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Characterization And Functional Analysis Of USP14

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CHARACTERIZATION AND FUNCTIONAL ANALYSIS OF USP14

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ABSTRACT

The ubiquitin proteasome system (UPS) is essential for regulated protein degradation, a requirement for numerous neuronal process, including vesicle cycling, neurotransmitter release, spine morphology, and synaptic plasticity. Better understanding of UPS function in neurons will increase our knowledge of neurological diseases caused by alterations in ubiquitin signaling.

I utilized the *ataxia* (ax^J) mice as a tool to study the UPS in the nervous system. The ax^J mice have lowered expression of ubiquitin-specific protease 14 (Usp14) in all tissues; this decreased expression results from an intracisternal-A particle insertion into *Usp14*. The ax^J mice are phenotypically indistinguishable from wild type littermates at birth. By 2 weeks, ax^J mice are smaller in size and display a resting tremor. Muscle wasting and tremor become more pronounced until 6 weeks of age, and death occurs by 8 weeks. It was suggested that the ax^J phenotypes are caused by dystrophic Purkinje cell axons. However, the global loss of Usp14 indicates that the ax^J phenotypes result from multiple organ system failure.

To understand Usp14's function in neurons, I characterized the expression and activity of *Usp14* gene products**.** Loss of Usp14 results in a decrease in steady state levels of monomeric ubiquitin. Because Usp14's catalytic activity is stimulated by proteasome binding, loss of Usp14 on the proteasome implies altered proteasome function; evidence of which is strengthened by ubiquitin loss in ax^J mice. Furthermore, I specifically ex-

pressed Usp14 in *ax*^{J} neurons to test the hypothesis that the *ax*^{J} phenotype results from loss of Usp14 expression in these cells. Neuronal-specific expression of Usp14 rescued the premature death, muscle wasting, and tremor independently of Purkinje cells.

Here we demonstrate an essential requirement for Usp14 in the mammalian nervous system. Surprisingly, Usp14 appears dispensable in all tissues except neurons, indicating the UPS may have nervous system specific functions not required in other cell types. Our data also provide the first evidence for a primary defect in proteasome function as the underlying cause of a neurological disease in mammals. Through increasing our understanding of Usp14 in neurons, we can better understand UPS function in other diseases; illuminating new therapeutic targets for neurological disorders.

DEDICATION

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INTRODUCTION

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INTRODUCTION

The specific function of the ubiquitin proteasome system (UPS) is to provide the cell with a signaling pathway to control the activity of various cellular processes. The UPS accomplishes this task by covalently attaching ubiquitin to protein substrates. This post-translational modification can have multiple effects on a protein substrate; however, the best studied effect is regulated protein degradation by the 26S proteasome. The UPS derives its name from regulated protein degradation by the 26S proteasome despite the involvement of ubiquitin signaling independent of substrate degradation. By controlling protein turnover, the UPS directly influences such cellular processes as protein trafficking, receptor endocytosis, cell cycle control, synaptic growth and function, and DNA repair [1-8].

Early Studies on Ubiquitin Signaling

The initial studies on the 76 amino acid protein ubiquitin found that the protein could differentiate both T and B cells *in vitro* [9]. Goldstein *et al.* called this protein ubiquitous immunopoietic polypeptide (UBIP) because it was expressed in every tissue type they examined [9]. The group furthermore noted that ubiquitin is highly evolutionarily conserved both in structure and function. For example, UBIP purified from celery was able to induce the *in vitro* differentiation of B and T cells [9]. It was also found shortly thereafter that histone 2A is covalently modified on an internal lysine residue by a protein called A24, suggesting that A24 plays a role in gene regulation [10]. The se-

quence of protein A24 was finally elucidated by Hunt and Dayhoff in 1979 where they demonstrated that A24 and bovine ubiquitin share the same 37 N-terminal amino acid residues thus demonstrating that A24 and ubiquitin are the same molecule [11].

As the investigation of the UPS continued, numerous components of the pathway were identified. Ciehanover's lab in the 1970's began studying protein degradation in rabbit reticulocyte lysates and observed several unusual things. The first intriguing observation was that proteolysis required energy. Second, rabbit reticulocyte lysates could be separated into two different fractions (fraction I and fraction II), and only when these two fractions were combined together with ATP was protein degradation observed. This result indicated that protein degradation could not be attributed to a single protease but required at least two factors, in addition to ATP. Further studies demonstrated that Fraction I was the source of a heat stable polypeptide of approximately 8 kDa that was later identified as ubiquitin [12]. Fraction II was later found to contain not only the multisubunit protease called the proteasome but also all of the ubiquitin conjugation machinery. It was not understood until later that ATP supplied the energy for two processes in the UPS. One process is the covalent attachment of ubiquitin, the targeting signal for substrate degradation by the proteasome. The other process is the protein unfolding activity of the proteasome. These early studies were critical to our understanding of regulated protein degradation and have opened up an enormous new field in cell signaling.

The Ubiquitin-Proteasome System

Protein ubiquitination is a signaling cascade used in all eukaryotic cells and occurs through the orchestration of three different classes of enzymes: E1 activating en-

zymes, E2 conjugating enzymes, and E3 ubiquitin ligases. There is also a newly found class of ubiquitin elongation factors called E4 ubiquitin ligases, the function of which will be explained later. Each of these enzymes plays a different role in ubiquitin modification of protein substrates, as illustrated in figure 1. Although ubiquitin modification can have many effects on modified substrates, the best characterized is its role as a signal for 26S proteasome degradation. This review will focus mainly on the ubiquitination machinery, the catalytic activities of the 26S proteasome, and the enzymes responsible for removal of ubiquitin modification.

E1/E2 Enzymes

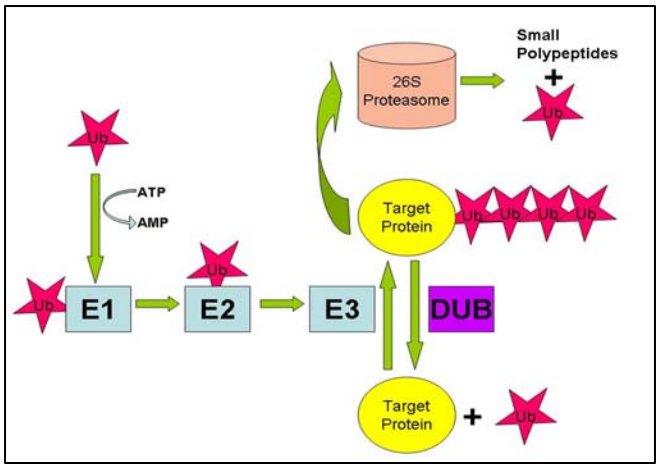

Figure 1: The Ubiquitination Pathway. Proteins are modified with ubiquitin chains and degraded by the proteasome into small peptides. Ubiquitin is recycled back into the cytosol by deubiquitinating enzymes on the 26S proteasome.

The E1 activating enzyme is the first enzyme to start the process of ubiquitination by covalently binding ubiquitin monomers from the cytosol in an ATP dependent reaction. The E1 functions to transfer the ubiquitin to the E2 conjugating enzymes. To transfer a ubiquitin moiety to an E2, the E1 enzyme undergoes a multistep, ATP dependent process. The E1 must hydrolyze 2 molecules of ATP to AMP and pyrophosphate and bind two ubiquitin monomers [13]. It should be noted that the E1 is the only enzyme involved in ubiquitin modification that hydrolyzes ATP. The other ATP dependent activities involved in ubiquitin dependent

protein degradation are localized to the 26S proteasome. After the attachment of the second ubiquitin moiety has occurred, the thioester linked ubiquitin on the E1 is then passed to a cysteine residue on the E2 conjugating enzyme through a trans-thiolation reaction [14, 15].

The function of the E2 is to accept the transfer of ubiquitin from an E1 enzyme and, typically via an E3 dependent mechanism**,** attach the ubiquitin to a protein substrate. E2s add an additional layer of complexity to the UPS. For example, studies in yeast have suggested that E2s have considerable differences not only in their affinity for different E3 ligases but also in their catalytic activity. The E2 in conjunction with the E3 ubiquitin ligase then assists in the covalent linkage of ubiquitin to a protein substrate. What is also particularly fascinating about E2 ligases is that it has recently been discovered that some E2s can directly catalyze the monoubiquitination of substrates independent of E3 ubiquitin ligases depending on the protein substrate [16]. It was proposed that bypassing the E3 ligases in favor of direct substrate monoubiquitination by an E2 functions specifically to regulate the activity of these monoubiquitinated substrates that contain ubiquitin interacting motifs (UIM). This regulation occurs because the monoubiquitin provides a *cis* activating inhibitory signal that binds to the UIM thereby preventing the ubiquitin interacting motif from binding to other ubiquitinated substrates in *trans*. It will be interesting to see if direct substrate ubiquitination is a general characteristic of E2 ligases thus increasing the complexity of the ubiquitin cascade. Furthermore, the specificity of these E2s for monoubiquitination of substrates containing a UIM, suggests the presence of a consensus sequence for ubiquitination, possibly the UIM itself. Finding a consensus sequence or a conserved structural characteristic in this example may suggest that other E2

enzymes are able to recognize specific protein sequences either for direct substrate ubiquitination or for transfer of ubiquitin to an E3 ligase.

E3 Ligases/E4 Processivity Factors

To date, the known genes encoding E3 ligases number in the hundreds and are thought to provide substrate specificity for the UPS through the recognition of proteins for ubiquitination. E3 ligases fall into three different classes based on their structure. The three classes are the Really Interesting New Gene (RING) finger E3 ligases, the U-box E3 ubiquitin ligases, and the Homologous to the E6-AP Carboxyl Terminus (HECT) domain E3 ligases. Although these three E3 ligase classes are divergent in structure, suggesting different mechanisms of specificity, the way in which they catalyze the transfer of ubiquitin can be split into two different categories. RING finger and U-box E3 ligases do not form a covalent attachment to ubiquitin; instead, they act as a scaffold to bring the E2 and substrate together to allow direct transfer of ubiquitin from the E2 [17-19]. The HECT domain E3 ligases on the other hand first form a covalent attachment to ubiquitin before transferring the ubiquitin to the substrate [15]. Although the E3 ubiquitin ligases can be classified on their mechanism of ubiquitin transfer, it is presently unclear as to what generates their substrate specificity. As the mechanism for E3 substrate specificity is uncovered, it is possible that the E3 ubiquitin ligases will be further subdivided based on other criteria.

The E1, E2, and E3 ubiquitin ligases are the enzymes that are classically thought to be the preeminent enzymes for protein ubiquitination. The above discussion of the UPS only demonstrates the addition of a single ubiquitin to a substrate. However, many

substrates of the UPS have polyubiquitin chains attached to them. Polyubiquitin chains are created through the sequential covalent attachment of the c-terminal glycine of one ubiquitin molecule onto one of the seven internal lysine residues on another ubiquitin molecule. The mechanism for chain elongation has not been fully elucidated; however, there are several possible mechanisms for chain generation. One possibility is that ubiquitin chain generation can occur through repetitive action of the E1, E2, and E3 cascade. The repetitive action model of polyubiquitination may however become self-limiting because chain length could sterically inhibit E2/E3 substrate binding thus preventing enzymatic activity. It has been demonstrated however, that HECT domain E3 ligases are capable of attaching preassembled polyubiquitin chains to a substrate [20]. Recently, a new class of E4 ubiquitin processivity factors has been discovered that, at present, is poorly understood in terms of its mechanism of action. These processivity factors are collectively referred to as E4 ubiquitin ligases, or ubiquitin chain assembly factors. E4s are thought to catalyze ubiquitin chain elongation and are dependent on the E1, E2, and E3 components of the UPS. It is critical to note that E4s do not interact with the protein substrate. Rather, the E4s bind directly to substrate bound ubiquitin chains and catalyze the continued elongation of those chains [21]. To date, E4s have been shown to be important for several different cellular and developmental processes, including cardiac development, nervous system function, and cell stress survival [21, 22].

The functional significance of poly-ubiquitination is dependent mainly on the type of branching found in the poly-ubiquitin chains. Ubiquitin contains 7 lysine residues at amino acid positions 6, 11, 27, 29, 33, 48, and 63 that are available for polyubiquitin chain formation. Ubiquitin can be covalently attached by its c-terminal glycine to any

lysine residue in another ubiquitin molecule to produce polyubiquitin chains of different linkages [21, 23]. So far, K48 and K63 are the best studied polyubiquitin chains in terms of ubiquitin signaling. K63 linkages have been demonstrated to be important for cellular response to DNA damage, TrkA receptor internalization and signaling, and lysosomal degradation of substrates [1, 24-26]. K48 linkages of 4 or more ubiquitin moieties has been demonstrated as a sufficient signal for protein degradation at the 26S proteasome [27, 28]. It has been hypothesized that the different linkages can change the electrostatic surface presentation of ubiquitin. The change in surface morphology influences the ability of other proteins to bind polyubiquitinated substrates thus allowing for different functional consequences for ubiquitin chains [29, 30]. As the ubiquitin signaling mechanisms of the other types of ubiquitin linkages are elucidated, undoubtedly our understanding of the complexity and regulation of the UPS will increase.

Specificity for Ubiquitination

A major question in the ubiquitin signaling field is the mechanism of specificity for ubiquitination. The specificity for ubiquitination may be achieved by a wide variety of mechanisms that can be dependent on other cell-signaling pathways. In some instances, a substrate must be primed by another post-translational modification such as phosphorylation before ubiquitination occurs. Some E3s like the anaphase promoting complex and Skp1p-Cdc53p-F-box protein complexes contain multiple subunits. Some of these subunits, while not directly involved in the ubiquitination reaction, are believed to guide substrate recognition by binding to protein consensus sequences. It should be noted that, although there are some instances in which ubiquitination specificity is guided

by degrons, *cis* acting amino acid sequences that direct substrate ubiquitination and degradation, or other post-translational modifications, a universal ubiquitination consensus sequence or signal has not been discovered. The lack of a generalized ubiquitination signal highlights the specificity and regulation of the UPS.

Ubiquitin-Specific Proteases

In general, cell signaling is a reversible process. In the case of protein phosphorylation, phosphate groups are added to substrates by the action of protein kinases and are removed by the action of protein phosphatases. Ubiquitin modification is similarly reversible and can be removed from proteins by a class of enzymes called deubiquitinating enzymes (DUBs). Protein deubiquitination serves several essential functions in the cell, including liberating ubiquitin from translational fusion peptides and polyubiquitin chains, recycling ubiquitin from proteasome targeted substrates, and controlling the rate of protein degradation [31, 32].

DUB Class	Characteristics	Substrates	Size
UCH	-Blocking loops -Positionally conserved C, D, H catalytic residues	-Small monoubiquitinated substrates -Ubiquitin translational fusion proteins	20-30 kDa
UBP	-Conserved Cys and His boxes	-Large poly-ubiquitinated proteins -Poly-ubiquitin chains	50-300 kDa
Otubains	-OTU domain	-Poly-ubiquitin chains	30 kDa
MPN Domain	-MPN domain that contains polar residues -Zinc binding	-Poly-ubiquitin chains	35 kDa
Josephin Domain	-Domain similar to papain-like cystine proteases	-Edit poly-ubiquitin chain length	42 kDa (Ataxin-3 only known enzyme of this family)

Table 1: Characteristics of Known Deubiquitinating Enzyme Classes

DUBs are separated into several different classes depending on their amino acid sequence. To date, five different classes of DUBs have been identified (Table 1). Of these, the ubiquitin C-terminal hydrolase (UCHs) and the ubiquitin-specific protease (UBPs) family members have been studied more thoroughly than the others; therefore, these particular DUBs will be discussed further here.

Ubiquitin C-Terminal Hydrolases

The UCHs are defined by the presence of a conserved catalytic triad consisting of three positionally conserved amino acid residues: cysteine, histidine, and aspartic acid. UCHs are different from the other DUB families in two critical aspects. One difference is that, although the catalytic triads of UCHs are in the correct orientation for activity, the enzyme contains intramolecular blocking loops that occlude the active site and prevent activity. Catalytic activation of UCHs is accomplished by the enzyme binding either to allosteric regulatory proteins or to the proper substrate; thus, effectively removing the blocking loops and granting access to the catalytic domain. Another critical difference of this class of DUBs is the size of the UCHs. UCHs are small (20-30 kDa) and throughout the class do not vary widely in size. It is thought that due to the small size of the UCHs, that this class of DUBs functions primarily in the processing of monomeric ubiquitin from translational fusion proteins and in maintaining homeostatic levels of ubiquitin expression through prevention of ubiquitin degradation [33].

UchL1

The role of UCHs in neurological function has been well studied in the *gad* mouse, which contains a deletion in *UchL1* resulting in a truncated protein that is rapidly degraded [34]. UchL1 is a highly abundant protein in the brain and its loss in the *gad* mice results in a phenotype consisting of ataxia and tremor as well as muscle wasting in the hind limbs; this *gad* mouse shares similar characteristics to the *ataxia* (ax^J) mouse phenotype [35]. The *gad* mice also show accumulation of β and γ synucleins in neurons,

as well as a loss of monomeric ubiquitin [36, 37]. Interestingly, the loss of monomeric ubiquitin is not caused by an increase in the amount of polyubiquitinated substrates but most likely results from lysosomal degradation of ubiquitin [37]. These data suggest that UchL1 functions to stabilize monomeric ubiquitin levels in the cell and possibly to rescue ubiquitinated protein substrates from lysosomal degradation by using its DUB activity to edit polyubiquitin chain length thus quenching the polyubiquitin signal for degradation. The loss of monomeric ubiquitin may decrease the cellular pool of ubiquitin available for ubiquitin conjugation and subsequent degradation and therefore may explain the accumulation of the two synuclein isoforms in the *gad* mouse. The loss of monomeric ubiquitin may also contribute to the phenotype of the *gad* mice independently of other biochemical defects such as loss of UchL1 activity or accumulation of β and γ synucleins. The involvement of ubiquitin loss in the *gad* phenotype could be resolved through the transgenic expression of ubiquitin in the *gad* mice.

The DUB activity of UchL1 can remove ubiquitin modifications from protein substrates. However, there is evidence that UchL1 is important in cell signaling independent of its catalytic function [38]. Sakurai *et al.* found that UchL1 hydrolase activity is dispensable for the generation of proper neuronal morphology. The high abundance of UchL1 in the nervous system, the loss of ubiquitin in the *gad* mice, and the ability of catalytically inactive UchL1 to induce neuronal morphology indicates that UchL1 has two important activities in the nervous system. One activity is the removal of ubiquitin modifications from protein substrates, which is dependent on UchL1's catalytic activity. The other function of UchL1 may be to bind to ubiquitin in the cell to improve ubiquitin pool stability via a catalytic independent manner. As indicated above, the loss of ubiq-

uitin in the *gad* mice may be a critical cause of the *gad* mouse phenotype. The stabilization of the ubiquitin pool by catalytically inactive UchL1 may be enough to rescue the *gad* mouse phenotype. To explore the importance of the catalytic independent function of UchL1, expression of such a construct could be driven in the nervous system of *gad* mice.

It is particularly interesting to view the noncatalytic function of UchL1 in the context of the entire class of UCH deubiquitinating enzymes. As mentioned above, the UCHs are typified by blocking loops that occlude the catalytic site of the enzyme. For a UCH to be activated the blocking loops must be removed; this requisite suggests that the UCH is non-functional otherwise. However, the data from Sakurai *et al.* suggest something else: The UCH enzymes may have additional functions independent of their catalytic activity [38]. It will be interesting to see if other UCHs also have critical catalytic independent functions similar to UchL1. Discovery of non-catalytic functions of UCHs would open the possibility of critical non-catalytic functions not only of other enzymes in the UPS but also of enzymes in other signaling cascades.

Ubiquitin-Specific Proteases

The ubiquitin specific proteases (UBPs) are another class of DUBs that are defined by the conserved regions of homology around their catalytic cysteine and histidine residues (Cys and His boxes) [39]. Besides their regions of homology, the catalytic residues, and three conserved intramolecular structures (fingers, palm, and thumb), the structure of the UBPs is highly variable; this diversity in protein structure is thought to play an important role in the recognition of protein substrates [30]. Unlike the UCHs that have

intramolecular blocking loops that regulate protein activity, the UBPs have a different regulatory mechanism. The catalytic domain of the UBPs are misaligned; as a result, protein catalysis is prevented until the UBPs come into the proper conformation during substrate binding [30]. These DUBs range in size from 50 to 300 kDa and are thought to function in removing ubiquitin from large polyubiquitinated proteins and in processing monomeric ubiquitin from free ubiquitin chains. Usp14, the DUB that is the focus of my work, is a member of the UBP family and will now be discussed in greater detail below.

Ubp6/Usp14

The use of yeast systems has generated a significant amount of information about Ubp6, the yeast orthologue of Usp14, which has been utilized to guide this body of work. Because of the conservation of UPS proteins between yeast and mammalian systems, the remainder of this review will refer to the yeast UPS proteins unless otherwise stated or unless the authors of the referenced papers examined the mammalian UPS counterparts.

Ubp6 was first identified by Park *et al.*, who demonstrated the specificity of Ubp6 toward peptides with short ubiquitin chains [40]. Park *et al.* hypothesized that the function of Ubp6 was to liberate *de novo* ubiquitin from translational fusion proteins or to deubiquitinate proteins destined for 26S proteasomal degradation [40]. However, not until Wyndham *et al.* demonstrated the presence of a ubiquitin-like domain (Ubl) on Ubp6 did the function of the protein start to be elucidated [41]. Although it has only loose sequence homology to ubiquitin, the Ubl domain does contain a ubiquitin fold, a structural domain conserved from ubiquitin. Since previous studies have demonstrated the pres-

ence of ubiquitin binding motifs on the proteasome, the Ubl domain indicates that the Ubp6 may function as part of the proteasome [42-44].

To confirm Ubp6 as a component of the proteasome, Verma *et al* demonstrated that Ubp6 co-purified with proteasomes; thus, solidifying a role for Ubp6 as a proteasome component [45]. Borodovsky *et al.* used a DUB active site probe to show that Usp14 is an active constituent of the proteasome [46]. Although they did not look at the effect that proteasome binding has on Usp14 activity, Borodovsky *et al*. examined the effect of 20S core particle (20S CP) inhibition on Usp14 activity. Borodovsky *et al*. found that inhibition of proteasome activity enhanced the catalytic activity of proteasomal bound Usp14. One possibility to explain this observation is that 20S CP inhibition can influence Usp14 activity through inter-molecular signaling. Alternatively, proteasome inhibition could signal for additional recruitment of Usp14 to the proteasome.

Soon after the Borodovsky *et al.* finding, Leggett *et al*. examined the effect that proteasome binding has on Ubp6 activity [46, 47]. Binding of Ubp6 to the proteasome is dependent on Ubp6's Ubl domain as was suggested by Wyndham *et al.* Following binding, Ubp6 showed a 300-fold increase in activity. Leggett *et al.* demonstrated *in vivo* the importance of this proteasome dependent activity. They found that yeast knockouts of Ubp6 (Ubp6 KO) were sensitive to growth on canavanine, an arginine analogue that increases the amount of protein misfolding resulting in an increase in the amount of substrates targeted for proteasome degradation. Furthermore, Leggett *et al.* also demonstrated that canavanine growth sensitivity could not be rescued through expression of Ubp6 lacking the Ubl domain. This finding by Leggett *et al.* is the first suggestion that Ubp6 has an *in vivo* function that requires binding to the proteasome.

The molecular mechanism of Usp14 activation was not clarified until the crystal structure was solved by Hu *et al.* [47, 48]. Much like the UCH class of DUBs, Usp14 has intramolecular blocking loops; such a regulatory mechanism had not been demonstrated on a UBP. In addition, like other UBPs, Usp14 has a misaligned catalytic domain that is then brought into proper conformation by substrate binding. The Hu *et al.* finding introduces an interesting regulatory mechanism for Usp14. The presence of blocking loops on Usp14 suggests that Usp14 requires an activator to render Usp14 catalytically competent. Furthermore, the loops indicate that Usp14 activity may be regulated on the proteasome through dynamic movement of the loops, possibly through post-translational modification.

26S Proteasome

As has been stated earlier, ubiquitin can have effects on multiple cellular processes. The fundamental role of a substrate-linked polyubiquitin chain is to serve as a recognition motif for the large multi-subunit protease known as the 26S proteasome. The 26S proteasome is a holoenzyme made up of two substructures: the 20S CP and the 19S regulatory particle (19S RP). The 19S RP subunits are illustrated in figure 2. The 20S CP is responsible for the degradation of proteins into short peptides. The 20S CP consists of 14 different subunits arranged in a layer of 4 rings of 7 subunits each: α7, β7, β7, α7. The β subunits contain the catalytic activity for protein degradation that includes trypsin, chymotrypsin

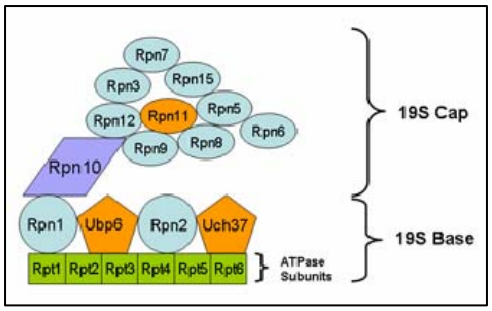

Figure 2: Schematic of the Yeast 19S Regulatory Particle.

and caspase-like activities [49]. The n-terminal tails of the α subunits provide a mechanism of regulatory control for 20S CP activity by occluding the lumen of the 20S CP and preventing substrate entry.

The 19S RP has four important functions for the degradation of polyubiquitinated proteins. These functions of the 19S RP are activation of the 20S CP, capture of polyubiquitinated substrates, deubiquitination of polyubiquitinated substrates, and unfolding of polypeptides. The 19S RP accomplishes activation of the 20S CP by binding to the 20S CP α subunits and moving the N-terminal tails of the α subunits out of the lumen thus opening the center of the 20S CP; the result is a catalytically active 26S proteasome [50-52]. The regulation of 20S CP activity through the α subunit N-terminal tails and the 19S RP prevents uncontrolled protein degradation by the proteasome.

Polyubiquitinated substrates are captured by the polyubiquitin chain receptor Rpn10, a subunit of the 19S RP; however, most polyubiquitinated proteins presented to the proteasome are globular and thus too bulky to enter into the narrow 20S CP lumen for catalysis [42, 44, 53]. The 19S RP is thought to be partly responsible for the chaperone activity that unfolds protein substrates and assists in the translocation of those substrates into the lumen of the 20S CP [54]. The chaperone activity of the 19S RP is confined to the ATPase subunits, Rpt subunits 1 through 6 [54, 55].

The polyubiquitin modification on protein substrates adds additional hindrance for the translocation of substrates into the 20S CP lumen. Removal of the ubiquitin modification releases this steric hindrance enabling entry into the proteasome for catalysis. To accomplish the function of ubiquitin removal from substrates, the 19S RP utilizes three different DUBs. The three DUBs that associate with the mammalian 19S RP are

Usp14, Uch37, and Poh1. Both Usp14 and Uch37 have been shown to be activated by proteasome binding [56, 57]. Although it has yet to be demonstrated, I suspect that Poh1 will also require proteasome binding for activation. The enzymatic activation of Usp14 and Uch37 by the proteasome indicates that these DUBs are prevented from hydrolyzing ubiquitin from substrates not intended for proteasomal degradation. Although all of these DUBs have been shown to hydrolyze ubiquitin chains, they appear to have different specificities for various polyubiquitin chain lengths *in vitro*. Rpn11/Poh1 catalyzes the removal of large chains of ubiquitin, and Uch37 has been shown to hydrolyze di-ubiquitin chains [57, 58]. Usp14 may catalyze the removal of short ubiquitin chains from substrates destined for 26S degradation [40]. In concert, these three 19S RP bound DUBs are able to regulate the removal of poly-ubiquitinated chains from substrates presented to the 26S proteasome. In addition to relieving steric hindrance for substrate entry into the 20S CP lumen, the activities of these DUBs are also required to recycle ubiquitin into the cellular pool, edit ubiquitin chains, and possibly rescue proteins from proteasomal degradation. Although it would appear that these DUBs would not possess substrate specificity, it is worthwhile to note that both Rpn11 and Uch37 are essential genes in yeast, although Ubp6 is not essential.

Proteasome Distribution in Neurons

A detailed study of the distribution of proteasomes in the CNS determined that while proteasomes are found within all neurons, there is much heterogeneity in the level and subcellular distribution amongst various neuronal populations. Motor neurons in the spinal cord and pyramidal cortical neurons of layer 5 contained the most abundant levels

of proteasomes in the CNS while the magnocellular portion of the red nucleus, the intermediate gray layer of the superior colliculus, the lateral vestibular nucleus, the deep cerebellar nuclei, the motor nuclei of the brainstem, and the reticular formation displayed significantly lower levels of proteasomes. In addition, while all neurons contain nuclear proteasomes, motor neurons contain proteasomes in the cytoplasm, dendrites, axon processes and in synaptic boutons. The nuclear distribution of proteasomes most likely reflects their role in modulating transcription and chromatin remodeling while the cytosolic proteasomes are likely to be essential for neuronal processes such as synaptic remodeling. These differences in proteasome expression among different neural populations indicate that the requirements of the UPS vary widely in the nervous system; this observation may grant insights into why some neuronal populations are particularly sensitive to mutations in the UPS.

Other Roles for Ubiquitin Signaling

The UPS has many important functions within the nervous system that are apart from its classical function in protein degradation. Such cellular duties highlight the versatility of ubiquitin signaling. As mentioned above, ubiquitin dependent protein degradation involves the polyubiquitination of substrates for efficient proteasome degradation. However, monoubiquitination is also an important post-translational cell signal. Monoubiquitination has been shown to be important in different cellular processes, including protein sorting into endosomes and receptor endocytosis; these finding demonstrate the versatility of ubiquitin modification [6, 59, 60]. As ubiquitination is studied further, undoubtedly new effects of this post-translational modification will be discovered.

UPS and Neurological Disease

The identification of Parkinson's disease (PD) mutations in components of the UPS clearly demonstrates the importance of ubiquitin signaling in the nervous system. PD is defined by tremor, rigidity, bradykinesia, and postural instability, all of which result from loss of dopaminergic neurons in the substantia nigra pars compacta and locus ceruleus. It is thought that two separate enzyme classes of the UPS, an E3 ubiquitin ligase and a DUB, are linked to PD.

The autosomal recessive form of PD is often associated with a mutation in the E3 ligase parkin. Parkin has been shown to interact with the proteasome; this finding suggests that at least one manifestation of PD can be attributed in part to proteasomal defects [61, 62]. It is interesting that the holoenzyme responsible for the degradation of polyubiquitinated protein substrates, the 26S proteasome, would harbor E3 ligases. It is customarily thought that proteins destined for degradation have already been ubiquitinated by the time they reach the 26S proteasome; however, it has recently come to light that proteins sent to the proteasome are not necessarily degraded by the proteasome. E3 ligases such as parkin may act to rescue substrates from protein degradation and may do so through the addition of polyubiquitin chains containing ubiquitin linkages not used as a signal for substrate degradation. Polyubiquitin chains formed through K48 linkages bind to ubiquitin receptors on the proteasome and attach the polyubiquitinated substrate to the 26S proteasome for degradation. Other ubiquitin linkages are not recognized by the proteasome's ubiquitin receptors. Because parkin is an E3 ligase that can associate with the proteasome and is known to produce poly-ubiquitin chains with K63 linkages,

parkin may assist in the attachment of polyubiquitin chains to proteasome bound substrates [63]. Parkin may accomplish this chain remodeling function by producing K63 linked polyubiquitin chains on substrates at the proteasome as DUBs are removing the K48 linked polyubiquitin chains. Parkin's production of K63 polyubiquitin linkages on a substrate at the proteasome may prevent the substrate from binding to the proteasome ubiquitin receptors that preferentially bind K48 ubiquitin linkages; as a result, a substrate is rescued from proteasome degradation. Mutations in parkin may therefore partly result in a proteasomal defect that causes an increased degradation of some parkin substrates because of the loss of parkin's function to rescue substrates from proteasomal degradation [64].

What is fascinating about PD is that there is evidence that not one but two different genes in the UPS may be responsible for PD. UchL1, a deubiquitinating enzyme that functions to stabilize monomeric ubiquitin levels, is considered to be a PD susceptibility gene in some patients with PD [34]. The link between UchL1 and PD is somewhat contentious. Healy *et al.* first reported that, in a study of the prevalence of the S18Y polymorphism of UchL1 in Caucasian patients with PD, they did not find the polymorphism to be protective against PD; therefore, they concluded that *UchL1* is not a PD susceptibility gene [65]. The Healy *et al.* results were countered by Tan *et al.*, who studied a population of Chinese patients with PD who carried the same S18Y polymorphism; the Tan *et al.* paper reported the polymorphism to be protective against PD thus concluding that UchL1 is a PD susceptibility gene. The study by Tan *et al.* was later corroborated by Carmine Belin *et al.* in their report on a population of Swedish patients [66, 67]. This

ethnic difference in the UchL1 polymorphism for PD susceptibility may result from a variety of factors, including environment, sample size, or different genetic modifiers.

PD highlights the importance of two separate components of the UPS in neurological function. First, PD illustrates that a mutated protein that normally exerts part of its effects at the proteasome can manifest in a neurological disorder. Second, PD indicates that enzymes that act independently of the proteasome but in the UPS can possibly cause neurological dysfunction as well.

Although this review detailed the role of UPS dysfunction in PD, there are several other UPS mutations that can cause neurological diseases with very different phenotypes from PD. UBE2A X-linked mental retardation (UBE2A-XLMR) is caused by a mutation in the E2 conjugating enzyme, UBE2A. UBE2A-XLMR is typified by multiple physical abnormalities as well as mental retardation, seizures, severe speech impairment, and white matter hypoplasia [68]. The mutation in UBE2A most likely prevents the ubiquitination of many downstream targets, possibly resulting in aberrant signaling cascades and accumulation of proteins. Mutations in the E6AP E3 ligase cause Angelman's syndrome, which results in mental retardation, unusual movements, and epilepsy [69]. The site of mutation on E6AP interferes with the thioester bond formation with ubiquitin. Therefore, it seems that this particular mutation in E6AP prevents the proper ubiquitination and degradation of substrates, possibly resulting in an accumulation of proteins that result in the Angelman's syndrome phenotype.

UPS Dysfunction in *ataxia* Mice

While there is a lack of a good PD mouse model, several spontaneous mouse mutations have been identified in components of the UPS that result in neurological disease. One such mouse model was identified by D'Amato and Hicks who gave the first characterization of the spontaneously occurring ax^J mouse mutation in 1965; these mice were later found to be deficient in the proteasomal bound DUB Usp14 [70, 71]. In their paper, D'Amato and Hicks discussed their study concerning the development of the phenotype and pathology of the ax^J mouse. At 2 weeks, they noted the onset of the phenotype, which included weakness and unsteadiness, especially a difficulty in the mice's righting their bodies after being turned on their side. D'Amato and Hicks found that, at 4 to 6 weeks, the animals were lying on their sides and moving through use of their forelimbs; this activity indicated severe hind limb problems that they found resulted from muscle wasting. Also, at about 3 weeks, the ax^J mice showed a pronounced tremor that was associated with movement and abated during rest.

What is particularly interesting about the D'Amato and Hicks paper is the neuropathology that developed in the brain and spinal cord as early as 2 weeks, when the first phenotypes were noted [70]. There are two structures in particular that D'Amato and Hicks examined that are very interesting because deficiencies in these areas can contribute to the ax^J phenotype. D'Amato and Hicks found that the overall size of the cerebellum was smaller than wild type; although the lobules were spared, white matter deficiency in the cerebellum was discovered. Furthermore, the Purkinje cell axons, which are the only output of the cerebellar cortex, were swollen and thick. What is particularly interesting is that, although the Purkinje cells were dysmorphic, the other substructures of

the cerebellum were unaffected. The deep nuclei, which receive input from the Purkinje cell axons, were normal. Furthermore, the climbing fibers were present and did not show any abnormalities in either morphology or number. The inferior olives, the olivocerebellar fibers, and the ventral spinocerebellar tract were all normal as well. Because the Purkinje cells are the only output of the cerebellar cortex, disease in these cells may explain the tremor and loss of motor coordination seen in the ax^J mice. Furthermore, abnormalities or injuries to the cerebellum are known to affect movement, especially balance and gait. Also, spinal cord disorders can produce characteristics similar to the ax^J phenotypes, including tremor, paralysis, and muscle wasting indicating the possibility of spinal cord involvement in the ax^J phenotype.

We are interested in studying ubiquitin signaling in the nervous system and have used the ax^J mice as a tool to investigate the ways in which changes in UPS can lead to neurological disease. The ax^J mice have a hypomorphic allele of *Usp14* due to the insertion of an Intracisternal-A Particle (IAP) into intron 5 of the *Usp14* gene [71]. The IAP insertion is retained during pre-mRNA processing and causes a 95% reduction in Usp14 levels in the ax^J mice [72]. The loss of Usp14 in ax^J mice results in early postnatal lethality, motor system defects, reduced levels of ubiquitin and synaptic deficiencies [71, 72]. The synaptic deficiencies in the mice include loss of paired pulse facilitation in the CA1 area of the hippocampus indicating a presynaptic defect in the ax^J mice. To lend further evidence to the presynaptic defect, miniature endplate potential, which measures single vesicle release at the neuromuscular junction, was decreased in frequency and increased in amplitude. The increased amplitude may be due to an increased quantal content of vesicles released from motoneurons [71]. Usp14 is able to bind to the proteasome; upon

doing so, this enzyme undergoes increased activity possibly as a result of conformational changes in the protein [40, 46-48, 72, 73]. Because the activity of Usp14 is clearly dependent on association with the proteasome, we hypothesize that the *ax*^{J} mice suffer from a defect in proteasome function. This work focuses on the characterization of Usp14, the proteasome associated DUB that is mutated in the *ax*^{J} mice. The striking phenotype of these mice, which consists of resting tremor, premature death, and muscle wasting, highlights the importance of Usp14 in proper growth and development and is the first example of a neurological disorder caused by a proteasome defect.

TRANSGENIC RESCUE OF *ATAXIA* MICE WITH NEURONAL-SPECIFIC EXPRESSION OF USP14

by

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Transgenic rescue of *ataxia* mice with neuronal-specific expression of Usp14

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Abstract

The *ataxia* mutation (*ax*^{J}) is a recessive neurological mutation that results in reduced growth, ataxia and hind-limb muscle wasting in mice. The *ax*^{J} gene encodes ubiquitin-specific protease 14 (*Usp14*), a deubiquitinating enzyme (DUB) that associates with the proteasome via its ubiquitin-like domain (Ubl) and is involved in processing ubiquitin chains. Analysis of *Usp14* gene products demonstrated that *Usp14* undergoes alternative pre-mRNA splicing to produce a full-length form of Usp14 that is capable of binding proteasomes and a form that contains a deletion in the Ubl domain. The full-length form of Usp14 is the only form that appears to be reduced in the *ax*^{J} mice. Transgenic rescue of the *ax*^{J} mice with neuronal-specific expression of Usp14 demonstrated that the full-length form of Usp14 was sufficient to restore viability and motor system function to the *ax*^{J} mice. Biochemical analysis showed that the ubiquitin-hydrolyase activity of this form of Usp14 is dependent upon the presence of proteasomes, and neuronal expression of full-length Usp14 was able to restore the levels of monomeric ubiquitin in the brains of *ax*^{J} mice. However, the *ax*^{J}-rescued mice still displayed the Purkinje cell axonal swellings that are seen in the *ax*^{J} mice, indicating that this cerebellar alteration is not the primary cause of the *ax*^{J} movement disorders. These results show that the motor defects observed in the *ax*^{J} mice are due to a neuropathic disease rather than a muscular disorder, and suggest that changes in proteasomal function may contribute to neurological dysfunction in the *ax*^{J} mice.

Introduction

The ubiquitin proteasome system (UPS) controls numerous cellular processes such as cell cycle progression and development by regulating the degradation of intracellular proteins. This regulation occurs in a temporal or spatial manner through the covalent attachment of a polyubiquitin chain to a substrate, which targets it for degradation by the proteasome (Glickman and Ciechanover, 2002). Alterations in the UPS have been hypothesized to contribute to defects in immune regulation (Gregory et al., 2003), cancer (Gregory et al., 2003; Kovalenko et al., 2003; Kondo and Kaelin, 2001), and chronic neurological diseases such as Parkinson's disease, Huntington's disease and Spinocerebellar ataxias (Dawson and Dawson, 2003; McNaught et al., 2001; Alves-Rodrigues et al., 1998).

The *ax*^{J} mutation is one of the few genetic lesions in the UPS that leads to neurological disease. This mutation results in reduced expression of Usp14 and causes several defects in mice, including ataxia and muscle wasting (D'Amato and Hicks, 1965). Unlike many chronic neurological diseases (Alves-Rodrigues et al., 1998), the *ax*^{J} mice do not have detectable ubiquitinated protein aggregates or display significant neuronal loss. Instead, these mice exhibit altered synaptic activity in both the CNS and PNS (Wilson et al., 2002). These findings support a direct role of the UPS in the regulation of synaptic function and demonstrate that synaptic defects may be a primary cause of neurological disease.

Usp14 is one of over 70 DUBs encoded in the mouse genome (Soboleva and Baker, 2004). DUBs are responsible for disassembling ubiquitin chains at the proteasome, cleaving ubiquitin precursors and editing ubiquitin side chains (Guterman and

Glickman, 2004), and are therefore essential for regulating protein stability and localization. Observations with Usp14 and its homologue Ubp6 indicate that one of the functions of Usp14 is to recycle ubiquitin at the proteasome (Leggett et al., 2002; Chernova et al., 2003, Anderson et al., 2005). Although Usp14 is one of three DUBs that have been shown to associate with mammalian proteasomes (Guterman and Glickman, 2004), the finding that loss of Usp14 results in neuronal dysfunction (Wilson et al., 2002) indicates that these proteasomal-bound DUBs act on specific substrates and are not redundant.

This study was initiated in order to better understand the *in vivo* function(s) of Usp14. We recently determined that *Usp14* is alternatively spliced to produce two forms of Usp14, one that is capable of binding to proteasomes and one that has a deletion in the proteasome-binding domain. To examine the effect of the ax^J mutation on the expression of these two spliced forms of Usp14, we characterized the expression of the Usp14 gene products in wild type and ax^J mice. Transgenic mice that specifically express Usp14 only in neurons were also constructed to determine the site of neuromuscular dysfunction in the ax^J mice. The Usp14 transgene was evaluated for its ability to complement the biochemical and behavioral defects of the ax^J mice in order to determine how Usp14 functions in the nervous system and to gain new insights into how alterations in the UPS lead to neurological disease.

Materials and Methods

Animals

Wild type C57BL/6J and Usp14^{axJ} mice (Jackson laboratories, Bar Harbor, ME) 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^{axJ} 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. Transgenic animals were generated by pronuclear injection of the *Thy1-Usp14LF* transgene into C57BL/6J fertilized eggs. 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.

Total RNA isolation

RNA was isolated from mouse brain using RNA-STAT (Tel-Test, Friendswood, TX).

Reverse transcription PCR

Mouse E7 and E15 cDNA libraries were purchased from Clontech (Mountain View, CA). The P28 library was constructed using the first stand synthesis kit from Invitrogen (Carlsbad, CA). Approximately 2 μL of reverse transcribed cDNA was used for PCR amplification in a Perkin Elmer 9700 thermocycler with platnium Pfx DNA polymerase (Invitrogen). Twenty PCR cycles were used to amplify cDNAs.

Two-dimensional gel electrophoresis

Total protein extracts were generated using the ReadyPrep Protein Extraction Kit (Bio-Rad, Hercules, CA). Proteasome extracts were precipitated prior to isoelectric focusing using the 2D gel cleanup kit (Amersham, Piscataway, NJ). ReadyStrip IPG strips (11 cm,

pH 4-7) were hydrated overnight with protein extracts. Linear focusing conditions were as follows: 250 V for 15 min, 8000 V for 2.5 h, 35000 total V h. Strips were equilibrated in a solution containing 6 M Urea, 0.375 M Tris-HCL, pH 8.8, 2 % SDS, 20 % glycerol, and 2 % DTT for 10 min with shaking. Strips were subsequently incubated for 10 min in a solution containing 6 M Urea, 0.375 M Tris-HCL, pH 8.8, 2 % SDS, 20 % glycerol, and 2 % iodoacetamide. Strips were then rinsed in 1X SDS PAGE running buffer and placed into a 4-15 % Criterion gel (Bio-Rad) and sealed with agarose.

Construction of *Thy1-Usp14LF* transgene

The full length *Usp14* cDNA, including the *Usp14* Kozak consensus sequence, was generated using RT-PCR and was cloned into the *Xho*I site of the p*Thy1.2* expression cassette (gift from Dr. Pico Caroni at the Friedrich Institut, Basel, Switzerland). The transgene was excised from the vector using *EcoR*I and *Nde*I and prepared for microinjection using standard procedures.

Antibodies

Antibodies to Usp14 were generated using recombinant Usp14 (amino acids 100-493) to immunize NZW rabbits (Covance, Radnor, PA). Usp14 polyclonal antisera from rabbit R138 was affinity purified and used for immunoblotting at a dilution of 1:2000. The JLA20 actin antibody and β-tubulin antibody were purchased from Developmental Hybridoma Core (Iowa City, IA) and were diluted 1:5000 and 1:2000, respectively. The control Rpt1 and Rpt4 monoclonal antibodies (Biomol, Plymouth Meeting, PA) and the Calbindin antibody (Swant, Bellinzona, Switzerland) were used at a 1:1000 dilution.

Isolation of mouse proteins

Mice 4 to 6 weeks of age and of appropriate genotype were sacrificed by CO_2 asphyxiation. Tissues were homogenized in 1 to 3 mL of homogenization buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 0.5 % SDS, 2 mM N-ethylmalemide, and Complete protease inhibitors from Roche (Indianapolis, IN). 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 (Rockford, IL).

Isolation of proteasomes

Proteasomes were isolated as described by Borodovski et al. (2001).

Immunoblotting

Proteins were resolved on either 8 % Tris-glycine gels or 4-20 % Tris-Glycine NUPAGE gels (Invitrogen) and transferred onto either nitrocellulose or PVDF membranes. The polyclonal Usp14 R138 antisera was diluted in PBS containing 3 % BSA. Primary antibodies were detected using an anti-mouse or anti-rabbit HRP conjugated antibody (Southern Biotechnology Associates, Birmingham, AL) 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). Each value represents the average and standard error from at least six blots using at least three different animals of each genotype.

Labeling of proteasome-associated DUBs

Approximately 5 μg of proteasomes were diluted into a solution containing 50 mM Tris, pH 7.5, 250 mM Sucrose, 1 mM DTT and 2 mM ATP. One μL of a 0.2 μg/μL solution of HA-Ub-VME was then added and the reaction was incubated at room temperature for 30 min to label active DUBs. 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 DUBs were detected by probing the blot with the 12CA5 anti-HA monoclonal antibody (Roche) and were visualized using Luminol reagents (Pierce). Approximately 50 ng of recombinant Usp14 (Biomol) was incubated with proteasomes at room temperature for 2 h prior to the addition of HA-Ub-VS.

Culture and staining of rat hippocampal neurons

Neurons were cultured as described in Price and Brewer (2001). E15 to E18 rat hippocampal neurons were dissociated, plated and allowed to grow in culture for 7 to 14 days before staining. Coverslips were washed 3 times with PBS containing 2.67 mM KCl, 1.47 mM KH_2PO_4, 137.93 mM NaCl, and 8.10 mM Na_2HPO_4. Cells were fixed for 20 min with 4 % paraformaldehyde in sodium phosphate buffer containing 100 mM $NaPO_4$.

After fixation, the coverslips were washed an additional 2 times with PBS. Cells were blocked in a solution containing 3 % normal goat serum (NGS) and 0.3 % Triton X-100 in PBS. Primary antibodies were diluted in blocking solution and applied to the coverslips overnight at 4 ºC. The next day, coverslips were washed 3 times in PBS containing 1 % NGS. Alexa Fluor secondary antibodies from Invitrogen were diluted 1:500 in 1 % NGS and applied to the coverslips for 1 h in the dark at room temperature. Coverslips were then washed in PBS containing 1 % NGS, followed by PBS containing 1 % NGS and 4',6-Diamidino-2-phenylindole (DAPI), and finally in PBS before being mounted and imaged.

Transfection of Rat Hippocampal Neurons

Neurons were transfected using the Amaxa rat hippocampal neuron transfection kit (Gaithersburg, MD) according the manufacturer's protocol.

COS7 Cell Culture and Transfection

COS7 Cells (African Green Monkey Kidney cells) were maintained in Dulbecco's Modified Eagle Medium (Life Technologies, Gaithersburg, MD) containing 10 % Fetal Bovine Serum, 50 U/mL penicillin and 50 μg/mL streptomycin (Invitrogen). Cells were maintained by passaging the cells every 3 days through trypsinization and subsequent replating of approximately 1 x 10^6 cells. For transfection, 9 x 10^5 COS7 cells were plated on a 100 mm tissue culture dish the day before transfection. Each 100 mm dish was transfected with 3 μg of the appropriate DNA construct using the Fu-Gene 6 transfection reagent (Roche) following the manufacturer's protocol. Cells were allowed to express the

construct for approximately 24 h before collection in a solution containing 200 mM Tris-HCL (pH 7.4), 750 mM NaCl, 5 % NP-40, 5 mM EDTA, and Complete protease inhibitors (Roche). Following collection, cells 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 BCA protein assay kit from Pierce.

Immunofluorescence

Mouse brains were submersion-fixed with either methacarn or 4 % PFA overnight at 4 ^{o}C followed by 70 % EtOH for 24 h. Tissues fixed in 4 % PFA were embedded in paraffin and 10 micron sections were cut for staining. Methacarn-fixed brains were cut on a vibratome at 100 μm. Sections were blocked at room temperature for 1 h in PBS containing 10 % normal goat sera, 1 % BSA, and 0.1 % Triton X-100. Primary and secondary antibodies were in diluted in PBS containing 2 % normal goat antisera, 0.1 % BSA, and 0.1 % Triton X-100. Images were acquired using an Olympus IX 70 inverted microscope equipped with epifluorescence optics. The camera used was a Retiga 1300 cooled CCD, firewire, high resolution, monochromatic camera purchased from Qimaging (Burnaby, British Columbia, Canada). We used an 83000 Pinkel filter set from Chroma Technology Inc. (Brattleboro, VT). A Mac 2000 electronic filter wheel with an automatic shutter separately housed the excitation filters. All image acquisition was performed using IPLab Spectrum imaging software from Scanalytics (Fairfax, VA). The UAB High Resolution Imaging Facility assisted in sample preparation and imaging.

Elevated beam assay

Assays were performed as described in Stanley et al. (2005). Mice were placed on 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 hind limbs slipped from the beam) for each trial.

Results

Usp14 is alternatively spliced

Analysis of reverse-transcribed PCR products generated using *Usp14*-specific primers demonstrated the presence of two *Usp14* transcripts that are developmentally regulated and are detected in both neuronal and non-neuronal tissues (Fig 1a). Cloning and sequencing of these *Usp14* transcripts demonstrated that they are generated by alternative splicing of exon 4, which encodes approximately 33 amino acids of the Ubl domain that is required for proteasome binding (Fig 1b). The cDNA of *Usp14* that contains exon 4 has been designated *Usp14LF*, and the cDNA that lacks exon 4 is referred to as *Usp14SF*. No other alternatively spliced cDNAs were identified for *Usp14*.

Protein expression of Usp14LF and Usp14SF

The predicted molecular weights of Usp14LF and Usp14SF are 56 kDa and 52 kDa, respectively. However, immunoblot analysis of whole brain extracts from wild type mice demonstrated the presence of two immunoreactive bands, one that migrated at 52

kDa and a second band that migrated at 66 kDa (Fig. 1c). The 52 kDa immunoreactive band was not detected in proteasome fractions from the brains of wild type mice (Fig. 1d) and was greatly reduced in the immunoblots from wild type E15 embryos (Fig. 1d), which produce only a small amount of alternatively spliced *Usp14SF* mRNA (Fig. 1a). Although the 66 kDa band could not be detected in the brain extracts from ax^J mice, wild type levels of the 52 kDa band were still observed in the ax^J mice (Fig. 1c). To test whether these two bands correlated with the two forms of Usp14 produced from the alternative splicing of exon 4 of *Usp14*, COS7 cells were transfected with mammalian expression vectors containing either *Usp14LF* or *Usp14SF*. Examination of protein extracts made from these transfected cells confirmed that Usp14LF migrates at approximately 66 kDa, whereas Usp14SF migrates at 52 kDa (Fig. 1d). In addition, when we compared the migration pattern of Usp14 from the proteasome fractions with recombinant Usp14LF, which was produced in bacteria and therefore lacks any post-translational modification, both samples exhibited the same migration pattern during electrophoresis (Fig. 1e).

To examine any potential post-translational modification to Usp14LF, such as phosphorylation, proteasome fractions from the brains of wild type mice were separated by 2D gel electrophoresis and immunoblotted with an antibody to Usp14. In these gels, Usp14 migrated at a range of pIs from approximately 5.0 to 5.1 (Fig. 1f). The predicted change in pI is approximately 0.05 pI units per phosphate group added to Usp14, indicating that Usp14LF may contain at least 3 to 4 phosphorylated residues.

Usp14LF is activated in the presence of proteasomes

Previous work on the yeast orthologue of Usp14, Ubp6, demonstrated a 300-fold increase in activity when Ubp6 associated with the proteasome (Leggett et al., 2002). To determine if Usp14 also requires proteasomes to activate its ubiquitin-hydrolyase activity, we examined the activity of recombinant Usp14LF in the presence and absence of proteasome fractions prepared from the brains of ax^J mice. In this assay, the ubiquitin-hydrolyase activity of Usp14 was examined by the ability of Usp14 to be labeled with HA-Ub-VS. Since HA-Ub-VS is an active-site probe, only catalytically active DUBs will be covalently modified by this probe (Borodovsky et al., 2001) and can be detected using an anti-HA antibody. As previously shown (Anderson et al., 2005), Usp14 was labeled with the HA-Ub-VS probe in the positive control lane of neuronal proteasomes isolated from wild type mice (Fig. 2). No activity of Usp14 against the HA-Ub-VME substrate was observed from proteasomes isolated from ax^J mice or from recombinant Usp14LF produced from bacterial cultures (Fig. 2). However, when recombinant Usp14LF was mixed with the proteasome fraction isolated from ax^J mice, we detected significant labeling of Usp14 (Fig. 2). To determine if the labeling was dependent on contact with proteasomes, and not due to a soluble factor in the preparations, the ubiquitin hydrolyzing activity was also measured from supernate fractions from the proteasome preparations of wild type mice both in the presence and absence of recombinant Usp14LF. Neither supernate fraction was able to activate the catalytic activity of Usp14 (Fig. 2), indicating that the ubiquitin-hydrolyzing activity was dependent upon the binding of Usp14 to the proteasomes.

Subcellular localization of Usp14 in neurons

We have previously shown that Usp14 co-fractionates with neuronal proteasomes (Anderson et al., 2005) and that loss of Usp14 results in synaptic transmission defects (Wilson et al., 2002), indicating that Usp14 may be located with proteasomes near the synapse. To determine the subcellular distribution of Usp14 in neurons, we examined Usp14 expression from cultured primary rat hippocampal neurons using indirect immunofluorescence. Similar to previous results (Mengual et al., 1996; Wojcik and Demartino, 2003), a predominately nuclear and perinuclear pattern was observed after staining neurons with antibodies to the 26S proteasome (Fig. 3a). Staining for the proteasome was also detected throughout the cytoplasm of the hippocampal neurons. The distribution of endogenous Usp14 overlapped the staining of the cytoplasmic proteasomes, with the most predominate signal observed in the perinuclear region (Fig. 3a).

To verify the distribution of Usp14 with a different antibody, we next examined the expression pattern from primary hippocampal neurons transfected with *myc*-tagged *Usp14LF*. The myc-tagged Usp14LF showed a similar perinuclear localization pattern as the endogenous Usp14, with some nuclear staining (Fig. 3b). In addition, cytosolic Usp14 staining was also detected throughout the cell soma and in the neurite processes (Fig. 3b).

Generation of Usp14LF transgenic mice

The ax^J mice suffer from a severe neuromuscular disorder characterized by tremor, muscle wasting and hind limb spasticity. We were therefore interested in determining if the defects in the ax^J mice were due solely to the loss of Usp14 expression in

the nervous system, or whether loss of Usp14 in muscle also contributed to disease in the *ax*^{J} mice. In order to determine if neuronal-specific expression of Usp14 could rescue the growth and motor defects of the *ax*^{J} mice, we cloned *Usp14LF* behind the pan neuronal *Thy1.2* promotor (Caroni, 1997) to generate *Thy1-Usp14LF* transgenic mice (Fig. 4a). We chose to express Usp14LF in these studies because our previous research demonstrated a role for Usp14 in recycling ubiquitin at the proteasome (Anderson et al., 2005), it is the predominate form of Usp14 detected in the brain (Fig. 1), and because it is the only form of Usp14 that shows reduced expression in the *ax*^{J} mice (Fig 1c).

Two independent founder lines, 9002 and 9045, were generated for the *Thy1-Usp14LF* transgene. Immunoblot analysis indicated that the *Thy1-Usp14LF* transgene was robustly expressed around postnatal day 7 and was maintained in each line until at least 20 weeks after birth (data not shown). Southern analysis of the founder lines demonstrated that both lines have a similar number of transgene insertions. No growth or behavioral defects were observed in either founder line, and both lines demonstrated similar expression patterns of the *Thy1-Usp14LF* transgene (data not shown). Although both lines yielded identical results throughout the study, only the results of the 9002 founder line are reported below.

To test the ability of the *Thy1-Usp14LF* transgene to rescue the defects observed in the *ax*^{J} mice, we generated mice that were homozygous for the *ax*^{J} mutation and contained the *Thy1-Usp14LF* transgene. These mice have been designated *ax*^{J} *Tg*. To ensure that the transgene only expressed Usp14LF in neuronal tissues, protein extracts were prepared from the brain, spinal cord, muscle, liver, kidney and heart of wild type, *ax*^{J} and *ax*^{J} *Tg* mice and immunoblotted with an antibody to Usp14. When compared with the

levels of Usp14LF expressed in the *ax*^{J} mice, increased expression of Usp14LF from the *Thy1-Usp14LF* transgene was only detected in the brain and spinal cord (Fig. 4b). These levels corresponded to a 4.5-fold increase of Usp14LF in the brain and a 6-fold increase in the spinal cord of the *ax*^{J} *Tg* mice (Fig. 4c), compared to the levels normally found in wild type mice.

Thy1-Usp14LF rescues the *ax*^{J} growth defects and lethality

To determine if the growth defects and juvenile death of the *ax*^{J} mice were rescued with the *Thy1-Usp14LF* transgene, body mass and survival rates were monitored for the *ax*^{J}, *ax*^{J} *Tg*, and wild type mice. Similar to previous results (Anderson et al., 2005), all of the *ax*^{J} mice died within 8 weeks of age (Fig. 5a). In contrast, all of the wild type and *ax*^{J} *Tg* mice survived to the end of the 20-week test period (Fig. 5a). A comparison of the body weights of the *ax*^{J} *Tg* mice with those of the *ax*^{J} and wild type mice demonstrated that neuronal-specific expression of Usp14LF was able to restore body weights to wild type levels (Fig. 5b), indicating that both the *ax*^{J} postnatal lethality and growth defects are due to loss of Usp14 in the nervous system. This improvement was evident by 6 weeks of age, the time at which the *ax*^{J} mice normally show a remarkable decrease in body weights compared to wild type mice (Fig. 5b), and continued throughout the 20 week test period.

Usp14LF interacts with proteasomes and restores the cellular levels of ubiquitin

To examine if the neuronal expression of Usp14LF rescued the biochemical defects observed in the *ax*^{J} mice, we first examined the proteasomes from wild type, *ax*^{J},

and *ax^J Tg* mice for the presence of Usp14. As we previously reported (Anderson et al., 2005), Usp14 was found in the proteasome fractions from wild type animals and was greatly reduced in the fractions from *ax^J* mice (Fig. 6a). Immunoblot analysis of *ax^J Tg* proteasomes demonstrated that transgenic Usp14LF was able to stably associate with neuronal proteasomes (Fig. 6a).

To determine if the proteasome-bound Usp14 was catalytically active, proteasomes from wild type, *ax^J*, and *ax^J Tg* mice were incubated with the HA-Ub-VME probe that is directed to the active-site of DUBs. Similar to previous results (Anderson et al., 2005), Usp14 and UCH37 were the only DUBs that labeled with the HA-Ub-VME probe in wild type mice (Fig. 6a). The *ax^J* proteasomes lacked Usp14 but retained wild type levels of UCH37 (Fig. 6a). In addition to stably associating with neuronal proteasomes, Usp14LF expressed from the *Thy1-Usp14LF* transgene was also catalytically active (Fig 6a).

To elucidate if we could increase the amount of Usp14 stably associated with the proteasome, we next measured the amount of Usp14 bound to proteasomes from wild type mice containing the *Usp14LF* transgene. Since the *Thy1.2* promoter has previously been shown to exhibit mosaic expression patterns (Caroni, 1997), proteasomes were prepared from the hippocampus, where expression of *Thy1-Usp14LF* is robust (Fig. 8k) and the level of proteasome-associated Usp14 could be evaluated in the wild type mice containing the transgene. There was a 2.5-fold increase in total Usp14 and a 3-fold increase in labeling of catalytically-active Usp14 in the proteasomes from wild type mice that contained the *Thy1-Usp14LF* transgene (Fig. 6b, wt *Tg*) as compared with proteasomes from

wild type mice, indicating that neuronal proteasomes are capable of binding more Usp14 than what is normally observed in the wild type mice.

To determine if transgenic expression of *Thy1-Usp14LF* restored the levels of monomeric ubiquitin in the ax^J mice, we examined the steady state levels of ubiquitin in wild type, ax^J, and ax^J *Tg* mice. Similar to previous results (Anderson et al., 2005), there was a 30% loss of monomeric ubiquitin in ax^J brain extracts as compared to controls (Fig. 6c). In contrast, immunoblot analysis of hippocampal extracts showed that the levels of monomeric ubiquitin were restored to wild type levels in the ax^J *Tg* mice (Fig. 6c), indicating that the Ubl-containing form of Usp14 is sufficient for the proper maintenance of ubiquitin levels in the brain.

Thy1-Usp14LF rescues the motor defects of the ax^J mice

The ax^J mice display severe hind limb paralysis and muscle wasting by 8 weeks of age, which is easily demonstrated by their inability to traverse an elevated beam. In order to determine if the motor system defects observed in the ax^J mice were rescued by the *Thy1-Usp14LF* transgene, we measured the performance of the wild type, ax^J, and ax^J *Tg* mice on the elevated beam assay described by Stanley et al. (2005). In this assay, all of the 8 week old wild type animals were capable of traversing the beam without any falls and all of the ax^J mice fell off the beam (Fig. 7a). Examination of the ax^J *Tg* mice demonstrated that transgenic expression of Usp14LF specifically within neurons was able to restore the ability of the ax^J mice to traverse the elevated beam (Fig. 7a). However, when we measured the number of times the hind feet of the animals slipped from the elevated beam during the trial, the ax^J *Tg* mice demonstrated an increase in the number of foot

slips as compared to wild type controls (Fig. 7b), possibly indicating the presence of cerebellar abnormalities in the *ax^J Tg* mice that were not rescued by the *Thy1-Usp14LF* transgene.

Thy1-Usp14LF is not detected in Purkinje cells of *ax^J Tg* mice

To determine if the *Thy1-Usp14LF* transgene restored Usp14 expression in the various layers of the cerebellar cortex, immunostaining was used to examine the expression pattern of Usp14LF in the cerebellum of the *ax^J Tg* mice. Our Usp14 antisera could not detect the endogenous Usp14 in the tissues from wild type mice, but could readily detect Usp14LF when it was overexpressed in the cerebellum of the *ax^J Tg* mice (Fig. 8c). Although high levels of Usp14 expression were observed in the granule cell layer, expression of Usp14LF from the *Thy1-Usp14LF* transgene was not detected in the Purkinje cell layer of the *ax^J Tg* mice (Fig. 8c). The only other neuronal population that lacked detectable expression of the *Thy1-Usp14LF* transgene was the CA3 pyramidal cells of the hippocampus (data not shown).

When we looked at the distribution of Usp14 in the brain, transgenic expression of *Thy1-Usp14LF* resulted in a mosaic pattern of expression of Usp14LF in the cortex (Fig. 8e-h) and fairly uniform expression in the hippocampus (Fig. 8i-l). In agreement with previous results (Fig. 3), Usp14 was detected primarily in the cytosol of the cortical and hippocampal pyramidal neurons (Fig. 8g and k).

ax^J Tg mice display Purkinje cell axonal swellings

Previous studies on the *ax^J* mice reported the presence of Purkinje cell axonal swellings that were hypothesized to be responsible for the motor defects of the *ax^J* mice (D'Amato and Hicks, 1965). Because Usp14LF was not detected from the *Thy1-Usp14LF* transgene in the Purkinje cells of the *ax^J Tg* mice, indirect immunofluorescence microscopy was used to examine the Purkinje cell axons of wild type, *ax^J*, and *ax^J Tg* mice to determine if the Purkinje cell pathology was still present in the cerebellum of the *ax^J Tg* mice. In agreement with previous studies (D'Amato and Hicks, 1965), large varicosities could be detected along the Purkinje cell axons of the *ax^J* mice as early as 3 weeks of age (Fig. 9a). Surprisingly, not only was the Purkinje cell axonal pathology still present in the *ax^J Tg* mice at 8 weeks of age (Fig. 9b), but the number of the swellings increased between 8 and 12 weeks of age (data not shown), indicating that this pathology can not be responsible for the overt motor defects of the *ax^J* mice. However, the remaining Purkinje cell pathology may be related to the increased number of foot slips in the *ax^J Tg* mice.

Discussion

The results presented in this study have uncovered several new and important aspects of Usp14. *Usp14* encodes a ubiquitin specific protease that is ubiquitously expressed in mammalian tissues (Anderson et al., 2005) and is thought to regulate ubiquitin levels at the proteasome. By using transgenic animals that specifically express Usp14 within neurons, we have demonstrated that the postnatal lethality, stunted growth, and overt motor defects in the *ax^J* mice are solely due to the loss of Usp14 expression in the nervous system. Although our previous studies identified an IAP insertion into intron 5

of Usp14 in the *ax*^{J} mice, which suggested that the *ax*^{J} phenotype was due to the loss of Usp14 expression, the *ax*^{J} mutation was the only known mutant allele of Usp14, and it is known that IAP insertions can have long-range effects on gene expression. The transgenic complementation of the *ax*^{J} mice with a *Usp14* cDNA therefore provides genetic proof that the *ax*^{J} mutation resides in Usp14. This work has also demonstrated that, although Usp14 is expressed in a variety of tissues in mammals, Usp14 has an essential, non-redundant function in the nervous system and that the *ax*^{J} neuromuscular defects are due to a neuropathy and not a muscular disorder.

In addition to the full length proteasome-bound form of Usp14LF, our studies have identified a novel splice form of *Usp14*, *Usp14SF*, that is expressed in both neuronal and non-neuronal tissues, is developmentally regulated and contains a 33 amino acid deletion in the Ubl domain. Consistent with the observation that the Ubl is required for stable association with the proteasome (Leggett et al., 2002; Chernova et al., 2003), Usp14SF was not able to fractionate with proteasomes from the brains of wild type mice (Fig. 1d). Surprisingly, the insertion of the intracisternal A- particle (IAP) into intron 5 of the *ax*^{J} *Usp14* (Wilson et al., 2002) does not appear to affect the expression of Usp14SF in the brains of the *ax*^{J} mice. Since the IAP exerts its effect by altering *Usp14* pre-mRNA splicing, it is possible that the IAP has a much greater effect on processing the *Usp14LF* pre-mRNA. The finding that the IAP insertion can exert variable effects on Usp14 protein levels is supported by our observation that the levels of Usp14LF are only modestly reduced in the testes of the *ax*^{J} mice, while they are greatly reduced in brain and muscle (Anderson et al., 2005).

Immunoblot analysis of brain extracts from wild type mice demonstrated the presence of two immunoreactive Usp14 bands that migrate to the same positions as the Usp14 proteins from extracts prepared from COS7 cells transfected with the Ubl-containing *Usp14LF* construct and the alternatively spliced *Usp14SF* construct. We estimate that the steady-state level of Usp14LF is approximately 5 times greater than that of Usp14SF. Although Usp14SF migrates according to its predicted molecular weight, Usp14LF migrates significantly slower than its predicted molecular weight of 56 kDa. Comparison of the migration patterns of the 2 forms of Usp14 with recombinant Usp14LF clearly demonstrated that the altered migration of Usp14LF is due to the presence of the Ubl domain. Ubiquitin domains are extremely stable and therefore may be less susceptible to denaturation, resulting in reduced migration in our gels.

While our studies using 2D gel electrophoresis suggest that Usp14LF is phosphorylated, phosphorylation does not appear to contribute to the reduced migration of Usp14LF in our gels. However, the phosphorylation of Usp14 could have important effects on the activity of Usp14. Recent studies have shown that the Usp14 homologue in Arabidopsis can interact with calmodulin and may therefore be phosphorylated by CaMKII (Moon et al., 2005). Examination of the Usp14 protein sequence revealed a potential CaMKII phosphorylation position at amino acids 428-431. In addition, the crystal structure of Usp14 was recently solved and shown to contain two loops which block the catalytic domain of Usp14 (Hu et al., 2005). These loops were hypothesized to be displaced following proteasome binding to allow for the activation of Usp14. Since the CaMKII site is located in one of these blocking loops, phosphorylation of Usp14 by CaMKII may

provide a mechanism to regulate the activity of Usp14 on the proteasome in a calcium dependent manner.

Although a specific mechanism of proteasome malfunction has not been shown in the *ax*^{J} mice, our data, along with Ubp6 studies in yeast and proteasome inhibition studies in EL4 cell lines (Legget et al., 2002; Borodovsky et al., 2001) is consistent with the requirement of Usp14LF to bind to the proteasome in order to become catalytically active. In this study, we have shown that recombinant Usp14LF becomes catalytically active only in the presence of proteasomes, and that cell extracts devoid of proteasomes are unable to activate the ubiquitin-hydrolyase activity of Usp14. Approximately 50% of Usp14LF was found within the neuronal proteasome fraction of wild type mice (Fig. 2), and over-expression of Usp14LF resulted in an increase in the amount of Usp14LF that associates with neuronal proteasomes (Fig 6a), suggesting that not all proteasomes contain Usp14LF. Therefore, by controlling the association of Usp14LF with the proteasome, it may be possible to modulate the deubiquitinating activity of the proteasome and thus control protein turnover in a temporal or spatial manner.

Our studies have also indicated that Usp14 is predominately a cytoplasmic protein. This was unexpected because previous reports demonstrated that the Usp14 homologue, Ubp6, is predominately a nuclear protein in yeast (Chernova et al., 2003). In addition, while some neuronal proteasomes are found in the cytoplasm, neuronal proteasomes have been shown to be localized primarily to the nucleus (Mengual et al., 1996; Wojcik and DeMartino, 2003). The cytoplasmic distribution of Usp14 therefore suggests that cytoplasmic proteasomes may have a different profile of associated DUBs than nuclear proteasomes and that they may therefore regulate different functions or activities at the

proteasome. Cytoplasmic proteasomes are found in the cell soma, dendrites and axons as well as synaptic terminals, where they have been suggested to regulate the stability of proteins involved in the function of synapses (Mengual et al., 1996; Bingol and Schuman, 2005; Aravamudan, 2003; Speese et al., 2003). The demonstration that Usp14 is localized to the cell soma and neurites in primary hippocampal neurons and in brain sections is consistent with our previous findings that loss of Usp14 alters synaptic activity (Wilson et al., 2002) and suggests that Usp14 may have a direct role in modulating synaptic function.

The rescue of the neuromuscular defect in the ax^J mice by neuronal specific expression of Usp14LF also supports our studies that indicate Usp14 functions at the presynapse (Wilson et al., 2002). Since our *Thy1-Usp14LF* construct expresses only the Ubl-containing form of Usp14, these experiments also suggest that the essential function of Usp14 is likely to be on the proteasome. Although it is formally possible that rescue of the ax^J mice is due to a proteasome independent function, we believe that our data are more consistent with a model whereby Usp14 functions on the proteasome. Our previous studies on the ax^J mice, and those on the Usp14 homologue in yeast, demonstrated that Usp14/Ubp6 functions to recycle ubiquitin at the proteasome (Anderson et al., 2005; Legget et al., 2002; Chernova et al., 2003). Examination of the ax^J *Tg* mice in this study (Fig. 6c) demonstrated that the Ubl-containing form of Usp14 is essential for maintaining ubiquitin levels in the brain. Because Usp14 is predominately a cytosolic protein, it suggests that the activities associated with the 19S subunit may vary with its subcellular location and that the 30% loss of monomeric ubiquitin levels is likely to be due to defects in cytosolic proteasomes. Whether there are potential focal sites of ubiquitin depletion in

the *ax*^{J} mice, such as at the synapse, is not known. The requirement of Usp14 to disassemble ubiquitin side chains at the proteasome may therefore be necessary for maintaining the local concentration of ubiquitin at sites of intense UPS activity, or to rescue proteins from degradation by removing their ubiquitin side chain. Given that Usp14 is expressed in a variety of tissues, and that loss of Usp14 results in decreased levels of ubiquitin in most tissues, either the nervous system is uniquely sensitive to decreased ubiquitin levels, or Usp14 has another function(s) in the nervous system.

Our behavioral tests demonstrated that, although the *Thy1-Usp14LF* transgene restores most of the motor system defects of the *ax*^{J} mice, mild coordination defects still exist in the *ax*^{J} *Tg* mice. Examination of Usp14LF expression patterns in mice demonstrated that the only neuronal populations that did not express detectable levels of Usp14 were the CA3 hippocampal pyramidal cells and Purkinje cells of the cerebellum. The lack of expression of the *Thy1-Usp14LF* transgene in the Purkinje cells is consistent with the increase in the number of foot slips on the elevated beam test and with the presence of the Purkinje cell axonal swellings in the *ax*^{J} *Tg* mice. Although the initial description of the *ax*^{J} mice demonstrated the presence of Purkinje cell axonal swellings, which were suggested to be causal to the *ax*^{J} motor defects, our data do not support a primary role of the Purkinje cell axonal pathology in the *ax*^{J} muscle wasting, tremor and ataxia. However, given the robust expression of Thy1-Usp14LF in the granule cell layer and the remaining Purkinje cell disease, the inability to correct the Purkinje axonal swellings indicates that Usp14 acts in a cell autonomous fashion. Further investigation into the expression patterns of the *Thy1-Usp14LF* transgene should provide important insights into the dysfunctional neuronal circuit(s) in the *ax*^{J} mice.

It is becoming increasingly clear that changes in the activity of the UPS play an important role in both synaptic plasticity and disease (Karpova et al., 2006; Patrick, 2006; Yi and Ehlers, 2005; Speese et al., 2003; Wilson et al., 2002). It is therefore possible that Usp14 has evolved a specialized function in neurons to regulate the ubiquitin side chain length of proteins bound to the proteasome. Since proteins with short ubiquitin chains are thought to be poor substrates for the proteasome (Guterman and Glickman, 2004), Usp14 may be involved in the release and rescue of proteins from proteasomal degradation. An essential question that remains is whether Usp14 exhibits substrate specificity or acts globally on proteins targeted to the proteasome. Given that there appears to be selective neuronal vulnerability to the ax^J mutation, we believe that it is likely that Usp14 functions in the processing of specific substrates. In addition, since Usp14 resides on the regulatory particle of the proteasome, it is poised at a critical position to modulate protein degradation and to influence processes vital to maintaining neuronal viability.

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Figure Legends

Figure 1. *Usp14* is alternatively spliced. (A) The left panel depicts *Usp14* RT-PCR products from tissues of adult (postnatal day 64) mice and the right panel shows the *Usp14* RT-PCR products from E7 and E15 embryos and whole brain from postnatal day 28 (P28) wild type mice demonstrating the 2 forms of *Usp14* produced from the alternative splicing of exon 4. Primers flanking exon 4 were used for amplification of reverse transcribed cDNAs. (B) Schematic diagram of the cDNAs isolated from brain. The full length cDNA is referred to as *Usp14LF* and the splice variant lacking exon 4 (asterisk) is referred to as *Usp14SF*. (C) Immunoblot analysis of 6-week old wild type and ax^J brains demonstrating the two forms of Usp14. Blots were probed with polyclonal antisera to Usp14 and re-probed for α-actin as a loading control. (D) Immunoblot analysis demonstrating the different migration patterns of Usp14LF and Usp14SF expressed from either wild type E15 embryos, COS7 cells transfected with expression vectors for *Usp14LF* or *Usp14SF*, and neuronal proteasome fractions from wild type (P28) mice. Blots were probed with polyclonal antisera to Usp14. (E) Immunoblot of Usp14 showing the similar migration pattern between recombinant Usp14 and Usp14 found in neuronal proteasomes from wild type mice. Blots were probed with polyclonal antisera to Usp14. (F) 2D electrophoresis gel of proteasome fractions from wild type mice immunoblotted with polyclonal antisera to Usp14.

Figure 2. Usp14 is catalytically active only in the presence of proteasomes. The top panel indicates the catalytically active DUBs detected in wild type and ax^J proteasome fractions in the presence and absence of recombinant Usp14. The preparations were incubated with HA-Ub-VS and probed with the anti-HA antibody 12C5. The locations of the two proteasome-bound DUBs are indicated to the right. The middle panel depicts total Usp14 detected by polyclonal Usp14 antisera. The blots were also probed with an antibody to the 19S proteasome component Rpt4 as a control.

Figure 3. Usp14 is a cytosolic protein in primary neuronal cultures. (A) Rat primary hippocampal neurons were stained with DAPI (blue) and with antibodies to Usp14 (green) and 26S proteasomes (red). (B) Primary rat hippocampal neurons were transfected with *myc*-tagged *Usp14LF* and stained with DAPI (blue) and a 9E10 anti-myc antibody to detect the myc-tagged Usp14LF (green). Red boxes depict the neurite processes of the transfected neurons.

Figure 4. The *Thy1-Usp14LF* transgene is expressed only in the nervous system. (A) A full length *Usp14* cDNA was cloned into the *Thy1.2* expression cassette to allow for neuronal-specific expression of Usp14LF. (B) Tissue extracts from wild type (wt), ax^J and ax^J *Tg* mice were prepared and immunoblotted with anti-Usp14 polyclonal antibodies to detect Usp14 levels. Blots were probed with an antibody to β-tubulin as a loading control. (C) Quantitation of immunblots from 5 sets of wt, ax^J and ax^J *Tg* animals showing the fold increase of Usp14LF expression in the tissues.

Figure 5. *Thy1-Usp14LF* rescues the postnatal lethality and reduced growth in ax^J mice. (A) Survival curves for wild type (wt), ax^J and ax^J *Tg* mice (n=5). (B) Body weights were obtained over a 20 week period for wt, ax^J and ax^J *Tg* mice (n=5). Error bars indicate SE.

Figure 6. *Thy1-Usp14LF* associates with proteasomes and restores the levels of ubiquitin in ax^J mice. (A) The left panel shows the immunoblot of proteasome fractions from the brains of wild type (wt), ax^J and ax^J *Tg* mice probed with polyclonal Usp14 antisera. The blots were also probed for the 19S proteasomal component Rpt4 as a positive control for proteasomes. The right panel shows catalytically active DUBs from neuronal proteasome fractions from wild type (wt), ax^J and ax^J *Tg* mice that were labeled using HA-Ub-VME and immunoblotted with the anti-HA Mab 12CA5. The identities of the known DUBs are indicated at the right. (B) Immunoblot analysis of hippocampal proteasome fractions from wt mice and wt mice containing the *Usp14LF* transgene (wt *Tg*) probed for Usp14 and the control 19S proteasomal component Rpt1 (right panel) and active DUBs labeled with HA-Ub-VME and immunoblotted with the anti-HA Mab 12CA5 (left panel). Graphs depict quantitation of the blots. (C) Immunoblot analysis of hippocampal extracts measuring levels of conjugated and monomeric ubiquitin in wt, ax^J and ax^J *Tg* mice. As loading controls, blots were re-probed with a monoclonal antibody to α-actin.

Figure 7. The *Thy1-Usp14LF* transgene rescues the ax^J motor dysfunction. Elevated beam walking assay of wild type (wt), ax^J and ax^J *Tg* mice demonstrating that neuronal-specific expression of Usp14LF greatly reduces the number of falls per trial seen in the ax^J mice (n=4). (B) The frequency of front or rear paw slips off the elevated beam is greater in ax^J *Tg* mice than in wild type mice. Error bars indicate SE.

Figure 8. Expression patterns of *Thy1-Usp14LF* in the CNS. Usp14 (red) staining in the cerebellum (A, C), cortex (E, G) and hippocampus (I, K) of wild type (wt) and ax^J *Tg* mice. Endogenous Usp14 is not detected in wild type tissues. DAPI staining is shown in blue.

Figure 9. Purkinje cell axonal pathology in ax^J and ax^J *Tg* mice. (A) Indirect immunofluorescence microscopy was used to examine Purkinje cell axons from ax^J and wild type (wt) mice during development using calbindin (red) antibodies and DAPI (blue). (B) Indirect immunofluorescence microscopy demonstrated that ax^J *Tg* mice have a similar Purkinje cell disease as the ax^J mice. Arrowheads indicate the presence of normal Purkinje cell axons in wt mice, and arrows represent abnormal Purkinje cell axons in ax^J and ax^J *Tg* mice.

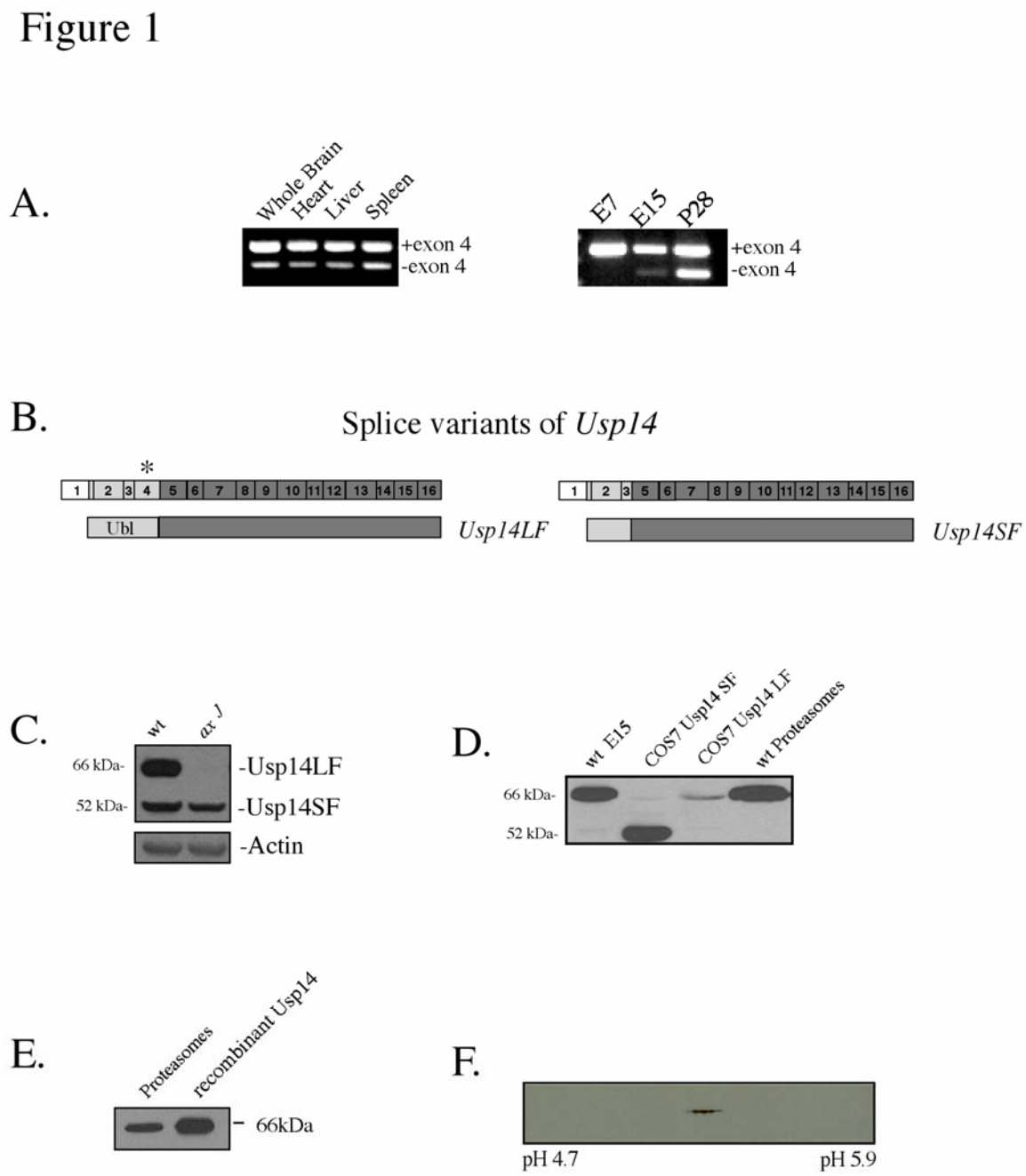


Figure 1. *Usp14* is Alternatively Spliced.

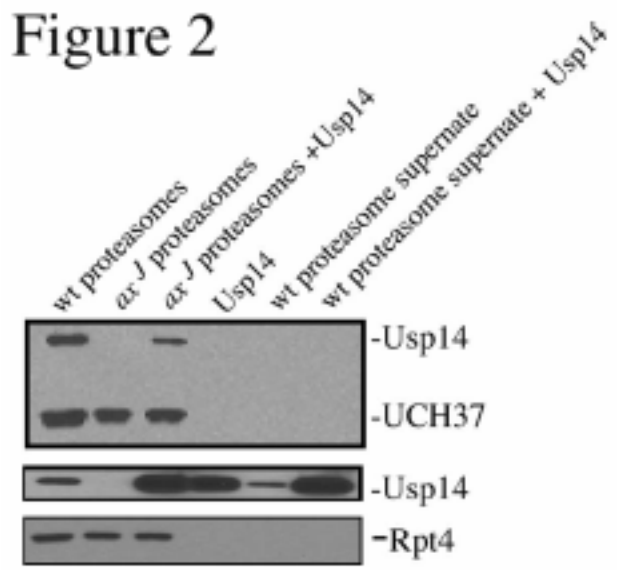


Figure 2. Usp14 is Catalytically Active in the Presence of Proteasomes.

Figure 3

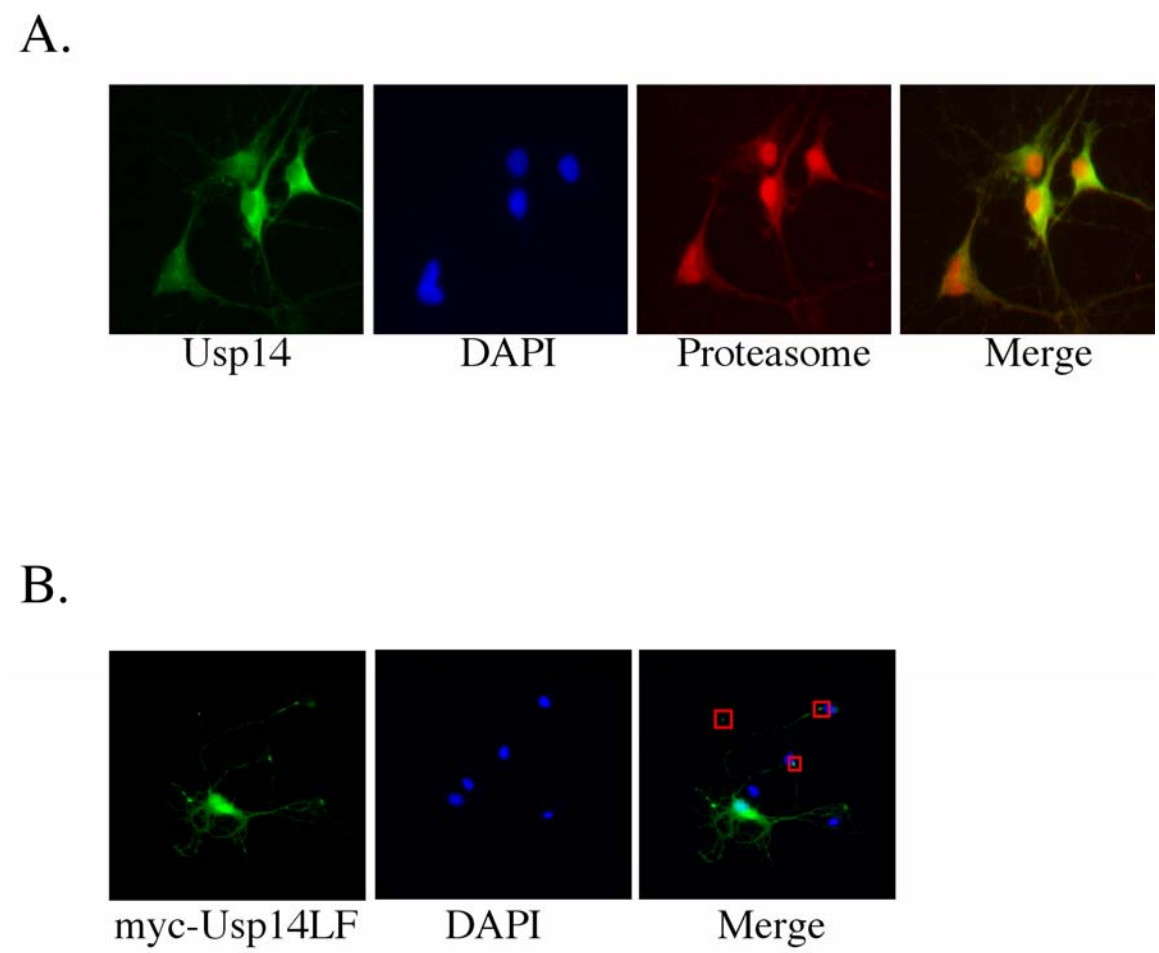


Figure 3. Usp14 is a Cytosolic Protein in Primary Neuronal Cultures.

Figure 4

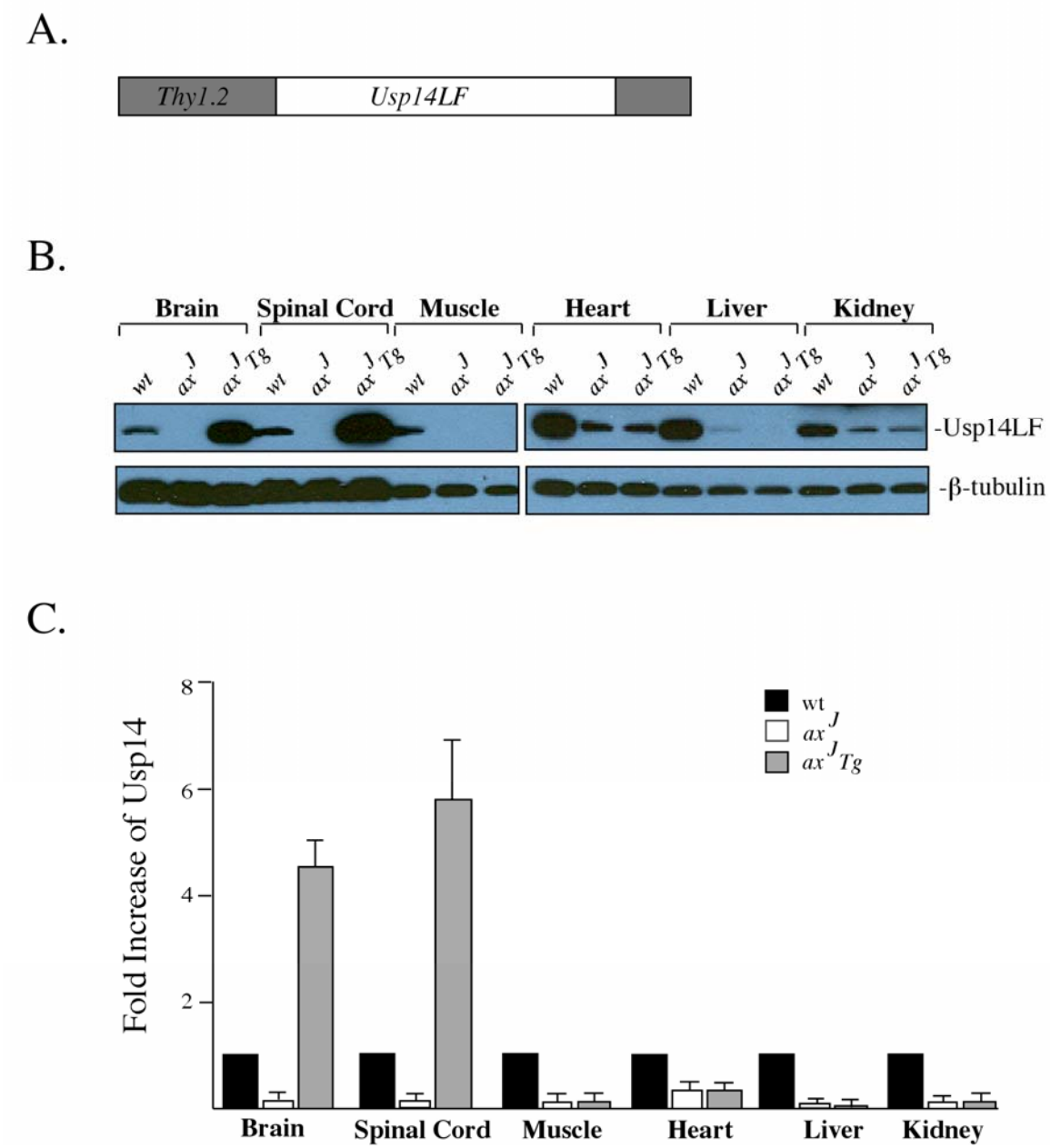


Figure 4. The *Thy1-Usp14LF* Transgene is Expressed Only in the Nervous System.

Figure 5

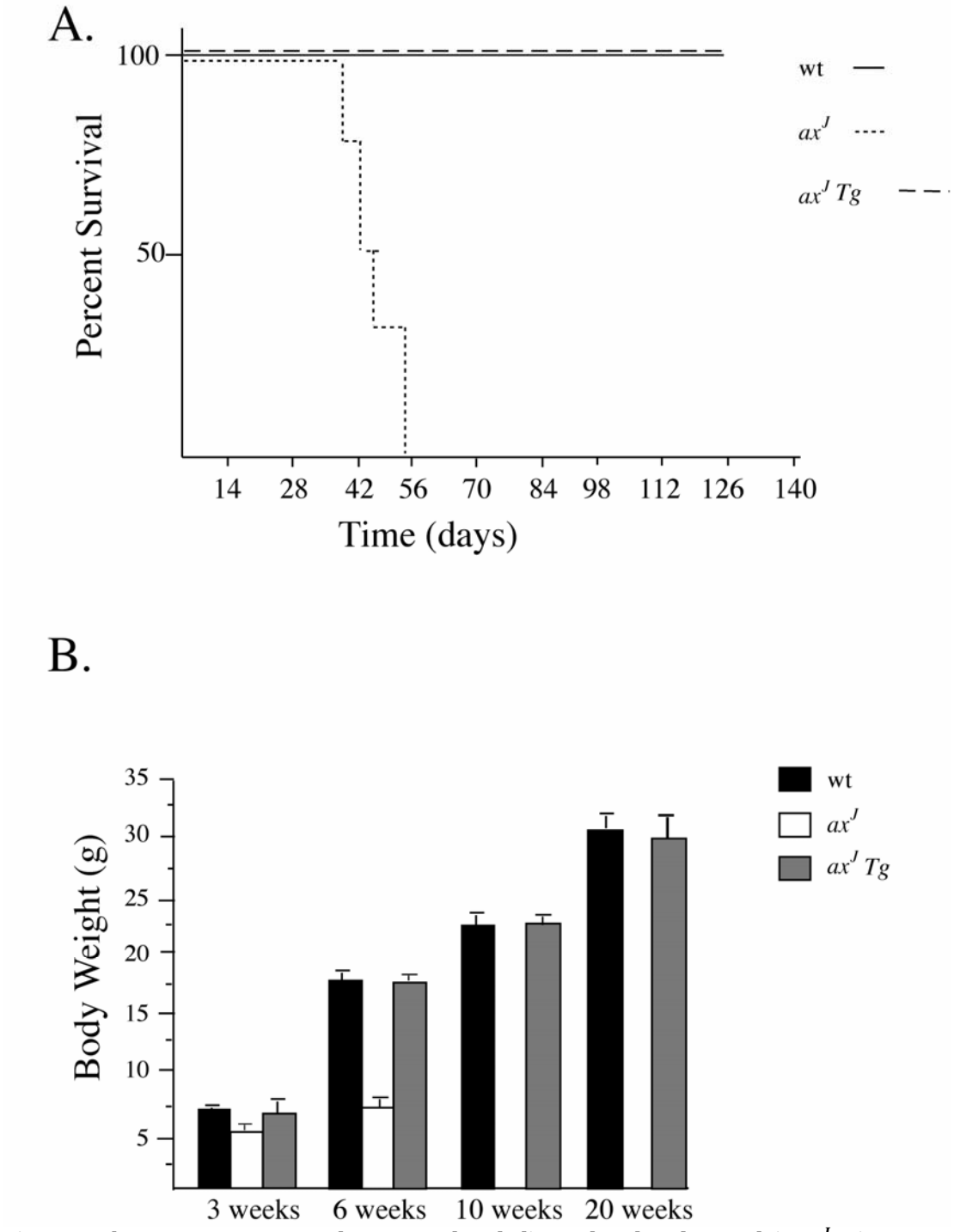


Figure 5. *Thy1-Usp14LF* Rescues the Postnatal Lethality and Reduced Growth in *ax*^{J} Mice.

Figure 6

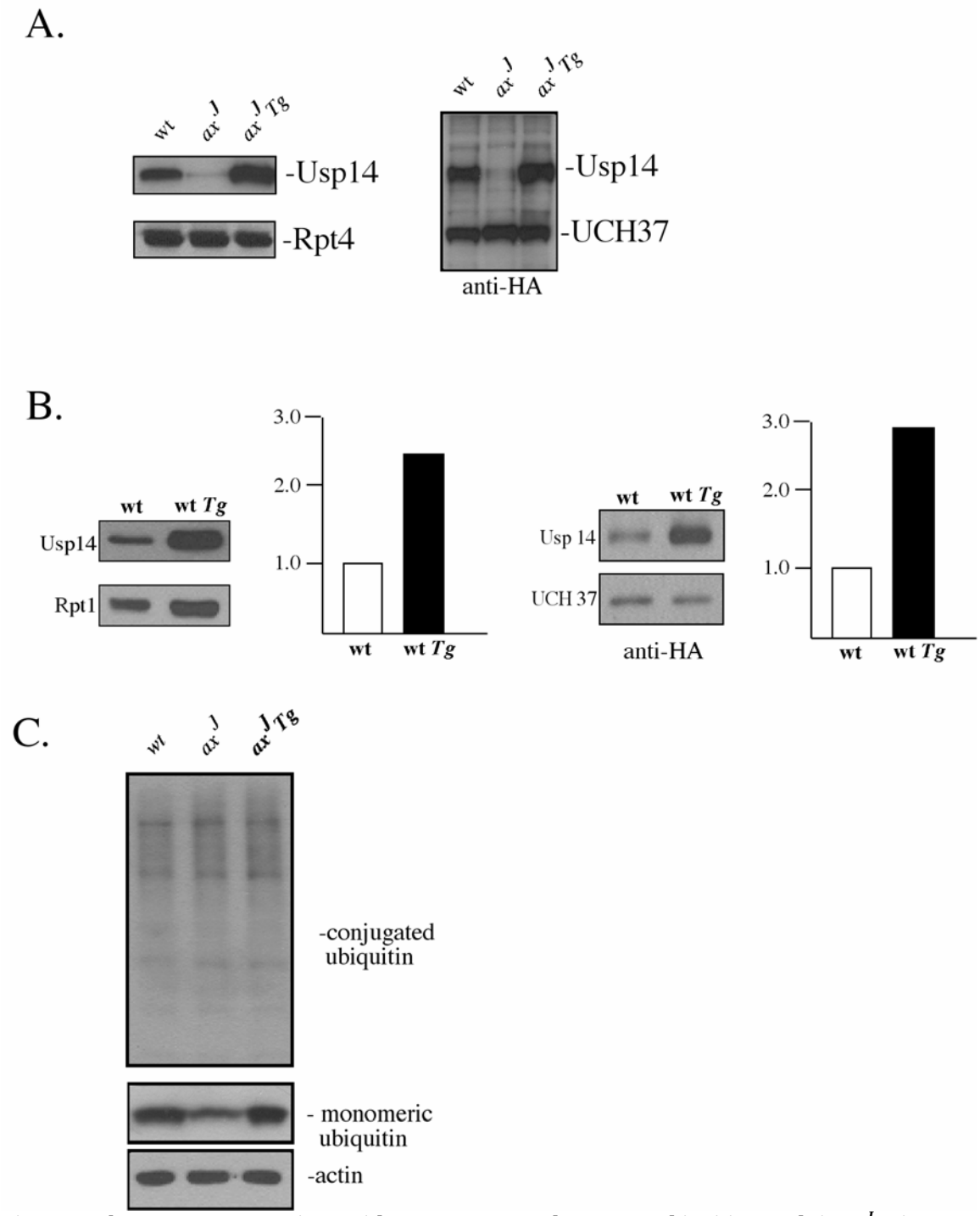


Figure 6. *Thy1-Usp14LF* Associates with Proteasomes and Restores Ubiquitin Levels in *ax^J* Mice.

Figure 7

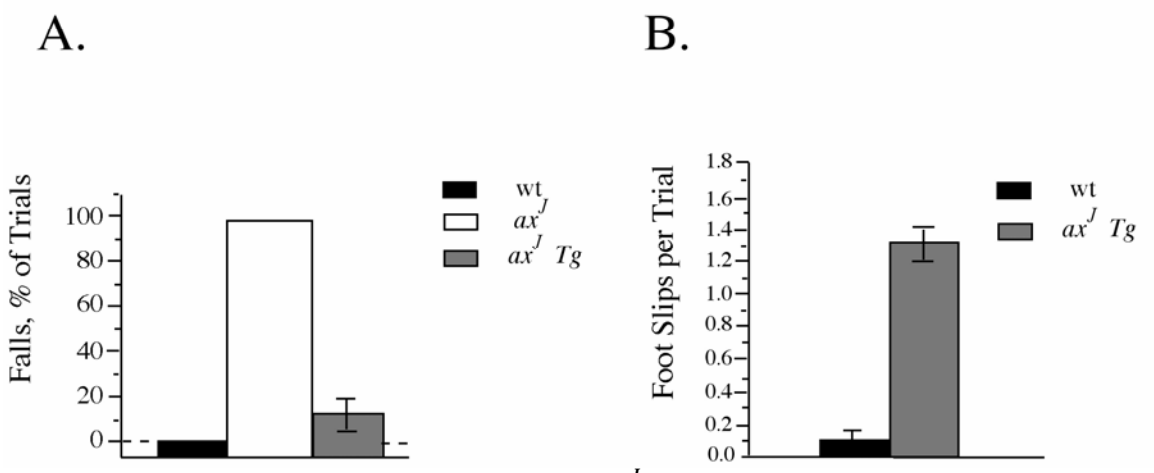


Figure 7. The *Thy1-Usp14LF* Transgene Rescues the *ax*^{J} Motor Dysfunction.

Figure 8

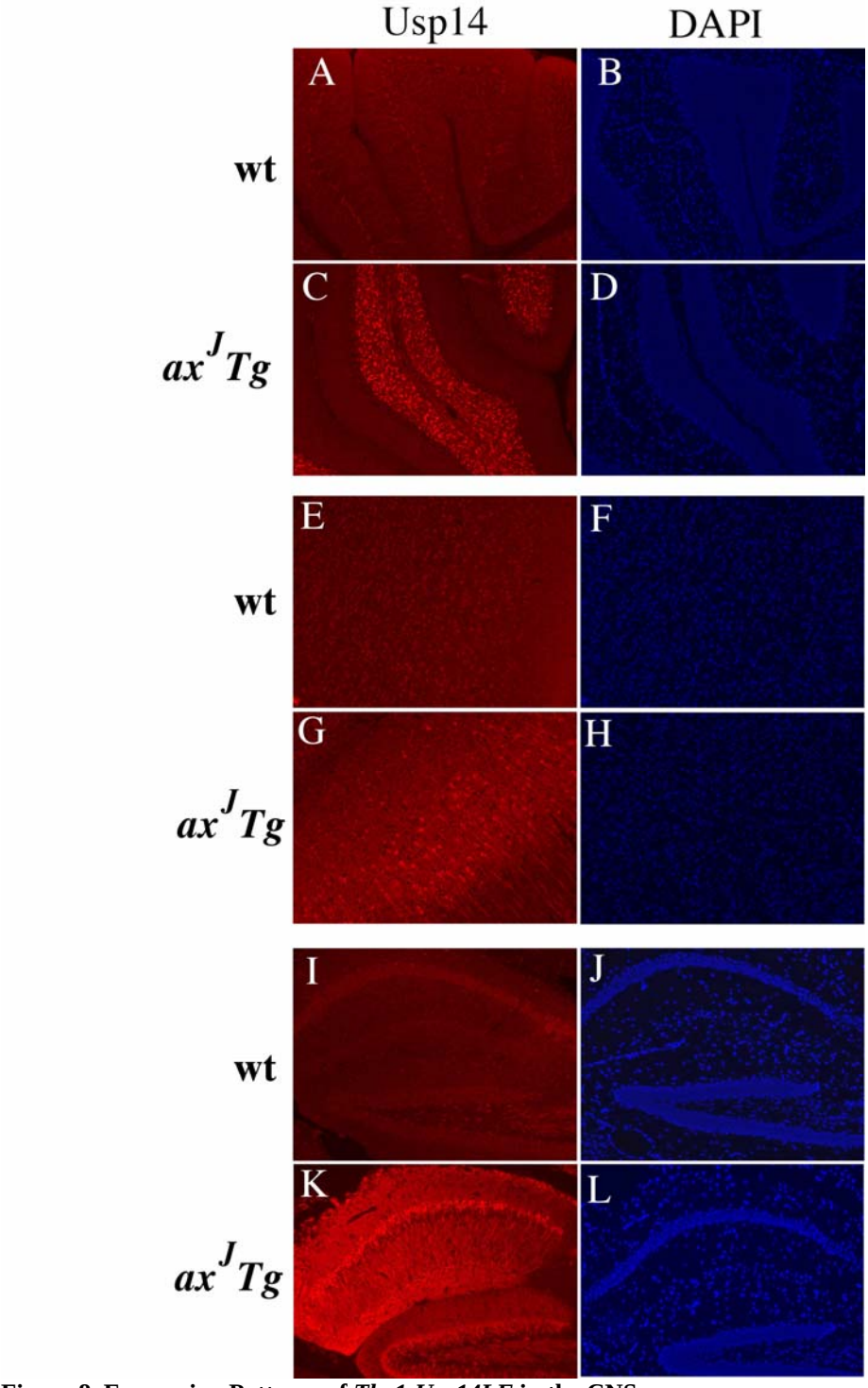
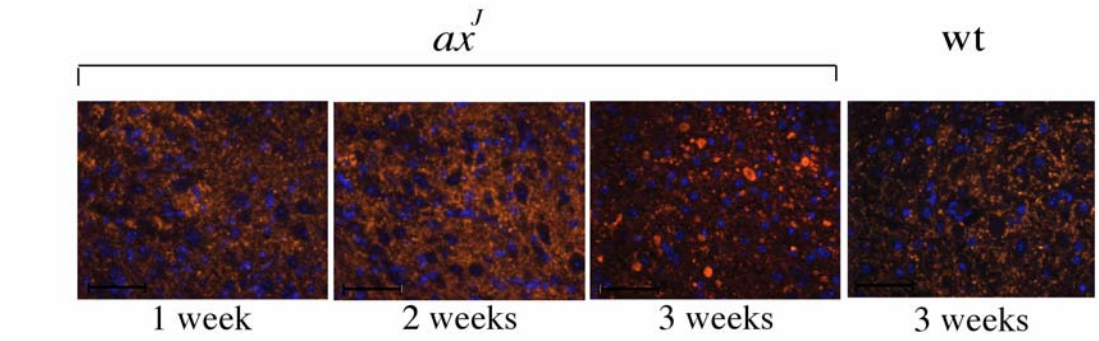


Figure 8. Expression Patterns of *Thy1-Usp14LF* in the CNS.

Figure 9



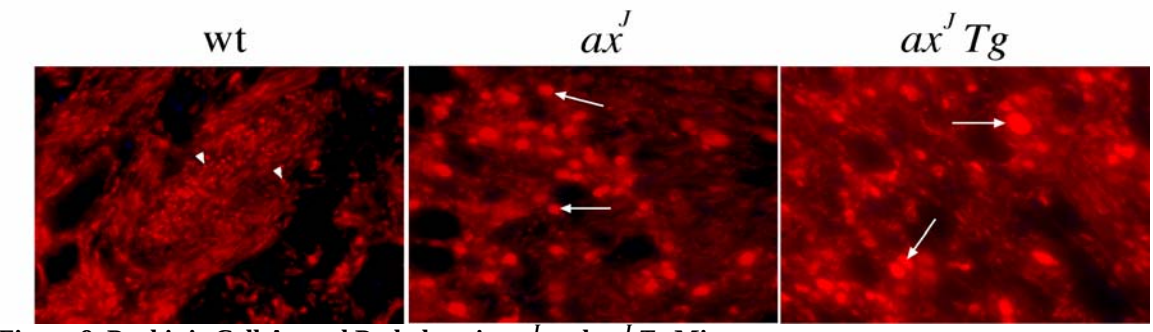


Figure 9. Purkinje Cell Axonal Pathology in *ax*^{J} and *ax*^{J} *Tg* Mice.

SUMMARY

The UPS plays an essential role in the regulation of neuronal function. The UPS is now being recognized as a significant regulatory pathway that controls disparate neuronal processes such as receptor endocytosis, vesicle pool size, cell cycle, protein degradation, synaptic dynamics, long term potentiation, and axonal growth [6, 74-85]. This work focuses on the characterization of Usp14, the proteasome associated DUB that has reduced expression in the ax^J mice [71]. The phenotypes of these mice, which consists of resting tremor, premature death, and muscle wasting, highlights the importance of protein deubiquitination in proper growth and development.

The ax^J Mutation

The cloning of the ax^J mutation demonstrated that Usp14 is critical to proper nervous system function in mice [71]. The ax^J mutation arose spontaneously through the transposition of an IAP into intron 5 of the *Usp14* gene. During pre-mRNA processing of the ax^J transcript, the IAP is retained in the mRNA because of the presence of cryptic splice sites in the IAP. Approximately 5% of the transcripts are correctly spliced and produce functional Usp14. When the ax^J mRNA transcript is translated, a stop codon in the IAP causes premature termination. The result of the IAP insertion is an approximately 90% reduction in the levels of Usp14 protein.

Because there is still some functional Usp14 produced in the ax^J mice, we procured Usp14 knockout (Usp14 KO) embryonic stem cells and attempted to generate adult

mice with a completely null allele of Usp14; thus, enabling us to study an animal with complete deletion of Usp14. The critical importance of Usp14 is further highlighted in the Usp14 KO mouse. As was discussed in the introduction, although Ubp6 KO yeast have decreased levels of monomeric ubiquitin and show growth sensitivity to various drugs, the knock out yeast are still viable in normal conditions. Usp14 KO mice on the other hand die *in utero* thus demonstrating that this DUB has evolved to perform absolutely essential functions for viability. These essential functions, as will be explored in this text, most likely concern Usp14's role as an enzymatic component of the 26S proteasome and an essential regulator of ubiquitin levels in neurons.

Due to the early death of the USP14 KO mice at E12, we relied on the ax^J mice to examine the function of Usp14 in the nervous system. My studies explored in greater detail the Usp14 gene products and how they function in the nervous system. Since there has been extensive work done on Ubp6, we will utilize those studies including work on Ubp6 KO yeast to facilitate our studies on Usp14, especially in our biochemical analysis [70-72].

Usp14 Transcripts and Protein

To characterize the function of Usp14, we first examined the *Usp14* transcripts produced at various time points in prenatal and postnatal tissue. Analysis of the mRNA and protein expression of Usp14 showed the presence of two splice forms of Usp14. One form is Usp14 long form (Usp14LF), which encodes the full length form of the protein. The other form is called Usp14 short form (Usp14SF), which is created by removal of exon 4 during pre-mRNA processing. The alternative splicing of exon 4 removes part of

the Ubl domain of Usp14. Exon 4 of *Usp14* encodes an important conserved structural feature called the ubiquitin fold. This ubiquitin fold is critical for the ability of Usp14 to bind to its receptor on the proteasome [47, 86]. Interestingly, although Usp14LF is expressed at the same level throughout development, the Usp14SF is not fully expressed until postnatal day 28. The expression time points of Usp14LF and Usp14SF indicate some interesting things about the function of the 2 forms of Usp14. Since Usp14LF is expressed at the same level throughout the development of the animal, this form most likely has a generalized cellular function in the organism. Usp14SF, on the other hand, is expressed at a low amount at E15 and increases in expression until P28. The time course of Usp14SF expression indicates that this form may have a very specific role in ubiquitin signaling that is independent of the proteasome. Multi-tissue Western blot analysis reveals that Usp14 is present in all tissue types tested. Western blot analysis of multiple tissue systems from ax^J mice demonstrated that Usp14LF is knocked down in all tissue types. In contrast, we have not been able to detect any changes in expression of Usp14SF in the ax^J mice. The wild type expression levels of Usp14SF most likely results from the removal of the IAP during pre-mRNA processing and indicates differential effects of the IAP on the alternative pre-mRNA processing of Usp14. The specific loss of Usp14LF demonstrates that Usp14SF is not associated with the ax^J phenotype and that the proteasome binding of Usp14LF is likely to be an absolute requirement for nervous system function.

Usp14 Localization in Neurons

In order to discern the localization of Usp14 in neurons, we produced monoclonal and polyclonal antibodies to the catalytic domain of Usp14. Since the catalytic domain is shared between the two different forms of Usp14, I was unable to discern the localization differences between Usp14SF and Usp14LF. However, immunostaining of cultured rat hippocampal neurons using these antibodies demonstrated that endogenous Usp14 localized to the cytoplasm and extended into the neurites. Although our Usp14 antibodies would not allow us to examine any localization differences between endogenous Usp14SF and Usp14LF, we attempted to circumvent this problem by transfecting myc-tagged Usp14 constructs in neurons. We expressed myc-tagged Usp14SF and Usp14LF constructs in cultured rat hippocampal neurons. I found that the myc-Usp14SF construct did not transfect or express to an appreciable level in our culture system. The low expression level of myc-Usp14SF could indicate that the cellular level of this form of Usp14 is carefully regulated. On the other hand, transfection of myc-Usp14LF into neurons resulted in robust expression and a localization pattern similar to that of endogenous Usp14. The data concerning the localization of Usp14 give us further insight into the potential cellular function of the enzyme. It is possible that, because Usp14 is localized to the neurites and cytoplasm of neurons, Usp14 could have a direct effect on synaptic activity.

Usp14 Co-Localization with the Proteasome

The previous *in vitro* data showing the activation of Ubp6 by the proteasome prompted us to examine the co-localization of Usp14 with the proteasome in neurons

[47]. On the basis of the co-fractionation of Usp14 with the proteasome examined by Western blot, where less than 100% of cellular Usp14LF fractionates with the proteasome, and none of the Usp14SF co-fractionates, I hypothesized that endogenous Usp14 would partially co-localize with the proteasome in neuronal cultures when examined by immunohistochemistry. I stained cultured rat hippocampal neurons for Usp14 and the 26S proteasome and found that there is not 100% co-localization between Usp14 and the proteasome. This lack of co-localization is exemplified by the high intensity staining of proteasomes in the nucleus but a low amount of Usp14 staining in that same cellular organelle. Immunofluorescence corroborated the subcellular fractionation data demonstrating that some cellular Usp14 is not bound to the proteasome. The subcellular fractionation data in particular suggest that not all proteasomes have Usp14LF bound to them indicating that Usp14LF may be a transiently interacting proteasomal subunit. In conjunction with the synaptic defects seen in the ax^J mice, these data indicate something interesting about the cellular regulation of Usp14. One, this observation indicates that not all proteasomes are equivalent and that Usp14 is involved in controlling ubiquitin signaling events in the cytoplasm. Two, these data suggest that there are two pools of Usp14, a proteasomal bound pool and a non-proteasomal bound pool. It is possible that the non-proteasomal bound pool of Usp14 requires a cell signal to induce association with and activation by the proteasome; such a cell signal may be caused by a post-translational modification of Usp14.

Post-Translational Modification of Usp14

Many signaling cascades rely on post-translational modification. 2D gel analysis is a technique that can detect post-translational modification of a protein since modifications will produce electro-mobility shifts in the protein during isoelectric focusing. We examined proteasomes using 2D gel analysis for evidence of such post-translational modifications on Usp14. 2-D gel analysis of proteasomes, and immunoblotting for Usp14, revealed an electro-mobility shift for Usp14 in the first dimension. The presence of 5 Usp14 immunopositive spots of decreasing pI on the 2D gel is consistent with Usp14's being phosphorylated at multiple sites. To verify that the electro-mobility shift of Usp14 is due to phosphorylation, phosphatases could be used to remove the phosphorylation events. If the Usp14 immunopositive spots on the 2D gel are phosphorylation events, the removal of the phosphate groups would result in all the Usp14 immunopositive spots to condense into one spot on the 2D gel. Phosphorylation of Usp14, as will be demonstrated later, is not absolutely necessary for *in vitro* association and activation by the proteasome; however, *in vivo*, phosphorylation may provide an additional important regulatory mechanism for the activity of Usp14 in relation to the proteasome. Phosphorylation may function to change the avidity of Usp14's interaction with the proteasome or function as an additional layer of proteasome associated regulation that increases or decreases the time that Usp14 spends in either the active or the inactive conformation.

A search for phosphorylation consensus sequences revealed several different putative phosphorylation sites on Usp14 that include the kinases Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase C, and casein kinase II, all of which

have known presynaptic functions and thus fit with the observed presynaptic defects of the ax^J mice [87-91]. Interestingly, a CaMKII phosphorylation site was identified on one of Usp14's blocking loops, and this finding indicates that CaMKII phosphorylation may directly regulate the activity of Usp14. Phosphorylation of Usp14's blocking loop by CaMKII may increase the amount of time that Usp14 spends in the active state on the proteasome or may regulate the association of Usp14 with the proteasome. To test this experimentally, we can use *in vitro* kinase reactions to phosphorylate Usp14 with CaMKII. The phosphorylated Usp14 can be added back to ax^J proteasomes, and the activity can be studied by using a vinylsulfone HA-tagged ubiquitin probe (HA-Ub-VS) that can irreversibly label DUBs in an activity dependent manner. DUBs labeled with the HA-Ub-VS probe can be examined by Western blot with the use of anti-HA antibodies that detect if the HA-Ub-VS molecule is covalently attached to the active site cysteine of the DUB. Presence of immunopositive HA bands indicate a catalytically active DUB. It has been demonstrated that synaptic activity can rapidly produce global changes in the ubiquitinated state of proteins in the synapse [92]. Chen *et al*. demonstrated that these changes are dependent on calcium and are not affected by proteasome inhibitors, suggesting that the mechanism that changes the ubiquitinated protein profile at the synapse is due to deubiquitinating enzymes and not due to 26S proteasome degradation [92]. Because CaMKII activity is induced during synaptic activity in response to the influx of calcium into the synapse, this particular kinase may phosphorylate Usp14's blocking loop to increase Usp14's catalytic activity. The induction of Usp14's catalytic activity in response to CaMKII phosphorylation may provide a mechanism that allows for Usp14 to contribute to the global changes in ubiquitinated proteins demonstrated in the Chen *et al*. paper.

To further study the effect that these proposed phosphorylation sites have on Usp14, the putative phosphorylation residues on Usp14 could be mutated. Mutation of these residues could be used to prevent phosphorylation. Alternatively, the putative phosphorylation residues could be replaced with aspartic acid, which can function as a constitutively active phosphorylation residue by mimicking the negative charge of a phosphate group. The mutant Usp14 constructs could be used to generate recombinant protein and used in the *in vitro* kinase reactions mentioned above. Also, to examine the contribution that mutations in the proposed phosphorylation sites have on Usp14 activity *in vivo*, the constructs could be transgenically expressed in ax^J mice. The 2-D gel analysis and the phosphorylation consensus sequences found on Usp14 suggest that, *in vivo*, the activity of Usp14 may be regulated by multiple kinase signaling cascades possibly as a result of synaptic activity.

Activity of Usp14

Because of the data suggesting that Usp14 may be a transiently interacting proteasome subunit, we wanted to test the catalytic activity of both proteasome bound and unbound Usp14. To examine the activity of these different Usp14 fractions, we allowed recombinant Usp14 to associate with proteasomes enriched from ax^J mice and then carried out activity dependent labeling with the HA-Ub-VS probe. Labeling of recombinant Usp14 was detected in the proteasome enriched fraction from ax^J mice, whereas recombinant Usp14 alone did not label. Endogenous Usp14 retained in proteasome free supernatant generated during proteasome purification was also tested for activity. Usp14 in the

supernatant fraction was not labeled with HA-Ub-VS; these findings indicate that the activators of Usp14 co-sediment in the proteasome fraction.

Our proteasome purification methods do not produce a pure fraction of proteasomes. Therefore, the possibility exists that Usp14 is being activated by other proteins that co-purify with the proteasome. This problem can be addressed by using different techniques to improve proteasome purity. Fast protein liquid chromatography is one method of obtaining highly purified samples of biologically active enzymes. Another method uses affinity capture by the epitope tagging of proteasomal subunits [47]. Using these protocols, we can obtain pure ax^J proteasomes for use in testing the activation of Usp14 by proteasome binding. These experiments can also be used to discover potential nonproteasomal activators of Usp14. Since our standard proteasome purification method produces a pellet that contains a stimulator of Usp14 activity, we can use fast protein liquid chromatography or affinity purification detailed above to remove the proteasomes from this pellet. The proteasome void pellet fraction can then be tested for its ability to activate Usp14. If this proteasome void fraction is able to activate Usp14, we can then sequence the proteins contained in it to identify candidate Usp14 activating proteins.

Enzymatic Activities of the ax^J 26S Proteasome

We were interested in thoroughly examining the enzymatic activity of the 26S proteasome for defects that could help explain the ax^J phenotypes. Previous data showing the increased activity of Usp14 in response to 26S proteasome inhibition suggested a possible enzymatic coupling between Usp14 and the 20S CP [46]. Similarly, the enzymatic coupling could work in the opposite direction such that loss of Usp14 could influ-

ence 20S CP activity. To address this concern, we tested the ability of the 20S CP from ax^J mice to degrade model substrates [72]. We found that there was no change in the rate of model substrate degradation by the ax^J 20S CP when compared to the wild type 20S CP; this finding indicates that a putative proteasomal defect would be localized to the 19S RP.

Because Usp14 is only one of three DUBs active on the 19S RP, I wanted to examine the other enzymes on this substructure for normal function in the ax^J mice. The 19S RP contains two other deubiquitinating enzymes in addition to Usp14: Uch37 and Poh1. To examine the 19S RP for changes in enzymatic activity in response to loss of Usp14, I applied HA-Ub-VS to purified ax^J proteasomes and assayed for labeling of the associated DUBs. This experiment revealed that there were no changes in labeling of 19S RP associated Uch37 and thus demonstrated that loss of Usp14 does not affect the levels or the enzymatic activity of this proteasome bound DUB or result in the recruitment of other HA-Ub-VS sensitive DUBs to the proteasome.

Because Poh1 is not labeled by the HA-Ub-VS probe, we are unable to rule out changes in Poh1 activity in response to loss of Usp14. However, recent evidence demonstrating the coupling of enzymatic activity in yeast between Ubp6 and Rpn11, the yeast homologue of Poh1, indicates that loss of Usp14 may affect Poh1 activity. It was recently found by Hanna *et al.* that Ubp6 functions to delay proteasome degradation in an activity independent manner; the group hypothesized that Ubp6 delays proteasome degradation of substrates so that Rpn11 can assist in the removal of large chains of ubiquitin [58]. The data demonstrating the inverse relationship between Ubp6's catalytic activity

and Rpn11's catalytic activity suggests that Ubp6 can negatively regulate the deubiquitinating activity of Rpn11 on the proteasome.

Although these experiments were performed in budding yeast, which lacks Uch37, Hanna *et al*. demonstrated the interesting possibility that loss of Usp14 in the *ax*^{J} mice may enhance Poh1 activity. As mentioned above, Poh1 is not labeled by the HA-Ub-VS probe, possibly due to differences in ubiquitin chain length specificity. Therefore, to test for any changes in Poh1 activity in response to loss of Usp14, one could examine the degradation rate of a model ubiquitinated substrate like cyclin B. Cyclin B is a substrate of the UPS with a short half-life; furthermore, it can be polyubiquitinated *in vitro* [58]. Polyubiquitinated cyclin B could be applied to proteasomes purified from *ax*^{J} and wild type mice. Once the polyubiquitinated cyclin B is added to the different proteasomes, the rate of polyubiquitin chain disassembly and of proteasome degradation of cyclin B can be monitored by Western blotting. If loss of Usp14 increases Uch37 or Poh1 activity, then there should be faster polyubiquitin chain liberation and faster cyclin B degradation by proteasomes lacking Usp14. Since Poh1 is thought to assist in the removal of large ubiquitin chains from substrates, the generation of free ubiquitin chains in this assay would be indicative of Poh1 activity. The faster polyubiquitin chain liberation from cyclin B in response to loss of Usp14 on the *ax*^{J} proteasome would then suggest enzymatic coupling between Usp14 and Poh1.

Loss of Monomeric Ubiquitin in *ax*^{J} Mice

To determine if Usp14 is required for maintaining ubiquitin levels in mammals, and if defects in ubiquitin-recycling contribute to the neuronal deficiencies observed in

the *ax*^{J} mice, we measured the levels of monomeric ubiquitin in wild type and *ax*^{J} mice. A 30-40% reduction in the levels of monomeric ubiquitin was observed in *ax*^{J} brain, liver, heart and spleen, suggests that, in mammals, Usp14 is required for maintaining levels of ubiquitin. Because the nervous system displays altered pathology when the levels of Usp14 are reduced, whereas none of the non-neuronal tissues that have altered Usp14 levels in the *ax*^{J} mice have exhibited any pathological defects, neurons may be more sensitive to the ubiquitin depletion resulting from the loss of Usp14. Given that synaptic activity likely places special demands on the UPS in neurons, it is not completely surprising that neurons may have different requirements for the UPS than other tissues. Perturbations in the UPS can have a variety of effects on synaptic transmission. Inhibition of the proteasome in neurons results in an increase in fluorescent styrylpyridinium dye staining which is used to visualize the size of the vesicle pool in the presynapse [77]. Willeumier *et al*. suggest that the proteasome is used as a homeostatic regulator of synaptic function. The proteasome most likely accomplishes its homeostatic role by regulating the stoichiometric ratio of synaptic proteins, the importance of which has been demonstrated in the *Drosophila* neuromuscular junction. Proteasome inhibition in the Speece *et al*. study caused an accumulation of DUNC-13, a protein important for vesicle priming, in *Drosophila* neuromuscular junctions [93]. The role of the UPS in synaptic function is not just localized to the presynapse. Receptor endocytosis in the postsynaptic compartment, specifically demonstrated with receptor tyrosine kinases and glutamate receptors, also utilizes ubiquitin signaling [6, 94]. Furthermore, as will be discussed below, Ehlers found changes in the ubiquitinated state of proteins in the postsynaptic compartment in response to synaptic inhibition and stimulation [78]. The stability of the ubiquitin pool

possibly occurs through Usp14's removal of ubiquitin from substrates that are being degraded by the proteasome; therefore, loss of Usp14 would allow the concurrent degradation of substrates and their attached ubiquitin through the proteasome resulting in the observed loss of monomeric ubiquitin.

The loss of monomeric ubiquitin in the *ax*^{J} mice suggests that the primary defect in the *ax*^{J} mice may result from the decrease in the cellular ubiquitin pool. Because many neurological processes utilize ubiquitin, it is possible that loss of ubiquitin could affect a variety of neuronal activities. There has been some recent work highlighting the importance of dynamic regulation of ubiquitin signaling in the synapse. Ehlers found ubiquitin mediated, activity dependent remodeling of the synapse in an *in vitro* hippocampal cell culture system [78]. What is particularly interesting about Ehlers's work is that he found bidirectional, proteasome dependent remodeling of the synapse in response to synaptic activity: Proteins that were increased during synaptic stimulation were found to be decreased during inhibition, and proteins decreased during synaptic stimulation were increased during inhibition. A few years later, Bingol and Schuman highlighted the dynamic role of the proteasome during neuronal activity [95]. Utilizing the photobleaching of a GFP tagged proteasome subunit, Bingol and Schuman found that synaptic activity induced entry of the proteasome into the dendritic spines and delayed proteasome egress from the spines. The results of these two studies raise some interesting questions about the ways in which ubiquitin levels could affect synaptic communication. Although we see a global loss of ubiquitin in *ax*^{J} neurons, the results of the studies by Ehlers and by Bingol and Schuman indicate that synaptic activity may exacerbate ubiquitin loss in *ax*^{J} neurons; thus, resulting in the observed deficits in synaptic communication. Since *ax*^{J}

proteasomes lack Usp14 which results in ubiquitin pool instability, thus suggesting increased degradation of ubiquitin by the proteasome, recruitment of Usp14 deficient proteasomes to ax^J neural spines may further depress the localized ubiquitin environment. It is possible that the synapse functions as a discrete cellular site which has unique demands upon the UPS. If synapses are sites of intense rapid protein ubiquitination and turnover, then ubiquitin depletion may be extremely high in these regions in the ax^J mice and result in alterations in protein stability at the synapse.

Presently we do not know the extent, if any, of ubiquitin depression in the ax^J synapses or if proteasomal degradation in response to synaptic activity is changed in the mutant animals. A definitive answer on synaptic ubiquitin levels in the ax^J mice could be obtained through purification of whole synaptosomes and Western blotting for ubiquitin levels. Both presynaptic and postsynaptic fractions could also be purified for Western blotting for ubiquitin to examine even more localized ubiquitin deficiencies.

Given our finding of loss of monomeric ubiquitin in the ax^J mice, we utilized previous studies examining the loss of monomeric ubiquitin in Ubp6 KO yeast to guide us to an interesting experiment with the ax^J mice. The growth defect observed when Ubp6 KO yeast are challenged with canavanine, anisomycin, and cycloheximide is most likely caused by an increase in proteasomal load [73]. Because of the loss of monomeric ubiquitin in the Ubp6 KO yeast, the cell cannot properly degrade UPS dependent substrates and the sensitivity to the various drugs develops; however, the drug sensitivity can be rectified by the transgenic overexpression of ubiquitin. The question arises of whether the ax^J phenotype can similarly be reversed through transgenic expression of ubiquitin. To address this question in the ax^J mice, we created a transgenic mouse with neuronal

specific expression of ubiquitin; this expression was driven by the *Thy1* promoter (*Thy1-Ub*). The expression of the *Thy1-Ub* in ax^J mice restored the loss of monomeric ubiquitin in the brain and spinal cord of the ax^J animals. The neuronal specific expression of ubiquitin in the ax^J mice also partially rescued the tremor, muscle wasting, and premature death associated with the loss of Usp14. The *Thy1-Ub* ax^J mice also suggest that the decrease of monomeric ubiquitin originates from the loss of Usp14LF; therefore, Usp14LF is required to maintain the ubiquitin pool in neurons. In addition, the *Thy1-Ub* ax^J mice furthermore demonstrate that due to the continued depression of ubiquitin in non-neuronal tissues, that proper maintenance of the ubiquitin pool is absolutely critical for normal neuronal function. It is unclear why neurons in particular seem to have a narrow tolerance for changes in the ubiquitin pool; however, this narrow tolerance may have developed due to cell wide or synapse specific demands of the UPS in synaptic communication.

Neural Circuits Involved in the ax^J Phenotype

Defects in the cerebellum alone can result in the loss of motor coordination and failure to upright similar to that seen in the ax^J mice. The Purkinje cell degeneration (*pcd*) mice demonstrate the importance of Purkinje cells in motor coordination through complete knockout of the Purkinje cells, which results in a phenotype consisting of ataxia and reduction in body weight [96]. The ataxia that Mullen *et al.* found in the original description of the *pcd* mice was caused by the loss of Purkinje cells in the cerebellum [96]. D'Amato and Hicks suggested that the ax^J phenotype is also due to a cerebellar defect [70]. D'Amato and Hicks arrived at this conclusion through matching the loss of motor

coordination with cerebellar pathology progression as Mullen *et al.* had done in characterizing the *pcd* mice. The characterization of the ax^J mice by D'Amato and Hicks proceeded as follows. At 2 weeks, the onset of unsteadiness and weakness was seen in the ax^J mice, and D'Amato and Hicks found that Purkinje cell axonal swellings were already present at that time. By 3 to 4 weeks, the deficiency in the cerebellar white matter was already apparent, although D'Amato and Hicks did not note any myelin disease or phagocytes. The Purkinje cell axonal swellings were also pronounced at 3 weeks which perfectly correlated with the onset of tremor and ataxia in the ax^J mice, this observation suggested a predominant involvement of the Purkinje cells in the ax^J phenotype. What is also interesting is that the two authors did not detect any cell death in the ax^J cerebellum; this finding suggests that, if cell death did occur, it was a protracted event that occurred in just a few cells at any one time.

The findings of D'Amato and Hicks argue for a cerebellar involvement in ax^J phenotype progression [70]. However, the researchers also examined the spinal cord where they found white matter loss at 3 weeks and sparse swellings in the axis cylinders that did not appear in the spinocerebellar tracts, the tracts responsible for carrying proprioceptive information to the brain. If it is assumed that the axonal swellings are indicative of neurological dysfunction, swellings in the descending motoneuron axis cylinders are a particularly interesting finding that opens the possibility that the ax^J phenotype may not involve the cerebellar circuit but may possibly involve a motoneuron circuit. In addition, D'Amato and Hicks also noted changes in muscle size, indicating that the ax^J mice may suffer from a muscular disorder.

Transgenic Expression of Usp14 in *ax*^{J} Neurons

My hypothesis is that loss of Usp14 expression in the nervous system causes the *ax*^{J} phenotype; if this hypothesis proves true, the phenotype is caused by a neuropathy. To test this hypothesis, I generated a mouse that transgenically expresses Usp14LF only in the nervous system. Usp14LF was chosen instead of Usp14SF because of the specific loss of Usp14LF in the *ax*^{J} mice. Also, the loss of monomeric ubiquitin likely results from abnormal ubiquitin recycling on the proteasome, where Usp14LF is known to be active. The expression of Usp14LF was driven by the neural specific *Thy1* promoter (*Thy1-Usp14LF*). The *Thy1-Usp14LF* transgene was crossed into the *ax*^{J} mice to restore expression of Usp14LF in the nervous system of the *ax*^{J} animals. *ax*^{J} mice that contain the *Thy1-Usp14LF* transgene are referred to as *ax*^{J} Tg animals. After the *ax*^{J} Tg mice were created, expression of the *Thy1-Usp14LF* transgene was examined. Multiple tissue Western blot analyses of the *ax*^{J} Tg mice indicated that the *Thy1-Usp14LF* transgene is only expressed in brain and spinal cord; thus, demonstrating that this experimental procedure is able to create an *ax*^{J} mouse with increased expression of Usp14LF in the nervous system but maintained reduced expression of Usp14 in all other tissue types.

Next, I wanted to examine the proteasomes of the *ax*^{J} Tg mice to look at the effect that the *Thy1-Usp14* transgene has on the proteasome. We must determine if transgenically expressed Usp14 can complement the biochemical defects in the *ax*^{J} mice. If transgenically derived Usp14LF does bind to the proteasome, it must also be demonstrated that the biochemical defects of the *ax*^{J} mice were restored; such a demonstration would further strengthen the argument that the *ax*^{J} phenotype is caused by a proteasomal defect.

Analysis of the *ax*^{J} Tg proteasomes showed that the transgenically expressed Usp14LF can bind to the proteasome and that Usp14LF is active on the proteasome as indicated by positive HA-Ub-VS labeling. Western blotting was used to examine total brain extract for monomeric ubiquitin; this examination revealed restoration of the monomeric ubiquitin pool and thus strengthened the argument of an essential function for Usp14 in maintaining cellular ubiquitin levels.

As mentioned above, the tremor and ataxia in addition to the Purkinje cell axonal swellings seen in the *ax*^{J} mouse led to the description of the animal as having a predominantly cerebellar deficit. The *Thy1-Usp14LF* transgene did rescue the premature death, tremor, ataxia, and muscle wasting seen in the *ax*^{J} mice. It was presumed that because of the rescue of the *ax*^{J} phenotype, and the proposed cerebellar involvement in the phenotype, transgene expression was abundant in the cerebellum and corrected the Purkinje cell pathology. Examination of the *ax*^{J} Tg mice by using a balance beam assay revealed an interesting observation. Although the *ax*^{J} Tg mice were able to transverse the balance beam without falling, we noted a significant number of foot slips. It is likely that these foot slips are indicative of motor coordination problems due to persistent cerebellar dysfunction. Examination of the Usp14LF transgene expression profile by immunofluorescence indicates that transgene expression is not restored in the Purkinje cells of the cerebellum. Concordantly, the Purkinje cell axonal swellings seen in the cerebellum of the *ax*^{J} mice were not restored either. Since the Purkinje cells are the only output of the cerebellar cortex, the continued motor coordination problems combined with the persistence of Purkinje cell axonal swellings in the *ax*^{J} Tg mice, demonstrate that the cerebellum, although possibly contributing to the motor coordination problems of the *ax*^{J} mice,

is not the neural circuit causing the overt *ax*^{J} phenotype of muscle wasting, tremor, and premature death.

To determine what neural circuits could account for the reversal of the *ax*^{J} phenotype, I examined the expression of the Usp14 transgene in other neuronal populations. As mentioned above, the description of the *ax*^{J} animals by D'Amato and Hicks indicated spinal cord pathology in the *ax*^{J} mice [70]. Also, defects in the spinal cord would be consistent with the muscle wasting and tremor seen in the *ax*^{J} mice. This observation prompted us to examine the spinal cords of the *ax*^{J} Tg mice for transgenic expression of Usp14. Immunofluorescence staining of the spinal cord in the *ax*^{J} Tg mice showed restoration of Usp14LF expression throughout the spinal cord. Furthermore, the recent unpublished discovery in our lab of axonal swellings and deinnervated neuromuscular junctions in the *ax*^{J} mice makes it likely that the motoneuron circuit is the primary site of dysfunction in the *ax*^{J} mice.

Overall, the transgenic rescue of *ax*^{J} mice with *Thy1-Usp14LF* demonstrated several important principles of Usp14's role in the *ax*^{J} phenotype. First, although there is global loss of Usp14 in all tissues of the *ax*^{J} mouse, the *ax*^{J} Tg mice show that the *ax*^{J} phenotype results from a specific neuropathy. The unique requirement for Usp14 in the nervous system is consistent with a specific role that Usp14 plays in synaptic transmission, a cellular function not exhibited in other organ systems. Second, the *ax*^{J} Tg mice and the *Thy1-Ub ax*^{J} mice also demonstrate a critical role for ubiquitin in the nervous system. These two transgenic animals have depressed ubiquitin levels in all tissues except the nervous system; however, the premature death, muscle wasting, and tremor is reversed. These data also suggest that tight maintenance of the ubiquitin pool is essential

for normal neural function. Third, the *ax*^{J} Tg mice demonstrate that Usp14 must act cell intrinsically to rescue cellular pathology. Finally, although the expression of the *Thy1-Usp14LF* transgene in the brain is 4 to 6 times the level of endogenous Usp14 in wild type brains, these *ax*^{J} Tg mice do not show any additional phenotypes resulting from increased Usp14 expression; thus, indicating tight enzymatic regulation of Usp14. If Usp14's activity was not closely regulated we would expect that wild type mice that contain the transgene and have 4 to 6 times the normal expression levels of Usp14 to show a phenotype. A phenotype in the wildtype animals that contain the transgene could result from increased stabilization of proteins that are normally degraded through the 26S proteasome. Initial studies on wild type animals that contain the transgene suggest that these animals do not show any behavioral defects or changes in synaptic transmission (data not published).

The *ax*^{J} Tg mouse will be of great benefit to the study of Usp14 in the mammalian nervous systems. First, restoration of Usp14 in the nervous system will allow us to examine not only the neural requirement of Usp14 for the *ax*^{J} phenotype but also the possibility that Usp14 is required in other tissue systems. Second, although the *Thy1* promoter is neuron specific, it does not express in all neural subtypes; therefore we have an opportunity to study the specific circuits important for the *ax*^{J} phenotype [97]. Finally, the mosaic nature of the *Thy1* promoter also means that we can potentially use this mouse to dissect the presynaptic and postsynaptic functions of Usp14 by using electrophysiology to record synaptic activity between *ax*^{J} and *ax*^{J} Tg neuron pairs.

Our studies of the *ax*^{J} mice have shown that mice lacking Usp14 have a pronounced defect in synaptic transmission. Defects in synaptic transmission have been

measured in the central nervous system showing loss of paired pulse facilitation in the CA1 to CA3 synapse in the hippocampus. Synaptic transmission measured in the peripheral nervous system at the neuromuscular junction also demonstrated defects. Miniature end plate potentials, which measures spontaneous single vesicle release, at the neuromuscular junction in the ax^J mice are decreased in frequency but doubled in size [71]. Furthermore, the quantal content of the vesicles released from ax^J motoneurons was decreased approximately 50% in the mutant mice. These defects in synaptic transmission are thought to be the underlying cause of the neurological disease in the ax^J mice. I suggest that the synaptic defect in the ax^J mice could result from the following possible mechanisms. As mentioned above, the loss of ubiquitin stability in the ax^J mice suggests that, because of the loss of Usp14, ubiquitin is being improperly degraded by the proteasome. It is possible that the underlying synaptic deficits in the ax^J mice may be caused by increased degradation of important synaptic proteins. This analysis may seem counterintuitive since global decreases in monomeric ubiquitin should indicate that less ubiquitin is available for polyubiquitin chain formation, normally a requirement for the efficient signaling of substrates to the proteasome, and that, therefore, the rate of protein turnover is lower. However, previous studies indicate that monoubiquitination is a sufficient signal for *in vitro* proteasome dependent degradation of proteins [98-100]. If Usp14 predominately removes short ubiquitin chains and possibly monomeric ubiquitin, it may be conceivable that monoubiquitinated proteins sent to the proteasome that are substrates for Usp14, and are normally rescued from degradation by the proteasome, are being degraded in ax^J mice. The improper degradation of monoubiquitinated substrates could potentially occur because, although Poh1 and Uch37 are also proteasome bound DUBs that

could rescue such improperly degraded proteins, their specificity may not recognize the same ubiquitin chain length as Usp14. Therefore, improper synaptic protein degradation may occur in the ax^J mice if such monoubiquitinated substrates targeted to the proteasome are not rescued due to loss of Usp14 on the proteasome. The idea that Usp14 can function to rescue specific substrates from degradation is strengthened by the partial phenotype restoration of ax^J mice with nervous system specific expression of ubiquitin. If the ax^J phenotype was due only to destabilization of the ubiquitin pool, then restoration of ubiquitin levels should completely restore the ax^J phenotype. Since the restoration of ubiquitin levels in the ax^J mice does not completely rescue the phenotype, this suggests that loss of Usp14 does destabilize specific substrates that contribute to the ax^J phenotype and that Usp14 functions in part to rescue these substrates from proteasome degradation.

Alternatively, Usp14 may function to stabilize the ubiquitin pool in a way that is independent from rescuing ubiquitin from substrates bound for 26S proteasomal degradation. At present, ubiquitin is considered a stable cellular protein, with a low rate of basal turnover. The stability of ubiquitin may partly be due to the action of the three DUBs on the 19S RP that may function to prevent the degradation of substrate free ubiquitin monomers or chains; thereby, maintaining the ubiquitin pool and contributing to ubiquitin stability. The loss of Usp14 may result in increased substrate free ubiquitin turnover since the loss of Usp14 activity on the 26S proteasome would reduce rescue of this ubiquitin from degradation by the proteasome.

Inert, epitope tagged ubiquitin could be used to test for increased turnover of free ubiquitin. As mentioned earlier, normally ubiquitin can form chains, which is achieved through the attachment of ubiquitin's c-terminal glycine to any internal lysine residue on

another ubiquitin molecule. In order to test the rate of substrate free ubiquitin turn over in neurons, the ubiquitin molecule must be rendered unable to attach to other wild type ubiquitin molecules or to lysine residues on protein substrates. Such an inert ubiquitin molecule could be produced by mutating its c-terminal glycine residue and internal lysine residues. An n-terminal epitope tag could be joined to the inert ubiquitin construct for visualization during Western blotting. To determine if this construct is a reasonable surrogate for ubiquitin, the construct could be made into a HA-Ub-VS probe. If the modified ubiquitin probe can label active deubiquitinating enzymes then this suggests that the inert ubiquitin construct is acceptable for measuring the basal degradation rate of monomeric ubiquitin. The inert ubiquitin construct could be transgenically expressed in wild type and *ax*^{J} neurons; Western blotting for the epitope tag can be used to examine the basal rate degradation of this transgenically derived ubiquitin construct. If increased turnover of the inert ubiquitin is seen in the *ax*^{J} neurons, this would suggest that Usp14 in part functions to maintain ubiquitin levels through regulating the basal degradation of free ubiquitin. Furthermore, such a function for Usp14 would indicate that the loss of ubiquitin due to decreased expression of Usp14 could prevent important ubiquitin signaling events at the synapse thus leading to the observed synaptic defects.

Usp14's Role in the Nervous System

Overall, the study of the *ax*^{J} mice demonstrates several important concepts that have improved our knowledge of UPS function in the nervous system. The first concept is that there are differences in the UPSs of different tissue types. This observation is strengthened by the *ax*^{J} Tg mice, which have a reversal of the *ax*^{J} phenotype despite per-

sistent non-neuronal loss of Usp14; thus, demonstrating an absolute necessity of Usp14 in the nervous system but not in other tissue systems. These differences in the UPSs of various tissue systems are possible because of changes in UPS gene expression and the requirements of various UPS components in different tissues. Such differences in the expression levels of UPS components are demonstrated by the difference of Usp14 expression among various tissue types [56]. These changes in the expression of UPS components most likely underlie important idiosyncrasies in UPS function among tissues.

The other concept that was found in the study of the ax^J mice is that particular neuronal circuits are more susceptible to loss of Usp14 than others. This finding reflects that even within neurons, different neuronal populations have varying UPS requirements. Although loss of Usp14 occurs in all neurons, only Purkinje cells, spinal cord tracts, and motoneurons showed pathology. The sensitivity of particular neuronal populations to different disease states is not unique to Usp14. Other diseases such as PD, Pick's disease, and amyotrophic lateral sclerosis are all examples in which a specific neuronal population is affected and known to cause the disease. Many different factors could account for the susceptibility of various neuronal subpopulations. Differential metabolic requirements, gene transcription, number of excitatory and inhibitory synapses, and vesicle release probability may all contribute to the sensitivity of particular neurons to different disease states.

Motoneurons in particular, one of the neuronal populations suspected to play a role in the ax^J phenotype, have a high metabolic requirement that may increase proteasomal load. As mentioned earlier, yeast knockouts of Ubp6 have growth defects when challenged with canavanine [73]. It is possible that, in comparison with other neuronal

populations, the higher metabolic activity of motoneurons coupled with their high expression of 26S proteasomes results in an increased amount of proteins being sent to the proteasome for degradation; therefore, culminating in a deeper depression of monomeric ubiquitin levels than other neuronal populations in the *ax*^{J} mice. The higher loss of monomeric ubiquitin may cause malfunction in the motoneurons of the *ax*^{J} mice that result in the *ax*^{J} phenotype.

I suggest that the synaptic dysfunction discovered in the hippocampus of the *ax*^{J} mice, a neural population that does not show any gross pathology, further demonstrates the importance of ubiquitin pool regulation on a subcellular scale [71]. The ubiquitin requirement in a neuron is most likely not homogenous across the entire cell. Certain subcellular compartments may have a higher ubiquitin demand. The synapse, as indicated at length above, requires ubiquitin for proper biological function. The natural activity of the synapse may increase proteasome recruitment and UPS load; the loss of Usp14 would then decrease the stability of the synaptic monomeric ubiquitin pool. Unlike the Purkinje cells and motoneurons, the neurons of the hippocampus do not show any overt pathology; however, the UPS demands in the synaptic environment may be high enough to deplete the localized monomeric ubiquitin pool and therefore lead to the generation of the synaptic defects seen in the *ax*^{J} mice. In closing, the *ax*^{J} mice provide the first evidence for a primary defect in proteasome enzymatic activity as the underlying cause of a neurological disease in mammals. Additional studies of the *ax*^{J} mice will elucidate not only the function of the UPS in the nervous system but also the exquisite differences in the UPS among different neural populations.

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APPENDIX: NOTICE OF IACUC APPROVAL

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

NOTICE OF APPROVAL

DATE: August 28, 2007

TO: Scott M. Wilson, Ph.D.
SHEL 914 2182
FAX: 934-6571

FROM: Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: Title: The Role of Usp14 in Regulating Neuronal Function
Sponsor: NIH
Animal Project Number: 070806744

On August 28, 2007, the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) reviewed the animal use proposed in the above referenced application. It approved the use of the following species and numbers of animals:

Species	Use Category	Number in Category
Mice	B	100
Mice	A	260

Animal use is scheduled for review one year from August 2007. Approval from the IACUC must be obtained before implementing any changes or modifications in the approved animal use.

Please keep this record for your files, and forward the attached letter to the appropriate granting agency.

Refer to Animal Protocol Number (APN) 070806744 when ordering animals or in any correspondence with the IACUC or Animal Resources Program (ARP) offices regarding this study. If you have concerns or questions regarding this notice, please call the IACUC office at 934-7692.

Institutional Animal Care and Use Committee
B10 Volker Hall
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