Published in final edited form as:

Neurosci Lett. 2015 November 16; 609: 182–188. doi:10.1016/j.neulet.2015.10.031.

Caspase-Dependent Degradation of MDMx/MDM4 Cell Cycle Regulatory Protein in Amyloid β -induced Neuronal Damage

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Abstract

MDMx/MDM4 is a negative regulator of the p53 tumor suppressor protein and is necessary for survival in dividing cells. MDMx is also expressed in postmitotic neurons, with prosurvival roles that are independent of its extensively described roles in carcinogenesis. We and others have shown a role for MDMx loss in neuronal death *in vitro* and *in vivo* in several neurodegenerative diseases. Further, we have recently shown that MDMx is targeted for proteolytic degradation by calcium-dependent proteases, calpains, in neurons *in vitro*, and that MDMx overexpression provided partial neuroprotection in a model of HIV-associated neurodegeneration. Here, we assessed whether amyloid β (A β)-induced MDMx degradation occurred in Alzheimer's Disease (AD) models. Our data shows an age-dependent reduction in MDMx levels in cholinergic neurons within the cortex of adult mice expressing the swedish mutant of the amyloid precursor protein, APP in the Tg2576 murine model of AD. *In vitro*, A β treatment of primary cortical neurons led to the caspase-dependent MDMx degradation. Our findings suggest that MDMx degradation associated with neuronal death occurs via caspase activation in neurons, and that the progressive loss of MDMx protein represents a potential mechanism of A β -induced neuronal death during disease progression in AD.

Keywords

MDMx; MDM4; Alzheimer's Disease; amyloid β ; caspase

1. Introduction

Alzheimer's Disease (AD) is an age-associated neurodegenerative condition and a leading cause of dementia, affecting 5.2 million Americans [1–3]. AD is characterized by the presence of extracellular senile plaques composed predominantly of amyloid- β (A β), intracellular neurofibrillary tangles (NFTs) composed of hyperphosphorylated tau protein,

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Conflict of interest: The authors declare no competing financial interests.

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and progressive synaptic loss and neuronal death throughout the cortex and the hippocampus [4, 5]. Previous evidence from studies conducted in postmortem human tissue and in transgenic animals has led to the “amyloid hypothesis” of AD which states that the aberrant metabolism of amyloid precursor protein (APP) is a source of extracellular A β accumulation, which in turn leads to subsequent pathological changes such as tau hyperphosphorylation, synaptic damage, and neuronal death [6–8]. Studies examining the relationship between A β and neuronal pathology suggest that the accumulation of A β within neurons precedes plaque deposition, and intraneuronal A β is suggested to be more toxic than extracellular forms [9, 10]. In addition, while A β is the major component of extracellular amyloid plaques, soluble oligomeric forms of A β have been implicated as key mediators of AD pathogenesis [11, 12]. Biomarker studies suggest that these pathological markers develop in a sequential manner; amyloid pathology appears earliest, and is followed by the accumulation of NFTs, and the resultant synaptic damage and neuronal death underlie the clinical presentation including progressive memory loss and dementia [13].

While the precise role of amyloid in the pathogenesis of AD is not completely understood, A β is shown to contribute to neuronal damage via several mechanisms. A β has been shown to interact with lipid membranes, affecting multiple intracellular compartments, including the endoplasmic reticulum, mitochondria, and the endosomal-lysosomal system [14]. A β can also induce endoplasmic reticulum stress, leading to disruption of the intracellular calcium homeostasis [15–19]. A β has also been shown to impair mitochondrial function, leading to oxidative stress and activation of the apoptotic pathways [20]. In addition, A β can interact with various neuronal surface receptors at the synapse, leading to synaptic damage and neuronal death [5, 8, 21, 22]. Calpain activity is also implicated in the progression of AD, likely through intracellular calcium dysregulation [23, 24]. AD models show evidence of calpain activation, leading to increased CDK5 activity, resulting in tau hyperphosphorylation and neuronal death [25–28]. One other key factor linked to A β -induced neuronal damage is the activation of caspases and subsequent apoptosis. Multiple models show caspase-mediated neuronal damage caused by A β [18, 19]. In addition, several pro-apoptotic proteins have been implicated in neuronal death in AD models, including Bax and p53 [29]. p53 is an intriguing target in AD, due to its role in mediating A β -induced cell death. While studies have shown increased p53 in AD patient brain tissue, in AD models, there is mounting evidence for p53’s role in A β generation [30–34].

Multiple studies have shown roles for several cell cycle proteins including p53, E2F1 and MDMx in a variety of neurodegenerative diseases *in vitro* and *in vivo* [31, 33, 35–37]. Specifically, multiple studies have shown that pro-survival roles of MDMx in neurons. First, *mdmx*^{-/-} mice are embryonically lethal with massive neuronal loss and delayed CNS development, which is rescued in *mdmx*^{-/-}/*p53*^{-/-} mice [38–41]. In addition, *in vitro*, a number of neurotoxic stimuli induce caspase-mediated proteolytic degradation of MDMx in post-mitotic neurons, while MDMx overexpression provides neuroprotection [42]. In addition, MDMx knockdown precipitates neuronal death implicating MDMx as a necessary factor for neuronal survival [42]. Finally, we have previously shown that MDMx degradation in response to calpain activation was contributing to neuronal death in an *in vitro* model of HIV-associated neurotoxicity [43].

In this study, we sought to determine the effects of amyloid on neuronal MDMx using *in vitro* and *in vivo* models. We assessed MDMx in the Tg2576 mouse model of A β -mediated neurodegeneration *in vivo*, and in primary neurons exposed to A β *in vitro*.

2. Materials and Methods

2.1. Reagents and Chemicals

The following vendors were used for the indicated antibodies: Abcam: acetylcholinesterase (ChAT); Cell Signaling Technology: cleaved caspase-3; Covance: microtubule-associated protein (MAP2); Jackson ImmunoResearch Labs: goat anti-rabbit FITC and goat anti-mouse Cy3 antibodies; Millipore: amyloid precursor protein (APP, mab314); Santa Cruz Biotechnologies: MDMx (H-130); Sigma: actin (A2066); ThermoFisher Scientific: horse radish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies. The following vendors were used for the indicated chemical reagents: Bachem: A β 1–40 peptide; Bio-Rad: Bradford protein assay dye, Polyvinylidene fluoride (PVDF) membrane, prestained broad range molecular weight ladder; Life Technologies: 4–12% Bis-Tris gels, Dulbecco's Modified Eagle's Medium (DMEM), Neurobasal media and B27 supplement; Peptide International: Poly-L-Lysine; Scytek Labs: Normal Antibody Diluent; Sigma: bovine serum albumin (BSA), Cytosine β -D-arabinofuranoside, dithiothreitol (DTT), Fast Green FCF, protease inhibitor cocktail, phenylmethanesulfonylfluoride (PMSF), roscovitine, staurosporine; ThermoFisher Scientific: enhanced chemiluminescence (ECL) reagent; Tocris: Q-VD-OPh.

2.2. Animals

Female hemizygous Tg2576 mice harboring the amyloid precursor protein Swedish (APP_{swe}) mutation (K670N/M671L) and female wild-type (w.t.) mice of the same genetic background (BL6SJL/J) were used at the indicated ages. Female animals were used due to differences in amyloid pathogenesis previously observed in the Tg2576 line [44]. All animals were housed at the University of Pennsylvania the Perelman School of Medicine in accordance with the Institutional Animal Care and Use Committee (IACUC) protocols. Whole cell protein lysates were prepared from the frontal cortices using RIPA buffer (10 mM Tris-Cl, pH 8.0, 1 mM ethylenediamine tetraacetic acid (EDTA), 0.5 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl) supplemented with PMSF and the protease inhibitor cocktail, followed by brief sonication to dissociate tissue and centrifugation at $40,000 \times g$ for 30 min at 4°C. Bradford assay was used to determine the protein concentration in the collected supernatants, and were stored at -80°C until immunoblotting.

2.3. Primary Neuronal Cultures

Rat embryos from embryonic day 17 were harvested from timed-pregnant Sprague-Dawley adults euthanized by CO₂ inhalation and cervical dislocation, in accordance with the Institutional IACUC protocols. Primary cortical neuroglial cultures were prepared, as described previously, and cells were plated at a density of 2×10^6 cells in 60 mm petri dishes or at a density of 1×10^5 cells per well in 96-well tissue culture plates coated with poly-L-lysine [45]. The cultures were maintained in neurobasal media with B27 supplement

at 37°C with 5% CO₂. 10 µM Cytosine β-D-arabinofuranoside was added to cultures 72 hours post-plating to deplete non-neuronal cells, and the media was replenished with 50% fresh media every 7 days until treatments.

2.4. In vitro amyloid-β toxicity model

Aβ 1–40 was prepared as described previously [46]. Briefly, Aβ 1–40 peptide was reconstituted in 1% NH₄OH, and immediately diluted to 1 mg/ml in sterile PBS to be stored at –20°C until use. For treatments, the peptide solution was first diluted to 0.2 ml/ml in neurobasal media, was vortexed and sonicated for 1 min, followed by incubation under gentle agitation for 48 hours at 37°C, to induce aggregation and formation of higher-molecular weight oligomers. The resultant solution was used for treatments of primary neuronal cultures at a final concentration of 10 µM.

2.5. Immunoblotting

Immunoblotting for *in vitro* studies was performed as described previously. Briefly, primary neuronal lysates were collected by scraping cells in lysis buffer containing 50 mM Tris-HCl, 200 mM NaCl, 0.5% NP-40, 10% glycerol, 1 mM DTT, and 80 µM KCl. Following centrifugation at 20,000 × *g* for 20 min at 4°C, protein concentrations were determined using the Bradford method. 10–30 µg protein per treatment was separated via SDS-PAGE in 4–12% Bis-Tris gels under denaturing conditions, following standard protocols for immunoblotting. The membranes were visualized using ECL reagent and autoradiography.

2.6. Immunohistochemistry

Formalin-fixed/paraffin embedded tissue sections that are 6 µm thick were used to detect MDMx and ChAT expression using immunofluorescent detection, as described previously [43, 47]. Deparaffinization was performed using histoclear, endogenous peroxidase activity was blocked with 3% H₂O₂ in methanol and antigen unmasking was achieved with target retrieval solution at 95°C for 1 h. Sections were blocked with 10% normal goat serum in PBS, and primary antibodies at empirically defined dilutions (ChAT: 1:50 or MDMx: 1:1,000, MAP2: 1:1,000, DAPI: 1:4,000) were incubated overnight at 4°C. Tyramide amplification, described previously [47], was used for immunohistochemistry of ChAT. Slides were mounted in Citifluor AF1, and for each slide, 10 randomly selected areas at high-magnification (600X) were captured by laser confocal microscopy on a Bio-Rad Radiance 2100 equipped with Argon, Green He/Ne, Red Diode, and Blue Diode lasers (Biorad, Hercules, CA). The images were used to count MDMx- and ChAT-positive neurons in a double-blinded fashion. Averages are expressed as mean ± SEM.

2.7. Data analysis

An unpaired Student's *t*-test was used for comparisons between two groups. ANOVA with Tukey's post-hoc analysis was used for comparisons across multiple groups/conditions. All experiments shown were replicated in at least 3 independent experiments. Averages are expressed as mean ± SEM. Prism 5.0 (GraphPad; La Jolla, CA) was used for all data analysis. *p* values less than 0.05 were interpreted as statistically significant.

3. Results

3.1. MDMx protein expression is reduced in the frontal cortex of aged Tg2576 mice

We have previously demonstrated the loss of MDMx in the post-mortem frontal cortex of patients with HIV-Associated Neurocognitive Disorders (HAND), and in the cortex of macaques in a simian model of lentiviral encephalitis. In this study, we assessed whether a similar change in MDMx was occurring in an animal model of AD by determining MDMx protein levels in Tg2576 transgenic mice. These animals, which express the Swedish mutant APP (APP^{swe}), display age-dependent deficits in learning and memory, increased cortical A β _{1–40} and A β _{1–42} levels and amyloid plaque formation in the cortex and limbic system, thus recapitulating several clinical and neuropathological findings of AD [48–50]. We first performed immunofluorescent staining on formalin-fixed and paraffin-embedded slides from 14-month-old Tg2576 and wild-type (w.t.) mice to assess MDMx expression in the frontal cortex. As seen in images captured by confocal immunofluorescence microscopy in Figure 1A, we found that MDMx immunostaining was primarily localized to the soma and nuclei of cortical neurons that were labeled with an antibody for the microtubule-associated protein 2 (MAP2), in 14-month-old w.t. mice. However, there was a significant loss of MDMx expression within the neurons in the frontal cortex of 14-month-old Tg2576 mice compared to the w.t. mice of the same age (quantified in Figure 1B). Importantly, we did not find any significant differences in the number of MDMx-positive neurons between 4-month-old Tg2576 and 14-month-old w.t. mice, suggesting that the loss of MDMx in Tg2576 mice is due to the neuropathology arising from APP^{swe} mutation in aged mice, as reported previously. Further, we confirmed by immunoblotting that there was a significant decrease in the levels of MDMx protein in 14-month-old Tg2576 mice compared to w.t. mice of the same age (Figure 1C, D). Overall, these findings demonstrate that age-dependent amyloid pathology observed in Tg2576 mice is associated with reduced levels of MDMx in the frontal cortex.

3.2. Loss of MDMx-expressing cholinergic neurons in aged Tg2576 mice

In AD, the cholinergic neuronal population is preferentially lost in the frontal cortex and hippocampus [51]. To assess whether MDMx expression is also reduced in this neuronal population in Tg2576 mouse, we immunofluorescently labeled MDMx, choline acetyltransferase (ChAT) and nuclei (DAPI) (Figure 2A), and counted the number of ChAT-positive as well as ChAT/MDMx double-positive neurons in 4- and 14-month-old Tg2576 and 14-month-old w.t. mice (Figure 2B–D). We found a significant reduction in the number of cholinergic neurons in 14 month-old Tg2576 mice compared to 14-month-old w.t. and 4-month-old Tg2576 animals (Fig. 2B), paralleling data from AD patients [51]. In addition, we found a significant reduction in the number of ChAT/MDMx double-positive neurons in 14-month-old Tg2576 mice compared to 14-month-old w.t. and 4-month-old Tg2576 animals (Figure 2C). To account for the overall loss of ChAT-positive neurons occurring during aging in Tg2576 mice, we examined the percentage of ChAT/MDMx double-positive cells in these animals. As seen in Figure 2D, while there was a significant reduction in the percentage of ChAT-positive cells expressing MDMx in 14-month-old Tg2576 mice compared to w.t. mice of the same age, there was no significant change in the percentage of double-positive cells between 14-month-old w.t. and 4-month-old Tg2576 mice, suggesting

a loss of MDMx-expressing cholinergic neurons during aging in Tg2576 mice following the emergence of amyloid deposition extensively documented in this model.

3.3 A β -mediated MDMx degradation in neurons is caspase-dependent

Previous research in dividing cells and in neurons has shown that caspases can directly cleave MDMx at a DVPD caspase-recognition site located at amino acids 361–364, leading to its degradation [42, 52]. As extracellular A β has been shown to induce caspase activation in neurons, we hypothesized that A β -induced caspase activity leads to MDMx protein loss in primary neurons [18, 19, 46]. To this end, we utilized an *in vitro* model in which primary rat cortical neurons were treated with A β _{1–40} [46]. As seen in Figure 3A, 10 μ M A β treatment for 48 hours led to approximately 40% loss in MAP2-positive neurons. In these cultures, immunoblotting revealed that A β induced a reduction in MDMx protein over a similar time course (Figure 3B). Importantly, A β led to an increase in cleaved caspase-3, indicative of caspase activation, while pretreatment of neurons with Q-VD-OPh (OPH), a pan-caspase inhibitor blocked caspase activation and reversed the reduction in MDMx protein in A β -treated cultures (Figure 4A). We also determined that staurosporine, which induces apoptosis, similarly led to decreases in MDMx levels with concomitant increases in cleaved caspase-3 levels (Figure 4B) [53]. Together, these results confirm earlier reports showing that MDMx is degraded upon caspase activation, and further show that A β -mediated MDMx degradation in neurons is caspase-dependent.

4. Discussion

In our study, we show the loss in the number of MDMx-expressing neurons as well as a decrease in MDMx protein levels in the cortex of mice in a transgenic model of age-dependent amyloid pathology [49, 54]. A previous study observed a small, but significant increase in MDMx mRNA in patients with AD, but this change did not associate with changes in plaque density or Braak staging [55]. Our results suggest that MDMx regulation may be occurring primarily at the protein level in the CNS, as we and others have reported previously [42, 43]. The reductions in protein expression and increases in mRNA levels suggest that a compensatory mechanism may be at play. Future studies assessing the transcript and protein levels of MDMx in the Tg2576 mouse and postmortem AD human tissue will address this potential mechanism.

Our results indicate that the cholinergic neurons in the frontal cortex in mice express MDMx, and that this neuronal population, which is particularly vulnerable to damage in AD, is lost with age in Tg2576 mice [51]. Damage to, and the loss of, cholinergic neurons in mouse models of AD is consistent with previous observations [56]. Our study has started to identify MDMx loss in neurons in an age-dependent amyloid toxicity model, and our results suggest that A β may be inducing MDMx degradation in neurons of the frontal cortex, including cholinergic neuronal populations [51]. Future studies investigating the changes in cortical MDMx expression over the lifespan of these mice will reveal the temporal relationships between the loss of MDMx, amyloid pathology, and the cognitive and behavioral outcomes in this model.

We show, in agreement with previous studies, that A β induces caspase-mediated degradation of MDMx *in vitro*, and confirm that MDMx is a target in a variety of cellular stress signals. The mediator of MDMx degradation is dependent on the insult and the class of proteases subsequently activated by that stress, including caspases, calpains and the proteasome, as shown previously in neurons and in dividing cells [42, 43, 52]. Caspase and calpain activation are not mutually exclusive mechanisms leading to neuronal death, and our results suggest that MDMx degradation may be one point of crosstalk between these two pathways, as MDMx loss is associated with calpain-dependent neuronal damage [43, 53].

Several models have demonstrated pro-survival functions of MDMx in neurons. The genetic deletion of MDMx results in embryonic lethality with severe CNS defects [40], and siRNA knockdown leads to neuronal apoptosis [42]. In addition, we recently reported that overexpression of MDMx in primary neurons confers partial protection from excitotoxic death [43]. Importantly, we have shown that primary neurons treated with SJ-172550, a pharmacological inhibitor that blocks MDMx-p53 interaction, undergo a calpain-dependent neuronal death, [43]. Benosman et al. have shown that downregulation of MDMx led to increased p53 transcriptional activity and resulted in neuronal death, while Soares et al. recently demonstrated that the interruption of MDMx:p53 interaction lead to mitochondrial membrane depolarization, accumulation of reactive oxygen species and apoptosis in dividing cells [57]. The dysregulation of p53 has been shown as a contributor to neuronal death *in vitro* and *in vivo* [33]. Intriguingly, p53 can regulate the gamma-secretase complex [58] and the expression of APP [59]. All together, these suggest MDMx:p53 interaction as an important component of neuroprotective roles of MDMx, and raise the possibility that A β -induced MDMx loss can lead to increased p53 activity, leading to altered APP expression and/or processing, and perpetuating a progressively increasing production of A β . Thus, future studies investigating the roles of MDMx in different subcellular compartments and downstream pathways, including p53-mediated apoptotic pathways, in cycling cells and postmitotic neurons are needed to address these reported differences.

5. Conclusion

Our studies show a loss of MDMx in a murine model of amyloid-mediated neurodegeneration, as well as caspase-mediated MDMx loss in an *in vitro* model of AD, and further demonstrate the loss of MDMx protein in neuronal populations affected in human disease. Further understanding of the role of MDMx in neuronal survival will provide novel CNS-specific targets for the development of neuroprotective interventions in AD and other neurodegenerative diseases.

Acknowledgments

This work was supported by the following grants: NIH NS41202 (K.J-S) and F31-NS074942 (C.W.Z).

We would like to thank Margaret Maronski for the preparation of primary neuronal cultures. We thank Drs. Michelle Erickson and Patrick Gannon for critical discussions. We also thank Drs. Virginia M.-Y. Lee and Jenna Carroll, and the Center for Neurodegenerative Disease Research (CNDR) at the University of Pennsylvania for tissue samples and helpful discussions.

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Highlights

- Age-dependent reduction in MDMx levels in cholinergic neurons in Tg2576 murine model of AD
- Caspase-dependent degradation of MDMx in neurons exposed to amyloid beta in vitro

Figure 1. MDMx expression is reduced in the frontal cortex of aged Tg2576 mice

A. Formalin-fixed, paraffin-embedded tissue sections from the frontal cortex of w.t. and Tg2576 mice from indicated ages were immunostained for MDMx (green), MAP2 (red), and DAPI (blue). Sections were visualized by laser confocal microscopy and images were quantified for MDMx expression. Representative images of one case per group are shown.

B. Quantification shows the reduction in MDMx-positive neurons in 14-month old Tg2576 mice compared to 14-month-old w.t. and 4-month-old Tg2576 group. Values are expressed as mean $\pm$ S.E.M. Significance determined by One-Way ANOVA with Tukey's post-hoc analysis, *** p < 0.001, ns: not significant, n = 5/group.

C. Whole cell tissue lysates prepared from fresh-frozen tissue sections from the frontal cortex of 14-month-old w.t., 14-month-old and 4-month-old Tg2576 mice were immunoblotted for MDMx, APP and actin (loading control).

D. Quantification of MDMx expression. Values are expressed as mean $\pm$ S.E.M. Significance determined by Student's t-test, ** p < 0.01.

Figure 2. Loss of MDMx-expressing cholinergic neurons in aged Tg2576 mice

Formalin-fixed, paraffin-embedded tissue sections from the frontal cortex of w.t. and Tg2576 mice from indicated ages were immunostained for MDMx (green), ChAT (red), and DAPI (blue). Representative images captured laser confocal microscopy from one case per group are shown. **B.** Quantification shows a significant reduction in the number of ChAT(+) neurons in 14-month old Tg2576 mice compared to 14-month-old w.t. mice. **C.** There is a significant reduction in the number of ChAT/MDMx double-positive (+) neurons in 14-month old Tg2576 mice compared to 14-month-old w.t. mice. **D.** The analysis shows that the percentage of ChAT/MDMx double-positive neurons are significantly reduced in 14-month-old Tg2576 mice compared to 14-month-old w.t. as well as 4-month-old Tg2576 mice. Values are expressed as mean $\pm$ S.E.M. Significance determined by One-Way ANOVA with Tukey's post-hoc analysis, ** $p < 0.01$, *** $p < 0.001$, ns: not significant, $n = 5$ /group.

Figure 3. Aβ induces MDMx degradation in primary neurons

A. Primary rat cortical neurons at 14 days in vitro (DIV) were treated with 10μM Aβ or vehicle for 24 hours, followed by immunostaining for MAP2 to assess neuronal viability. Values are expressed as mean $\pm$ S.E.M. Significance was determined by One-Way ANOVA with Tukey's post-hoc analysis, *** p <0.001. **B.** 14DIV pure cortical neurons were treated with 10μM Aβ, 10μM NMDA or vehicle for 4, 24 or 46 hours and whole cell protein were collected for immunoblotting (n=2, vehicle: PBS).

Figure 4. Aβ-mediated MDMx loss is caspase-dependent

A. 14DIV primary cortical neurons were pre-treated with 50 μM Q-VD-OPh (OPH) for 2 hours, followed by treatment with 10 μM Aβ for 24 hours. Whole cell protein lysates were immunoblotted for MDMx, cleaved caspase-3 and actin. A representative blot from three biological replicates is shown. **B.** 14DIV primary neurons were treated with 1 μM staurosporine for 2, 8 or 16 hours, followed by immunoblotting of whole cell protein lysates for MDMx, cleaved caspase-3 and actin. A representative blot from two biological replicates is shown.