if this interaction of ATM and VAMP2 depends on ATM kinase activity. To address this question, VAMP2 was first immunoprecipitated from the cytoplasmic fraction of mouse brain and then examined the precipitates on Western blots with an antibody against [S/T]Q phosphorylated sites – the canonical ATM target sequence. A strong band of staining was observed (Figure 2C). There are two potential ATM phosphorylation sites in VAMP2: T35 and S75. The issue of whether either one or both of these sites are the crucial targets of phosphorylation was tested by constructing non-phosphorylatable alanine substitution mutants at these two sites. The S75 mutation did not alter the [S/T]Q phosphorylation signal as compared to the wild-type. However either T35 mutation or the T35/S75 double-mutation lost all their phospho-[S/T]Q signals and their ability to immunoprecipitate ATM (Figure 2D). Synapsin-I was also found to interact with ATM in protein immunoprecipitation from mouse brain (Figure 2E). It was then tested if Synapsin-I is a phosphorylation target of ATM. A strong band of [S/T]Q phosphorylation signal was observed in protein immunoprecipitations from the wild type (+/+) but not Atm^{tm1Awb} (−/−) mouse brain extract (Figure 2F).

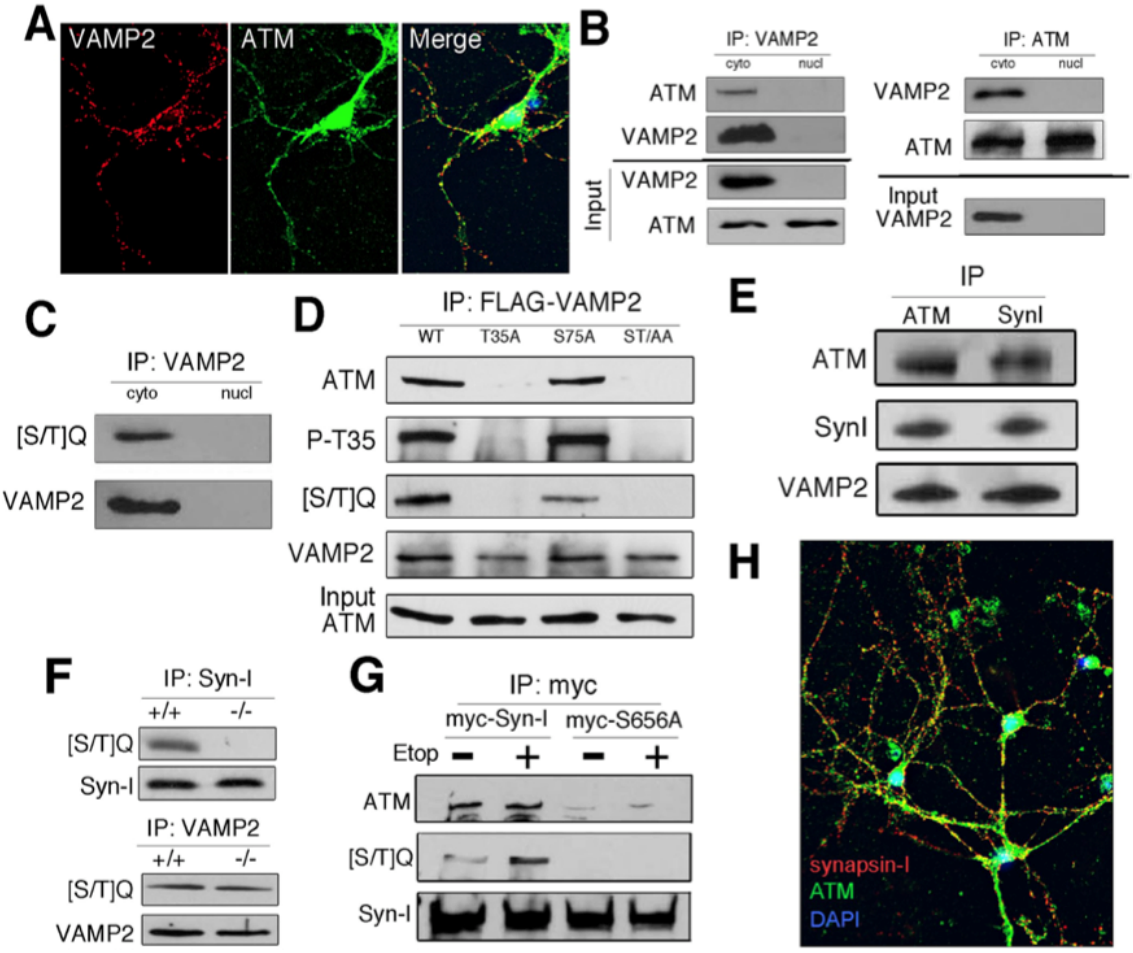

Figure 2. VAMP2 and Synapsin I are novel binding partners. A) VAMP2 and cytoplasmic ATM co-localization. Cultured cortical neurons were stained with antibodies against ATM (green) or VAMP2 (red). B) VAMP2 physically interacts with cytoplasmic ATM in mouse brain. Immunoprecipitations of cytoplasmic (cyto) and nuclear (nucl) extracts from 4–6 weeks old mouse brain were probed with the antibodies indicated. C) Neuronal VAMP2 immunoprecipitation is labeled with phospho-[S/T]Q antibody. D) VAMP2 Thr35 is phosphorylated in N2a cells, which is necessary for binding to ATM. The T35A and T35A/S75A alanine-substitution mutants lose virtually all of their phospho-T35 (P-T35) signal (2nd row), their phospho-[S/T]Q signal (3rd row) and their ability to immunoprecipitate ATM (1st row). E) Synapsin-I and ATM are physically associated. Synaptosomal protein extracts from adult mouse brain were immunoprecipitated and blotted with ATM synapsin-I or VAMP2 antibodies. F) Synapsin-I, but not VAMP2, is a enzymatic target of ATM. Cytoplasmic protein extracts from wild type (+/+) or Atm^{tm1Awb} (−/−) mouse brain were immunoprecipitated with either synapsin-I (top) or VAMP2 (bottom) and blotted with [S/T]Q antibody. G) S656 is critical for ATM binding. Myc-tagged wild type and S656A synapsin-I were overexpressed in N2a cells, immunoprecipitated with anti-myc antibody and blotted with anti-ATM or anti-[S/T]Q. H) ATM and synapsin-I have unique but overlapping distributions in primary neuronal cultures.
<Figure adapted from (Li et al., 2009)>

Serine 656 of synapsin-I is a strong theoretical candidate for an ATM phosphorylation target. A S656 non-phosphorylatable alanine substitution mutation lost the phospho-[S/T]Q signal and its ability to immunoprecipitate ATM (Figure 2G). Synapsin-I and ATM partially co-localize in cultured cortical neurons where they are found in a punctate pattern along the neurites (Figure 2H).

Cytoplasmic ATM modulates spontaneous synaptic vesicle release

Synapsin-I and VAMP2 are well known synaptic vesicle proteins found in pre-synaptic nerve terminals. Both proteins are important modulators of synaptic transmission, making us postulate that cytoplasmic ATM might also be involved in the modulation. To test this hypothesis, genetically engineered ATM mutant mice (Atm^{tm1Awb}) were acquired from the Jackson Laboratory. Atm^{tm1Awb} mice were first generated in Dr. Wynshaw-Boris's lab in 1996. The ATM gene was disrupted by using an insertion vector containing the *neo* resistance gene to produce a truncation mutation at approximately the site corresponding to that commonly mutated in ATM in many human beings with A-T (Barlow et al., 1996). FM4-64 dye was used as a tracer to monitor synaptic vesicle cycling from cultured neurons derived from either wild type or homozygous Atm^{tm1Awb} mutant mice (Figure 3A). There was no significant difference in FM4-64 dye uptake between Awb/Awb and wild type neurons (data not shown). By contrast, spontaneous dye release was significantly slower in the Awb/Awb cultures than in wild type (60% of the FM4-64 dye was lost after 25 min from wild-type neurons, Atm^{tm1Awb} neurons lose only 30% of their dye in the same period of time ($n = 6$, $p < 0.01$)). A similar slower releasing pattern was also observed from ATM siRNA treated

wild type neurons (Fig. 3A). Thus in the absence of ATM, spontaneous vesicle release was much reduced in culture.

In the previous set of experiments it was observed that the T35 site but not S75 site on VAMP2 was significantly phosphorylated in normal situation, and the blocking of VAMP2 T35 phosphorylation resulted in the dissociation of ATM from VAMP2 (Fig. 2D). It was then determined if this separation of ATM from VAMP2 could also alter synaptic vesicle cycling. Non-phosphorylatable alanine substitutions at either the T35 site or the S75 site or both we constructed, and they were expressed as Flag-tagged derivatives in N2a cells, which were then differentiated with dibutyryl1-cAMP. FM4-64 dye was then used to monitor synaptic vesicle cycling in wild type cells or point-substitution mutant cells. S75A-VAMP2-GFP mutant and wild type cells showed similar vesicle cycling kinetics (59% destained after 30 min) as observed in wild type neuronal cultures (Figure 3B). In contrast, T35A-VAMP2-GFP mutant or double substitution mutant cells showed much slower vesicle cycling rate (25% destained, N=3, $p < 0.05$) which resembled the finding in ATM -/- mice neuron cultures (Figure 3B). Taken together, these experiments indicate that cytoplasmic ATM can modulate synaptic vesicle recycling. And the mechanism of this regulation is probably through the interaction with VAMP2 by phosphorylation on its T35 site.

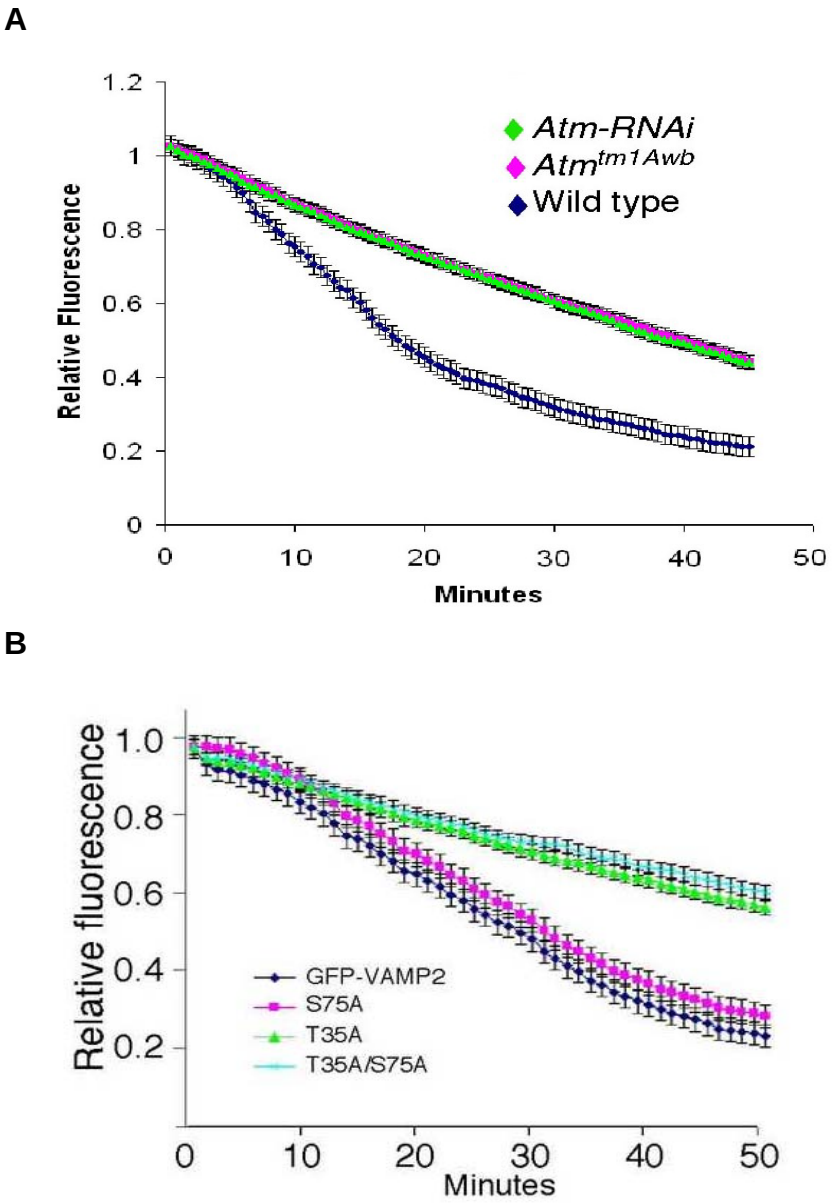

Figure 3. Synaptic Vesicle Recycling Are Defective. (A) FM4-64 dye release kinetics is less rapid in Atm-deficient cells. In cultures that lack ATM (small interfering RNA [siRNA], green; Atm^{tm1Awb}, red), spontaneous release kinetics are slowed compared to wild-type (blue). Whereas 60% of the FM4-64 dye is lost after 25 min from wild-type cells, siRNA-treated or Atm^{tm1Awb} neurons lose only 30% of their dye in the same period of time ($n = 6$, $p < 0.01$). (B) VAMP2 Phosphorylation mutant delayed FM dye release. Differentiated N2a loaded with FM4-64 dye destain with different kinetics when they are overexpressing wild type (GFP-VAMP2) single or double S/T->A substitution mutants. T35A- and T35AS75A-VAMP2-GFP kinetics are slowed (25% destained after 30 min) compared to WT or S75A-VAMP2-GFP (59% destained, N=3, $p< 0.05$). Graphs represent averages of three independent experiments. Error bars represent standard deviation.
<Figure adapted from (Li et al., 2009)

Chapter 2. Cytoplasmic ATM regulates synaptic transmission in Atm^{tm1Awb} knockout mice

The previous chapter showed that the cytoplasmic ATM binds to two important synaptic vesicle proteins: Synapsin-I and VAMP2. When this association was interrupted either by eliminating ATM protein or by a point mutation on VAMP2 to block its phosphorylation, thus making VAMP2 unable to bind ATM, spontaneous unstimulated synaptic vesicle release was significantly reduced. Proper synaptic vesicle release is a critical step during synaptic transmission. These alterations of activity suggested that synaptic transmission might also be compromised if cytoplasmic ATM proteins were defective.

To investigate this hypothesis i chose to examine Theta-Burst induced Long-Term Potentiation (TBS-LTP) in the hippocampal Schaffer Collateral pathway (CA3->CA1) to study the synaptic transmission efficacy and plasticity. Using hippocampal TBS-LTP as our experimental model has three advantages: 1) the TBS-LTP in the Schaffer Collateral pathway is a well studied and documented experimental model and widely used to examine the synaptic transmission and plasticity; 2) no neurodegeneration in the hippocampus was reported in ATM deficient mice or A-T patients, thus any decrease of LTP observed in the hippocampus will not be confounded by neuron loss; 3) TBS-LTP is performed in acutely prepared hippocampal brain slices. Though it is still an in-vitro system, the results obtained should relate to findings in-vivo. TBS-LTP recordings were made by placing both stimulating and recording electrodes at the CA1 stratum radiatum of a hippocampal

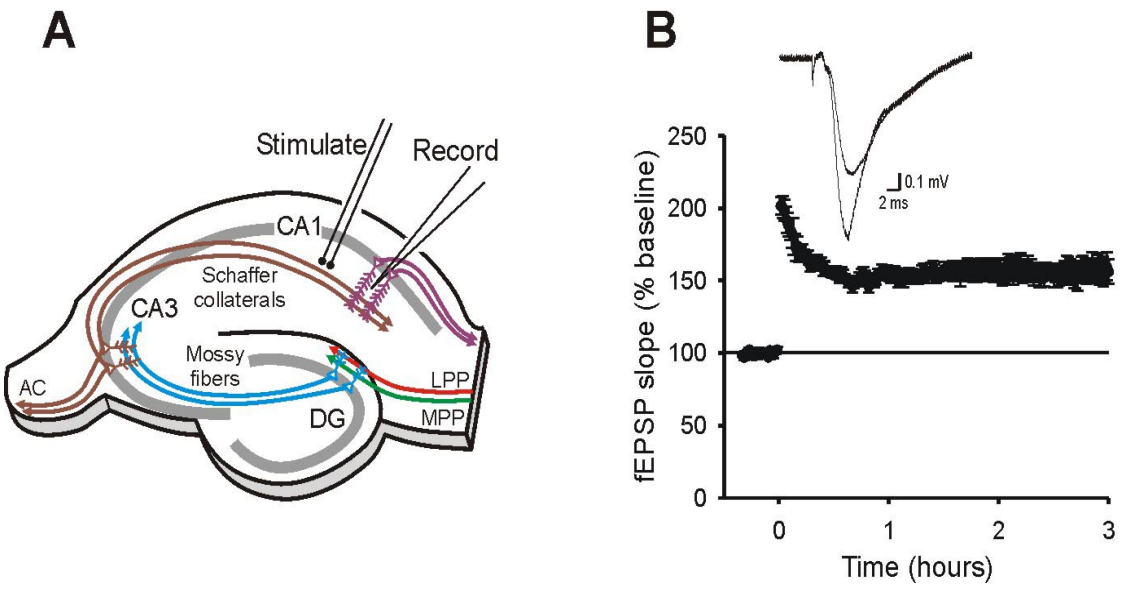

Figure 4. Extracellular recordings of field EPSPs (fEPSPs) are made with ACSF-filled glass electrodes (5–10 µm tip diameter). (A) For recordings of CA1 activation by Schaffer collateral stimulation, recording and stimulating electrodes are both placed in stratum radiatum. Test stimuli (0.1 ms) are delivered with a bipolar platinum/iridium stimulating electrode at 1 min intervals except for specialized protocols that elicit changes in synaptic strength. Each experiment is begun by obtaining input-output relationships to establish the strength of baseline synaptic transmission. A Grass S8800 stimulator connected to a Grass PSIU6 photoelectric stimulus isolation unit is used to deliver a series of increasing intensity constant current pulses. Current magnitude is adjusted to elicit responses ranging from just-suprathreshold to near maximal. Following this, stimulus intensity is adjusted to evoke fEPSPs 30–40% of maximum, typically 30–40 µA. To elicit LTP, theta burst stimulation (TBS) is used. A single TBS consists of 12 bursts of 4 100 Hz pulses spaced 200 ms apart. Response magnitude is quantified using the slope of the field potential. (B) A sample time course of LTP elicited by theta burst stimulation (TBS) showing the long lasting increase in the field excitatory postsynaptic potential (fEPSP) slope. Inset: sample fEPSPs illustrating the pre- and post- responses to TBS stimulation.

slice, and then recording the ensemble CA1 neuronal responses (field potential) elicited by different protocols of stimuli (Fig. 4).

Cytoplasmic ATM deficiency reduced TBS-LTP

Acutely prepared hippocampal slices from either wild type or homozygous Atm^{tm1Awb} mutant mice were subjected to TBS-LTP tests. Consistent with our expectations, the LTP observed from the mutant slices during the 3-hour period was dramatically reduced as compared to their wild type littermates (WT = 156% ± 8.1% measured at 175–180 min after TBS stimulation, n = 12, Mutant = 113% ± 5%, n = 6; $p < 0.01$, Fig. 5B). Baseline synaptic transmission, on the other hand, did not differ between the wild type and mutant animals as indicated by the similar input-output curves (Fig. 5A). This result demonstrates that cytoplasmic ATM does have a role in regulation of synaptic transmission plasticity. However the mechanism of this modulation is yet to be known.

LTP expression and maintenance involve both pre- and post-synaptic mechanisms (Bliss and Collingridge, 1993, Maren and Baudry, 1995). At this stage we don't know the detailed mechanism how ATM affects the LTP. However, based on the fact that ATM associates with Synapsin-I and VAMP2, which both are presynaptic proteins, we highly suspect this impairment occurs at the presynaptic terminals. And the lack of change in the baseline level of synaptic transmission indicates that the basic synaptic vesicle releasing function is still operating normally in the Atm^{tm1Awb} mutant mice.

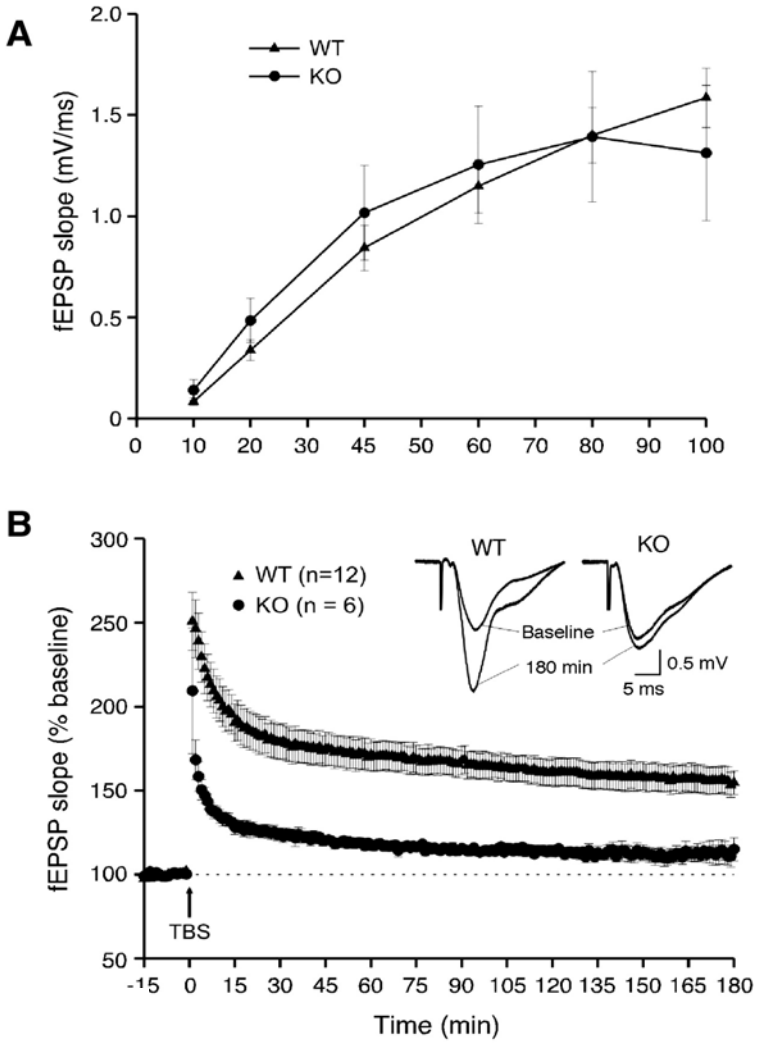

Figure 5. Long-Term Potentiation is defective in Atm-deficient brains (A) Input-output relations showing that baseline transmission was not significantly different between wild-type and Atm^{tm1Awb} mice. (B) Schaffer collateral long-term potentiation (LTP) is dramatically reduced in Atm^{tm1Awb} mice. The time course of LTP elicited by theta burst stimulation (TBS) shows the expected increase in the field excitatory postsynaptic potential (fEPSP) slope (156% ± 8.1% measured at 175–180 min after TBS stimulation, n = 12) in wild-type slices, but not in slices from Atm^{tm1Awb} mice (113% ± 5%, n = 6; $p < 0.01$). Inset: sample fEPSPs from wild-type (WT) and Atm^{tm1Awb} (KO) recordings illustrating the different responses to TBS stimulation.
<Figure adapted from (Li et al., 2009)>

Blocking Cytoplasmic ATM kinase also reduced TBS-LTP

In the biochemistry experiments, it was shown that the T35 point mutation on VAMP2 had three significant consequences. First, T35A but not S75A mutant blocked the VAMP2 phosphorylation, suggesting T35 is the primary phosphorylation site for VAMP2 (Fig. 2D). Second, immunoprecipitation with ATM antibody reveals that ATM does not bind to the T35A-VAMP2 mutant, suggesting that ATM binds primarily to the phosphorylated form of VAMP2. And third, over-expression of the VAMP2 T35A mutant in N2a cells was associated with a slowdown in the rate FM4-64 dye release from differentiated N2a cells, which resembled the dye release deficiency observed in Atm^{tm1Awb} mutant mice neuron cultures (Fig. 3A&B).

Taken together, it suggests that the association between cytoplasmic ATM with phosphorylated Synapsin-I and VAMP2 is crucial to ensure normal regulation of synaptic transmission, and the phosphorylation of Synapsin-I and VAMP2 by ATM's kinase activity might play a crucial role in the process. To test this hypothesis, i utilized an ATM kinase inhibitor – KU-55933. KU-55933 is a potent and highly selective ATM kinase inhibitor (Hickson et al., 2004). It is a pyranone compound that can permeate through cell membranes and act as an ATP-competitive inhibitor of ATM. Using an end concentration of 10 uM it displays excellent selective inhibition of ATM kinase activity. It inhibits ATM-dependent cellular protein phosphorylation following ionizing radiation (IR) and sensitizes cells with wild-type ATM, but not mutant ATM, to the cytotoxic effects of IR and DNA-damaging agents (Hickson et al., 2004, Eaton et al., 2007, Crescenzi et al., 2008).

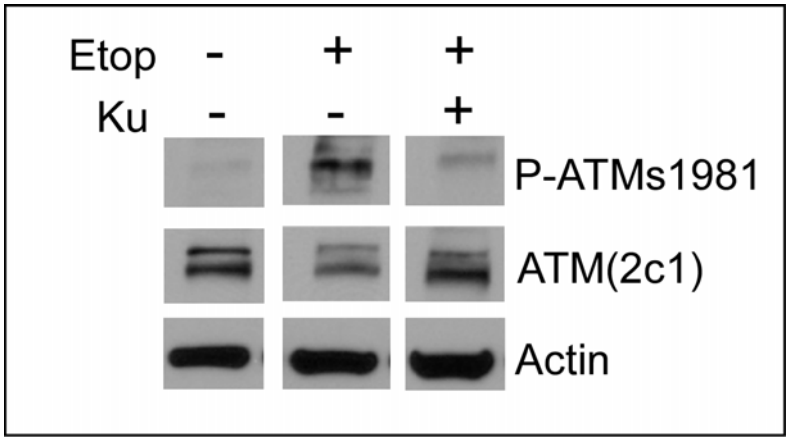

Figure 6. KU-55933 blocked ATM kinase activity in hippocampal slices. Etoposide treatment (20 uM) for one hour activated ATM kinase activity in hippocampal slices (center lane). Co-application of KU-55933 (10 uM) abolished this activation (right lane).

To test whether KU-55933 was able to penetrate into brain slices efficiently and then inhibit the kinase activity of ATM inside neurons, freshly prepared hippocampal slices were treated for an hour with 20 uM Etoposide (to elicit the ATM dependent DNA damage response, Fig. 6, center lane), 20 uM Etoposide and 10 uM KU-55933 (right lane), or vehicle only (left lane). Then all three groups of slices were blotted with 2C1A1 antibody (ATM total protein), or with phospho-specific antibody against ATM phospho-S1981. Etoposide treatment clearly activated the ATM kinase activities (center lane). And co-application of KU-55933 abolished this activation (right lane).

Next KU-55933 was used to test the importance of ATM kinase activity in the modulation of synaptic transmission. TBS-LTP was recorded from acutely prepared wild-type hippocampal slices either pre-treated with KU-55933 (10 uM) or with vehicle for an hour. The result is shown in Figure 7. KU-55933 treatment significantly reduced LTP (103% ± 7.6%, $n = 5$, $p < 0.05$, Fig. 7B) whereas LTP from vehicle treatment group remained the same as that observed in wild-type slices (195% ± 29.6%, $n = 6$, Fig. 7B). More interestingly, KU-55933 treatment also dramatically reduced I/O response curve comparing to vehicle controls (Fig. 7A). This effect on baseline transmission was not observed in the Atm^{tm1Awb} mutant mice TBS-LTP experiment.

In the inhibitor study, TBS-LTP was clearly abolished by the inhibition of ATM kinase activity. This result further confirmed our postulation that ATM's kinase activity is crucial for cytoplasmic ATM's modulation on synaptic transmission. Though the detailed mechanism for this is still yet to be known, we hypothesize that the phosphorylation of Synapsin-I and VAMP2 facilitates them to bind with cytoplasmic ATM. And the association of ATM with Synapsin-I and VAMP2 helps to bring these

proteins together into a hetero-protein-complex in the presynaptic terminal. As both synapsins and VAMP2 are key regulators of synaptic vesicle release. This spatial adjacency may help to increase the synaptic vesicle release efficiency. Thus cytoplasmic ATM, though not directly involved in synaptic vesicle release machinery, is very likely to play a central modulatory role of regulating synapsin and VAMP2 to fine-tune the synaptic transmission efficacy. And when the association of this hetero-protein-complex was disrupted either by ATM mutation or kinase inhibition, the synaptic vesicles can still be released but the efficiency was much reduced.

The reduction in baseline transmission by ATM kinase inhibition however was a novel finding. When tested in Atm^{tm1Awb} mutant mice, the baseline synaptic transmission showed no difference from the wild type counterparts. So why does inhibition of ATM kinase result in the reduction of the basal level of synaptic transmission? There are a couple of potential explanations for this discrepancy. First, a problem common to all genetic mutation studies, is that when a mutation is introduced into the initial stage of fertilized eggs, the organism may be able to compensate for the deficit through other alternative pathways during the development. However the acute block of a protein's function leaves the organism little chance for a similar compensation. Second, we have not yet checked the phosphorylation status of synapsin-I and VAMP2 in the Atm^{tm1Awb} mutant mice. It is possible that these proteins are still phosphorylated by other kinases or maybe a residual ATM left from the mutation. To test these possibilities, a more careful examination of ATM and other related proteins as well as their phosphorylation levels in the Atm^{tm1Awb} mutant mice are needed.

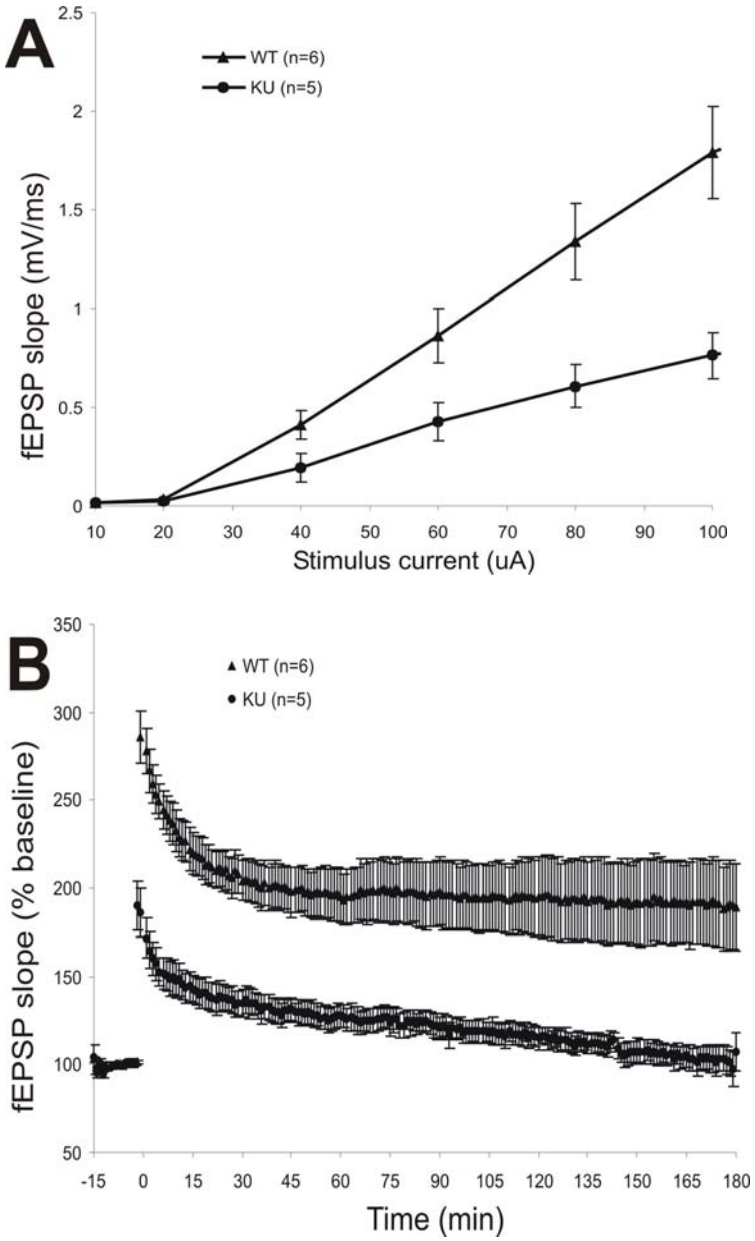

Figure 7. Long-Term Potentiation is defective in KU-55933 treated hippocampal slices. (A) Input-output relations showing that baseline transmission was significantly reduced in KU-55933 treated slices as compared to vehicle treated ones. (B) Schaffer collateral long-term potentiation (LTP) is dramatically reduced in KU-55933 treated slices. The time course of LTP elicited by theta burst stimulation (TBS) shows the expected increase in the field excitatory postsynaptic potential (fEPSP) slope (195% ± 29.6% measured at 175–180 min after TBS stimulation, n = 6) in vehicle treated slices, but not in KU-55933 treated slices (103% ± 7.6%, n = 5; $p < 0.05$).

Atm^{tm1Awb} mutants express a residual amount of ATM inside brain

Recently, a more thorough biochemical examination of ATM protein level and kinase activity was performed on Atm^{tm1Awb} mutant mice (Li et al., 2011). Quite intriguingly, it revealed that the Atm^{tm1Awb} message undergoes a novel, brain-specific splicing event that leads to the production of a truncated ATM protein in brain only. This brain-specific splicing has never been documented in ATM knockout mice models or human A-T patients, and the methods used by the neurons to achieve this unusual splice pattern are unknown. Western blots of wild-type and Awb/Awb spleen extracts, thymus extracts, and brain extracts were probed with three different ATM antibodies: 2C1A1, 5C2, and Y-170 (Fig. 8A). In wild-type mice, the three ATM antibodies each recognized a band corresponding to full-length ATM in every tissue (lane 1, 4, 7). The Awb mutant mice, however exhibited a more complicated expression pattern. There was not any ATM expression in the Spleen or Thymus tissue extracted from Awb mutant mice (lane 2, 5). But in the Brain tissue a protein band very close to the wild-type ATM protein showed up in the lanes with 2C1A1 and 5C2 antibody recognition, whereas Y-170 antibody failed to exhibit that protein (lane 8). This result indicates the ATM protein expressed in Awb mutant mice brain has a very similar size as the wild-type protein, only missing a small segment around the Y-170 epitope region. An additional piece of important information that we can get from this western result is that production of this mutated ATM is greatly reduced compared to protein levels in the wild-type tissue.

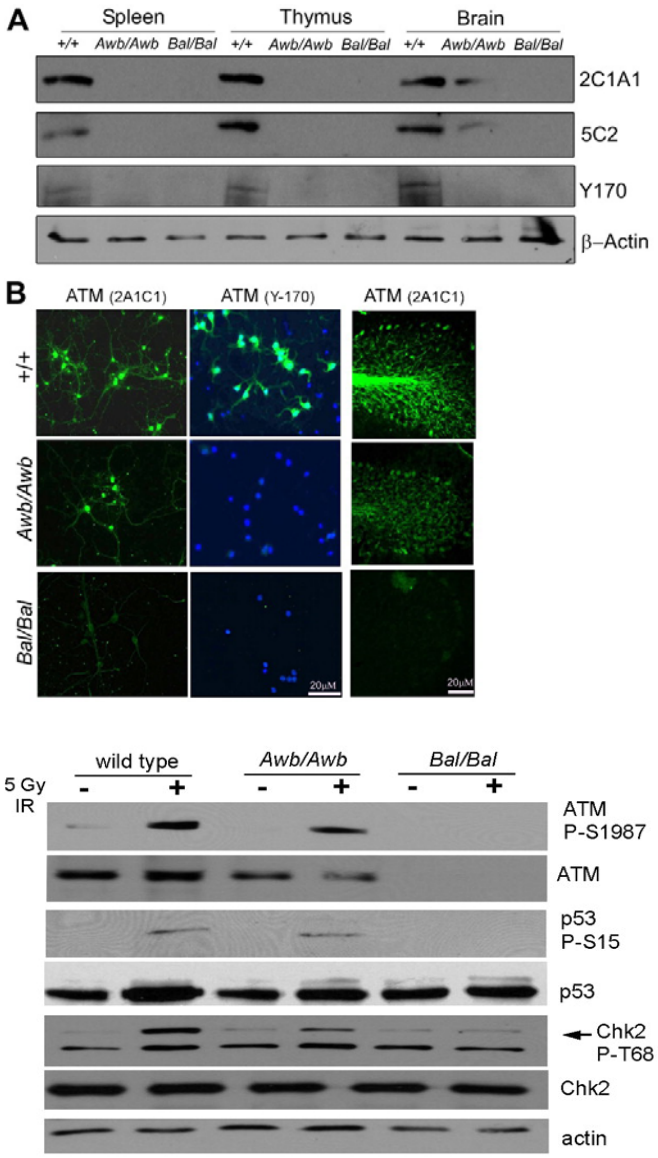

Figure 8. *Atm*^{tm1Awb} but not *Atm*^{tm1Bal} produces a brain-specific mutant ATM protein. ***A***, Western blots of wild-type, *Awb/Awb*, and *Bal/Bal* spleen extracts, thymus extracts, and brain extracts were probed with three different ATM antibodies: 2C1A1, 5C2, and Y-170. Actin levels were used as a loading control. ***B***, Immunofluorescence of cultured E16.5 cortical neurons immunostained with either 2C1A1 (left column) or Y-170 (middle column) ATM antibody. The images in the right column are cryostat sections of cerebellar cortex immunostained with 2C1A1 ATM antibody. The genotypes of the cultures or sections are indicated on the left. No staining of *Bal/Bal* cells was found with any ATM antibody, *in vivo* or *in vitro. Awb/Awb* cells stain with 2C1A1 but not Y-170. ***C***, ATM^{AWB} protein retains kinase activity. Mice of wild-type, *Bal/Bal*, and *Awb/Awb* genotypes were exposed to 5 Gy whole-body ionizing radiation (IR) (**+**) or left untreated (−). Six hours later, mice were killed and cerebellar extracts subjected to Western analysis with phospho-specific antibodies against ATM phospho-S1987, p53 phospho-S15, or Chk2 phospho-T68. In wild type, all substrates showed evidence of radiation-induced labeling. In *Bal/Bal* mice, by contrast, none was labeled after IR-induced DNA damage. Despite their genotype, *Awb/Awb* mice showed a robust activation of all three substrates after ionizing radiation.
<Figure adapted from (Li et al., 2011)>

Immunocytochemistry also confirms this finding. Cultured E16.5 cortical neurons from either wild-type mice or Awb mutant mice were immuno-stained with either 2C1A1 (left column) or Y-170 (middle column) ATM antibody (Fig. 8B). The images in the right column are cryostat sections of cerebellar cortex immunostained with 2C1A1 ATM antibody. 2C1A1 antibody stained both wild-type and Awb/Awb cells, in vivo or in vitro. But Y-170 antibody only stained wild-type cells.

In addition to demonstrating the presence of ATM in Awb mutant mice, a functional assay was also done and it was determined that the ATM protein found in Awb mutant mice retains kinase activity (Fig. 8C). Mice of wild-type and Awb/Awb genotypes were exposed to 5 Gy whole-body ionizing radiation (IR) (+) or left untreated (−). Six hours later, mice were killed and cerebellar extracts subjected to Western analysis with phospho-specific antibodies against ATM phospho-S1987, p53 phospho-S15, or Chk2 phospho-T68. In wild type, all substrates show evidence of radiation-induced labeling. Despite their genotype, Awb/Awb mice also showed a robust activation of all three substrates after ionizing radiation.

Taking these findings together, Atm^{tm1Awb} mutant mice do express ATM at a very low level in brains only, and the mutated ATM protein still contains kinase activity and is capable of reacting to DNA damage. Though expressed at a much lower level as compared to wild-type ATM proteins, this residual protein and its kinase activity could potentially be account for the difference observed in baseline level synaptic transmission between Awb mutant mice brains and the acute kinase inhibitor treatment studies (Fig. 5A & 7A).

Chapter 3. Atm^{tm1Bal} knockout mice showed varied phenotypes

In the previous chapter, the TBS-LTP experiments clearly showed that cytoplasmic ATM indeed is important in regulating synaptic transmission, and this regulation is related to its kinase activity. Furthermore, the basal level of synaptic transmission in Atm^{tm1Awb} mutant mice was unaffected, whereas the acute inhibition of ATM kinase function reduced the baseline synaptic transmission. Then the more careful examination of Atm^{tm1Awb} mutant mice revealed that residual amount of ATM is still expressed in the mutant mice, but in brains only. And the ATM in the mutant mice is missing a small segment contains the Y-170 antibody epitope, but still remains kinase activity and is capable of reacting to DNA damage. This residual ATM and its kinase activity could potentially account for the normal baseline transmission observed in the Atm^{tm1Awb} mutant mice, but it cannot totally rule out the possibility of a parallel developmental compensation. To test the latter hypothesis, we acquired a second line of ATM mutant mice (Atm^{tm1Bal}).

Atm^{tm1Bal} mutant is a total null mutation

Atm^{tm1Bal} was engineered by Xu et al. in 1996 (Xu et al., 1996). Different from Awb knockouts, Atm^{tm1Bal} has a complete deletion of 58th exon which contains the PI3-Kinase domain (Xu et al., 1996). The deletion results in a total null mutation of the protein. Western assay revealed no ATM protein staining from Bal/Bal Spleen, Thymus or Brain tissue extract with any ATM antibody (2C1A1, 5C2, and Y-170, Fig. 7A, lane 3, 6, 9). The absence of the ATM protein is also verified by immunocytochemistry (Fig. 8B). In accordance, Bal/Bal mice showed no ATM kinase downstream targets activation

Table 1: Comparisons between Atm^{tm1Awb} and Atm^{tm1Bal} mutant mice

Atm^{tm1Awb}	Atm^{tm1Bal}
The 1st ATM mutant mice, generated in 1996	Second ATM knockout mutant mice, also generated in 1996
Constructed by Barlow et al. (Wynshaw-Boris lab)	Constructed by Xu et al. (Baltimore lab)
178 bp in 38th exon (corresponding to many human A-T patients mutations) was disrupted, producing a truncation mutation	Disruption of two exons encoding the PI3-Kinase domain)
A unique RNA splicing results in a novel brain-specific mRNA production	No Stable mRNA is made in brain
Small amount of ATM is made that is nearly normal in size and retains detectable kinase activity	No production of ATM observed
Growth retardation	Growth retardation
Mild neurological deficits	No clear neurological dysfunction
No cerebellar degeneration	Purkinje and granule neurons degeneration observed in cerebella, and other neuron degeneration in neocortex
Male and female infertility	Male and female infertility
Hyper-sensitivity to γ-irradiation	Hyper-sensitivity to γ-irradiation
Immune defects: development of thymic lymphomas and early death (< 4 months)	Immune defects: development of thymic lymphomas and early death (< 4.5 months)
The only commercially available mutation from Jackson Labs (JAX™) and a well studied model	Gift from Dr. Xu

Table content synthesized from (Barlow et al., 1996, Xu et al., 1996, Li et al., 2011)

after IR-induced DNA damage (Fig. 8C). For a more detailed comparison between the two ATM knockout mutant mice, please refer to Table1.

Atm^{tm1Bal} mutants exhibited mild neurological deficit

It was previously reported that the ATM mutant mice exhibited only mild ataxia symptoms, such as altered gait pattern and more difficulty in keeping balance on the rota-rod (Xu et al., 1996). In order to assess the degree of deficit expressed in the Atm^{tm1Bal} mutant mice, hind-paw footprint analysis was used to determine the degree of ataxia in the mutant mice (Barlow et al., 1996). The hind paws were first dipped in ink, and then the mouse was placed at an open end of a tunnel. The mouse was allowed to walk to the other end of the tunnel, where it would be retrieved and placed into its home cage. To characterize the walking pattern of each mouse, i measured the average distance between each stride (stride length). Mutant mice had significantly shorter ($p < 0.05$) stride lengths compared to the wild-type controls (Figure 9A), which in part could be due to their smaller size. However, the maximum difference in stride lengths (longest stride to shortest stride) was significantly greater ($p < 0.05$) in mutant mice (Figure 9B), which should be independent of size. The stepping pattern of the mutant mice (as shown by the example recording in Figure 9C) was less consistent than in wild-type mice, which indicates ataxia.

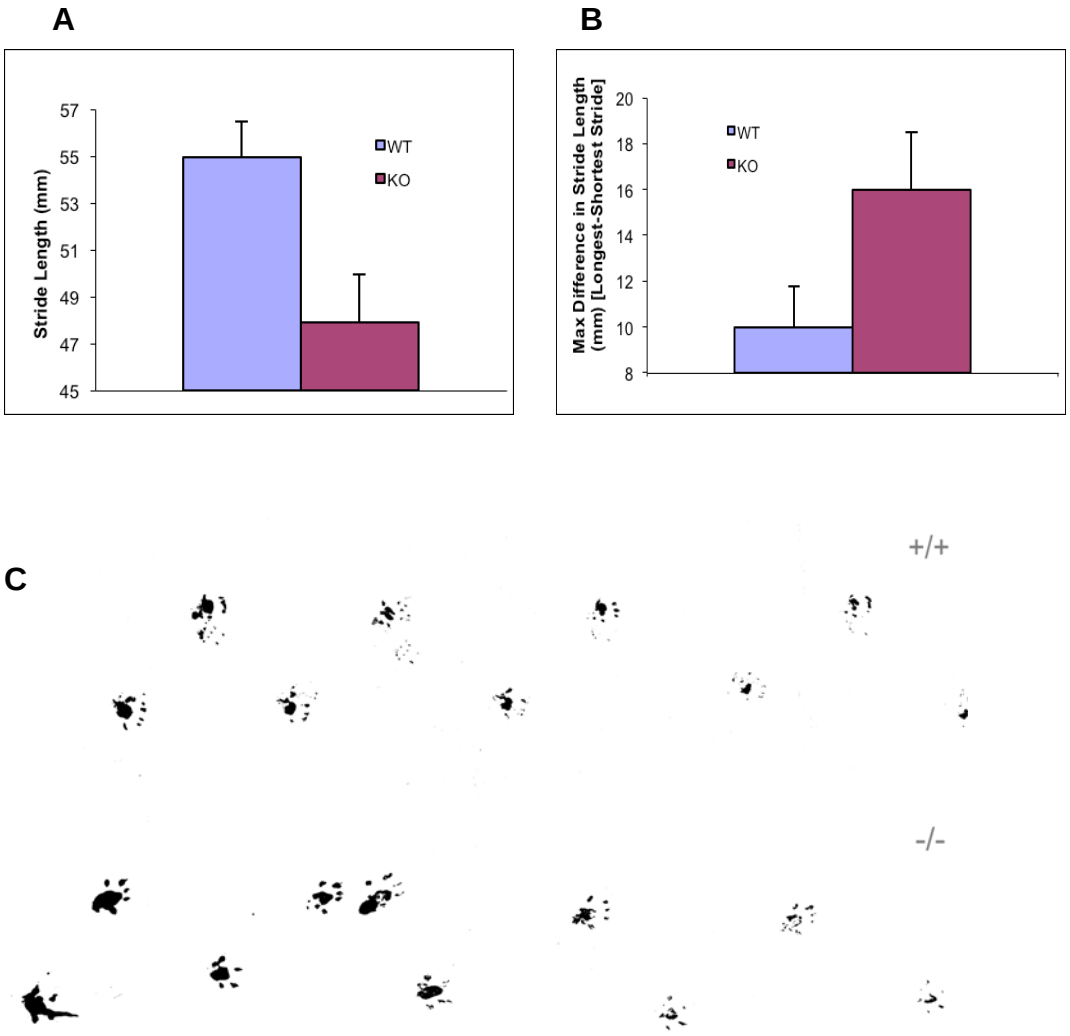

Figure 9. Atm^{tm1Bal} mice show behavioral abnormalities in the hind-paw footprint test. (A) The average stride length was longer for wild-type mice (54.9 ± 1.52, n = 10) than for the mutant mice (47.9 ± 2.03, n = 14; $p < 0.05$, one-tailed test). (B) Wild-type mice had more consistent stride lengths, as revealed in a significantly shorter maximum difference in stride length value (10 ± 1.74, n=10) compared with that seen with mutant mice (16 ± 2.50, n=14; $p < 0.05$, one-tailed test). In (C), representative footprint patterns from a wild-type and a mutant mouse are shown. Top, wild-type mouse (+/+); bottom, Atm-disrupted mouse (−/−).

TBS-LTP was unaltered in Atm^{tm1Bal} mutants

Next i examined TBS-LTP in the Atm^{tm1Bal} mutant mice. Acutely prepared hippocampal slices from either wild type or homozygous Atm^{tm1Bal} mutant mice were subjected to TBS-LTP tests. Quite unexpectedly, LTP observed from the mutant tissue during the 3-hour period was almost the same as compared to their wild type littermates (WT = 182% ± 13.3% measured at 175–180 min after TBS stimulation, n = 10, Mutant = 176% ± 11.5%, n = 10, Fig. 10B). And there was also no deficit in baseline synaptic transmission as both genotypes had similar input-output curves (Fig. 10A).

This TBS-LTP result from the Atm^{tm1Bal} mutant mice is very different from the substantial LTP deficit that we observed in Atm^{tm1Awb} mutants, even though both mice strains are ATM knockouts. One interpretation is that there may be considerable diversity in the phenotypes even among the different ATM mutant mice types. Correspondingly, there are many mutation variants observed in human A-T patients. A genetic study done in Germany showed there are as many as 46 different types of ATM mutations among the 66 A-T families they surveyed (Sandoval et al., 1999). It is possible that these different mutations may result in different neurological phenotypes, and that the specific manifestation of symptoms may depend on how ATM participates in the regulation of synaptic transmission. The exact mechanism of this regulation is still not known, but the next set of experiments shed light on facets of synaptic transmission that are compromised in the Atm^{tm1Bal} mutant mice.

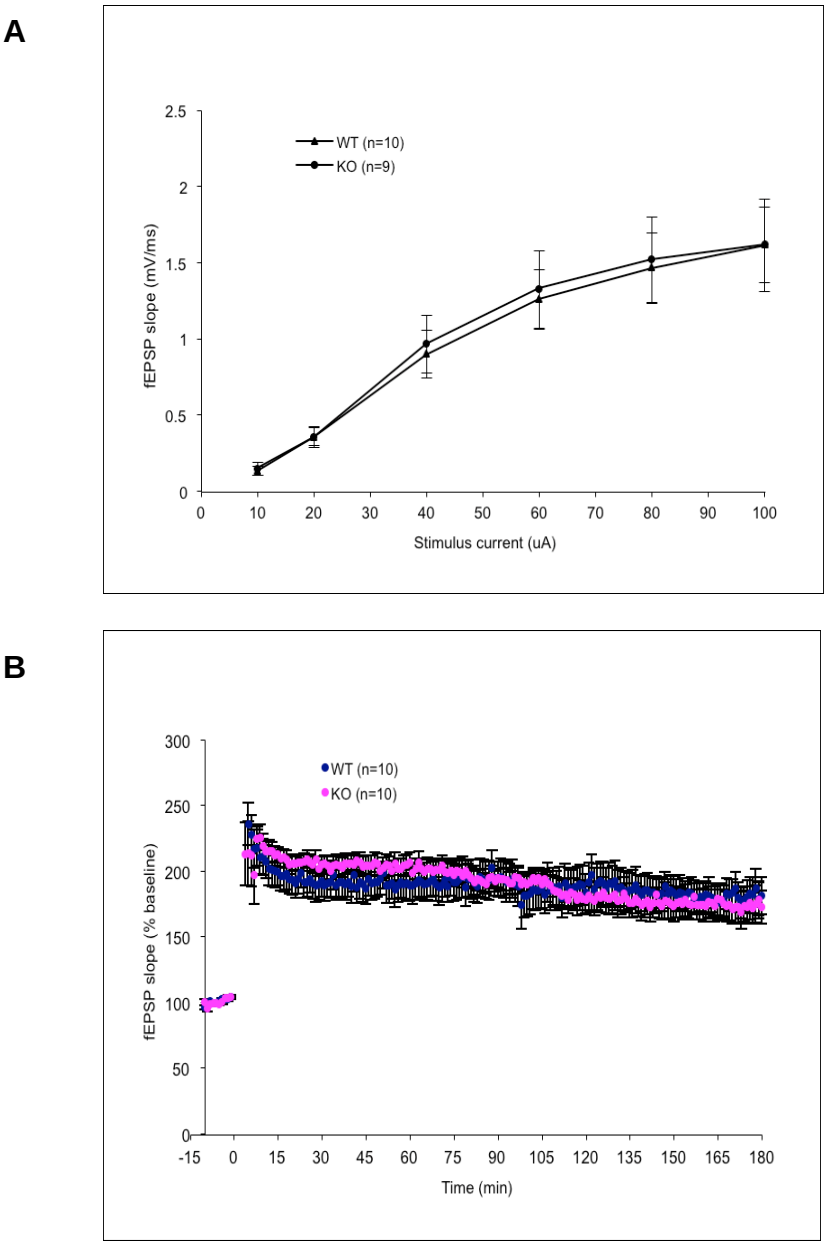

Figure 10. Long-Term Potentiation is unchanged in Atm^{tm1Bal} hippocampus (A) Input-output relations showing that baseline transmission was not significantly different between wild type and Atm^{tm1Bal} mice. (B) Schaffer collateral long-term potentiation (LTP) is the same in Atm^{tm1Bal} mice as in wild type mice. The time course of LTP elicited by theta burst stimulation (TBS) shows the expected increase in the field excitatory postsynaptic potential (fEPSP) slope (182% ± 13.3% measured at 175–180 min after TBS stimulation, n = 10) in wild-type slices, and in slices from Atm^{tm1Bal} mice (176% ± 11.5%, n = 10).

Presynaptic NTs release in Atm^{tm1Bal} knockout mice is impaired

So far the interaction between cytoplasmic ATM with synapsin-I and VAMP2 remains as the best candidate mechanism for ATM's regulation of synaptic transmission, which implicates the regulation primarily occurs at the presynaptic locus of action. Thus at this stage I focused on electrophysiological studies that provide information about presynaptic mechanisms. As the long-term potentiation study did not reveal any difference between knockouts and wild types, we shifted the focus toward the short-term plasticity. Both synaptic fatigue and paired-pulse facilitation are good methods that examine the presynaptic neurotransmitter release during the short-term plasticity (Zucker, 1989). Thus i performed these studies with the Atm^{tm1Bal} mutant mice.

Synaptic responses during TBS were recorded to quantify synaptic fatigue from both wild type and Atm^{tm1Bal} knockout mice. To enable comparison between the different recordings, the first evoked field potential from individual TBS burst 2, 6, 7, 11 and 12 were normalized to the very first response in the first burst and then plotted as an index of synaptic fatigue. Hippocampal slices prepared from adult Atm^{tm1Bal} knockout mice showed much more pronounced synaptic fatigue in CA1 synapses during TBS compared with wild-type mice (Fig. 11 A). In wild type animals, there was a dramatic increase in the size of the fEPSP when comparing the first response in the first TBS burst to the first response in the second TBS burst, a trend still evident in TBS bursts 6 and 7. In the Atm^{tm1Bal} knockout mice, however, the first response in the second burst not only failed to show an increase, there was on average a clear decrease (Fig. 11 A, 1.15±0.02, 1.21±0.03, 1.17±0.03 for +/+ mice during TBS burst #2,6,7; 0.93±0.03, 1.06±0.04, 1.03±0.04 for -/- mice; $p<0.05$).

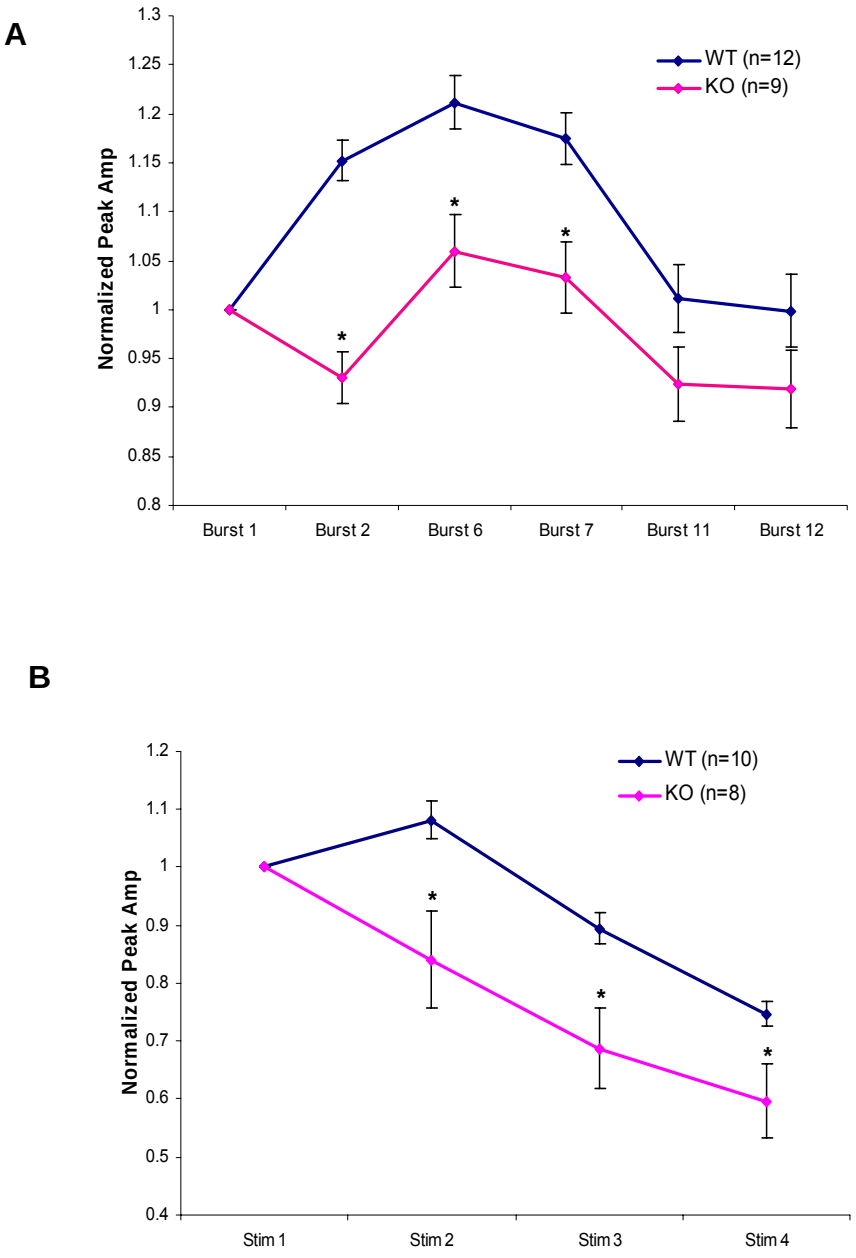

Figure 11. Pronounced synaptic fatigue in CA1 synapses during Theta-Burst stimulation in Atm^{tm1Bal} knockout mice (A) Summary of synaptic fatigue in Atm^{tm1Bal} mice between the different bursts in TBS. The peak amplitude of the 1st stimulation response from TBS burst 2,6,7,11&12 are normalized to the first stimulation response from the 1st burst. **, Significantly different from +/+; p<0.05*. n = number of recordings. (B) Summary of synaptic fatigue in Atm^{tm1Bal} mice within a single burst in TBS. **, Significantly different from +/+; p<0.05. n = number of recordings.*

Interestingly, the knockout animal did show an increased response in bursts 6 and 7, but it was not large enough to put the overall response on par with the wild type animals. Next I compared responses within the individual TBS bursts, and found that the Atm^{tm1Bal} knockout mice also had more significant synaptic fatigue from individual stimulations within each burst. Wild type mice showed a small increase of the EPSP amplitude in the 2nd response followed by a gradual decreasing of the EPSPs. However knockout mice showed a consistent synaptic depression through all four stimuli (Fig. 11 B, 1.08±0.03, 0.89±0.03, 0.75±0.02 for +/+ mice; 0.84±0.08, 0.69±0.07, 0.60±0.06 for -/- mice; $p<0.05$).

The reduced responsiveness during TBS stimulation, although typically attributed to presynaptic mechanisms, could potentially result from changes in the postsynaptic neurotransmitter receptor sensitivity. I therefore examined Paired-pulse facilitation (PPF) which is almost universally thought to be a presynaptic phenomenon. The magnitude of PPF was calculated as the ratio of field potential slopes in response to two successive stimulation pulses. The magnitude of PPF is expected to vary as a function of the time between the two stimuli. PPF at 10, 25, 50, 100, 150, 200, 300 msec intervals were measured both in Atm^{tm1Bal} knockouts and wild-type mice. Both wild type and Atm^{tm1Bal} knockout animals showed some degree of PPF at intervals between 25 and 200 msec, but compared to wild type, the Atm^{tm1Bal} mice exhibited impaired PPF in all inter-pulse intervals of 50 msec and greater (Fig. 12 A&B; 1.34±0.02, 1.33±0.02, 1.23±0.02, 1.15±0.02, 1.06±0.01 for +/+ mice at PPF stimulus interval 50, 100, 150, 200 300 msec; and 1.24±0.02, 1.22±0.02, 1.14±0.01, 1.07±0.01, 0.99±0.01 for -/- mice; $p<0.05$).

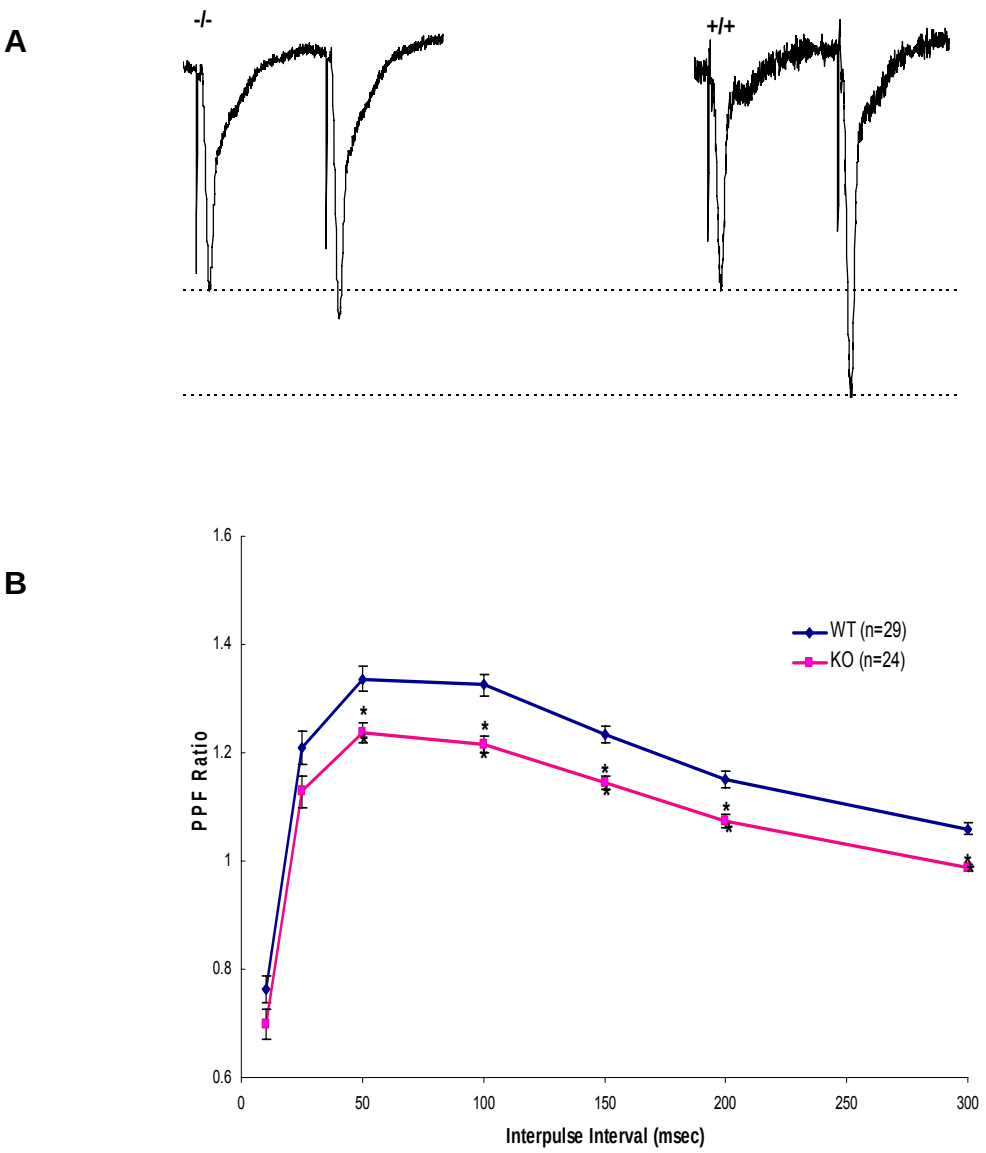

Figure 12. Impairment of Paired-Pulse Facilitation (PPF) in Atm^{tm1Bal} Mice (A) Examples of EPSPs elicited during PPF in hippocampal slices from Atm^{tm1Bal} mice. Note the significant decrease of PPF in the -/- mice. . (B) Plot of PPF at different interpulse intervals. PPF:1.34±0.02, 1.33±0.02, 1.23±0.02, 1.15±0.02, 1.06±0.01 for +/+ mice at stimulus interval 50, 100, 150, 200 300 msec; and 1.24±0.02, 1.22±0.02, 1.14±0.01, 1.07±0.01, 0.99±0.01 for -/- mice; **, Significantly different from +/+; p<0.05. n = number of recordings*.

This is interesting in that the longer intervals are in some ways comparable to the time between individual bursts in the TBS stimulation protocol, and the PPF data correlate quite nicely with what would be predicted from the synaptic fatigue analysis. Overall, the findings suggest strongly that the Atm^{tm1Bal} knockout animals show a decrease in presynaptic vesicle release properties, specifically mobilization of vesicles under conditions of repetitive presynaptic activation.

To summarize the findings to date, Atm^{tm1Bal} mice contain a total null ATM mutation with no protein observed in brain or any other tissue, and the absence of DNA damage responses confirmed this finding. Atm^{tm1Bal} mice showed a mild ataxia phenotype (comprised hind foot gaits) as reported before. Totally unexpectedly, there was no TBS-LTP difference observed between Atm^{tm1Bal} mutant and wild-type mice. The reason behind this is still under investigation. Meanwhile the two short-term plasticity tests (synaptic fatigue and paired-pulse facilitation) both revealed a more profound synaptic depression in the Atm^{tm1Bal} knockout mice. This most likely reflects the lack of cytoplasmic ATM which has altered presynaptic synaptic vesicle release properties, potentially making neurotransmitter release in response to repetitive stimulation less efficient.

Chapter 4. Future directions

In previous three chapters I have presented the specific cytoplasmic ATM distribution in the central nerve cells. ATM associates with synapsin-I and VAMP2 in the presynaptic nerve terminals, most likely through its kinase action. While knocking down ATM or using an inhibitor to acutely block the kinase activities both resulted in a dramatically reduced TBS-LTP in the hippocampus slices, a total ATM knockout (Atm^{tm1Bal}) showed no impairment of LTP at all. More subtle electrophysiology studies showed that the presynaptic neurotransmitter release efficacy under the repetitive stimulation was reduced with ATM ablation. These novel findings have raised two questions: 1) How does the clear deficit in synaptic vesicle release observed in the STP experiments relate to the lack of deficit in LTP study? 2) Why does the complete ATM knockout show an apparently less severe synaptic transmission phenotype than the mice with residual ATM protein of incomplete function?

A similar scenario at synapsin-I knockout mice studies

To elucidate the first question, I'd like to draw on the synapsin knockout studies as an analogous comparison. Biochemical and histological studies clearly showed that synapsins are important associating proteins that tether synaptic vesicles to each other and to the cytoskeleton thus forming an ready-to-use reserve SVs pool (Greengard et al., 1993). And in the neurons of synapsin knockout mice, the SVs at the synaptic terminal become more dispersed. However, electrophysiology studies showed no LTP deficit but some mild PTP, PPF and synaptic depression abnormalities in the Syn-I, Syn-II, and the double-knockout mice (Rosahl et al., 1995). It was originally proposed that the different

synapsin isoforms have compensated for the loss of synapsin-I and II proteins (Rosahl et al., 1993, Rosahl et al., 1995). However a later similar study of synapsin-I knockout mice but using quantitative fluorescence imaging dye (FM 1-43) revealed a different story (Ryan and Smith, 1995, Ryan et al., 1996). The researchers reported that both the number of vesicles able to release their contents during brief trains of action potentials and the total recycling vesicle pool are significantly reduced (60-70%) at synapses in hippocampal cultures derived from mice lacking synapsin-I compared to their wild-type counterparts. However the kinetics of endocytic reuptake of vesicle membrane appear to be identical in the knockout and wild-type mice. The researchers argued that the reason for not seeing defects in the electrophysiology studies were mainly because electrophysiological assays of synaptic transmission generally measure the successful exocytotic events from a large and indeterminate number of synaptic inputs. As a result, many presynaptic details, such as release probability on a per terminal basis, as well as post-exocytotic events in the synaptic vesicle, remain hidden.

Actually using the similar dye tracing technique, combined with the assist of more advanced electron microscopy and live imaging, more exciting findings were coming out from synapsins studies (Gitler et al., 2008, Fornasiero et al., 2012, Orenbuch et al., 2012). It has been shown that the reserve pool of SVs at the synaptic terminals is not a static organization. Instead, a subset of functional synaptic vesicles is dynamically shared between adjacent terminals by lateral axonal transport during intense synaptic events. Synapsins are essential to maintain the size and the dynamic organization of SVs in the reserve pool. And an additional synapsin-independent mechanism, whose molecular

substrate remains to be clarified, targets SVs to synaptic boutons at rest and might be outpaced by activity.

TBS-LTP becomes defective in Atm^{tm1Bal} mutant mice when TBS is reduced

Taken together, studies from synapsin-I knockout mice have suggested us that in some electrophysiology studies, e.g. LTP, when the stimuli are over-saturated, it can mask the fundamental deficits in the synaptic transmission such as SV release probability changes. This can certainly be adapted to our studies of ATM knockouts. One way to find out is to examine the SVs mobility using dye-tracing microscopy. Alternatively, I have attempted a different approach here by lowering the overall stimulation intensity to be around the sub-threshold level in LTP induction, then test for differences between mutant and wild-type mice. Indeed after reducing the TBS from 12 stimulation bursts to just 2, a difference in LTP between Atm^{tm1Bal} knockout mice and the wild-type counterparts started to emerge. A sample experiment of the TBS-LTP recording from the original stimulation protocol and the new reduced intensity protocol is shown in figure 13. As 12*TBS induced LTP from both knockout and wild-type mouse are very similar to what we have observed before (Fig. 10B), the new 2*TBS stimulation protocol produced different results from knockout and wild-type animals. Though wild-type mouse still exhibited comparable LTP as the ones induced by 12*TBS, the 2*TBS-LTP in Atm^{tm1Bal} knockout went almost back to baseline level showing the deficit in synaptic transmission efficacy. Clearly, more recordings need to be performed to confirm this result.

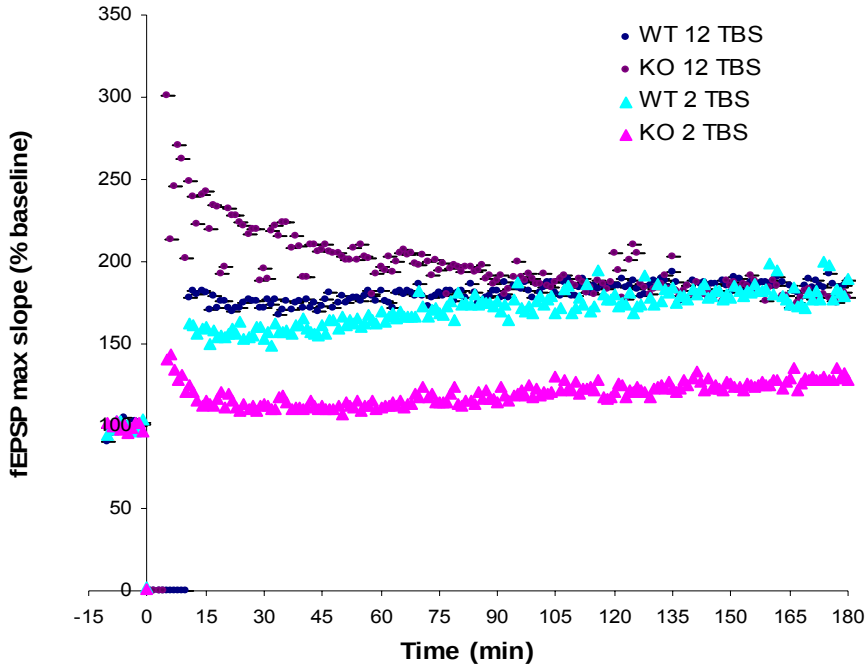

Figure 13. Different TBS intensity made a difference in inducing LTP from wild type and Atm^{tm1Bal} mouse. 12*TBS elicited robust LTP from both genotypes, the 2*TBS only produced LTP in the wild type but not knockout mouse.

ATR, a good candidate to compensate for the cellular functions of ATM

On one hand the lower stimulation intensity TBS protocol was able to demonstrate a deficit in LTP in the Atm^{tm1Bal} knockout mice; on the other hand this phenomenon still suggests that the Atm^{tm1Awb} mutant mice have a more severe neurological disorder phenotype than Atm^{tm1Bal}, because the 12*TBS-LTP protocol still would not produce LTP in the Atm^{tm1Awb} mouse hippocampus. The mechanism accounting for the different phenotype between the ATM deficient mice models is still not known, however we postulate that developmental compensation might play a role. As Atm^{tm1Bal} is genetic null mutation, the organism probably employs proteins that share similar function as ATM to compensate for the absence of the protein. But in Atm^{tm1Awb} mutant mice since ATM protein is still expressed, though at a much lower level and the protein is also truncated comparing to the wild type ones, these residual ATM mutant proteins could potentially act as a dominant negative to the system, preventing the activation of other alternative rescue pathways, thus producing a more severe phenotype.

Though this is a wild guess of what is really going on with those mutant mice, there is good evidence for an alternative to the ATM protein that may provide a basis for compensation. The candidate is Ataxia-Telangiectasia and Rad3-related protein (ATR). Similar to ATM, ATR is also a PI3-kinase family protein that responds to DNA damage. ATM and ATR share similar genome surveillance functions, and upon detection of DNA damage, they will halt the cell cycle and initiate DNA repair (in the case of minor DNA damage) or programed cell death (in the case of severe damage). The primary difference between the two molecules is the type of DNA damage signals that they respond to. ATM primarily responds to Double Strand DNA damages caused by ionizing radiation;

ATR typically reacts to UV radiation and hydoxyurea caused damages (Osborn et al., 2002, Kumagai and Dunphy, 2006). Another big difference is that ATM deficient mice are viable and only exhibit mild neurological symptoms, whereas ATR-null mice are not viable. In humans, mutation of the ATR protein causes Seckel syndrome (O'Driscoll et al., 2003).

Nonetheless, there are two particular reasons that make ATR a good candidate to substitute for ATM function. First, our own study has shown (Li et al., 2009) that ATR is also present in neuronal cytoplasm (Fig. 14D) and the function of this cytoplasmic ATR is largely unknown in the scientific literature. Second, similar to ATM, cytoplasmic ATR was found to co-precipitate with synapsin-I and VMP2, which indicates a similar or redundant function as cytoplasmic ATM (Fig. 14 E). Moreover, it has been shown that ATM preferentially phosphorylates synapsin-I, whereas the ATR is the primary kinase that phosphorylates VAMP2 (Fig. 14 A, B &C). Certainly the data are too preliminary to prove that ATR indeed acts as compensatory factor during ATM's absence, but it is certainly worth further investigation.

While preliminary in nature, the results described in this section serve as good supplemental material to my research work on cytoplasmic ATM. They also point to new lines of exciting research which will hopefully further expand our understanding of the function and mechanism of cytoplasmic ATM.

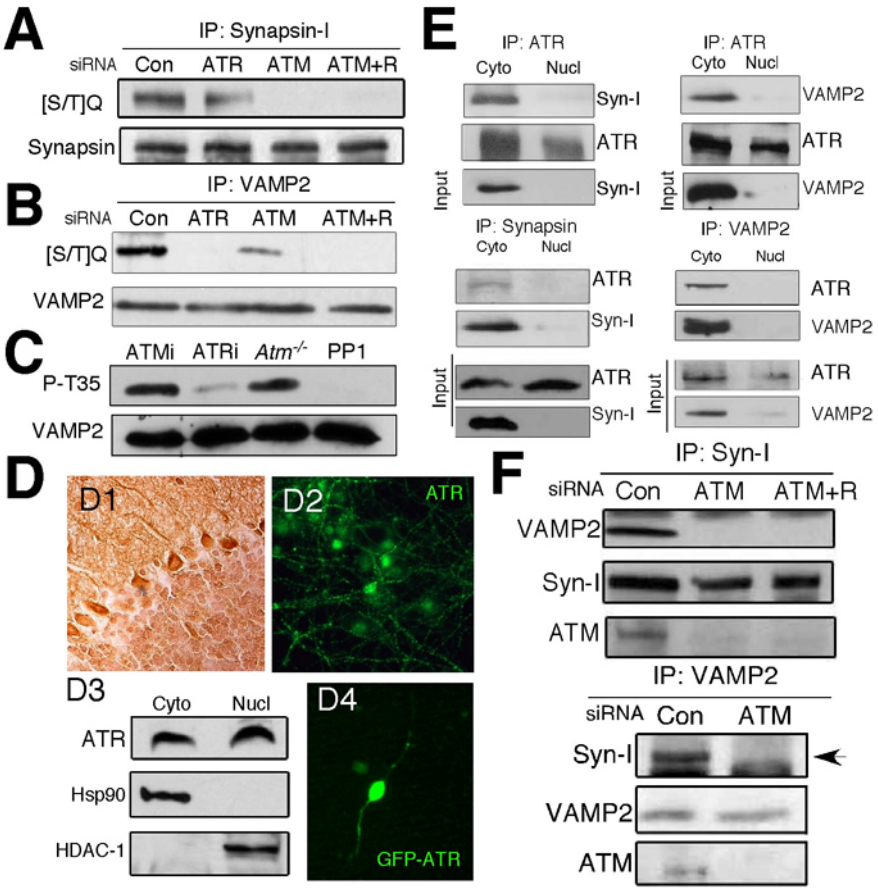

Figure 14. ATM/ATR link VAMP2 and synapsin-I. ATM phosphorylates synapsin-I. Cortical cultures were transfected with the siRNA species indicated. Immunoprecipitation from cytoplasmic extracts was performed with either anti-synapsin I (A) or anti-VAMP2 (B, C) and blotted with anti-phospho-[S/T]Q (A, B) or a phospho-T35-specific antibody (C). ATM knockdown reduces the synapsin-I phosphorylation while ATR knockdown reduces VAMP2 phosphorylation, specifically on T35. D) ATR is found in neuronal cytoplasm. (D1) ATR immunostaining of mouse cerebellar sections. (D3) Subcellular ATR distribution in cultured cortical neurons. Immunostaining of endogenous ATR (D2) and localization of exogenous GFP-ATR (D4) in cultured neurons. E) Cytoplasmic ATR in neurons partially co-localizes with synapsin-I and VAMP2. Immunoprecipitation of ATR pulls down both synapsin-I (top left) and VAMP2 (top right). The reverse immunoprecipitations are also effective. F) ATM forms a link between synapsin-I and VAMP2. Introduction of siRNA to ATM disrupts association of VAMP2 with synapsin-I in immunoprecipitations.
<Figure adapted from (Li et al., 2009)>

DISCUSSION

In this dissertation I provide evidence that ATM, which has a unique cytoplasmic distribution specific to neurons, modulates synaptic transmission most likely through its interaction with two important presynaptic proteins at the nerve terminals: synapsin-I and VAMP2. The mechanism of this modulation is probably related to the phosphorylation of synapsin-I and VAMP2 that makes them able to associate with cytoplasmic ATM as a hetero-protein complex, and then subsequently regulates the synaptic vesicle mobility.

Cytoplasmic distribution of ATM in neurons

It has been reported previously that in neuronal or neuron-like cells, ATM localization was found to be predominantly cytoplasmic (Oka and Takashima, 1998, Barlow et al., 2000, Boehrs et al., 2007). Additional support of this observation is provided by three lines of evidence: antibody staining showing the significant ATM existence in the cytoplasmic subcellular fractionation; immunocytochemistry revealing the cytoplasmic distribution of ATM in both neurons or neuron-like cells culture and in cryostat brain sections; and exogenous GFP-ATM fusion protein expressed in neurons or neuron-like cells. The prominence of this cytoplasmic component is cell type specific as neither spleen, nor thymus, nor cell lines from non-neuronal origin have significant amounts of ATM outside their nucleus.

We are the first group to report that cytoplasmic ATM co-immunoprecipitates with two well-known presynaptic proteins: synapsin-I and VAMP2. Moreover, synapsin-

I and VAMP2 are very likely to be the phosphorylation targets of ATM, as point mutation of the [S/T]Q motif (ATM PI-3 kinase phosphorylation motif) on both proteins would block the protein from binding with ATM. Co-immunoprecipitation has also indicated that synapsin-I actually forms an association with VAMP2 through the link of ATM, as introduction of siRNA to ATM disrupts the association. Whether this association is crucial for cytoplasmic ATM's regulation of synaptic transmission still needs more experimental verification.

Another noteworthy finding is that cytoplasmic ATM showed no evidence of activation after exposure to ionizing radiation. The autophosphorylation site S1981 was clearly activated by IR inside the nucleus, which indicates that nuclear ATM responds to DNA damage. In contrast, cytoplasmic ATM showed no signs of activation (phospho-S1981). It has been suggested that ATM kinase activity unrelated to the phosphorylation of S1981 exists (Pellegrini et al., 2006, Daniel et al., 2008). And the ATM-dependent phosphorylation of synapsin-I at S656 would tend to support this suggestion. This findings implies that cytoplasmic ATM may be regulated by a completely different mechanism than nuclear ATM. Again more experimental investigation is needed for further elucidation.

Cytoplasmic ATM modulates synaptic transmission

Neurons use regulated secretion at specialized synaptic contacts to transmit information during patterns of electrical activity. Modulation of the release of the synaptic vesicles (SVs) during action potential firing is thought to underlie major forms of plasticity necessary for nervous system function. Determining the cellular processes

that regulate vesicle exocytosis at presynaptic terminals is thus of central interest to neurobiology. Although much progress has been made in identifying important components that participate in synaptic vesicle trafficking and secretion [for reviews see: (Sudhof, 1995, Ting and Scheller, 1995)], the ambiguous assignment of these molecules to specific events in the presynaptic terminal still remains a major challenge.

Previous studies have reported that both synapsins and VAMP2 are important modulators of SV mobility in the pre-synaptic terminal. The classical view if synapsins is that they are a family of SV association proteins that tether SVs together and to the cytoskeleton and form a ready for release reserve pool of SVs at the nerve terminal (Sudhof et al., 1989, Greengard et al., 1993). In addition, VAMP2 serves as a member of the SNARE protein group for docking SVs to the presynaptic terminal membrane and priming them for release (Sollner et al., 1993a, Jahn and Sudhof, 1999, Jahn and Scheller, 2006). Recent research progress has further shown that synapsins might also be important in regulating the lateral axonal transport of SVs and maintaining a highly dynamic pool of SVs at the nerve terminal (Fornasiero et al., 2012, Orenbuch et al., 2012).

However our research showing the interaction between the cytoplasmic ATM with synapsin-I and VAMP2 and its involvement in the regulation of synaptic transmission (Li et al., 2009) is a novel finding. Not only did neural cells from ATM deficient mice exhibit a deficiency in spontaneous vesicle release, LTP from the mutant mice hippocampus was also dramatically reduced. LTP has long been considered as the cellular substrate for memory formation and storage (Teyler and Discenna, 1984, Bliss and Collingridge, 1993). Thus the impairment of this important form of synaptic

plasticity could potentially result in many neurological defects such as the ones that have been observed in A-T patients. As there is no baseline synaptic transmission difference between the ATM deficient mice and the wild-types observed in the LTP study, it more suggests a regulatory role for cytoplasmic ATM in neuronal activity rather than a central function in synaptic connectivity.

More careful comparison of the synaptic transmission efficacy between ATM mutant mice and the wild type counterparts revealed that synaptic fatigue and paired-pulse facilitation were also impaired in the knockout mice. Both synaptic fatigue and PPF are considered as presynaptic plasticity mechanism that relates to SV release regulation. This fits our working model that cytoplasmic ATM interacts with synapsin-I and VAMP2 and modulates synaptic vesicle release. Further, the kinase inhibitor KU-55933 study showed the similar LTP impairment as the ones we have observed in one of the ATM mutant mouse model. This indicates that ATM's kinase activity indeed is very important in its regulation of the synaptic transmission. Synapsin-I and VAMP2 are certainly top two candidates on the phosphorylation list.

Our hypothesized model that the association of cytoplasmic ATM with synapsin-I and VAMP2 regulates the synaptic vesicle mobility in the presynaptic terminals can explain well the LTP deficit that we observed in ATM mutant mice. If the synaptic vesicle release mechanism has been compromised by the lack of ATM, then the presynaptic terminals cannot sustain a high level of transmitter release during the high frequency repetitive stimulation, thus resulting in the difficulty in eliciting LTP. However, our results can not totally rule out a possible parallel postsynaptic mechanism of cytoplasmic ATM during LTP. Immunocytochemistry study has also revealed robust

staining of ATM in the cell soma and dendrites, while its function there is not yet discovered (Fig. 1&2). To better elucidate the function of cytoplasmic ATM, one interesting experiment that can be tried in the near future is to test the PPF and synaptic fatigue in the Atm^{tm1Awb} mutant mice. As Atm^{tm1Awb} mutant mice showed much more severe LTP deficit than the Atm^{tm1Bal} mutant mice (Fig. 5&10), if the regulation of synaptic transmission occurs solely at the presynaptic locus, then we would predict a much worse PPF and synaptic depression be observed in Atm^{tm1Awb} mutant mice. On the contrary, if the PPF and synaptic depression in Atm^{tm1Awb} mutant mice remain comparable with Atm^{tm1Bal} mutant mice, then it would strongly suggest additional mechanism of ATM at postsynaptic terminal or soma area exists that also contributes to LTP. Either way, the results from this study would help us better understand the mechanism of cytoplasmic ATM's involvement in synaptic transmission regulation.

Phenotype difference observed between ATM mutant mouse models

Another interesting finding revealed by our research is that different ATM mutant mouse models showed different TBS-LTP responses in the electrophysiological observations. One way to interpret this difference is coming from the developmental compensation in the mutant mice. When a protein is completely missing during the development, it is more likely to be replaced by other alternative molecules by the system to compensate for the function loss. However in the other scenario when a protein is mutated, e.g. truncation, the protein will remain there but not with the full capacity function. Thus the system may not be likely to initiate other compensatory pathways. Depending on how severely the protein's function is reduced, this type of mutation may

actually cause more damage to the system than the complete absence of the protein, almost the same as the dominant-negative approach in the genetic studies. We think the difference we observed between the two mutant mouse lines reflects this mechanism, because indeed in the Atm^{tm1Awb} mutant mice the trace amount of ATM was detected by antibodies. But the sequence of this protein reveals it is a truncated protein missing a Y-170 epitope domain on the protein.

Our finding with regard to heterogeneity of phenotype actually reiterates clinical observations from human A-T patients. Given that ATM is a large protein (~370 KD) with produced by a gene that contains 66 exons and spans across ~6000 base pairs along the chromosome, it is a no surprise that many mutations could occur in the human genome. In a genetic study surveying the ATM gene in A-T patients living in Germany, it has revealed that there were 46 different ATM mutations and 26 sequence polymorphisms from the 66 patients being surveyed (Sandoval et al., 1999). Thus a huge degree of variance among A-T patients can be anticipated. Moreover, the study has also indicated that the majority of the mutants were protein truncations. This confirmed previous report that the absence of full-length ATM protein is the most common molecular basis of A-T (Becker-Catania et al., 2000). It is traditionally considered that null mutation tends to lead to more severe neurological deficits. However, a recent clinical report contradicts that view (Alterman et al., 2007). Two siblings were clinically diagnosed as mild A-T, but no ATM protein was detected in the patients' cells, and the cellular phenotype of these patients was indistinguishable from that of classical ATM-null cell lines. This is almost exact the same scenario that we observed from Atm^{tm1Bal} mutant mice. Thus our research with different types of ATM mutation mice together

with the human clinical study suggest that null mutation of ATM may result in less severe neurological phenotypes than partial truncation mutations. Once this is confirmed, it may bring important directions for clinical intervention of A-T disease.

Y-170 epitope

In a parallel study done by our collaborator using biochemical methods to examine the difference between Atm^{tm1Awb} and Atm^{tm1Bal} mutant mice (Li et al., 2011), it has been shown that the *Awb* mutation leads to a unique splicing event in brain only, the end result of which is production of an ATM protein that is nearly normal in size and retains detectable kinase activity. This new ATM^{AWB} protein was recognized by commonly used ATM 2C1A1 antibody, but could not be detected by Y-170 antibody indicating the corresponding epitope is missing.

More interestingly, as pointed out in that paper, the absence of Y-170 domain on ATM protein is also common in human A-T patients. Among the four A-T brain samples they examined, all of them showed detectable ATM by 2C1A1 antibody in their Purkinje cells, and all samples showed negative for Y-170 antibody detection.

In contrast with our finding that Atm^{tm1Awb} mice actually had a more severe neurological defects as indicated by the near complete lost of LTP in hippocampus, the Atm^{tm1Bal} mutant mice with null ATM mutation showed LTP indistinguishable from wild-type. This would argue that certain CNS-specific functions, that may well be central to the neurobiological symptoms of A-T, require the Y-170 region of the ATM protein to be carried out. Further, the clear response of *Awb/Awb* neurons to IR suggests

that this actvitity of ATM is most likely distinct from its function as a DNA damage response protein.

It has been estimated that only <10% of the sequence of the ATM gene has been well studied (Shiloh and Kastan, 2001). Thus ATM could have many, yet unknown, additional functions. My study on the cytoplasmic ATM has added to our knowledge, yet there are still much that needs to be double-checked and explored. However, the novel synaptic transmission modulation function of cytoplasmic ATM presented by my study has shown a really promising new direction for ATM research. With the additional insights that these experiments have provided, I hope that it can bring useful hints to clinical treatment and prevention of the devastating A-T disease, and also help to broaden our understanding about ATM as a key regulator of critical cellular functions, both in the nucleus and cytoplasm.

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1. Li H, Hader AT, Han YR, Wong JA, Babiarz J, Ricupero CL, Godfrey SB, Corradi JP, Fennell M, Hart RP, Plummer MR, Grumet M. Isolation of a novel rat neural progenitor clone that expresses dlx family transcription factors and gives rise to functional gabaergic neurons in culture. Dev Neurobiol. 2012 Jun;72(6):805-20.

2. Li J, Han YR, Plummer MR, Herrup K. Cytoplasmic ATM in neurons modulates synaptic function. Curr Biol. 2009 Dec 29;19(24):2091-6.

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APPENDIX

Paper published with Dr. Plummer

Li H, Hader AT, Han YR, Wong JA, Babiarz J, Ricupero CL, Godfrey SB, Corradi JP, Fennell M, Hart RP, Plummer MR, Grumet M. Isolation of a novel rat neural progenitor clone that expresses dlx family transcription factors and gives rise to functional gabaergic neurons in culture. Dev Neurobiol. 2012 Jun;72(6):805-20.

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Brief Communications

The Glial or Neuronal Fate Choice of Oligodendrocyte Progenitors Is Modulated by Their Ability to Acquire an Epigenetic Memory

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The identity of any cell type is determined by the specific pattern of gene expression. We show here that the ability of oligodendrocyte progenitors to acquire the identity of myelin-expressing cells or choose alternative fates is dependent on the activity of histone deacetylases. Using gene expression profiling, electrophysiological recordings, transplantation studies, and pharmacological inhibition, we demonstrate that specified NG2$^+$ oligodendrocyte progenitors are plastic cells, whose decision to initiate an oligodendrocytic rather than astrocytic or neuronal program of gene expression requires the establishment of an epigenetic identity that is initiated by histone deacetylation.

Key words: gene; oligodendrocyte; lineage; histone modification; neurogenesis; gliogenesis

Introduction

Cell replacement strategies using multipotential progenitors and stem cells are of great therapeutic significance for a wide variety of clinical disorders. For this reason, elucidating the mechanisms that regulate cell fate decisions after birth is of critical importance. In this manuscript, we use oligodendrocyte progenitors (OPCs) to address this question in the postnatal brain. OPCs were originally identified as the precursors of oligodendrocytes, the myelin-forming cells of the brain and spinal cord. Recent studies have shown that these cells have the ability to revert to stem-like cells (Kondo and Raff, 2000, 2004) and generate interneurons (Belachew et al., 2003; Aguirre and Gallo, 2004; Dayer et al., 2005). The mechanisms underlying the decision of an oligodendrocyte progenitor to become a myelinating oligodendrocyte or choose a distinct neural lineage are only partially understood. It is well accepted that the identity of distinct cell types is affected by extracellular factors, whose signaling pathways modulate the expression of critical transcription factors. However, distinct cell types need to retain their own unique "memory" while responding to the extracellular environment (Shen et al., 2007). This memory is engraved in the cell's genome and is achieved by a series of posttranslational modifications of histones and DNA methylation (Buszczak and Spradling, 2006). Among the posttranslational modifications, acetylation of lysine residues in the N-terminal tail of nucleosomal histones (mediated by histone acetyl transferases) is associated with transcriptionally competent chromatin (Ashraf and Ip, 1998; Strahl and Allis, 2000). Histone deacetylation, in contrast, is catalyzed by histone deacetylases (HDACs) and is functionally associated with transcriptional repression (Csordas, 1990; Rice and Allis, 2001). Because persistent histone acetylation in progenitors prevents the establishment of the mature oligodendrocytic phenotype (Shen et al., 2005), we asked whether this posttranslational modification is also responsible for alternative fate choice of these cells.

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Materials and Methods

Immunocytochemistry of primary cultures of oligodendrocyte progenitors. Oligodendrocyte progenitors were isolated from the cortex of neonatal rats using a positive selection strategy with antibodies against A2B5$^+$, followed by incubation with anti-Ig magnetic microbeads (50 nm in size) and separation with a high-gradient magnetic field generated by the permanent magnet in the MACS Separator unit (Miltenyi Biotec, Auburn, CA). The immunoselected progenitors were cultured on Permanox chambers as described previously (Liu et al., 2003) and characterized using immunocytochemistry. The A2B5$^+$ immunoselected population was composed of 99 ± 0.5% NG2$^+$ cells and 94 ± 1% PDGF receptor α-positive cells and thereby represented a pure population of oligodendrocyte progenitors (supplemental Fig. S1, available at www.jneurosci.org as supplemental material). These cells were induced to differentiate into oligodendrocytes by removing the mitogens from the medium and culturing them in the presence or absence of the HDAC inhibitors trichostatin A (TSA; 10 ng/ml) or valproic acid (VPA; 1 mM). The ability of oligodendrocyte progenitors to generate distinct cell types was further tested by culturing them in stem cell differentiation (SCD) medium (DMEM/F-12 plus 1 mM glutamine, 1% FBS, 25 mM glucose, and B27 supplement). For immunofluorescence, cells were incubated with antibodies against vi-

Functional Differentiation of a Clone Resembling Embryonic Cortical Interneuron Progenitors

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ABSTRACT: **We have generated clones (L2.3 and RG3.6) of neural progenitors with radial glial properties from rat E14.5 cortex that differentiate into astrocytes, neurons, and oligodendrocytes. Here, we describe a different clone (L2.2) that gives rise exclusively to neurons, but not to glia. Neuronal differentiation of L2.2 cells was inhibited by bone morphogenic protein 2 (BMP2) and enhanced by Sonic Hedgehog (SHH) similar to cortical interneuron progenitors. Compared with L2.3, differentiating L2.2 cells expressed significantly higher levels of mRNAs for *glutamate decarboxylases* (*GADs*), *DLX* transcription factors, *calretinin*, *calbindin*, *neuropeptide Y* (*NPY*), and *somatostatin*. Increased levels of *DLX-2*, *GADs*, and *calretinin* proteins were confirmed upon differentiation. L2.2 cells differentiated into neurons that fired action potentials *in vitro*, and their electrophysiological differentiation was accelerated and more complete when cocultured with developing astroglial cells but not with conditioned medium from these cells. The combined results suggest that clone L2.2 resembles GABAergic interneuron progenitors in the developing forebrain.** © 2008 Wiley Periodicals, Inc. Develop Neurobiol 68: 1549–1564, 2008

Keywords: **neuronal progenitor; v-myc; L2.2; RG3.6; GABA**

INTRODUCTION

Multipotent neural stem/progenitor cells (NSPC) undergo lineage restrictions before they become mature cell types in the central nervous system (CNS) (Lu et al., 2002; Rowitch et al., 2002; Noble et al., 2004). CNS radial glial cells (RG) are NSPC (Hartfuss et al., 2001; Gotz et al., 2002; Noctor et al., 2002) that acquire glial restricted precursor (GRP) marker A2B5/4D4 or neuronal restricted precursor (NRP) marker 5A5/PSA-NCAM both *in vivo* and *in vitro* as they transition to different lineage-restricted precursors (RP) (Li et al., 2004). These RP markers have been used to isolate antigenically defined populations of cells from developing CNS that have different capacities for differentiation (Mayer-Proschel et al., 1997; Rao and Mayer-Proschel, 1997;

Additional Supporting Information may be found in the online version of this article.

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BDNF Modulation of NMDA Receptors Is Activity Dependent

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Crozier RA, Bi C, Han YR, Plummer MR. BDNF modulation of NMDA receptors is activity dependent. *J Neurophysiol* 100: 3264–3274, 2008. First published October 8, 2008; doi:10.1152/jn.90418.2008. Brain-derived neurotrophic factor (BDNF), a potent modulator of synaptic transmission, is known to influence associative synaptic plasticity and refinement of neural connectivity. We now show that BDNF modulation of glutamate currents in hippocampal neurons exhibits the additional property of use dependence, a postsynaptic mechanism resulting in selective modulation of active channels. We demonstrate selectivity by varying the repetition rate of iontophoretically applied glutamate pulses during BDNF exposure. During relatively high-frequency glutamate pulses (0.1 Hz), BDNF application elicited a doubling of the glutamate current. During low-frequency pulses (0.0033 Hz), however, BDNF evoked a dramatically diminished response. This effect was apparently mediated by calcium because manipulations that prevented elevation of intracellular calcium largely eliminated the action of BDNF on glutamate currents. To confirm *N*-methyl-D-aspartate (NMDA) receptor involvement and assess spatial requirements, we made cell-attached single-channel recordings from somatic NMDA receptors. Inclusion of calcium in the pipette was sufficient to produce enhancement of channel activity by BDNF. Substitution of EGTA for calcium prevented BDNF effects. We conclude that BDNF modulation of postsynaptic NMDA receptors requires concurrent neuronal activity potentially conferring synaptic specificity on the neurotrophin's actions.

INTRODUCTION

A central tenet of Hebbian synaptic plasticity holds that synaptic strengthening requires concurrent pre- and postsynaptic activity. This assertion encompasses the concept of use or activity dependence and provides insight into processes ranging from refinement of synaptic connectivity during development to learning and memory. In the visual system, for example, interventions that inhibit the firing of retinal ganglion neurons can disrupt segregation of afferent fibers into cortical ocular dominance columns (Stryker and Harris 1986).

In many systems, the signal underlying activity dependence is the opening of the *N*-methyl-D-aspartate (NMDA) glutamate receptors. NMDA receptor antagonists can disrupt development of visual pathways (For review, see Debski and Cline 2002) and can prevent forms of associative synaptic plasticity such as hippocampal long-term potentiation (LTP) (for review, see Bliss and Collingridge 1993). Physiological features of the receptor, such as calcium permeability and voltage-dependent magnesium block (Mayer and Westbrook 1987) make it ideal to signal neuronal activity, leading to the description of this receptor as a "coincidence detector."

NMDA receptor involvement alone, however, cannot explain the multifunctional changes observed in the nervous system. One candidate for additional modulation of synaptic plasticity is the neurotrophin brain-derived neurotrophic factor (BDNF) (for review, see Chao 2003; Lu 2004; McAllister et al. 1999; Poo 2001; Tyler et al. 2002). In slices of visual cortex, BDNF requires glutamate receptor activity to promote alterations in dendritic morphology (McAllister et al. 1996). Injection of BDNF into cat visual cortex in vivo inhibited ocular dominance column formation in the vicinity of injection but not elsewhere (Cabelli et al. 1995), and blockade of endogenous ligand signaling via TrkB, the BDNF receptor, also inhibited ocular dominance column formation (Cabelli et al. 1997). In accord with this are studies demonstrating the activity dependence of BDNF release (Balkowiec and Katz 2000, 2002; Kolarow et al. 2007; Kuczewski et al. 2008; Magby et al. 2006).

Regulation of NMDA receptor-dependent synaptic plasticity by BDNF implies a link between TrkB activation and NMDA receptor activity. Recent studies have revealed such an interaction that may be critical for manifestation of activity dependence (Arvanian and Mendell 2001; Crozier et al. 1999; Garraway et al. 2005; Jarvis et al. 1997; Kerr et al. 1999; Legrand et al. 2005; Levine et al. 1998; Song et al. 1998). We have used glutamate receptor agonists (Levine et al. 1998) and antagonists (Crozier et al. 1999) to show that the predominant postsynaptic effect of BDNF is to enhance selectively the NMDA component of glutamate current. Previous studies, however, have not addressed this critical question: will BDNF enhancement of NMDA receptor availability occur in the absence of neuronal activity? In experiments done to date, glutamate receptors were co-activated with BDNF presentation. If BDNF was instead applied in the absence of glutamate receptor activation, would subsequent testing reveal increased NMDA receptor activity or would it be unaffected? If the former, then BDNF modulation is not use-dependent and would not be categorized as contributing to Hebbian plasticity. If the latter, the conclusion would be the opposite. To address this issue, we employed two different stimulation protocols: an "activity" protocol consisting of relatively high-frequency glutamate stimulation and a "low activity" protocol with low-frequency stimulation. Our results indicate that NMDA receptor opening and elevation of intracellular calcium are critical for postsynaptic enhancement of NMDA receptor activity by BDNF and that the NMDA receptors themselves are sufficient to provide the calcium influx.

METHODS

Cell culture

High-density hippocampal cultures were grown as previously described (Levine et al. 1995). In brief, time-mated pregnant Sprague-

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Report

Cytoplasmic ATM in Neurons Modulates Synaptic Function

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Summary

ATM is a PI 3-kinase involved in DNA double-strand break repair. ATM deficiency leads to ataxia-telangiectasia (A-T), a syndrome of cancer susceptibility, hypersensitivity to ionizing radiation, immune deficiency, and sterility [1, 2]—phenotypes that can straightforwardly be attributed to a defective response to DNA damage. Yet patients with A-T also suffer from ataxia, speech defects, and abnormal body movements [3–5]—neurological phenotypes whose origins remain largely unexplained. Compounding the discordance, *Atm* mutations in mouse interfere with DNA repair but have only mild neurological symptoms [6–9], suggesting that the link between DNA damage and the death of neurons can be broken [10–12]. We find that in neurons, ATM protein has a substantial cytoplasmic distribution. We show that in Atm^{tm1Awb} mice, hippocampal long-term potentiation is significantly reduced, as is the rate of spontaneous vesicular dye release, suggesting a functional importance of cytoplasmic ATM. In the cytoplasm, ATM forms a complex with two synaptic vesicle proteins, VAMP2 and synapsin-I, both of which must be phosphorylated to bind ATM. Also, cytoplasmic ATM physically associates with the homologous PI 3-kinase, ATR. The neurological symptoms of ataxia-telangiectasia may thus result from defective nonnuclear functions of ATM not associated with DNA repair.

Results

ATM Distributes in Neuronal Cytoplasm and Fails to Respond to DNA Damage

ATM is best known for its critical role in the DNA damage response where, after autophosphorylation on serine 1981 (S1981), the ATM dimer dissociates into two catalytically active monomers [13]. Although ATM is a predominantly nuclear protein, studies have shown significant amounts of cytoplasmic ATM protein in neurons, the function of which has not been completely identified [14–18]. We performed subcellular fractionations of tissue extracts from mouse brain, spleen, and thymus (Figure 1A). All three tissues had nuclear ATM (lanes 4–6), whereas in spleen and thymus, cytoplasmic ATM was nearly undetectable (lanes 2 and 3). By contrast, in brain tissue, cultured neurons, or N2a cells (Figure 1C, lanes 1 and 2), significant quantities of cytoplasmic ATM were present. Antibody specificity was confirmed by the absence of a band in Atm^{tm1Awb} tissues (see Figure S1C available online). Immunohistochemistry of cryostat sections (Figures 1Ba and 1Bb; Figures S1A and S2B) and of cultured cells (Figures 1Dd and 1De) was consistent with these findings. A similar nuclear/cytoplasmic distribution was found with a GFP-ATM fusion protein, transiently expressed in either cortical neurons (Figure 1Da) or N2a cells (Figure 1Db). Non-neuronal cells such as NIH 3T3 and HeLa had predominately nuclear ATM (Figure 1C, lanes 3 and 4; Figure 1Dc). Further, in NIH 3T3 cells (Figure 1Df), GFP-ATM was overwhelmingly nuclear. Finally, ATM could be found in association with synaptic vesicles and synaptic membranes in synaptosomal fractions from mouse brain (Figure 1E). Thus, four independent lines of evidence support the existence of neuronal cytoplasmic ATM.

The neuron-specific occurrence of cytoplasmic ATM suggests a function distinct from its role in the DNA damage response. If primary neuronal cultures are exposed to 5 Gy of radiation, the phospho-S1981-ATM epitope appears in the nuclei of neurons or N2a cells as reported previously [19], but not in the cytoplasm (Figure 1F). These results were verified with etoposide (Figure 1G; Figure S2) and in vivo with ionizing radiation (Figure S3A). ATM activation in the nucleus was demonstrated by the enhanced phosphorylation of two ATM targets, p53 and Chk2 (Figure S3B). Thus, neither irradiation nor topoisomerase inhibitors activate cytoplasmic ATM, suggesting that its function is unrelated to the DNA damage response.

ATM Deficiency Causes Dysfunction of Synaptic Vesicle Cycling and Long-Term Potentiation

The cytoplasmic and/or synaptosomal presence of ATM in neurons prompted us to ask whether ATM might play a role in synaptic function. We recorded from in vitro slices of adult wild-type and Atm^{tm1Awb} hippocampus but found no deficit in baseline synaptic transmission. Both genotypes had similar input-output curves (Figure 2A), although there was a tendency for the mutants to show a plateau in responsiveness at lower stimulus currents than wild-type mice. We next tested the consequences of ATM deficiency on long-term potentiation (LTP) at the Schaffer collateral-CA1 synapse. Examination of theta burst stimulation (TBS)-induced LTP revealed that Atm^{tm1Awb} mice have a considerable deficit as compared to wild-type animals (Figure 2B). In control mice, TBS produced a maintained elevation in synaptic strength; the same stimulation protocol produced only a modest long-term increase in Atm^{tm1Awb} mice (Figure 2B). Previous studies indicate a defect in exocytosis in lymphocytes of ataxia-telangiectasia (A-T) patients [20]. This led us to question whether a defect in vesicle recycling might occur in *Atm* mutant neurons. We monitored FM4-64 dye uptake and release from cultured neurons derived from either wild-type or Atm^{tm1Awb} mice. There was no significant difference in FM4-64 dye uptake between Atm^{tm1Awb} and wild-type neurons or wild-type neurons treated with RNA interference (RNAi) (data not shown). By contrast, spontaneous dye release was significantly slower in Atm^{tm1Awb} and *Atm* small interfering RNA (siRNA)-treated cultures than in wild-type, untreated dishes (Figure 2C; Movies S1–S3). Thus, in the absence of ATM, spontaneous vesicle release is reduced in culture, and the ability to establish and maintain LTP is significantly impaired.

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Isolation of a Novel Rat Neural Progenitor Clone that Expresses *Dlx* Family Transcription Factors and Gives Rise to Functional GABAergic Neurons in Culture

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ABSTRACT: **Gamma-aminobutyric acid (GABA) ergic interneurons are lost in conditions including epilepsy and central nervous system injury, but there are few culture models available to study their function. Toward the goal of obtaining renewable sources of GABAergic neurons, we used the molecular profile of a functionally incomplete GABAergic precursor clone to screen 17 new clones isolated from GFP$^+$ rat E14.5 cortex and ganglionic eminence (GE) that were generated by viral introduction of v-myc. The clones grow as neurospheres in medium with FGF2, and after withdrawal of FGF2, they exhibit varying patterns of differentiation. Transcriptional profiling and quantitative reverse transcriptase polymerase chain reaction (RT-PCR) indicated that one clone (GE6) expresses high levels of mRNAs encoding *Dlx*1, 2, 5, and 6, glutamate decarboxylases, and presynaptic proteins including neuropeptide Y and somatostatin. Protein expression confirmed that GE6 is a progenitor with restricted differentiation giving rise mostly to neurons with GABAergic markers. In cocultures with hippocampal neurons, GE6 neurons became electrically excitable and received both inhibitory and excitatory synapses. After withdrawal of FGF2 in cultures of GE6 alone, neurons matured to express βIII-tubulin, and staining for synaptophysin and vesicular GABA transporter were robust after 1–2 weeks of differentiation. GE6 neurons also became electrically excitable and displayed synaptic activity, but synaptic currents were carried by chloride and were blocked by bicuculline. The results suggest that the GE6 clone, which is ventrally derived from the GE, resembles GABAergic interneuron progenitors that migrate into the developing forebrain. This is the first report of a relatively stable fetal clone that can be differentiated into GABAergic interneurons with functional synapses.** © 2011 Wiley Periodicals, Inc. Develop Neurobiol 72: 805–820, 2012

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