Published in final edited form as:

Mol Cell Neurosci. 2008 December ; 39(4): 539–548. doi:10.1016/j.mcn.2008.07.028.

Differential effects of Usp14 and Uch-L1 on the ubiquitin proteasome system and synaptic activity

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Abstract

The ubiquitin proteasome pathway has been implicated in the pathogenesis of many neurodegenerative diseases, and alterations in two different deubiquitinating enzymes, Uch-L1 and Usp14, result in neurological phenotypes in mice. We identified a new mutation in Uch-L1 and compared the roles of Uch-L1 and Usp14 in the ubiquitin proteasome system. Deficiencies in either Uch-L1 or Usp14 result in decreased levels of ubiquitin, suggesting that they both regulate ubiquitin stability in the nervous system. However, the effect of ubiquitin depletion on viability and onset of symptoms is more severe in the Usp14-deficient mice, and changes in hippocampal synaptic transmission were only observed in Usp14-deficient mice. In addition, while Usp14 appears to function at the proteasome, Uch-L1 deficiency resulted in up-regulation of lysosomal components, indicating that Uch-L1 and Usp14 may differentially affect the ubiquitin proteasome system and synaptic activity by regulating different pools of ubiquitin in the cell.

Introduction

The ubiquitin proteasome system is essential for the selective degradation of intracellular proteins (Glickman and Ciechanover, 2002). Proteins that are selected for degradation by the proteasome acquire a poly-ubiquitin side chain which allows them to be trafficked to and engaged by the proteasome (Johnson et al., 1995). Once bound to the proteasome, the ubiquitinated substrate is unfolded, deubiquitinated and threaded into the proteasome, where it is cleaved into small peptides. In contrast to cytosolic proteins, many membrane proteins are trafficked to lysosomes following mono-ubiquitination, which provides a signal that can function for sorting at the trans-Golgi network and at the plasma membrane (Piper and Luzio, 2007).

The mammalian genome encodes over 90 deubiquitinating enzymes (Nijman et al., 2005). These proteases are thought to play important roles in modulating ubiquitin signaling by controlling ubiquitin chain length, processing of ubiquitin fusion proteins and recycling of

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ubiquitin from proteins targeted to the proteasome (Lam et al., 1997; Guterman and Glickman, 2004). Deubiquitinating enzymes are divided into five families based on the structure of their catalytic domains. These families consist of the ubiquitin-specific proteases (USPs), ubiquitin C-terminal hydrolases (UCHs), Otubain proteases (OTUs), Machado-Joseph disease proteases (MJDs) and a subset of metalloproteases (Nijman et al., 2005).

Very few animal models exist to study the function of deubiquitinating enzymes *in vivo* (Wilson et al., 2002; Saigoh et al., 1999; Ritche et al., 2002; Niendorf et al., 2007). In two of these models, mutations in either *Uch-L1* in *gad* mice or *Usp14* in *ax^J* mice result in neuromuscular disease (Saigoh et al., 1999; Wilson et al., 2002) and destabilization of ubiquitin in the nervous system (Osaka et al., 2003; Anderson et al., 2005).

The *ax^J* mutation disrupts expression of the proteasomal ubiquitin-specific protease Usp14 (Wilson et al., 2002). The *ax^J* mice first present with ataxia and tremor at 3 weeks of age. By 6 weeks of age, the mice are no longer mobile and exhibit reduced muscle mass and hind limb rigidity. The *ax^J* mice also show depressed levels of ubiquitin in the nervous system (Anderson et al., 2005). While the mechanism for ubiquitin loss in the Usp14 deficient mice is not known, work on the Usp14 orthologue in yeast (Legget et al., 2002; Hanna et al., 2003) and on Usp14 in mice (Anderson et al., 2005; Crimmins et al., 2006) indicates that increased ubiquitin turnover occurs at the proteasome. The loss of ubiquitin in the *ax^J* mice does not result in any significant increase in neuronal cell death or deposition of ubiquitin protein aggregates. Instead, loss of Usp14 results in synaptic transmission abnormalities in both the peripheral and central nervous systems (Wilson et al., 2002). The *ax^J* mice die by 8 weeks of age.

The *gad* mouse contains a deletion of exons 7 and 8 of the *Uch-L1* gene (Saigoh et al., 1999). These mice display sensory ataxia by 3 months of age and motor ataxia by 4 months of age, which correlates with the formation of spheroid bodies in axon nerve terminals of the gracile nucleus (Mukoyama et al., 1989). Ubiquitinated deposition of synuclein has also been observed in the gracile nucleus of the *gad* mice (Wang et al., 2004). Further analysis of these mice demonstrated that loss of Uch-L1 results in decreased levels of ubiquitin (Osaka et al., 2003). In addition, when Uch-L1 is over-expressed in cell culture, the level of monomeric ubiquitin is also increased in the cells (Osaka et al., 2003). The mechanism underlying ubiquitin loss in the *gad* mice has been suggested to be due to lysosomal degradation, which is consistent with findings linking Uch-L1 to lysosomal storage diseases (Osaka et al., 2003; Liu et al., 2002; Bifsha et al., 2007).

In this report, we describe a new mouse mutation, *nm3419*, in the *Uch-L1* gene that results in loss of Uch-L1 protein expression and subsequent neurological defects. As our research focus is on the role of deubiquitinating enzymes in the nervous system, the identification and characterization of this new *Uch-L1* mutation presented our laboratory with a unique opportunity to compare the biochemical and physiological changes that occur following the loss of two different deubiquitinating enzymes on the neuronal ubiquitin proteasome system.

Results

nm3419 mice contain a mutation in the Uch-L1 locus

The recessive neurological mutation *nm3419* spontaneously arose on a BALB/cJ mouse line at the Jackson Laboratory and the mice begin to exhibit signs of motor ataxia by 4 weeks of age. To identify the mutated gene associated with the neurological phenotype, we constructed a genetic map of the *nm3419* interval by outcrossing the *nm3419* mutation to C57BL/6J mice. Linkage analysis of the offspring localized the *nm3419* mutation to a 19.2 cM region on mouse chromosome 5 (Fig. 1a). The physical distance separating the closest proximal and distal markers was determined to be 30 Mb (Fig. 1b), which defined the *nm3419* critical region. Since

this genomic region contained the *Uch-L1* gene (Fig. 1b), we examined the *Uch-L1* gene in the *nm3419* mice for any DNA sequence changes. Sequence analysis of the *Uch-L1* PCR products showed that part of exon 6 was deleted and that intron 6 sequence could be found within the *Uch-L1* cDNA (Fig. 2a). Subsequent genomic PCR analysis revealed a 795 bp intragenic deletion that results in the removal of the final 24 bp of exon 6 and the first 771 bp of intron 6 from the *Uch-L1* gene in the *nm3419* mice (Fig. 2b and c). As a result of the mutation, 99 bp of intron 6 are included in the resulting mRNA and a cryptic splice donor within intron 6 is used to splice the mRNA to exon 7. The included intron 6 sequence encodes 13 novel amino acids before a premature termination codon is encountered, resulting in the loss of the final 78 amino acids of the Uch-L1 protein.

Phenotypic analysis of *nm3419* mice

To begin our studies of the *nm3419* mice, we first analyzed growth, survival and motor coordination of the mice compared with wild type controls. Homozygous *nm3419* mice were born in Mendelian fashion and no changes in viability were detected until approximately 80 days after birth (Fig. 3a). All homozygous *nm3419* mice died within 200 days after birth. The decline in viability of the *nm3419* mice correlated with a significant decrease in body mass (Fig. 3b).

In order to determine the onset of motor defects in the *nm3419* mice and measure changes in motor coordination compared with wild type controls, we examined the ability of the mice to traverse an elevated beam (Fig. 3c). At 4 weeks of age, we began to observe an increase in the number of falls from the elevated beam in the *nm3419* mice as compared to controls. This loss of coordination continued to increase with age and, by 16 weeks of age, the *nm3419* mice could no longer cross the elevated beam (Fig. 3c).

nm3419 results in loss of Uch-L1 expression and activity

In order to determine the effect of the genomic deletion on Uch-L1 expression, we performed immunoblot analysis to measure the level of Uch-L1 in the *nm3419* mice compared to wild type controls. A protein band that was immuno-reactive with Uch-L1 polyclonal sera was detected in extracts from the cerebrum of wild type and *ax^J* mice, but was absent in the extracts from the *nm3419* mice (Fig. 4a).

To determine if loss of Uch-L1 altered the activity of other deubiquitinating enzymes or increased the levels of active deubiquitinating enzymes in the *nm3419* mice, we performed labeling studies using a Ub-VME active site probe for deubiquitinating enzymes. This probe covalently modifies active deubiquitinating enzymes within the extract with an HA tag. Examination of Ub-VME treated extracts from wild type brains revealed several HA reactive bands, including one band which migrated at 25 kDa and was believed to be Uch-L1 (Fig. 4b). This band was not present in Ub-VME treated brain extracts from the *nm3419* mice, and there did not appear to be any newly labeled deubiquitinating enzymes or increased labeling of endogenous deubiquitinating enzymes in the *nm3419* extracts as compared to the extracts from wild type controls (Fig. 4b).

Altered expression of components of the ubiquitin proteasome system in *nm3419* and *ax^J* mice

To examine if the *Uch-L1* mutation alters the levels of ubiquitin in the *nm3419* mice, we compared the levels of ubiquitin from cerebrum extracts from wild type and *nm3419* mice (Fig. 4a). Since we have also shown that *Usp14*-deficient mice have depressed levels of ubiquitin in the nervous system (Anderson et al., 2005), extracts from *ax^J* mice were included as a control for decreased ubiquitin levels. Immunoblot analysis showed a 20% decrease in monomeric ubiquitin in the *nm3419* mice and a 35% reduction in the level of monomeric ubiquitin in the

ax^J mice compared to wild type controls (Fig. 4a), demonstrating that loss of either Uch-L1 or Usp14 results in destabilization of ubiquitin levels in mice. Similar to what we previously reported for the *ax^J* mice (Anderson et al., 2005), no changes were detected in the patterns of ubiquitinated protein conjugates between the *nm3419* and wild type mice (data not shown), indicating that there is not a major change in the global levels of ubiquitinated proteins in the mice.

The stability of substrates of the ubiquitin proteasome system has been shown to increase when ubiquitin levels become limiting. The regulatory subunit of cAMP dependent protein kinase (PKA) is one substrate whose stability has been suggested to be linked to ubiquitin levels (Hegde et al., 1997; Gong et al., 2006). To examine if the loss of ubiquitin in either the *nm3419* or *ax^J* mice induced stabilization of PKARII α , we measured the level of PKARII α in brain extracts from wild type, *nm3419*, and *ax^J* mice. No significant change in the level of PKARII α was observed in the hippocampus or cortex of either the *nm3419* or *ax^J* mice as compared to levels found in the brains of wild type controls (Fig. 4c).

To determine if expression of Usp14 and Uch-L1 may be regulated, in part, by the steady state levels of ubiquitin in neurons, immunoblots from wild type, *ax^J* and *nm3419* mice were examined for any compensating changes in Uch-L1 and Usp14 protein expression. While no increase in Uch-L1 expression was detected in the *ax^J* mice, there was a 15% increase in the level of full-length Usp14 in the *nm3419* brains as compared to wild type controls (Fig. 4a).

To examine if loss of Uch-L1 or Usp14 resulted in changes in expression of other deubiquitinating enzymes, we performed quantitative PCR (qPCR) on RNA from the brains of wild type, *nm3419*, and *ax^J* mice. Consistent with the protein results (Fig. 4a), qPCR analysis of *Usp14* and *Uch-L1* mRNA demonstrated that *Usp14* is up-regulated in the *nm3419* mice (Fig. 4d). No change in expression of *Uch-L3*, *Uch-L5*, or *Poh1* was detected in the *nm3419* mice or the *ax^J* mice compared to wild type controls (Fig. 4d). While no changes were detected in the level of Uch-L1 protein in the Usp14-deficient mice (Fig. 4a), we did detect a significant increase in the level of *Uch-L1* mRNA (Fig. 4d), suggesting that both *Usp14* and *Uch-L1* expression may be regulated, in part, by ubiquitin levels.

Loss of Uch-L1 does not lead to detectable changes in Ub-AMC hydrolysis levels

Uch-L1 is one of the most abundant proteins in neurons. To determine if genetic loss of *Uch-L1* eliminates a large proportion of the deubiquitinating enzyme activity measured by Ub-AMC hydrolysis, we compared Ub-AMC hydrolysis activity from hippocampal extracts from wild type and *nm3419* mice. Kinetic analysis of Ub-AMC hydrolysis rates demonstrated that there was robust Ub-AMC activity in wild type hippocampal extracts (Fig. 5a). Similar wild-type levels of Ub-AMC activity were detected from hippocampal extracts from *nm3419* heterozygous and homozygous mice (Fig. 5a), demonstrating that the Ub-AMC activity was not dependent on the amount of Uch-L1 found in the extracts (Fig. 5b). Both wild type and Uch-L1-deficient extracts were sensitive to the Ub-C-terminal hydrolase inhibitors NEM (Fig. 5a) and ubiquitin aldehyde (data not shown), demonstrating that the reactions were specific for cysteine proteases. Similar to the *nm3419* extracts, no change in Ub-AMC hydrolysis activity was detected in extracts from Usp14-deficient mice as compared to controls (data not shown), indicating that loss of Uch-L1 or Usp14 has no detectable effects on the total hippocampal Ub-AMC hydrolysis activity in the mice.

Loss of Uch-L1 does not lead to hippocampal synaptic defects in *nm3419* mice

Previous studies have indicated that protein turnover by the proteasome is an essential contributor to synaptic plasticity (DiAntonio et al., 2001; Cline, 2003; Speese et al., 2003; Zhao et al., 2003). To determine if loss of Uch-L1 alters hippocampal synaptic transmission

in *nm3419* mice, we measured paired-pulse facilitation (PPF) to examine short-term plasticity at the hippocampal Schaffer collateral synapses. These synapses were chosen for study because the CA3-CA1 hippocampal synapses are extremely well characterized and demonstrate forms of both short-term and long-term enhancement of synaptic activity. In addition, a recent report demonstrated that the *gad* mice have a deficit in theta burst induced long-term potentiation (LTP) at CA3-CA1 synapses (Sakurai et al, 2008). Examination of input/output curves demonstrated that there were no significant changes in baseline synaptic transmission in the *nm3419* mice as compared to wild type controls (Fig. 6a). In addition, the loss of Uch-L1 did not result in any significant differences in short-term synaptic plasticity, as measured by PPF, in the *nm3419* mice as compared to wild type controls for the five inter-stimulus intervals examined (Fig. 6b).

To examine long-lasting changes following synaptic activation, we measured LTP of hippocampal synapses using theta burst stimulation to induce LTP in wild type and *nm3419* hippocampal slices. As expected, there was a robust synaptic enhancement in the wild type mice that lasted over 1 h after stimulation (Fig. 6c). The hippocampal slices from the *nm3419* mice showed a similar strong enhancement of synaptic activity that also lasted over 1 h (Fig. 6c), indicating that loss of Uch-L1 does not impair LTP induced by theta burst stimulation.

Usp14 is essential for PPF but not theta burst induced LTP

Since our first description of the *ax^J* mice only analyzed LTP induction after high-frequency tetanic stimulation (Wilson et al., 2002), we decided to re-examine the *ax^J* hippocampal LTP using theta burst stimulation. Analysis of baseline synaptic transmission did not reveal any differences between controls and *ax^J* slices (Fig. 7a). As previously demonstrated (Wilson et al., 2002), there was a significant loss of PPF in the *ax^J* mice as compared to wild type controls (Fig. 7b). Following theta burst stimulation, there was a trend towards a reduction in the initial amount of post-tetanic potentiation (PTP) in the *ax^J* mice compared to wild type controls, although the difference was not statistically significant. However, no difference in the amount of theta burst LTP was observed in the *ax^J* mice relative to wild type controls 1 h after the stimulus (Fig. 7c). These results indicate that Usp14 and Uch-L1 are dispensable for induction of LTP at CA3-CA1 hippocampal synapses.

Usp14 and Uch-L1 are dispensable for maintenance of long-term depression (LTD) at CA3-CA1 synapses

The enduring weakening of synaptic activity by LTD, combined with LTP, is thought to underlie the cellular mechanisms of learning and memory. Since the *gad* mice have recently been reported to display learning and memory deficits (Sakurai et al., 2008), we investigated if Uch-L1 or Usp14-deficient mice have altered hippocampal LTD. Following low-frequency stimulation (1 Hz for 15 min), Schaffer collateral synapses in hippocampi from wild type mice demonstrated a significant reduction in the synaptic response, which stabilized at approximately 80% of baseline (Fig. 6d). The LTD from the *nm3419* mice was 82% of baseline (Fig. 6d) and from Usp14-deficient *ax^J* mice was 79% of baseline (Fig. 7d), suggesting that Uch-L1 and Usp14 were not required for the maintenance of LTD at the CA3-CA1 synapses. However, we did observe a significant difference in synaptic responses immediately following low-frequency stimulation in the *ax^J* mice (Fig. 7d).

Expression of proteasomal and lysosomal peptidases in *nm3419* and *ax^J* mice

To examine if the ubiquitin depletion in the *nm3419* or *ax^J* mice had an effect on either of the two major protein degradation pathways, we compared the expression of proteasomal and lysosomal components from *nm3419*, *ax^J* and wild type control mice (Fig. 8). qPCR of reverse-transcribed RNA from *nm3419* brains did not demonstrate any significant difference in

expression of either the 19S proteasome subunits Psmd2 and Psmc6 or the 20S proteasome subunits Beta1 and Beta 2 as compared to the levels of expression detected in the brains of wild type control mice (Fig. 8a). In contrast, when we examined the expression of these proteasomal components in the *ax^J* mice, there was a significant increase in the levels of the 20S Beta 1 and Beta 2 subunits compared to the levels found in wild type mice (Fig. 8a). No changes in the 19S proteasome subunits Psmd2 or Psmc6 were observed the *ax^J* mice.

When we measured the expression of the major lysosomal proteases expressed in neurons, no changes in the expression of cathepsins D and L were detected in the *ax^J* mice (Fig. 8b), and expression of cathepsin B was not significantly changed in either the *nm3419* or *ax^J* mice as compared to controls. In contrast, both the cathepsins D and L mRNAs were significantly increased in the *nm3419* mice as compared to controls (Fig. 8b), indicating that the alterations in the lysosomal proteases may be due to changes in the pool of ubiquitin that is utilized for lysosomal trafficking rather than a response to absolute levels of ubiquitin in the brain.

Discussion

The ubiquitin proteasome system has been shown to regulate numerous cellular functions by controlling protein degradation. Both increases and decreases in protein turnover have been implicated in several genetic forms of neurodegeneration (Alves-Rodrigues et al., 1998; Anderson et al., 2005; Rubinsztein, 2006; Miller and Wilson, 2003; Yi and Ehlers, 2007) and early effects of protein mishandling lead to changes in synaptic function. In some of these cases (Gilchrist et al., 2005), changes in the free ubiquitin pool have been implicated as the cause of the decreased protein turnover and subsequent synaptic abnormalities. In this study, we have identified a new mutation in *Uch-L1*, one of the gene products thought to regulate ubiquitin pools in neurons, and have analyzed the effect of loss of Uch-L1 on the ubiquitin proteasome system and synaptic function.

Although the phenotype of the *nm3419* mice is similar to the *gad* mice, we have noticed an earlier onset of motor system abnormalities in the *nm3419* mice compared to what has been reported for the *gad* mice (Mukoyama et al., 1989). The *nm3419* mice first display motor incoordination at 4 weeks of age, as measured by elevated beam analysis. Motor defects increased in severity over the next 4 weeks and, by 16 weeks of age, the mice were unable to successfully cross the elevated beam. By approximately 20 weeks of age, we observed a significant decrease in viability, and most of the *nm3419* homozygous mice died by 28 weeks of age.

In contrast to the Uch-L1-deficient mice, the neurological defects due to loss of the deubiquitinating enzyme Usp14 in the *ax^J* mice are much more severe. The *ax^J* mice are unable to cross the elevated beam by 3 weeks of age, and they die by 8 weeks of age. Therefore, whereas the *nm3419* mice should serve as a valuable model for examining chronic neurological disease due to impairment in the ubiquitin proteasome system, the *ax^J* mice may provide a model for examining the ubiquitin proteasome system during development.

The *nm3419* mutation appears to result in complete loss of expression of the Uch-L1 protein and, similar to what is observed in *gad* mice (Saigoh et al., 1999), the *Uch-L1* mutation in the *nm3419* mice results in approximately a 20% reduction in the levels of monomeric ubiquitin compared to controls. When we used activity based probes to determine if loss of Uch-L1 or Usp14 expression resulted in genetic compensation in other deubiquitinating enzymes, we observed increased levels of *Usp14* mRNA and protein in the Uch-L1 deficient mice and increased *Uch-L1* mRNA in the *ax^J* mice. These increases in *Usp14* and *Uch-L1* may be in response to lowered ubiquitin levels, as the orthologue of Usp14 in yeast has been shown to increase in expression upon ubiquitin depletion (Hanna et. al., 2007). In support of this idea,

qPCR analysis of deubiquitinating enzymes not believed to participate in ubiquitin stability did not show any significant alterations in expression in the *nm3419* or *ax^J* mice as compared to controls. Therefore Usp14 and Uch-L1 may be coupled to transcriptional programs that regulate ubiquitin levels in neurons. While their exact role is unclear, it is likely that both proteins are involved in key roles in maintaining ubiquitin homeostasis by controlling ubiquitin turnover.

Recent studies on drug inhibitors of Uch-L1 have indicated that Uch-L1 is responsible for greater than 60% of the deubiquitinating enzyme activity in the hippocampus, as measured by Ub-AMC hydrolysis (Gong et al., 2006). We therefore examined the Ub-AMC hydrolysis activity in the hippocampus of wild type, *nm3419* heterozygous and *nm3419* homozygous mice. In these studies, no Uch-L1 expression was seen in the homozygous *nm3419* mice and the level of Uch-L1 was reduced 50% in the heterozygous *nm3419* mice compared to controls. However, there was no correlation between the levels of Ub-AMC hydrolysis and the amount of Uch-L1, indicating that either Uch-L1 is not the major deubiquitinating enzyme contributing to the observed Ub-AMC hydrolysis activity in the hippocampus, or the genetic compensation observed in the *nm3419* mice has restored the deubiquitinating activity level back to control levels. Another possibility for the discrepancy between our genetic ablation studies and the drug inhibition studies of Uch-L1 may be that the LDN57444 drug inhibitor that was used to inhibit Uch-L1 (Gong et al., 2006) may act on other deubiquitinating enzymes and not be specific for Uch-L1.

Our studies are consistent with previously published results that demonstrate that loss of Uch-L1 causes a decrease in ubiquitin levels in neurons (Osaka et al., 2003). Since the ubiquitin proteasome system has been shown to be required for the maintenance of LTP (Fonseca et al., 2006), we examined several forms of synaptic plasticity in the hippocampus of *nm3419* mice. In contrast to recent reports demonstrating LTP defects in the *gad* mice (Sakurai et al., 2008), no differences in short-term or long-term plasticity were detected between wild type and *nm3419* mice. The lack of a theta burst induced LTP deficit at synapses in the *nm3419* hippocampi could be due to numerous subtle differences in experimental protocol or possible differences in the genetic background between the *nm3419* and *gad* mice. However, since Uch-L1 is likely to function at the level of protein stability and is located in the cytoplasm, it is unclear how loss of Uch-L1 in the *gad* mice would directly affect the transcription-dependent phase of LTP.

While we saw no alterations in short-term plasticity in the *nm3419* mice, the *ax^J* mice demonstrated changes in short term plasticity similar to what we previously reported (Wilson et al, 2002) in that there was a loss in PPF at the Schaffer collateral synapses from *ax^J* mice compared to controls. Because changes in short-term plasticity are usually presynaptic (Dobrunz and Stevens, 1997; Zucker and Regehr, 2002), this result is consistent with a required function for Usp14 in the presynaptic neuron. The amount of short-term potentiation following the theta burst stimulation was also reduced in the *ax^J* mice, although the effect was not statistically significantly. While our previous studies demonstrated depressed post-tetanic potentiation following 100 Hz tetanic stimulation (Wilson et al, 2002), the absence of a change in short-term plasticity after LTP induction in this study is likely to be due to differences in the stimulus used for LTP induction. Interestingly, we did observe an increased short-term depression of synaptic transmission in the *ax^J* mice following the low-frequency stimulation used to induce LTD. These changes in synaptic short-term plasticity in the *ax^J* mice are likely to be caused by presynaptic changes, such as alterations in the synaptic release machinery, as opposed to changes in the post-synaptic neuron. Further studies will be required to determine how loss of Usp14 results in these particular synaptic abnormalities.

When we examined the *nm3419* mice for compensating changes in components of the ubiquitin proteasome system, we only found changes in the level of *Usp14* mRNA. In contrast, we observed significant increases in mRNA expression in the lysosomal proteases *CathepsinD* and *CathepsinL* in the *nm3419* mice compared to wild type controls. Previous studies have shown that Uch-L1 can prevent apoptosis in cells treated with lysosomal protease inhibitors (Bifsha et al., 2007). In addition, Uch-L1 has been shown to have ubiquitin ligase activity and can generate K-63 linked side chains which have been shown to be a signal for degradation of membrane proteins by the lysosome (Liu et al., 2002). Since ubiquitination/deubiquitination of proteins has been suggested to modulate the transport of proteins through the endosome, our data and those of others (Bifsha et al., 2007; Liu et al., 2002) support a possible role for Uch-L1 in ubiquitin signaling in the lysosomal pathway. Using ubiquitin transgenic mice, we are currently examining if restoration of ubiquitin levels can correct the motor defects observed in the *nm3419* mice.

Unlike Uch-L1, Usp14 is a proteasome-associated deubiquitinating enzyme. Our qPCR studies showing up-regulation of 20S proteasome components are consistent with Usp14 functioning at the level of the proteasome. This up-regulation of the 20S proteasome components may compensate for a decreased rate of protein turnover due to ubiquitin loss in the *ax^J* mice. This idea is consistent with the recent observation that loss of ubiquitin in yeast results in alterations in proteasome levels (Hanna et al., 2007).

The data from this study clearly demonstrate the importance of deubiquitinating enzymes in the nervous system. The finding that loss of both Uch-L1 and Usp14 result in neurological phenotypes demonstrates the sensitivity of the nervous system to altered ubiquitin metabolism. However, the outcome of these mutations is much more severe in the Usp14-deficient mice. One possible explanation for the increased severity of the Usp14 mutation is that Usp14 and Uch-L1 may function at different times during the life span of the animal. Usp14 and Uch-L1 may also function in different subcellular regions of the neuron and therefore control the stability of different pools of ubiquitin. For example, changes in synaptic ubiquitin levels could explain the presence of synaptic transmission defects in the *ax^J* mice. While this paper supports an essential role for deubiquitinating enzymes in modulating ubiquitin stability, these enzymes are also likely to have essential roles in modulating substrate stability. The identification of possible protein substrates for these and other deubiquitinating enzymes will be an important next step in understanding the role of protein deubiquitination in neurons.

Experimental Methods

Animals

Wild type C57BL/6J, *Usp14^{ax^J}* and *Uch-L1^{nm3419}* mice (Jackson laboratories, Bar Harbor, ME, USA) have been maintained in our breeding colony at the University of Alabama at Birmingham, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Homozygous *Usp14^{ax^J}* mice (which we refer to as *ax^J* mice) were generated by intercrossing *ax^J/+* siblings and could be phenotypically identified by 3 weeks of age. Homozygous *Uch-L1^{nm3419}* mice were generated by intercrossing heterozygous Uch-L1 siblings. All biochemical and electrophysiological studies were completed using *Uch-L1^{nm3419}* that had been maintained on a C57BL/6J background. All research complied with the United States Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals, and adhered to principles stated in the *Guide for the Care and Use of Laboratory Animals*, United States National Research Council.

Mapping of the nm3419 mutation

To map the *nm3419* mutation, *nm3419*/+ heterozygous mice were intercrossed to generate F2 animals for genetic linkage studies. Tail DNAs from 12 affected and 14 unaffected F2 animals were genotyped for 148 small nucleotide polymorphisms (SNPs). All affected animals show linkage to SNP rs3691920 on chromosome 5. Primers 1 TGGACGGCTGTGTGTGCTAATG and primer 2 GTCATCTACCTGAAGAGAGCCAAG flank the genomic deletion of Uch-L1 in the *nm3419* mice and were used for genotyping.

Elevated beam assay

Assays were performed as described in Stanley et al. (2005). Mice were placed in the middle of a 60 cm rod that was 2 cm in diameter and was elevated 30 cm above the bench by metal supports. Mice were allowed to walk to a platform located at the end of the beam. Each trial consisted of 5 repetitions of this assay. Mice were videotaped to quantitate the number of falls and foot slips (one or both hindlimbs slipped from the beam) for each trial.

Antibodies

Antibodies to Usp14 were described previously (Anderson et al., 2005). Usp14 polyclonal antisera from rabbit 4457 was used for immunoblotting. The Uch-L1 AB1761 antibody and the PKARII α AB1613 antibody were purchased from Chemicon (Temecula, CA, USA). Mab1510, which was used to recognize monomeric ubiquitin, was purchased from Chemicon. Antibodies PW8805, which recognizes poly-ubiquitinated conjugates, and PW8810, which recognizes both poly- and mono-ubiquitinated conjugates, were purchased from Biomol (Plymouth Meeting, PA, USA). The anti-HA Mab 12CA5 was purchased from Roche (Indianapolis, IN, USA). Monoclonal antibodies to GAPDH (Abcam, Cambridge, MA, USA) and Actin (Developmental Hybridoma bank, Iowa City, IA, USA) were used as controls.

Isolation of mouse proteins

Mice 4 to 6 weeks of age and of appropriate genotype were sacrificed by CO₂ asphyxiation. Tissues were processed as described (Gong et al., 2006). Briefly, tissues were homogenized in 1 to 3 mL of T-PER tissue extraction buffer from Pierce (Rockford, IL, USA) containing Complete protease inhibitors from Roche. Following homogenization, tissues were sonicated for 10 s and then centrifuged at 17000 g for 10 min at 4°C. Supernates were removed and immediately frozen at -80°C. Protein concentrations were determined by using the Bicinchoninic Acid (BCA) protein assay kit from Pierce.

Immunoblotting

Proteins were resolved on either 4-12% Bis-Tris gels or 4-20% Tris-Glycine NUPAGE gels (Invitrogen, Carlsbad, CA, USA) and transferred onto either nitrocellulose or PVDF membranes. Primary antibodies were diluted in PBS containing 3% BSA at a dilution of 1:1000 for Usp14, 1:2000 for ubiquitin Mab1510, 1:2,000 for Uch-L1 and 1:5,000 for the HA antibody. Primary antibodies were detected using an anti-mouse or anti-rabbit HRP conjugated antibody (Southern Biotechnology Associates, Birmingham, AL, USA) and Luminol reagents (Pierce).

Quantitation of immunoblots

Blots were scanned using a Hewlett Packard Scanjet 3970 and quantitated using UN-SCAN-IT software (Orem, UT, USA). Each value represents the average and standard error from six blots using three different wild type and mutant animals.

Ub-AMC hydrolysis

Ubiquitin-AMC (Boston Biochem, Boston, MA, USA) was used for measuring deubiquitinating enzyme activity from the T-PER protein extracts mentioned above. Briefly, 10 μ g of hippocampal extract was diluted into UCH reaction buffer (50 mM Tris-HCL, pH 7.6, 0.5 mM EDTA, 5 mM DTT, and 0.5 mg/mL ovalbumin). To start the reaction, Ub-AMC was added to a final concentration of 400 nM. Fluorescence intensity was measured at an excitation wavelength of 380 nm and an emission wavelength of 460 nm at 37°C. Intensity was recorded at 1 minute in tervals for 30 minutes. N-ethylmaleimide (Sigma, St. Louis, MO, USA) was added at a final concentration of 10 mM as a negative control for Ubiquitin-AMC.

Labeling of deubiquitinating enzymes

Deubiquitinating enzymes were labeled as previously described (Borodovsky et al., 2002). Briefly, approximately 10 μ g of total protein extract was diluted in a solution containing 50 mM Tris, pH 7.5, 250 mM Sucrose, 1 mM DTT and 2 mM ATP. One microliter of a 0.2 μ g/ μ L solution of HA-Ub-VME was then added and the reaction was incubated at room temperature for 10 min to label active deubiquitinating enzymes. SDS sample buffer was then added and the reaction was boiled for 5 min. Proteins were resolved on a 4-12% Bis-Tris NUPAGE gel and were then transferred onto PVDF membranes. The labeled deubiquitinating enzymes were detected by probing the blot with the 12CA5 anti-HA monoclonal antibody (Roche) and were visualized using Luminol reagents (Pierce).

Quantitative PCR

Total RNA was isolated using RNA-STAT60 (Tel-Test, Friendswood, TX, USA) and 2 μ g of the RNA was then reverse transcribed using the Applied Biosystems GeneAmp Gold RNA PCR Reagent Kit (Foster City, CA, USA). Real-time PCR reactions were set up in triplicate using TaqMan gene assays and amplified in an Applied Biosystems Step-One instrument. $\Delta\Delta$ CCT curves were generated using 18S TaqMan gene assays as internal standards. Quantitative PCR results are shown as the standard deviation of 3 different amplifications from RNA that was reverse transcribed from 3 different mice. Individual gene assay kits were purchased from Applied Biosystems for each of the RNAs analyzed. Mm00495900_m1 (Uch-L1), Mm03040818_s1 (Uch-L3), Mm00497950_m1 (Uch-L5), Mm00451955_m1 (Poh1), Mm00458097_m1 (Usp14), Mm00663052_g1 (Psmc2), Mm00458820_m1 (Psmc6), Mm00515587_m1 (CtsD), Mm00515597_m1 (CtsL), Mm01310506_m1 (CtsB), Mm00650840_m1 (Beta 1), Mm00449477_m1 (Beta 2). Paired t-tests were conducted on relative quantity (RQ) values for each group to determine their significance.

Hippocampal slice preparation

Acute hippocampal slices were prepared from 4 to 6 week-old mice as previously described (Sun et al., 2005; Speed and Dobrunz, 2008). Mice were anesthetized with isoflurine, decapitated, and their brains were removed rapidly. 400 μ m coronal slices were prepared using a Leica VT1000 vibrating microtome. Slicing and dissection of the hippocampi were done in ice-cold (1–3°C) dissecting solution containing the following (in mM): 120 NaCl, 3.5 KCl, 0.7 CaCl₂, 4.0 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 glucose, bubbled with 95% O₂/5% CO₂, pH 7.35–7.45. Slices were stored at room temperature in a holding chamber containing the dissecting solution and bubbled with 95% O₂/5% CO₂ for >0.5 h before recording.

Slice Electrophysiology

Experiments were performed in a submersion recording chamber. Slices were perfused with extracellular recording solution composed of (in mM) 120 NaCl, 3.5 KCl, 2.5 CaCl₂, 1.3 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, and 10 glucose. The solution was bubbled with 95% O₂-5% CO₂, and the pH was adjusted to 7.35 using NaHCO₃. Recordings were done at 24.5–

25.0°C (room temperature). Extracellular field potentials were elicited with 100- μ s duration pulses via tungsten microelectrodes placed in the stratum radiatum. Dendritic field excitatory post-synaptic potentials (fEPSPs) were recorded using glass micropipettes (2–4 M Ω) filled with extracellular solution (described in the preceding text).

Experimental stimuli were set to an intensity that evoked a fEPSP that had a left slope of 50% (for LTP), and 80% (for LTD) of the maximum fEPSP needed for population spike generation. LTP was induced with theta burst stimulation consisting of a train of 3 stimuli delivered at 100 Hz, repeated 10 times at 5 Hz. The entire stimulation was repeated 3 times with 15 s between each repetition. Synaptic efficacy was monitored at 0.1 Hz for 0.5 h prior to and 1 h following theta burst stimulation. Slices that did not exhibit stable fEPSP slopes during the first 10 min of recording were excluded from the study. Normalized fEPSP slope values were averaged and plotted as the percent change from baseline. Input-output curves were measured before LTP induction to assess baseline synaptic transmission. Paired-pulse facilitation was measured at various interstimulus intervals (50, 100, 150, and 200, and 500 ms). Long-term depression (LTD) was induced by low-frequency stimulation (1 Hz stimulation applied for 15 min). Synaptic efficacy was monitored at 0.1 Hz for 10 min prior to and 1 h following induction of LTD. Slices that did not exhibit stable fEPSP slopes during the 10 min prior to LTD induction were excluded.

Acknowledgments

The authors would like to thank Dr. Lori McMahon (University of Alabama at Birmingham) for experimental assistance with LTP and LDP and Dr. Daniel Finley (Department of Cell Biology, Harvard Medical School) and Dr. J. David Sweatt (University of Alabama at Birmingham) for their helpful suggestions. This work was supported by the NIH/NINDS Grant R01NS047533 and the NIH Neuroscience Blueprint Core Grant NS57098 to the University of Alabama at Birmingham.

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Figure 1.
(A) Meiotic and (B) physical linkage maps of the *nm3419* critical region showing positions of single nucleotide polymorphism markers near the *nm3419* mutation and the location of several genes in the *nm3419* critical region.

Figure 2. Intragenic deletion in the *Uch-L1* genomic locus in *nm3419* mice. Diagram of (A) the cDNAs and (B) genomic sequences for the *Uch-L1* gene in wild type (wt) and *nm3419* mice. The horizontal arrows indicate the sites of primer sequence used to genotype the *nm3419* mice and the vertical arrows delineate the boundaries of the deletion. (C) Genomic PCR of wild type (+/+), heterozygous *nm3419* (+/-) and homozygous *nm3419* (-/-) mice. Primers used for PCR flank the site of the *Uch-L1* deletion.

Figure 3. Phenotypic analysis demonstrates increased mortality, decreased body mass and significant motor defects in the *nm3419* mice. (A) Survival curves for wild type (wt) and *nm3419* mice (n=8). (B) Body weights of wild type (wt) and *nm3419* mice were obtained over a 20 week period (n=5). (C) Elevated beam assay demonstrating number of falls per trial for 4, 8 and 16 week-old wild type (wt) and *nm3419* mice (n=5).

Figure 4a-c

A.

B.

C.

D.

Figure 4d

Figure 4.

Altered gene expression in the *nm3419* and *ax^J* mice. (A) Left panel, Immunoblot analysis of Uch-L1, Usp14 and ubiquitin expression in brain extracts from 6 week-old wild type (wt), *nm3419* (*nm*) and *ax^J* mice. GAPDH was used as a control for loading efficiencies. Right panel, quantitation of Uch-L1, full-length Usp14 (upper band) and ubiquitin levels in brain extracts. (B) Active-site labeling of deubiquitinating enzymes from wild type (wt) and *nm3419* (*nm*) brain extracts. The preparations were incubated with HA-Ub-VME and probed with the anti-HA antibody 12C5. Molecular weight markers (in kDa) are shown to the left and the position of Uch-L1 is indicated to the right. (C) Immunoblot analysis of PKARII α from cortical and hippocampal extracts from 6 week-old wild type (wt), *nm3419* (*nm*) and *ax^J* mice. The blots were probed for Actin as a loading control. (D) Quantitative PCR showing levels of neuronal deubiquitinating enzyme RNA in brains of wild type (wt), *nm3419* (*nm*) and *ax^J* mice. The levels of each deubiquitinating enzyme RNA is shown as the relative quantity versus the amount detected in wt controls.

Figure 5.

Total Ub-AMC hydrolysis activity is not affected by Uch-L1 protein levels. (A) Ub-AMC hydrolysis activity of hippocampal extracts isolated from wild type (+/+), *nm3419* heterozygous (+/-) and *nm3419* homozygous (-/-) mice. Extracts (10 μ g) were incubated with the deubiquitinating enzyme substrate Ub-AMC, and hydrolysis was measured by increased fluorescence over time. The addition of 10 mM N-ethylmaleimide (NEM) completely inhibited the reaction. N=3. (B) Immunoblots of hippocampal extracts from wild type (+/+), heterozygous *nm3419* (+/-) and homozygous *nm3419* (-/-) mice probed for Uch-L1. Blots were probed for GAPDH as a loading control.

Figure 6.

Loss of Uch-L1 does not alter synaptic transmission at Schaffer collateral synapses in the hippocampus of 6 week-old *nm3419* mice. (A) Baseline synaptic transmission in 6 week-old wild type (wt) and homozygous *nm3419* mice are similar. No changes in input/output curves were observed when comparing wt (n=5 animals) and *nm3419* mice (n=4 animals; $P>0.05$). (B) Paired-pulse facilitation is normal in *nm3419* mice. No significant differences were observed in the PPF ratio for 5 different inter-stimulus intervals. (C) Long-term potentiation was induced using theta burst stimulation. The magnitude of short-term potentiation after the theta burst stimulation (time 1) was not different between *nm3419* and wt control mice ($P>0.05$). There was no difference in the amount of LTP (1 h after theta burst stimulation, time 2) in *nm3419* mice (n=5 animals) as compared to wt controls (n=4 animals; $P>0.05$). (D) Long-term depression was induced using low-frequency stimulation (1 Hz for 15 min). There was no difference in the magnitude of short-term depression following the low-frequency stimulation (time 1) or in the magnitude of LTD (time 2) in *nm3419* mice (n=5 animals) as compared to wt controls (n=5 animals; $P>0.05$).

Figure 7.

Loss of Usp14 alters synaptic transmission in the hippocampus. (A) Baseline synaptic transmission in 6 week-old wild type (wt) and *ax^J* mice are similar. No changes in input/output curves were observed when comparing wt (n=5 animals) and *ax^J* mice (n=4 animals; $P>0.05$). (B) Paired-pulse facilitation is depressed in *ax^J* mice. Significant changes were detected in the PPF ratio for the first four inter-stimulus intervals ($P<0.05$). (C) LTP was induced using theta burst stimulation. The magnitude of short-term potentiation immediately after the theta burst stimulation (time 1) was not different between *ax^J* mice (n=4 animals) and wt controls (n=5 animals; $P>0.05$). There was no difference in the magnitude of LTP measured 1 h after theta burst stimulation (time 2) between *ax^J* mice (n=4 animals) and wt controls (n=5 animals; $P>0.05$). (D) The *ax^J* mice have increased synaptic short-term depression immediately following LTD induction using 1 Hz stimulation for 15 min (time 1, $P<0.05$). There is no difference in the magnitude of LTD expression in *ax^J* mice (n=5 animals) measured 1 h after stimulation (time 2) as compared to wt controls (n=5 animals; $P>0.05$).

Figure 8.

Expression of proteasomal and lysosomal proteases in the $nm3419$ and ax^J mice. (A) Quantitative PCR of the two 20S proteasomal genes (Beta1 and 2) and two 19S proteasomal genes (Psmc2 and Psmc6) in brain extracts from 5 to 6 week-old wild type (wt), $nm3419$ and ax^J mice. (B) Quantitative PCR of the lysosomal proteases Cathepsin D (CtsD), L (CtsL), and B (CtsB) from brain extracts of 6 week-old wild type (wt), $nm3419$ and ax^J mice.