Impaired disassembly of the axon initial segment restricts mitochondrial entry into damaged axons

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Abstract

The proteasome is essential for cellular responses to various physiological stressors. However, how proteasome function impacts the stress resilience of regenerative damaged motor neurons remains unclear. Here, we develop a unique mouse model using a regulatory element of the activating transcription factor (Atf3) gene to label mitochondria in a damage-induced manner while simultaneously genetically disrupting the proteasome. Using this model, we observed that in injury-induced proteasome-deficient mouse motor neurons, the increase of mitochondrial influx from soma into axons is inhibited because neurons fail to disassemble ankyrin G, an organizer of the axon initial segment (AIS), in a proteasome-dependent manner. Further, these motor neurons exhibit amyotrophic lateral sclerosis (ALS)-like degeneration despite having regenerative potential. Selectively vulnerable motor neurons in SOD1(G93A) ALS mice, which induce ATF3 in response to pathological damage, also fail to disrupt the AIS, limiting the number of axonal mitochondria at a pre-symptomatic stage. Thus, damage-induced proteasome-sensitive AIS disassembly could be a critical post-translational response for damaged motor neurons to temporarily transit to an immature state and meet energy demands for axon regeneration or preservation.

Keywords axonal transport; nerve injury; neurodegenerative disease; organelle; stress response

Subject Categories Molecular Biology of Disease; Neuroscience; Organelles

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Introduction

Nerve-injured motor neurons can regenerate through multiple stress responses, which require dynamic protein synthesis and degradation (Kiryu-Seo & Kiyama, 2011; Patodia & Raivich, 2012). The proteasome is thought to be involved in the post-translational stress response of damaged motor neurons; however, it remains unclear how proteasome function impacts the stress resilience of damaged motor neurons. Proteasomal dysfunction in motor neurons has been implicated in the pathology of amyotrophic lateral sclerosis (ALS) (Kabashi et al, 2012; Webster et al, 2017), a progressive neurodegenerative disease characterized by motor neuron loss, neuromuscular denervation, and axon degeneration (Taylor et al, 2016; Niijsen et al, 2017). Intriguingly, multiple stress responses observed in injured motor neurons are similarly activated in pathologically damaged motor neurons of ALS model mice (Kiryu-Seo et al, 2005; Lobsiger et al, 2007; Sun et al, 2015; Tyzack et al, 2017; Maor-Nof et al, 2021). We hypothesized that a proteasome-mediated stress-resilient mechanism exists in regenerative damaged motor neurons, which is lacking in damaged motor neurons prior to degeneration.

Alteration of axonal mitochondrial dynamics in response to damage is an early hallmark of both regeneration and degeneration (Patron & Zinsmaier, 2016; Sleigh et al, 2019). Mitochondria play pivotal roles in energy supply and metabolism that depend on cellular status (Han et al, 2020; Licht-Mayer et al, 2020). In neurons with long axons, such as motor neurons, mitochondria must be transported from the cell body to the axon. It is evident that successful regeneration requires enhanced transport and numbers of mitochondria in injured axons (Misgeld et al, 2007; Kiryu-Seo et al, 2010, 2016; Cartoni et al, 2016; Han et al, 2016). Increases in motile mitochondria or deletion of stationary mitochondria in injured axons can facilitate axon regeneration (Zhou et al, 2016; Han et al, 2020). Dysregulation of mitochondrial transport and quality has been observed during the very early onset of ALS...
degeneration (Bilsland et al., 2010; Magrane et al., 2014; Lin et al., 2017; Moller et al., 2017).

To clarify proteasome-mediated stress-resilient responses in damaged motor neurons, we developed a unique mouse system in which mitochondria are labeled with GFP (as a physiological indicator) and the proteasome is specifically ablated in nerve-injured motor neurons. In addition, we established ALS mice wherein mitochondria are selectively labeled in stress-responsive motor neurons at a pre-symptomatic stage without injury. To ensure responsiveness to damage, we employed the activating transcription factor (Atf3) gene as a regulatory element. ATF3 is robustly induced in injured neurons to elicit transcriptional reprogramming (Nakagomi et al., 2003; Seijffers et al., 2006; Chandran et al., 2016; Palmisano et al., 2019; Lu et al., 2020; Renthal et al., 2020). Intriguingly, ATF3 is also expressed in a specific subtype of motor neurons vulnerable to degeneration in an ALS mouse model (Vlug et al., 2005; Saxena et al., 2009; Morisaki et al., 2016).

Using the newly established mouse system, we identified ankyrin G (AnkG) as a crucial target of the proteasome in damaged motor neurons. AnkG acts as an organizer of the axon initial segment (AIS) by recruiting and assembling submembrane proteins, such as voltage-gated sodium channels and neurofascin-186 (Huang & Randahl, 2018; Leterrier, 2018). The AIS is a transition compartment between the soma and axon that functions as a selective filter for axonal vesicles in addition to generating action potentials. Traumatic injuries dismantle the AIS, while pathogenic conditions such as tau mutation can impair AIS plasticity, leading to hyperexcitability (Schafer et al., 2009; Marin et al., 2016; Sohn et al., 2019). The cysteine protease calpain is considered to be responsible for degradation of the AIS (Schafer et al., 2009; Zhao et al., 2020), although alternative degradation systems may also contribute to this event (Hamdan et al., 2020). The physiological significance and exact mechanisms of AIS morphological changes during brain damage and disease have not been well addressed.

Here, we demonstrate a new proteasome-mediated mechanism in vivo, in which damaged motor neurons temporarily dismantle the AIS in a proteasome-sensitive manner. Transient disassembly of the AIS facilitates mitochondrial entry into damaged axons. This regulatory mechanism of mitochondrial logistics may be a critical post-translational stress response that permits damaged motor neurons to produce enough energy for their regeneration or preservation of axon integrity.

### Results

#### Mouse model of proteasome impairment specifically in nerve-injured motor neurons

We used a hypoglossal motor nerve injury as a simple and elegant experimental model for motor neuron damage (Fig 1A). It has many experimental advantages; clear onset of damage, convenience of histological and biochemical comparison between control and injured motor neurons because hypoglossal motor neurons are densely located in bilateral neighboring hypoglossal nuclei, the regenerative ability of injured motor neurons, and numerous shared stress responses with ALS motor neurons (Nakagomi et al., 2003; Kiryu-Seo et al., 2005, 2006; Lobiger et al., 2007; Saxena et al., 2009; Sun et al., 2015; Maor-Nof et al., 2021).

To generate mouse wherein proteasome ablation occurs specifically in injured motor neurons, we used Atf3:BAC2 Tg mouse as a Cre driver mouse (Fig 1B). Atf3:BAC2 Tg mouse was designed to simultaneously express mitochondria-targeted green fluorescent protein (GFP) and Cre recombinase in an injury-specific manner, as driven by the Atf3 gene element within a bacterial artificial chromosome (BAC); multiple previous studies, including ours, have used ATF3 as a marker for nerve injury (Nakagomi et al., 2003; Seijffers et al., 2006; Kiryu-Seo et al., 2008). The initiation codon of Atf3 gene in BAC was replaced by that of GFP with the mitochondrial targeting sequence, so that Atf3:BAC2 Tg mice did not express exogenous ATF3. The Atf3:BAC2 Tg mouse is a different line from the Atf3:BAC Tg mice we previously reported (Kiryu-Seo et al., 2016). Thus, we first examined that Atf3:BAC2 Tg mice began to express...
Figure 1.
GFP in injured motor neurons at 20 h after injury and thereafter increased the number of GFP-positive cells. In total, 60–70% of ATF3-positive motor neurons expressed GFP following nerve injury (Fig EV1A–C).

By crossing Atf3:BAC2 Tg mouse with mouse floxed for Rpt3, an essential subunit of 26S proteasome for the protein degradation, we generated injury-induced proteasome-deficient mouse (Rpt3 CKO) (Figs 1B and EV1D–G). Injury-induced Rpt3-deficient mouse is a uniquely advantageous experimental tool to examine proteasome-mediated early stress responses in damaged motor neurons. Rpt3 CKO mice reached adulthood, whereas the previously reported Rpt3 knockout mouse died during early gestation (Sakao et al., 2000). We used Atf3:BAC2 Tg mice instead of Atf3:BAC Tg mice in this study, because the progeny of Rpt3 CKO mice crossed with Atf3:BAC Tg mice had a shorter life span. Atf3:BAC2 Tg mice and Rpt3 CKO mice exhibited identical numbers of choline acetyltransferase (ChAT)-positive hypoglossal motor neurons under the normal condition (Appendix Fig S1A and B). The expression of endogenous RPT3 protein was specifically abolished in GFP-positive injured motor neurons of Rpt3 CKO mice by 5 days after injury, whereas RPT3 protein expression was maintained in control motor neurons of Rpt3 CKO mice and both control and injured motor neurons of Atf3:BAC2 Tg mice (Fig 1C).

Injury-induced Rpt3-ablated motor neurons decrease numbers of mitochondria in axons

To clarify the cellular responses affected by proteasome deficiency, we acquired three-dimensional (3D) images of the transparent brains of Atf3:BAC2 Tg and Rpt3 CKO mice at 5 days after hypoglossal nerve injury, because these mice enabled us to observe GFP-labeled mitochondria in nerve-injured motor neurons, which are a good indicator of the cellular status (Fig 1D). Day 3–7 is the time point when dramatic change in protein expression occurs after injury. Three-dimensional images acquired by lightsheet microscopy clearly demonstrated global views of injured hypoglossal motor neuron morphologies in mice (Fig 1D; Movies EV1 and EV2). We found that the intensity of GFP signal was reduced throughout injured motor axons of Rpt3 CKO mice (Fig 1D and E). 3D images provided us the useful information, which ordinary histological sections missed. We further observed whole-mount peripheral injured axons of both Atf3:BAC2 Tg and Rpt3 CKO mice using a confocal microscopy with higher-resolution, to count numbers of mitochondria in injured axons more precisely (Figs 1F and EV2). The number of mitochondria in injured axons of Rpt3 CKO mice was significantly reduced compared with Atf3:BAC2 Tg mice (Fig 1H). Notably, this result was not due to unlabeled mitochondria in Rpt3 CKO mice because the results shown in Fig 1G confirmed that staining of cytochrome c (a mitochondrial marker) in injured axons of both mice was completely consistent with GFP-labeled mitochondria. When we examined lysosomes as one of the other cargos in injured axons, the distribution was not affected by Rpt3 deletion at least 5 days after injury (Fig EV2B–D), suggesting that mitochondria and lysosomes move separately by different mechanisms in response to damage. Thereafter, lysosomes in Rpt3-deficient injured axons were significantly increased at 10 days after injury, probably reflecting the impairment of axonal transport or endolysosomal-autophagic systems in severely degenerating axons. The total number of GFP-positive injured motor neurons was similar in Atf3:BAC2 Tg and Rpt3 CKO mice (Figs 1I and EV2E and F). Super-resolution microscopy further showed that mitochondrial density within soma of injured motor neurons seemed to be higher in Rpt3 CKO mice (Figs 1J and EV2G and H). Thus, our findings indicate the possibility that proteasome deficiency affects mitochondrial dynamics in injured axons at early time points after injury.

Injury-induced Rpt3-ablated motor neurons exhibit ALS-relevant degeneration, despite their regenerative potential

To further examine the cellular condition at 5 days after axotomy, we performed quantitative reverse-transcription PCR (qRT–PCR) for the expression of regeneration-associated genes (RAGs) using control and injured hypoglossal nuclei. Unexpectedly, the alteration of mRNA expression of RAGs after injury did not show any significant difference between Atf3:BAC2 Tg and Rpt3 CKO mice (Fig 2A). In addition, the expression patterns of representative regeneration-associated proteins, which were up- and downregulated, were similar in injured motor neurons of Atf3:BAC2 Tg mice and Rpt3 CKO.
Figure 2.
mice at 5 days after injury (Fig 2B). Downregulation of ChAT and NeuN proteins in Rpt3-deficient injured motor neurons is probably due to compensatory autophagy system. We next examined the fate of motor neurons after hypoglossal nerve injury. Thionine staining showed that motor neurons of Rpt3 CKO mice survived at 7 days after injury, similar to those of Atf3:BAC2 Tg mice; thereafter, those in Rpt3 CKO mice began to degenerate and died completely by 17 days after injury, while around 85% of injured motor neurons of Atf3:BAC2 Tg survived (Fig 2C and D). Microglial hyper-activation but not astrocyte was also observed at the proximity of the Rpt3-deleted injured motor neurons at 7 days after injury (Appendix Fig S1C and D). These results suggest that proteasome-mediated stress pathways are located at a critical point for further regeneration to proceed after regenerative programming. Rpt3-deleted injured motor neurons accumulated TDP-43 and p62 around 7 days after injury (Fig 2E). The accumulation of TDP-43 was shown in nucleus in our study, although it was generally exported and accumulated in the cytoplasm in ALS motor neurons. Its accumulation in nucleus may be a stress response rather than pathology, as previously described (Udan-Johns et al., 2014). Aggregation of p62, an autophagic marker, could indicate the compensation for proteasome impairment in injured motor neurons of Rpt3 CKO mice, although the ubiquitin-proteasome system (UPS) is a dominant protein degradation system in motor neurons (Tashiro et al., 2012). The injured axons of Atf3:BAC2 Tg mice, in which GFP-labeled mitochondria were transported from soma upon injury, reinnervated the muscle in tongue and initiated to reform neuromuscular junctions (NMJs) at 28 days after injury, while those of Rpt3 CKO mice did not (Fig 2F). Injured motor neurons in Atf3:BAC2 Tg mice also survived at 28 days after injury (Fig 2G). Collectively, Rpt3-deleted motor neurons have competence to regenerate during the initial stage of nerve injury response, but likely degenerate. Dysfunction of proteasome-mediated response during the initial stage may be involved.

**Axon injury-induced disappearance of the AIS is suppressed in Rpt3 CKO mice**

Based on 3D imaging results shown in Fig 1, we considered that proteasome dysfunction might affect the entry of mitochondria from the soma to injured axons at early time points after injury, a time when motor neuron death was not apparent (Fig 2). A transition compartment between the soma and axon is known as the AIS. Previous studies have reported the AIS changes its morphology in response to neuronal activity and neuronal insults (Yamada & Kuba, 2016; Huang & Rasband, 2018). We examined morphological changes in the AIS before and after hypoglossal nerve injury using higher-resolution cellular and subcellular 2D confocal images (Fig 3A and B). Immunohistochemistry for AnkG, an organizer of the AIS, demonstrated that the AIS disappeared in injured motor neurons of Atf3:BAC2 Tg mice at 5 days after injury, while the AIS remained in those of Rpt3 CKO mice (Fig 3C). As shown in Fig 3D and E, the difference of AIS number and length in injured motor neurons between Atf3:BAC2 Tg mice and Rpt3 CKO mice at day 5 was statistically significant. The number and length of the AIS were then recovered in injured motor neurons of Atf3:BAC2 Tg mice at 28 days after nerve injury, suggesting the reassembly of the AIS structure. AIS and GFP-positive injured motor neurons were not detected in Rpt3 CKO mice at 28 days after injury, because the injured motor neurons degenerated as shown in Fig 2C and D and F. The number of AIS in Atf3:BAC2 Tg mice was not completely recovered to the control level at 28 days after injury (Fig 3D), because all of the injured motor neurons in Atf3:BAC2 Tg mice did not regenerate in this model. These findings suggest that temporal disassembly of the AIS in injured motor neurons is a positive stress response mediated by the proteasome.

**AnkG is degraded by the ubiquitin-proteasome system**

We next attempted to clarify whether AnkG can be degraded by the UPS upon injury. We performed qRT–PCR using control and injured hypoglossal nuclei of wild-type mice collected at 5 days after axotomy, to examine transcriptional regulation of AnkG before and after injury (Fig 4A). The results showed that mRNA expression of AnkG was unaltered before and after injury, while that of Atf3 and Chat as positive and negative RAGs was dramatically up- and down-regulated, respectively. The longer AnkG splicing variant called giant AnkG, which is considered to be responsible for AIS formation, was also unchanged before and after injury. Thus, the regulation of AnkG at protein level is critical after nerve injury.

A computer search such as UbPred predicted that AnkG protein has multiple ubiquitination sites. To examine ubiquitination of AnkG, we performed a ubiquitination assay using cell lysates in which sequentially fragmented AnkG constructs were transfected into COS-7 cells (Fig 4B). A fragmented construct of AnkG was used for the ubiquitination assay because full-length AnkG was too long for this experiment. The results showed that most AnkG fragments were experimentally poly-ubiquitinated, suggesting that AnkG protein could be degraded by the UPS. Immunoblots of control and injured hypoglossal nuclei showed that both 480 and 270-kDa forms of AnkG were downregulated after injury, while injured nerves exhibited no signals for 480-kDa of AnkG and no obvious changes of 270-kDa and major 190-kDa AnkG between control and injured nerves (Fig 4C). A previous study reported that AnkG was degraded by the calcium-dependent protease calpain into ~ 95 and 72-kDa forms (Schafer et al., 2009). However, injured hypoglossal nuclei did not exhibit breakdown products, suggesting the possibility that proteasome is dominantly involved in this injury model. Immunohistochemical staining revealed that the broadly scattered dot-like signals of AnkG were still present in the injured side of the hypoglossal nucleus, while AnkG-positive AIS was lost in injured motor neurons of Atf3:BAC2 Tg mice (Fig 4D). These dot-like signals were assumed to be nodes of Ranvier (node), which exhibit similar protein components to the AIS. When we examined the nodes of hypoglossal motor nerves, both control and injured motor axons similarly exhibited signals for AnkG and Caspr, indicators of nodes and paranodes, respectively. Collectively, these results indicate that proteasome-mediated AnkG degradation occurred specifically in the AIS, but not in nodes of injured motor neurons.

**Nerve injury-induced disassembly of AIS enhances mitochondrial influx into axons**

We next evaluated the physiological significance of proteasome-dependent disassembly of the AIS in injured motor neurons. We
hypothesized that the absence of the AIS was beneficial for mitochondrial entry into injured axons. To evaluate this hypothesis, we focused on mitochondrial localization in the AIS region before and after hypoglossal nerve injury (Fig 5A). Atf3:BAC2 Tg mice exhibited mutually exclusive distribution of AnkG-positive AIS and mitochondria (Fig 5B). The AIS of Atf3:BAC2 Tg mice was gradually

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**Figure 3.** Injury-induced ablation of Rpt3 affects the disassembly of the AIS in injured motor neuron.

A Schematic diagram of the AIS in hypoglossal nerve injury model.

B Schematic showing the time course of motor neurons after nerve injury. Arrows indicate the time point at which the experiments were performed.

C Hypoglossal motor neurons of Atf3:BAC2 and Rpt3 CKO mice before and at 5 days (5d) and 28 days (28d) after injury, immunostained by AnkG and GFP. Arrows indicate the AIS stained by AnkG. Scale bars, 20 μm.

D Quantification of the number of the AIS in control (Cont) and injured (Inj) hypoglossal motor neurons of Atf3:BAC2 and Rpt3 CKO mice. Data are expressed as mean ± s.e.m. *P = 0.043, **P = 0.0162, ***P = 0.008, ****P < 0.0001, determined by one-way ANOVA followed by Tukey post hoc analysis, n.d., not detected, n = 5 mice for each group.

E Quantification of the AIS length in control (Cont) and injured (Inj) hypoglossal motor neurons of Atf3:BAC2 and Rpt3 CKO mice. Data are expressed as mean ± s.e.m. *P = 0.0087, **P = 0.0024, ***P = 0.0007, determined by one-way ANOVA followed by Tukey post hoc analysis, n.d., not detected, n = 5 mice for each group.

Source data are available online for this figure.
Figure 4. Proteasome is responsible for the AIS-specific degradation of AnkG in injured motor neurons.

A Graph represents fold change in mRNAs in injured hypoglossal (XII) nuclei compared with control hypoglossal nuclei of wild-type mice at 5 days after injury. Data are shown as the mean ± s.e.m. (n = 3 independent experiments).

B Ubiquitination assay of AnkG protein. The lysates of COS-7 cells expressing the indicated truncated AnkG tagged with GST and HA-Ubiquitin were pulled down with glutathione Sepharose beads, followed by western blotting with anti-HA and anti-GST antibodies.

C Western blot of hypoglossal nuclei (left) and nerves (right) at 5 days after injury with anti-AnkG, RPT3 and GAPDH antibodies. AnkG products of 190/270/480 kDa are indicated by arrowheads. The positions of the degraded products by calpain are shown by asterisks at ~95 kDa and 72 kDa.

D The expression of AnkG (red) and GFP (green) in the AIS of hypoglossal (XII) motor neurons and that of AnkG, GFP, and Caspr (blue) in nodes of hypoglossal motor nerves at 5 days after injury. Scale bars, 15, 5, and 1.5 μm from the top.

Source data are available online for this figure.
disassembled in response to injury and subsequently reassembled at 28 days after injury along with regeneration. In contrast, numerous GFP-labeled mitochondria appeared in the AnkG-disappeared region of the AIS after injury, while a limited number of mitochondria remained in the recovered AIS region at 28 days after injury. This was not an artificial effect of Atf3:BAC2 Tg mice because the widely described Thy1-Mito mouse, in which mitochondria are labeled by CFP in neurons under control of the Thy1 promoter, showed a similar relationship between the AIS and mitochondria (Fig EV3A–E). Unlike injured motor neurons in Atf3:BAC2 Tg mice, Rpt3-deficient injured motor neurons preserved the AIS at 5 days after injury and exhibited few GFP-labeled mitochondria in it (Fig SC–E). Correlatively, the number of mitochondria in axotomized motor nerves of Atf3:BAC2 Tg mice was significantly increased compared with normal nerves of Thy1-Mito mice and subsequently returned to a control level along with the AIS recovery (Fig 5F and G). However, numbers of mitochondria in Rpt3-deficient nerves did not show any injury-induced changes, suggesting that the presence or absence of the AIS is involved in regulating mitochondrial influx into injured axons.

To further address whether the loss of the AIS is directly involved in the increased number of mitochondria observed in injured axons or whether the two events are independent, we forced disruption of the preserved AIS structures in injured motor neurons of Rpt3 CKO mice. An AnkG shRNA was designed to suppress the larger splicing variant of AnkG. After injecting an AAV carrying AnkG shRNA into the tongue, it was retrogradely transported into motor neurons, whereby it suppressed AnkG expression (Figs 5H and EV4A and B). Our results showed that infection with AAV-AnkG shRNA disrupted the AIS, and numbers of GFP-labeled mitochondria in Rpt3-ablated injured motor neurons were increased (Fig SI–K). Infection with AAV-scramble shRNA affected neither the appearance of the AIS nor the localization of GFP-labeled mitochondria in Rpt3-deficient injured motor neurons. Infection with AAV-AnkG shRNA in uninjured motor neurons of Thy1-Mito mouse did not increase the number of GFP-labeled mitochondria in axons, suggesting that injury-induced driving forces are necessary to increase mitochondrial density in axons (Fig EV4C and D). Furthermore, we examined whether the knockdown of AnkG influences the survival of Rpt3-deficient injured motor neurons or not. The injection of AAV-AnkG shRNA delayed the death of Rpt3-deficient injured motor neurons, compared with that of AAV-scramble shRNA (Fig 5L and M). These results suggest that the AIS disassembly is necessary for injury-induced increase in mitochondrial influx into the axons to promote regeneration.

**Mitochondrial motility in injured axons is reduced in Rpt3 CKO mice**

Because two types of mitochondria, motile and stationary, are present in axons, we examined which type was responsible for the observed change in mitochondrial numbers in injured axons. For live imaging of mitochondrial flow, we used another nerve injury model: sciatic nerve injury (Fig 6A). Injured motor neurons after sciatic nerve injury showed similar responses of the AIS and mitochondria to those of hypoglossal nerve injury (Fig EV3C–E; Appendix Fig S2A and B). We performed live imaging of CFP- or GFP-labeled mitochondria in uninjured sciatic nerves of Thy1-Mito mice and injured sciatic nerves of both Atf3:BAC2 Tg mice and Rpt3 CKO mice (Fig 6B and C). Atf3:BAC2 Tg mice exhibited increased numbers of motile mitochondria in injured nerves compared with Thy1-Mito mice and Rpt3 CKO mice (Fig 6D and E). Increased numbers of motile mitochondria in injured sciatic nerves of Atf3:BAC2 Tg mice returned to control levels along with regeneration. Notably, stationary mitochondria were not significantly altered in any of these mice (Fig 6F). Nerve injury induces proteasome-dependent AIS degradation in motor neurons, which could be coupled with the increase in mitochondrial transport into axons under the presence of driving forces.
Figure 5.
Failure of AIS disassembly in ATF3-expressing ALS motor neurons at a pre-symptomatic stage

ATF3-expressing spinal motor neurons exist in ALS mice without injury (Vlug et al., 2005; Saxena et al., 2009; Morisaki et al., 2016). We crossed Atf3-BAC Tg mice with SOD1<sup>G93A</sup> (SOD1) ALS mice and generated Atf3-BAC;SOD1<sup>G93A</sup> (Atf3;SOD1) mice to selectively label ATF3-expressing motor neurons (Figs 7A and EV5A). We used Atf3;BAC Tg mice here, because Atf3:BAC Tg mice mimic the expression pattern of endogenous ATF3 more effectively than Atf3:BAC2 Tg mice. Atf3:SOD1 mice showed similar life span and age-dependent motor neuron death with SOD1 mice, suggesting that the Atf3:BAC transgene does not affect the disease phenotype of SOD1 mice (Fig EV5B–D). 3D image of transparent spinal cord in Atf3:SOD1 mouse clearly showed GFP-labeled motor neurons, while that of Atf3:BAC Tg mouse (littermate control) did not (Fig 7B;
Movie EV3). The expression of GFP was co-localized well with that of ATF3 in motor neurons of Atf3;SOD1 mouse (Fig 7C). Of ChAT-positive spinal motor neurons, GFP-expressing motor neurons were < 20% at P70 (pre-symptomatic stage; Fig 7D). These GFP-positive motor neurons appeared around P60, increased in number, and then degenerated along with age in Atf3;SOD1 mouse (Fig EV5E and F). Among the heterogenous spinal motor neuron pool, most of GFP-positive motor neurons in Atf3;SOD1 mice expressed ATF3 (Fig 7E). They also expressed matrix metalloproteinase-9 (MMP-9) or osteopontin (OPN), which are reportedly localized in vulnerable motor neurons (Figs 7E and EV5G) (Kaplan et al., 2014; Morisaki et al., 2016), suggesting that stress-responsive vulnerable motor neurons are selectively labeled by GFP in Atf3;SOD1 mice.

Given that ALS motor neurons reduce proteasome activity (Jenkins et al., 2020) and initiate to induce ATF3 at pre-symptomatic stage, we wondered whether the failure of AIS disassembly in injured motor neurons of Rpt3 CKO mice may occur in GFP-positive ALS motor neurons. We found that GFP-positive spinal motor neurons in Atf3;SOD1 mice retained the AIS and that the length of the AIS did not show any significant difference with that in the ChAT-positive spinal motor neurons in Atf3;BAC and SOD1 mice (Fig 7F and G). We next evaluated mitochondrial localization in the AIS region. Both traumatically damaged GFP-positive motor neurons in Atf3;BAC and pathologically damaged GFP-positive motor neurons in Atf3;SOD1 induce ATF3. However, pathologically damaged motor neurons failed to dismantle the AIS and exhibited few GFP-labeled mitochondria in it (Fig 7H–I). Concomitantly, GFP-positive motor neurons of Atf3;SOD1 mice did not increase the number of axonal mitochondria despite inducing ATF3. (Fig 7K and L).

We further confirmed that ATF3-inducing ALS motor neurons failed to disassemble the AIS using hypoglossal nerve injury model (Appendix Fig S3). The number of GFP-positive motor neurons of Atf3;SOD1 mice was originally much lower in the hypoglossal nucleus than in the spinal cord, probably because cranial motor neurons retained higher proteasomal activity than spinal motor neurons (An et al., 2019; Appendix Fig S3A–C). Almost all hypoglossal motor neurons in Atf3;SOD1 mice were labeled by GFP following nerve injury because of ATF3 induction. However, they did not disassemble the AIS nor increase mitochondria (Appendix Fig S3D and E). Collectively, the ATF3-positive ALS motor neurons lack or reduce proteasome-mediated stress responses, resembling injury-induced Rpt3-deficient motor neurons.

**Discussion**

In this study, the combination of motor nerve injury model with unique genetically engineered mice has clarified a new proteasome-mediated stress-resilient mechanism that is lacking in damaged motor neurons prior to degeneration. Injury-induced Rpt3-deficient motor neurons fail to activate proteasome-dependent AIS disassembly and exhibit ALS-relevant degeneration, despite remaining regenerative capacity. The functional consequence of the transient AIS disassembly after neuron damage has become clear in this study. Upon damage, proteasome-mediated disruption of the AIS facilitates mitochondrial entry into axons, perhaps to compensate for the increased energy expenditure associated with axon regeneration. Intriguingly, the failure of this proteasome-mediated mechanism against pathological damage might be associated with the vulnerability of ALS motor neurons (Fig 8).

**Transient AIS disassembly is critical for injured motor neurons to accelerate mitochondrial influx into axons**

Here, we defined that the presence or absence of the AIS alters mitochondrial density in injured axons. It has been established that mitochondrial supply is critical for injured axons and growth cones to acquire energy for reconstruction (Chamberlain & Sheng, 2019). However, the mechanism underlying enhanced mitochondrial transport has remained elusive. Furthermore, the physiological significance of AIS dismantling in injury and disease has been unclear, although AIS plasticity has been implicated in changes in neuronal excitability. This study is the first to examine the association between damage-induced disruption of the AIS and mitochondrial density.
Figure 7.
entry in vivo by taking advantage of a unique mouse model. Under normal conditions, the AIS acts as a selective barrier in which nuclear distribution element-like 1 (NDEL1) excludes non-axonal cargo (Farias et al., 2015; Jenkins et al., 2015; Kuijpers et al., 2016; Gumy et al., 2017). Strikingly, suppression of AnkG does not always influence axonal transport under normal conditions, partly because the proximal axonal region contributes to selective cargo transport in addition to the AIS (Farias et al., 2015; Jenkins et al., 2015). They are consistent with our result that the suppression of AnkG did not show any obvious change in mitochondrial number in axon under normal condition. However, the situation seems to be different upon injury, whereby the activation of mitochondrial transport-associated molecules such as dual-leucine zipper kinase 1 (Dlk1), armadillo repeat-containing X-linked 1 (Armcx1), mitochondrial Rho GTPases (Miro), and dynamin-related protein 1 (Drp1) and the modification of microtubules enhance mitochondrial localization in injured axons (Hellal et al., 2011; Benned-Jensen et al., 2016; Cartoni et al., 2016; Kiryu-Seo et al., 2016; Kalinski et al., 2019; Han et al., 2020). Under the presence of these powerful driving forces upon injury, the breakdown of the selective filter is efficient for enhanced mitochondrial entry into injured axons.

The absence of the AIS is usually observed in immature neurons. The AIS dismantling in response to injury may contribute to the temporal transition of injured motor neurons to an immature state at post-translational level, in order to acquire regenerative potential. Because immature neurons require higher mitochondrial motility in axon during development and progressively reduce it along with development (Lewis et al., 2016). Recent progresses have clarified that the transcriptional and epigenetic reprogramming makes injured neurons revert to immature state for regeneration (Chandran et al., 2016; Palmisano et al., 2019; Bradke et al., 2020; Lu et al., 2020; Renthal et al., 2020). In line with this, the proteasome-mediated disassembly of the AIS would be a post-translational mechanism after the transcriptional and epigenetic reprogramming. Injured motor neurons likely use this mechanism to meet urgent energy demand for regeneration. As we show in this study, the AIS
is reassembled at appropriate timing along with regeneration. The regulation mechanism of the AIS reassembly would also contribute to complete regeneration. Conclusively, the AIS disassembly and reassembly could be one of the key mitochondrial logistic mechanisms under emergency situations.

Proteasomal degradation is the major mechanism for AIS disassembly in damaged motor neurons

Using injury-induced Rpt3 CKO mice, we found that AnkG, the organizing component of the AIS, could be a proteasomal target upon injury. We showed that AnkG protein was indeed polyubiquitinated. The data are also supported by previous study showing that 26S proteasome dysfunction in neurons caused global accumulation of ubiquitinated proteins including AnkG (Ugun-Klusek et al., 2017). We do not rule out the possibility that other protein degradation systems, such as calpain and autophagy, participate in AIS loss in injured motor neurons or play a compensatory role in Rpt3-deficient injured motor neurons. Most notably, calpain is considered to be responsible for degradation of the AIS after brain damage (Schafer et al., 2009; Benned-Jensen et al., 2016; Zhao et al., 2020). One possible explanation is the different dominancy of protein degradation systems among neurons, because motor neurons are susceptible to proteasomal degradation rather than autophagy (Tashiro et al., 2012; Rudnick et al., 2017). Alternatively, differences in regenerative capacity between the central and peripheral nervous systems may reflect differences in degradation systems for the AIS. In central nervous system traumatic injury models, such as optic nerve injury and ischemia, both the AIS and nodes, which are composed of identical membranous proteins, seem to be disrupted (Marin et al., 2016); however, injured motor neurons in our injury model did not exhibit deteriorated nodes, perhaps because apparent demyelination did not occur. Taken together with these differences, it could be concluded that the proteasome is responsible for degradation of the AIS in damaged motor neurons. To regulate proteasome activity in an AIS-specific manner in damaged motor neurons, intrinsic or extrinsic local signaling such as signaling kinases and glial contact could be activated following injury (Yoshimura & Rashband, 2014; Baalman et al., 2015; Tamada et al., 2021). Otherwise, AIS-residing ubiquitin ligases, such as TRIM46, may participate in AIS-specific breakdown; although, thus far, TRIM46 reportedly has microtubule crosslinking activity, but not E3 ligase activity (van Beuningen et al., 2015).

Apart from disruption of the AIS, there likely exist further proteasome-mediated stress responses in injured motor neurons. Our previous study demonstrated that mitochondrial fission is a temporally adaptive response for nerve regeneration (Kiryu-Seo et al., 2016). Because mitochondrial proteins responsible for fusion/fission are regulated by degradation, the proteasome may be also involved in processes in injured motor neurons. Recently, Kong et al. (2020) identified an interaction between AMPK and the proteasome in axoplasm to promote degradation and drive regeneration (Kong et al., 2020). Considering that uninjured motor neurons maintain the AIS in the presence of the proteasome, Rpt3-deficient uninjured motor neurons which were previously reported would degenerate in different mechanisms from the failure of the AIS disassembly (Tashiro et al., 2012). Therefore, other multiple proteasome-dependent stress responses or injury-independent proteasome functions would also participate in resilience of damaged motor neurons.

Failure of AIS disassembly and subsequent mitochondrial insufficiency in vulnerable type of ALS motor neuron

Given that injury-induced Rpt3-ablated motor neurons exhibit ALS-like degeneration and that ALS motor neurons reduce the proteasome activity (Jenkins et al., 2020), we assumed that ALS motor neurons lack proteasome-mediated stress responses at presymptomatic stage, similar to injury-induced Rpt3-deficient motor neurons. Previous reports demonstrated that pathological ALS motor neurons show similar multiple stress responses to injured motor neurons, including ATF3 induction (Lobsiger et al., 2007). Notably, ATF3-positive motor neurons increase in number along with disease progression, or under the manipulation of disease-associated genes such as TANK-binding kinase 1 (TBK1), although it is unclear whether ATF3 drives survival or cell death mechanisms in pathological damaged motor neurons (Vlug et al., 2005; Seijffers et al., 2014; Morisaki et al., 2016; Gerbino et al., 2020). In this study, Atf3;SOD1 mice permitted selective visualization of ATF3-positive motor neurons by GFP at the pre-symptomatic stage. ATF3 is required for axotomy-induced reprogramming of injured neurons (Chandran et al., 2016; Renthal et al., 2020). Therefore, GFP-inducing SOD1 motor neurons might attempt to initiate regeneration programs but failed it. One of the reasons of the failure would be the lack of proteasome-mediated stress responses, which was demonstrated in this study.

We describe here that the proteasome-mediated disassembly of the AIS could be a stress response, which is somehow missing in GFP-positive SOD1 motor neurons. We did not find a significant change in AIS lengths in SOD1 motor neurons, although a previous study showed slightly shorter AIS structures in damaged motor neurons (Bonnevie et al., 2020). Instead, we found that GFP-positive SOD1 motor neurons had fewer mitochondria in the AIS and a concomitantly reduced number of axonal mitochondria, similar to injury-induced Rpt3-ablated motor neurons. Previous study using electron microscopy suggested the possibility that mitochondrial transport was impaired at the proximal axon in SOD1G93A mice and ALS patients (Sasaki & Iwata, 1996; Sasaki et al., 2005). Defective axonal transport of mitochondria is the earliest phenomena observed in ALS models and has been proposed to be causative in this disease (Magrane et al., 2014; Moller et al., 2017). Atf3;SOD1 mice and injury-induced Rpt3 CKO mice showed the possibility that the failure of proteasome-mediated AIS disruption would cause a defective supply of mitochondria from the cell body to the axon, at least in ATF3-positive motor neurons. Lin et al. (2017) have reported that mitochondria-anchoring protein syntaphilin is involved in the removal of damaged mitochondria from axon at early presymptomatic stage (Lin et al., 2017). Therefore, ALS motor neurons might require the replenishment of healthy mitochondria to axons via AIS disassembly, in cooperation with the removal of damaged mitochondria.

Overall, our mouse model system using unique character of Atf3 gene is a powerful tool for expanding our understanding of the molecular basis of damaged neurons prior to neurodegeneration. Using the system, we uncover the fundamental mechanism that the proteasome-dependent temporal breakdown of the AIS would be
beneficial for traumatologically and pathologically damaged motor neurons to supply mitochondrial energy to damaged axon through the transition to an immature state. This transient proteasome-mediated AIS disassembly could be a new machinery in the mitochondrial logistics system to keep the integrity of damaged motor neurons, and this system could be a new therapeutic target in ALS.

Materials and Methods

### Reagents and Tools table

| Reagent/Resource | Reference or Source | Identifier or Catalog Number |
|------------------|---------------------|-----------------------------|
| **Experimental Models** | | |
| C57BL/6NCrl (Mus Musculus) | SLC | N/A |
| SJL (Mus Musculus) | Charles River | N/A |
| Atf3-BAC Tg (Mus Musculus) | Kiryu-Seo et al (2016) | N/A |
| Atf3-BAC2 Tg (Mus Musculus) | This paper | N/A |
| Rpt3-flox (Mus Musculus) | Tashiro et al (2012) | N/A |
| B6SJ-L-Tg(SOD1-G93A)1Gur (Mus Musculus) | The Jackson Laboratory | JAX stock # 002726, RRID: IMSR_JAX:002726 |
| B6.Cg-Tg(Thy1-CFP/COX8A)2Lich (Mus Musculus) | The Jackson Laboratory | IMSR Cat# JAX:007967, RRID:IMSR_JAX:007967 |
| COS-7 cell | ATCC | Cat# CRL-1651 |

**Recombinant DNA**

| | | |
|------------------|---------------------|-----------------------------|
| Unmodified BAC clone | The BACPAC Resources Center at the Children’s Hospital Oakland Research Institute | RP24-318C6 |
| Rat AnkG (1-438, 439-837, 838-1156, 1157-1476, 1477-1908, 1909-2325, 2326-2622 aa) in pEF-BOS-GST | This paper | N/A |
| HA-Ubiquitin in pcDNA3 | Addgene | Addgene plasmid 18712 |
| MitoGFP-ires-Cre in pA51 | Kiryu-Seo et al (2016) | N/A |

**Antibodies**

| | | |
|------------------|---------------------|-----------------------------|
| Rabbit anti-GFP polyclonal (1:1,000) | MBL | Cat# 598, RRID:AB_591819 |
| Rat anti-GFP monoclonal (1:1,000) | Nacalai Tesque | Cat# O4404-84, RRID:AB_10013361 |
| Rabbit anti-Ank-G (Ankirin-G) polyclonal (1:500) | Frontier Institute | Cat# AnK-G-Rb, RRID:AB_2571661 |
| Mouse anti-Ankyrin G Monoclonal (4G3F8) (1:300) | Thermo Fisher Scientific | Cat# 33-8800, RRID:AB_2533145 |
| Rabbit anti-RPT3 polyclonal (1:500) | Enzo Life Sciences | Cat# BML-PW8175, RRID:AB_10541231 |
| Rabbit anti-TARDBP polyclonal (1:500) | Proteintech | Cat# 10782-2-A, RRID:AB_615042 |
| Mouse anti-TARDBP monoclonal (1:500) | Novus | Cat# NBP1-92695, RRID:AB_11005586 |
| Guinea pig anti-p62 polyclonal (1:500) | Progen | Cat# GP62-C, RRID:AB_2687531 |
| Goat anti-ECEL1 (N-20) polyclonal (1:1,000) | Santa Cruz Biotechnology | Cat# sc-11338, RRID:AB_2097871 |
| Mouse anti-NeuN monoclonal (1:1,000) | Millipore | Cat# MAB377, RRID:AB_2298772 |
| Rabbit anti-ATF-3 (C-19) polyclonal (1:1,000) | Santa Cruz Biotechnology | Cat# sc-188, RRID:AB_2258513 |
| Rabbit anti-ATF3 monoclonal (1:1,000) | Abcam | Abcam Cat# ab207434, RRID:AB_2734728 |
| Goat anti-ChAT polyclonal (1:1,000) | Millipore | Cat# AB144P, RRID:AB_2079751 |
| Rabbit anti-ChAT polyclonal (1:5,000) | Ichikawa et al (1997) | |
| Rabbit anti-Iba1 polyclonal (1:1,000) | Wako | Cat# 019-19741, RRID:AB_839504 |
| Goat anti-GFAP polyclonal (1:1,000) | Abcam | Cat# ab53554, RRID:AB_880202 |
| Rabbit anti-Caspr polyclonal (1:1,000) | Abcam | Cat# ab3415L, RRID:AB_869934 |
| Mouse anti-Neurofilament H monoclonal (1:1,000) | Covance | Cat# SMI-32P-100, RRID:AB_10719742 |
| Mouse anti-cytochrome c monoclonal (1:500) | Thermo Fisher Scientific | Cat# 45-6100, RRID:AB_2533821 |
| Goat anti-MMP-9 polyclonal (1:1,000) | Sigma-Aldrich | Cat# M9570, RRID:AB_1079397 |
| Goat anti-Osteopontin polyclonal (1:1,000) | R and D Systems | Cat# AF808, RRID:AB_2194992 |
| Rat ant-Lamp-1 monoclonal (1:1,000) | Santa Cruz Biotechnology | Cat# sc-19992, RRID:AB_2134495 |
Reagents and Tools table (continued)

| Reagent/Resource | Reference or Source | Identifier or Catalog Number |
|------------------|---------------------|-----------------------------|
| Mouse anti-GAPDH Monoclonal (6CS) (1:5,000) | Thermo Fisher Scientific | Cat# AM4300, RRID:AB_2536381 |
| α-Bungarotoxin, Alexa Fluor™ 594 conjugate (1:300) | Thermofisher | B13423 |
| GST-Tag Polyclonal (1:1000) | MBL | Cat# PM013, RRID:AB_291789 |
| Mouse Anti-HA.11 Monoclonal (1:1,000) | Covance | Cat# MMS-101P-1000, RRID:AB_291259 |
| Alexa Donkey anti-rat 488 (1:500) | Thermo Fisher Scientific | Cat# A-21208, RRID:AB_2535794 |
| Alexa Donkey anti-rabbit 488 (1:500) | Thermo Fisher Scientific | Cat# A-21206, RRID:AB_2535792 |
| Alexa Donkey anti-mouse 594 (1:500) | Thermo Fisher Scientific | Cat# A-21203, RRID:AB_141633 |
| Alexa Donkey anti-goat 647 (1:500) | Thermo Fisher Scientific | Cat# A-21447, RRID:AB_2535864 |
| Alexa Goat anti-rat 488 (1:500) | Thermo Fisher Scientific | Cat# A-11006, RRID:AB_2534074 |
| Alexa Goat anti-rabbit 594 (1:500) | Thermo Fisher Scientific | Cat# A-11012, RRID:AB_2534079 |
| Alexa goat anti-mouse 594 (1:500) | Thermo Fisher Scientific | A-11005, RRID:AB_2534073 |
| Alexa goat anti-guinea pig 594 (1:500) | Thermo Fisher Scientific | Cat# A-11076, RRID:AB_2534120 |

Oligonucleotides and sequence-based reagents

See Appendix Table S1

Chemicals, enzymes and other reagents

- ECL™ Prime Western Blotting System Amersham Cat# RPN2232
- SuperSeptMAce WAKO Cat# 197-15011
- RNase lipase tissue midi kit QIAGEN Cat# 74804
- SuperScript® III RNase H-Reverse Transcriptase Invitrogen Cat# 12188-085
- CUBIC-L Tokyo Chemical Industry Co., Ltd. Cat# T3740
- CUBIC-R Tokyo Chemical Industry Co., Ltd. Cat# T3741
- Mounting solution Tokyo Chemical Industry Co., Ltd. Cat# M3294
- Fast SYBR™ Green Master Mix Thermo Fisher Scientific Cat# 4385616

Software

- Fiji Image J Fiji NIH http://fiji.sc/
- GraphPad Prism version 7.0 Graphpad Software GraphPad Prism version 7.0 GraphPad Software, Inc https://www.graphpad.com/scientificsoftware/
- Imaris Bitplane Bitplane
- Zen black and blue Carl Zeiss Zeiss

Other

- AAV9-AnkG shRNA-mCherry - shRNA sequence 5’-GCGTTCCTCTTTAGATCTTTC-3’ Vector builder N/A
- AAV9-scramble shRNA Vector builder N/A
- Fv10i Olympus
- Lightsheet 7 Carl Zeiss
- SpinSR Olympus

Methods and Protocols

Animals

All animal protocols were carried out in accordance with the Nagoya university animal committee guidelines for the care and use of laboratory animals and were approved by the Nagoya university institutional animal care and use committee. All possible efforts were made to minimize suffering. Mice were housed in standard cages with ad libitum access to food and water under a 12-h light/dark cycle.

Atf3-BAC2 Tg mice were generated according to general bacterial artificial chromosome (BAC) modification protocols as previously described (Kiryu-Seo et al., 2016). The BAC clone RP24-318C6, containing the Atf3 locus, was purchased from the BACPAC Resources Center at the Children’s Hospital Oakland Research Institute (CHORI) (BPRC, California, USA). Briefly, a gene fragment of GFP from
*Aquorea coeruleens* (AcGFp) attached to the mitochondrial targeting sequence of cytochrome c oxidase subunit VIII, followed by an iRES, cre recombinase and SV40-polyA, was inserted into PL451 in front of the FRT-Neo-FRT cassette. The initiation codon of *Atf3* gene in BAC was replaced by that of GFP with the mitochondrial targeting sequence. BAC DNA was purified and injected into pronuclei from C57BL/6 oocytes (Institute of Immunology Co., Ltd., Tochigi, Japan) to generate Tg mice. The Atf3:BAC2 Tg mice were crossed with C57BL/6Ncr mice for at least seven generations. The hemizygous mice were fertile and viable without neurodevelopmental abnormalities.

To generate injury-induced Rpt3 CKO mice (Atf3:BAC2;Rpt3flox/flox), we carefully optimized cell-type-specific recombination (Luo et al., 2020). We crossed Atf3:BAC2 Tg mice with Rpt3flox/flox mice and maintained the colony as heterozygous Atf3:BAC2;Rpt3flox/+ mice. Rpt3flox/flox mice were provided by Drs. Tashiro and Takahashi (Kyoto University). Heterozygous Atf3:BAC2;Rpt3flox/+ mice were bred with homozygous Rpt3flox/flox mice to generate injury-induced Rpt3 CKO mice (Atf3:BAC2;Rpt3flox/flox; Fig EV1E–H). Homozygous Rpt3flox/flox mice were always maintained by crossing with Rpt3flox/flox, which had not crossed with cre driver mice to avoid unexpected germline recombination.

**Thy1-Mito** mice were purchased from Jackson Laboratory (Bar Harbor, ME; B6.Cg-Tg (Thy1-CFP/COX8A) S2Lich/J Stock No: 007967) and were maintained as a hemizygous colony.

To generate Atf3:BAC; SOD1G93A (Atf3:SOD1) mice, we initially crossed male S/J mice with female Atf3:BAC Tg mice (C57BL/6 background) which we reported previously (Kiryu-Seo et al., 2016) and obtained Atf3:BAC Tg mice with B6SJL background. The SOD1G93A mice (B6SJL-Tg(SOD1*G93A)*1Gur/J, stock number 002726, B6SJL background, Jackson Laboratory, Bar Harbor, ME) were crossed with Atf3:BAC Tg mice with B6SJL background to generate Atf3:SOD1 mice (Fig EV5A).

All mice were matched for age and sex. Experiments were performed on both sexes, with the exception of experiments involving Atf3:SOD1 mice. Male Atf3:SOD1 mice and the male littersmates carrying only Atf3:BAC transgene were used for experiment. Primer sequences for genotyping of Atf3:BAC Tg, Atf3:BAC2 Tg and Rpt3flox/flox mice are described in Appendix Table S1. Primer sequences and protocol for genotyping of Thy1-Mito and SOD1G93A mice are available through the Jackson Laboratory.

**Surgical procedures**

Animals of either sex at 8–10 weeks old were anesthetized under isoflurane. For hypoglossal nerve injury, the right hypoglossal nerve was exposed in the mid-thigh level. The nerve was carefully freed from surrounding connective tissue and then cut with a pair of scissors. The skin was closed with sutures.

**Immunochemistry**

Mice were perfused transcardially with 4% PFA–PBS, brain and spinal cord were dissected, washed with PBS, and processed according to CUBIC protocol (Tainaka et al., 2018). The samples were immersed in 1:1 water-diluted CUBIC-L (Tokyo Chemical Industry, M0374) for 1 day and then incubated with CUBIC-L with gentle shaking at 37°C for 4–5 days. After washing with PBS, the samples were immersed in 1:1 water-diluted CUBIC-R for 1 day and then in CUBIC-R with gentle shaking at room temperature for 1–2 days.

**Lightsheet microscopy**

The images are acquired with lightsheet 7 microscope (Zeiss, Germany). For CUBIC treated brain and spinal cord, images were captured using a 50 objective with digital zoom from 0.8 × to 2.5 × with digital zoom equipped with laser emitting 488 nm. The R lmatched sample was immersed in an oil mixture (RI = 1.525). Raw data were handled using a ZEISS Zen blue and black. 3D images were created in Imaris (Bitplane, Oxford Instruments, Belfast, UK).

**Counting of motor neurons**

For quantification of cell survival after axotomy, sections were stained with thionine. Thionine-stained hypoglossal motor neurons in injured and control sides were counted separately and at the

**Tissue clearing**

After mice were perfused transcardially with 4% PFA–PBS, brain and spinal cord were dissected, washed with PBS, and processed according to CUBIC protocol (Tainaka et al., 2018). The samples were immersed in 1:1 water-diluted CUBIC-L (Tokyo Chemical Industry, M0374) for 1 day and then incubated with CUBIC-L with gentle shaking at 37°C for 4–5 days. After washing with PBS, the samples were immersed in 1:1 water-diluted CUBIC-R for 1 day and then in CUBIC-R with gentle shaking at room temperature for 1–2 days.
identical level between animals as previously described (Kiryu-Seo et al., 2005). Data were presented as the percentage of surviving neurons on the injured side, compared with neurons on control side. For cell survival of GFP-positive injured motor neurons, the number of GFP-positive injured hypoglossal motor neurons at 5 days after injury was counted while that of ChAT-positive uninjured hypoglossal motor neurons was counted at the identical level between animals. Data were presented as the percentage of GFP-positive injured motor neurons compared with ChAT-positive uninjured motor neurons. For the cell number of uninjured hypoglossal motor neurons, ChAT-positive motor neurons in hypoglossal nucleus were counted at the identical level between animals. Data were shown as the cell number of unilateral hypoglossal nucleus per section. For counting GFP-positive hypoglossal motor neurons in transparent 3D images, serial 2D slices were used and motor neurons were carefully identified. Data were shown as the total number of GFP-positive motor neurons in hypoglossal nucleus.

RNA isolation and quantitative reverse-transcription PCR
Contralateral and ipsilateral hypoglossal nuclei at 5 days after injury were dissected from 7–10 mice. Total RNA was extracted using an RNaseasy Lipid Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. cDNA was synthesized using Super-ScriptIII (Thermo Fisher Scientific, Massachusetts, USA) following the standard protocols. Quantitative PCR was performed on the StepOnePlus™ Real-Time PCR System using primer pairs and SYBR Green PCR Master Mix (Thermo Fisher Scientific). Data were normalized to the GAPDH levels and calculated using the comparative threshold cycle method. Primer sequences are described in Appendix Table S1.

Immunoblotting
Contralateral and ipsilateral hypoglossal nuclei and nerves from 5–7 mice were dissected and homogenized in lysis buffer A containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 10 mM EDTA, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 5 µg/ml aprotinin, 1 mM PMSF, and 1 µg/ml leupeptin and centrifuged to collect the supernatant. Proteins were resolved by SDS–PAGE using 4–12% Bis-Tris gels (FUJIFILM Wako pure chemical corporation, Japan) and were transferred to PVDF membranes. Anti-AnkG (Frontier Institute, Cat# AnkG-Rb, RRID:AB_2571661), anti-RPT3 (Enzo Life Sciences, Cat# BML-PW8175, RRID:AB_10541231) and anti-GAPDH (Thermo Fisher Scientific, Cat# AM4300, RRID:AB_2536381) primary antibodies and were diluted 1:1,000–1:5,000. Signals were detected using ECL (GE Healthcare, Chicago, USA).

Ubiquitination assay
The cDNA fragments encoding truncated forms of rat AnkG were subcloned into pEF-BOS vector. Each GST-tagged AnkG plasmid and HA-tagged ubiquitin in pcDNA3 (addgene18712) were cotransfected into COS-7 cells. At 18 h after transfection, the cells were treated with 25 µM ALLN (Calbiochem, Merck, Darmstadt, Germany) for 6 h. The cells were lysed in lysis buffer B (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 0.5% NP-40, 1 mM DTT, 25 µM ALLN, 0.5 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 1 µg/ml leupeptin, 2 µg/ml antipain, and 10 µg/ml benzamidine). The cell lysates were incubated with Glutathione Sepharose 4B beads (GE Healthcare, Chicago, USA) for 2 h at 4°C, and the beads were subsequently washed three times with lysis buffer B. After washing, the beads were resuspended with SDS–PAGE sample buffer, and the samples were subjected to immunoblot analysis using anti-HA (Covance, Cat# MMS-101P-1000, RRID: AB_291259) and anti-GST (MBL, Cat# PM013, RRID:AB_591789) antibodies.

Image analysis of the fluorescently labeled AIS and mitochondria
All image analysis studies including length and number of the AIS, intensity ratio of GFP in the AIS, and fluorescent line intensity plots were performed by using Fiji software (NIH, Bethesda, MD, USA). Images were acquired on a confocal laser scanning microscope (Olympus FV10i, Tokyo, Japan) using a ×60 water-immersion objective (NA1.2). The length and number of AIS stained by AnkG was measured using z-stacked images. The number of the AIS per area with 1,024 × 1,024 pixel was counted. 20–30 neurons for AIS length and 3–6 areas for AIS number were measured in each mouse. For fluorescent intensity of GFP in the AIS region, we traced the line along the AIS region and subtracted the random fluorescence intensity in these images, to control for background signals. The mean intensities of GFP in the AIS were averaged and presented as the percentage of the intensity from Thy1-Mito mouse. 10–20 neurons from each mouse were measured. For fluorescent line intensity, the fluorescent intensity of GFP and AnkG in the AIS was plotted from the cell body to the axon along 1-µm-wide lines. Representative images and their corresponding plots are shown in figures. The disappeared AIS region after injury was identified when injured motor neurons retained very weak or fragmented signal of AnkG or when injured motor neuron extended the neurite, which exited hypoglossal nucleus and formed hypoglossal nerve bundle.

Super-resolution images of mitochondria in injured motor neurons were acquired by SpinSR10 (Olympus, Tokyo, Japan) using a x100 oil-immersion objective and deconvoluted. A single layer with highest resolution in the images was selected, and the fluorescent intensity of GFP in cell body was plotted along the line. Each peak represents the existence of mitochondria on a line within soma. The number of peaks on a line was counted as mitochondrial number from 5–7 neurons from each mouse.

In vivo imaging
Mice of either sex at 8–10 weeks old were anesthetized by i.p. injection of pentobarbital (45 mg/kg). To image axonal mitochondria in the sciatic nerve, we surgically exposed the uninjured and injured sciatic nerve without causing damage and positioned the mouse on an inverted laser scanning confocal microscope (Olympus FV10i) equipped with a temperature-controlled chamber at 37°C. Time-lapse movies were acquired on a confocal microscope equipped with a temperature-controlled stage at 37°C and with a ×60 water-immersion objective (NA1.2) (Olympus FV10i). Images were captured every 5 s and 100 frames were acquired in total for each recording, with laser power set to < 30% to minimize damage. Time-lapse images of mitochondrial dynamics were collected in a single focal plane at 1,024 × 1,024 pixel.

Quantification of axonal mitochondria
For whole-mount observation of mitochondria in nerves, hypoglossal and sciatic nerves were removed from mice, which were perfused transcardially with 4% paraformaldehyde (PFA)
PB. Subsequently, images of whole-mount axon were acquired on a confocal laser scanning microscope using ×60 water-immersion objective (Olympus FV10i). Digital Z stack images were obtained with sequential acquisition setting. The number of mitochondria in a single axon was counted using Fiji software. Data were presented as mitochondrial number in 50 μm² of axon. A 5–8 axons from each mouse were used for counting. To quantify mobile and stationary mitochondria, kymographs were generated using the Fiji software (NIH, Bethesda, MD, USA) as described previously (Kiryu-Seo et al, 2010). Motile mitochondria appeared as diagonal lines. Each mobile mitochondrion confirmed to be moving for six or more consecutive frames within the area was measured. Stationary mitochondrial sites were identified as vertical lines on the kymographs. The percentage of motile mitochondria of total mitochondria is defined in a given distance for 180 s per axons. Stationary mitochondria were counted per given distance of axon.

Adeno-associated viruses (AAVs)
The shRNA of AnkG was cloned into pAAV vector with the sequence, GCGTCCTATTTAGACTTTT, targeting a serine-rich region shared by both the 270-kDa and 480-kDa isoforms of mouse AnkG (Ye et al, 2020), followed by PGK promoter and mCherry (VectorBuilder, Chicago, USA). The scrambled shRNA followed by PGK promoter and mCherry was purchased from VectorBuilder. The plasmids were transfected into 293 cells to produce AAVs carrying serotype 9. The titers of all viral preparations were at least 1.0 × 10¹³ GC/ml. The AAVs were injected into the tongue of 6–8 week-old mice using Hamilton syringe at 3–4 weeks before hypoglossal nerve injury.

Statistical analysis
Results are expressed as mean ± SEM. Statistical analysis was performed using Prism? (GraphPad software). Unpaired t-test was used to compare two groups. One-way ANOVA was used for multiple comparisons, followed by Tukey post hoc analysis. Survival data were analyzed by long-rank test. P-values < 0.05 were considered significant.

Data availability
This study includes no data deposited in external repositories.

Expanded View for this article is available online.

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Author contributions
Sumiko Kiryu-Seo: Conceptualization; resources; data curation; formal analysis; supervision; funding acquisition; validation; investigation; visualization; writing – original draft; writing – review and editing. Reika Matsushita: Data curation; formal analysis; investigation. Yoshitaka Tashiro: Resources; writing – review and editing. Takeshi Yoshimura: Data curation; formal analysis; investigation; writing – review and editing. Yohei Iguchi: Resources; writing – review and editing. Masahisa Katsumo: Resources; writing – review and editing. Ryosuke Takahashi: Resources; writing – review and editing. Hiroshi Kiyama: Conceptualization; supervision; funding acquisition; writing – review and editing.

Disclosure and competing interests statement
The authors declare that they have no conflict of interest.

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Figure EV1. Injury-induced Atf3:BAC2 Tg mouse and Rpt3 CKO mouse.

A Time-dependent GFP expression in injured hypoglossal motor neurons of Atf3:BAC2 Tg mouse at 20, 36, and 48 h after injury.
B Representative image stained by ATF3 and GFP antibodies at 5 days following hypoglossal nerve injury of Atf3:BAC2 Tg mouse.
C The graph showing the percentage of GFP (++) or GFP (−) hypoglossal MNs in ATF3(+) MNs of control and injured sides at 5 days after injury of Atf3:BAC2 Tg mouse (n = 3 mice).
D Schematic of the breeding strategy to generate injury-induced Rpt3 CKO mouse.
E Diagram for Atf3:BAC2 transgene and Rpt3 gene. Arrows indicate the location of the genotyping primers (1)–(5) for Atf3:BAC2 Tg and Rpt3 CKO mouse. The gray triangles show loxP sites.
F Representative genotyping results using primers (1)–(4) to obtain Rpt3 CKO mouse. PCR products on the gel are amplified using indicated primer pairs.
G Representative genotyping using tail samples of adult Rpt3 CKO mice from #1 to #4 shows that the floxed Rpt3 alleles are not deleted.

Data information: Dashed lines outline hypoglossal nucleus in (A and B). XII, hypoglossal nucleus; cc, central canal. Scale bars, 150 μm in (A and B).
Source data are available online for this figure.
Figure EV1.
Figure EV2. GFP-labeled mitochondria in injured hypoglossal motor neurons of Atf3:BAC2 Tg and Rpt3 CKO mice.

A Whole-mount observation of hypoglossal nerve of Atf3:BAC2 Tg mouse without staining.
B Immunostaining of hypoglossal nerve at 5 and 10 days after injury, using anti-GFP, Lamp1, and neurofilament (NF) antibodies. Arrows show Lamp1 positive signals in injured nerve.
C Graph shows mitochondria number in hypoglossal (XII) axon at 5 days and 10 days after injury (n = 4 mice).
D Graph shows lysosome number in hypoglossal (XII) axon at 5 days and 10 days after injury (n = 4 mice).
E The GFP expression of injured hypoglossal motor neurons at 5 days following injury. XII, hypoglossal nucleus; cc, central canal.
F The percentage of GFP-positive injured motor neurons compared with ChAT-positive uninjured motor neurons (n = 3 mice per group).
G Super-resolution images of GFP-stained mitochondria in injured hypoglossal motor neuron. Stacked image of 2 µm thickness.
H Single layer image corresponding to boxed area in (G) and plot profile of the fluorescence intensity corresponding to the line in the image.

Data information: Dashed lines outline axon in (A) and hypoglossal nucleus in (E). Data are shown as the mean ± s.e.m. *P = 0.0247, **P = 0.0011 in (C), ***P < 0.0001 in (D), P = 0.7854 in (F), determined by one-way ANOVA followed by Tukey post hoc test. Scale bars, 300 µm in (A; left two panels) and 10 µm in (A; right panel), 5 µm in (B), 150 µm in (E), 4 µm in (G), and 2 µm in (H).
Source data are available online for this figure.
**Figure EV2.**

**A** Direct whole-mount observation of XII motor nerve

|           | Control | Injured 5d | mtGFP |
|-----------|---------|------------|-------|

**B** Injured XII nerve (proximal side)

|                  | Injured 5d | Injured 10d |
|------------------|------------|-------------|
| **Atf3:BAC2**    |            |             |
| **Rpt3 CKO**     |            |             |
| **GFP**          |            |             |
| **Lamp1**        |            |             |
| **NF**           |            |             |
| **Merge**        |            |             |

**C** Mitochondria No. / 5 μm axon

**D** Lysosome No. / 5 μm axon

**E** Atf3:BAC2

|                  | Control | Injured 5d |
|------------------|---------|------------|
| **GFP**          |         |            |

**Rpt3 CKO**

|                  | Control | Injured 5d |
|------------------|---------|------------|
| **GFP**          |         |            |

**F** % GFP (+ MNS) (Con A/ATTO647) (%)

**G** Atf3:BAC2

|                  |          |
|------------------|----------|
| **GFP**          |          |

**Rpt3 CKO**

|                  |          |
|------------------|----------|
| **GFP**          |          |

**H** Intensity (AU)

|                  |          |
|------------------|----------|
| **Atf3:BAC2**    |          |
| **Rpt3 CKO**     |          |
Figure EV3. Expression of CFP-labeled mitochondria in Thy1-mito-CFP Tg (Thy1-Mito) mouse before and after motor nerve injury.

A Immunostaining of hypoglossal nucleus (XII) of Thy1-Mito mouse using ATF3 (red) and GFP (green) antibodies at 5 days following hypoglossal nerve injury. XII, hypoglossal nucleus; cc, central canal.

B The localization of AnkG and CFP-labeled mitochondria in the AIS of uninjured and injured hypoglossal motor neurons of Thy1-Mito mouse.

C Immunostaining of the lumbar spinal motor neurons of Thy1-Mito mouse using ATF3 (red) and GFP (green) antibodies at 5 days following sciatic nerve injury.

D The localization of AnkG and CFP-labeled mitochondria in the AIS of uninjured and injured spinal motor neurons of Thy1-Mito mouse.

E Whole-mount observation of CFP-labeled mitochondria (mtCFP) in sciatic nerve.

Data information: Dashed lines outline hypoglossal nucleus (A), spinal cord (C) and axon (E). Closed arrowheads indicate the AIS, while open arrowheads indicate the region where the AIS is disrupted in (B and D). Scale bars, 50 μm (A and C) and 5 μm (B, D and E).
**Figure EV4. Suppression of AnkG in uninjured hypoglossal motor neurons.**

A  Schematic diagram for AAV injection to tongue.

B  Hypoglossal motor neurons labeled by mCherry (red) in injection side of AAVs. The decrease of AnkG (green) immunoreactivity is observed after the infection of AAV-AnkG shRNA but not AAV-scramble shRNA. Dashed lines show hypoglossal nucleus (XII). cc, central canal. Closed arrowheads indicate the AIS, while open arrowheads indicate the region where the AIS is disrupted.

C  CFP-labeled mitochondria in uninjured hypoglossal nerve of Thy1-Mito mouse after the infection of AAV-AnkG shRNA to tongue. Dashed lines show axon.

D  Graph shows the number of CFP-labeled mitochondria in uninjured hypoglossal (XII) axon of Thy1-Mito mouse before and after AAV infection. Data are shown as the mean ± s.e.m. (no significant difference ($P = 0.519$), determined by two-tailed Student’s t-test, $n = 3$ mice).

Data information: Scale bars = 100 μm (top), 20 μm (middle), and 5 μm (bottom) in (B) and 5 μm in (C).

Source data are available online for this figure.
Figure EV5. Characterization of Atf3:BAC;SOD1<sup>G93A</sup> (Atf3;SOD1) mouse.

A Schematic of the breeding strategy to generate Atf3;SOD1 mouse.
B Kaplan–Meier survival analysis (log-rank test, \( P = 0.4674 \)). The Atf3:BAC transgene has no significant effect on life span of SOD1<sup>G93A</sup>.
C Representative image of spinal motor neurons at 45, 70, and 130 days, immunostained with ChAT.
D Quantification of age-dependent ChAT-positive MN in the ventral horn of lumbar spinal cord. Data are shown as the mean ± s.e.m., no significant differences at same age, determined by unpaired t-test, \( n = 3 \) mice per group.
E The expression of GFP and ChAT in spinal motor neurons of Atf3;SOD1 at 45, 60, 90 and 130 days.
F The percentage (%) of GFP-positive MNs (black bar) of ChAT-positive MNs in Atf3; SOD1 mice. Data are shown as the mean ± s.e.m. \( n = 5 \) mice at each age.
G Immunostaining of spinal motor neurons in Atf3;SOD1 at P65 using GFP, MMP-9, OPN, and ChAT antibodies. Arrows denote GFP-positive motor neurons co-localized with MMP-9 or OPN.

Data information: Scale bar, 100 \( \mu \)m in (C), 50 \( \mu \)m in (E), and 20 \( \mu \)m in (G).
Source data are available online for this figure.
Figure EV5.
Mitochondrial entry into damaged axons is restricted by impaired disassembly of the axon initial segment

Sumiko Kiryu-Seo, Reika Matsushita, Yoshitaka Tashiro, Takeshi Yoshimura, Yohei Iguchi, Masahisa Katsuno, Ryosuke Takahashi, Hiroshi Kiyama

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Appendix Figure S1: Hypoglossal motor neurons and glial cells of Atf3:BAC2 Tg and Rpt3 CKO mice before and after nerve injury

Appendix Figure S2: The expression of GFP and AnkG in spinal motor neurons of Atf3:BAC2 Tg and Rpt3 CKO mice after sciatic nerve injury

Appendix Figure S3: The GFP-labeled hypoglossal motor neurons in Atf3:BAC;SOD1 mouse

Appendix Table S1: Primer list
Appendix Figure S1: Hypoglossal motor neurons and glial cells of Atf3:BAC2 Tg and Rpt3 CKO mice before and after nerve injury.

A. Uninjured hypoglossal nucleus immunostained by anti-ChAT antibody. XII, hypoglossal nucleus; cc, central canal; MNs, motor neurons. Dashed lines outline hypoglossal nucleus.

B. The graph showing the number of motor neurons in unilateral hypoglossal nucleus per section. Data are shown as the mean ± s.e.m. and no significance, determined by Student’s t-test (n= 3 mice per group).

C. Injured hypoglossal nucleus at 7 days after injury, immunostained by anti-GFP and -Iba1 antibodies. Microglial cells are more activated around injured hypoglossal motor neurons (MNs) of Rpt3 CKO mouse.

D. Injured hypoglossal nucleus at 7 days after injury, immunostained by anti-GFP and -GFAP antibodies.

Scale bars, 150 μ m in (A) and 30 μ m in (C and D).
Appendix Figure S2: The expression of GFP and AnkG in spinal motor neurons of Atf3:BAC2 Tg and Rpt3 CKO mice after sciatic nerve injury.
A. Schematic diagram of sciatic nerve injury model. The area surrounded by red box is observed in (B).
B. Spinal motor neurons of Atf3:BAC2 Tg and Rpt3 CKO mice before and at 5 days after injury, immunostained for AnkG and GFP. Arrows indicate the AIS stained by AnkG.
Scale bar, 30 μm.
Appendix Figure S3: The GFP-labeled hypoglossal motor neurons in Atf3;SOD1 mouse.

A. Uninjured hypoglossal motor neurons of Atf3;SOD1 mouse at 16 weeks old, immunostained by GFP, ATF3 and ChAT. Dashed lines outline hypoglossal nucleus (XII). cc, central canal.

B. Uninjured hypoglossal motor neurons of Atf3;SOD1 mouse at 16 weeks old, immunostained by AnkG- and GFP- antibodies.

C. The localization of the AIS and mitochondria in uninjured GFP-positive motor neurons of Atf3;SOD1 mouse at 16 weeks old.

D. Hypoglossal motor neurons of Atf3;SOD1 mouse (9 weeks old) at 5 days after injury, immunostained by AnkG- and GFP- antibodies.

E. The localization of AnkG and GFP-labeled mitochondria in the AIS of uninjured and injured hypoglossal motor neurons of Atf3;SOD1 mouse at 9 weeks old. Arrowheads indicate the AIS.

Scale bars, 100 μm in (A), 20 μm in (B and D), 5 μm in (C and E).
Appendix Table S1: Primer list

| Oligonucleotides | Primer sequences |
|------------------|------------------|
| Sprr1a-S         | TTGTGCCCCCAAAAACCAAG |
| Sprr1a-A         | GGCTCTGGTGCCCTTAGGTTG |
| Atf3-S           | ATGTCAGTCACCAAGTCTGAGGC |
| Atf3-A           | TGGATAAAGAGGTTCCCTCTGCTCTTCC |
| Gadd45a-S        | CTGCTGCTACTGGAAGGACGAC |
| Gadd45a-A        | CGACTTTCCCAGGCAAAAAACAAA |
| Cd44-S           | CACCATTGCCTCAACTGTG |
| Cd44-A           | TTGTGGGCTCTGAGTCTG |
| Fos-S            | CCATGATGTTCTCGGGG |
| Fos-A            | TGTCACGGTGAGGAAAGG |
| Gap43-S          | TGGTGTCAGCAGGCAAG | |
| Gap43-A          | GCTGTCAGCTCACCTTCT |
| Sox11-S          | CGACGACCTCATGTTGAC |
| Sox11-A          | GACGGGATAGGTTCC |
| Smad1-S          | GCTTCGTAAGGGGTTGG |
| Smad1-A          | CGGATTGAATAGATTG |
| Dine-S           | GTCTCTGAACTACGGGG |
| Dine-A           | GTAGGCTGAGGCTCCCATG |
| Rbfox3-S         | ATCGTAGAGGGAGG |
| Rbfox3-A         | GGGGCTGGCGTCCTG |
| Gapdh-S          | AGTGAAGGTCGCTG |
| Gapdh-A          | CGTGAAGGGATGAC |
| Pan AnkG-S       | CTAGAGTCCCAGCAG |
| Pan AnkG-A       | GCCTTTGCTCTGGATGAC |
| Giant AnkG-S     | CGAGTTCACATCTAGA |
| Giant AnkG-A     | TCACGATCTGGCTT |
| Chat-S           | GACAGCTAGGTTTGC |
| Chat-A           | CAGGAAGGCGG |

Genotyping primers

| Primer (1)  | CAATAAGTAGGAGTCAACTACAACGC |
| Primer (2)  | GACTTTCCCACACTATCCAACATC |
| Primer (3)  | TGAGCCTGATCAAGGTC |
| Primer (4)  | TAGAAGCCTAAGGGCA |
| Primer (5)  | TGCAATCCCTTGTCAAGGAGA |