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The Role of Autophagy in Nmnat-Mediated Protection Against Hypoxia-Induced Dendrite Degeneration

Yuhui Wen, R. Grace Zhai, and Michael D. Kim*

Department of Molecular and Cellular Pharmacology, University of Miami, Miller School of Medicine, Miami, FL 33136, USA

Abstract

The selective degeneration of dendrites precedes neuronal cell death in hypoxia-ischemia (HI) and is a neuropathological hallmark of stroke. While it is clear that a number of different molecular pathways likely contribute to neuronal cell death in HI, the mechanisms that govern HI-induced dendrite degeneration are largely unknown. Here, we show that the NAD synthase Nicotinamide mononucleotide adenylyltransferase (Nmnat) functions endogenously to protect *Drosophila* class IV dendritic arborization (da) sensory neurons against hypoxia-induced dendritic damage. Whereas dendrites of wild-type class IV neurons are largely resistant to morphological changes during prolonged periods of hypoxia (< 1.0% O₂), class IV neurons of *nmnat* heterozygous mutants exhibit significant dendrite loss and extensive fragmentation of the dendritic arbor under the same hypoxic conditions. Although basal levels of autophagy are required for neuronal survival, we demonstrate that autophagy is dispensable for maintaining the dendritic integrity of class IV neurons. However, we find that genetically blocking autophagy can suppress hypoxia-induced dendrite degeneration of *nmnat* heterozygous mutants in a cell-autonomous manner, suggestive of a self-destructive role for autophagy in this context. We further show that inducing autophagy by overexpression of the autophagy-specific kinase Atg1 is sufficient to cause dendrite degeneration of class IV neurons under hypoxia and that overexpression of Nmnat fails to protect class IV dendrites from the effects of Atg1 overexpression. Our studies reveal an essential neuroprotective role for endogenous Nmnat in hypoxia and demonstrate that Nmnat functions upstream of autophagy to mitigate the damage incurred by dendrites in neurons under hypoxic stress.

Keywords

Nmnat; autophagy; hypoxia-ischemia; dendrite; neurodegeneration; *Drosophila*

Introduction

Pathological alterations in dendritic structure are an early hallmark of brain injury in hypoxia-ischemia (HI). Transient ischemic episodes can induce rapid morphological changes in neurons, including extensive beading and swelling of dendrites, and the selective degeneration of dendrites during early ischemia likely serves as a precursor to neuronal cell

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*Corresponding author: Department of Molecular and Cellular Pharmacology, University of Miami, Miller School of Medicine, 1600 NW 10th Avenue, Miami, FL 33136, USA. Phone: +1 305 243 1690, Fax: +1 305 243 4555, mkim2@med.miami.edu.

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death (Hori and Carpenter, 1994; Hsu and Buzsaki, 1993; Ikonomidou et al., 1989; Kitagawa et al., 1989; Matesic and Lin, 1994). Whereas much attention has focused on the mechanisms underlying neuronal death following HI (Lipton, 1999), relatively little is known of how HI can induce dendritic damage.

Macroautophagy (hereafter referred to as autophagy) is a highly conserved, ubiquitous process that is one of the major pathways responsible for the bulk degradation of proteins and organelles in response to starvation and other cellular stresses (Levine and Klionsky, 2004). Basal levels of autophagy are essential for neuronal survival (Hara et al., 2006; Komatsu et al., 2006), and accordingly, autophagic dysfunction has been linked to a growing number of neurodegenerative disorders (Levine and Kroemer, 2008). Neurons show increased autophagy following cerebral ischemia (Adhami et al., 2006; Carloni et al., 2008; Nitatori et al., 1995; Puyal et al., 2009; Rami et al., 2008; Wen et al., 2008; Zhu et al., 2005); however, the question of whether elevated levels of autophagy are neuroprotective or directly contribute to HI-induced neuronal cell death remains largely unresolved (Nakka et al., 2008; Uchiyama et al., 2008).

Nmnat catalyzes a key step of NAD synthesis and the neuroprotective effects of Nmnat overexpression in response to injury and neurotoxic insults have been well documented (Coleman and Freeman, 2010). Further studies have provided evidence of an evolutionarily conserved role for endogenous Nmnat in axon and dendrite maintenance (Gilley and Coleman, 2010; Wen et al., 2011; Zhai et al., 2006). The recent finding that endogenous Nmnat is upregulated in the brains of adult flies exposed to hypoxia suggests that increasing Nmnat levels may serve as an adaptive response to hypoxic stress (Ali et al., 2011). We previously reported that Nmnat is required for maintaining dendritic coverage of *Drosophila* dendritic arborization (da) sensory neurons (Wen et al., 2011). In the present study, we set out to determine whether Nmnat functions endogenously to maintain the dendritic integrity of da neurons under hypoxic stress. We show that, while class IV neurons of wild-type larvae exposed to hypoxia (< 1.0% O₂) are largely resistant to morphological changes, class IV neurons of *nmnat* heterozygous mutants exhibit regression and fragmentation of dendrites under the same hypoxic conditions. This hypoxia-induced dendrite degeneration in *nmnat* heterozygous mutants is unrelated to apoptosis as expression of the caspase inhibitor p35 fails to rescue dendrite phenotypes associated with hypoxia. Surprisingly, we find that reducing *Atg1* function suppresses the dendrite degeneration observed in class IV neurons of hypoxia-exposed *nmnat* mutants and that cell-specific knockdown of autophagy-related genes rescues dendrite phenotypes associated with reduced *nmnat* function. We further demonstrate that overactivation of autophagy causes dendrite degeneration of class IV neurons under hypoxia and that overexpression of Nmnat fails to rescue autophagy-induced dendritic phenotypes. Our study supports a self-destructive role for autophagy in hypoxia and provides genetic evidence that *nmnat* functions upstream of autophagy to protect dendrites from damage induced by hypoxic stress.

Material and methods

Fly stocks

The *w¹¹⁸* strain was used for all wild-type controls. *nmnat*^{Δ4792} and *UAS-Nmnat* (Zhai et al., 2006), *UAS-p35* (Hay et al., 1994), *UAS-HSF-eGFP* (Yao et al., 2006), *UAS-Sima* (Zelzer et al., 1997), and *ppk-Gal4* (Kuo et al., 2005) are as previously described. *Atg1*^{Δ3D}, *UAS-Atg1*, *UAS-Atg5-RNAi*, *UAS-Atg7-RNAi*, *UAS-Atg12-RNAi*, and *UAS-mCherry-Atg8* flies were provided by Dr. Thomas Neufeld (University of Minnesota) (Scott et al., 2007; Scott et al., 2004). *UAS-GFP-huLC3* and *UAS-GFP-Atg5* (Rusten et al., 2004), *UAS-Cat*, and *UAS-SOD1* flies were obtained from the Bloomington Stock Center (Bloomington, IN).

Hypoxia treatment and live imaging

Embryos were collected for 4 hrs on grape juice agar plates supplemented with yeast paste and allowed to develop for 96 hrs at 25°C. Third-instar larvae aged 96-100 hrs after egg laying (AEL) (20-30 per genotype) were rinsed in water and transferred onto a new grape juice agar plate lacking yeast situated within a petri dish. The dish was placed within a plexiglass chamber and exposed to either room air (normoxia) or to < 1.0% O₂ (hypoxia) using a calibration mixture (4.94% CO₂ and N₂ Balance), which was constantly monitored using a Nuair O₂ Quickstick (Oxnard, CA). Larvae were then rinsed in water, mounted under a coverslip in 90% glycerol, and immediately imaged live using a Plan Apo 40x/1.3 NA objective on an Olympus FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan). Confocal image stacks (8-13 images per stack) were acquired at 800x800 pixel resolution and a step size of 1.0 µm and reconstructed into collapsed 2D images using ImageJ software (NIH, Bethesda, MD).

Quantitative and statistical analysis

The total number of dendritic branch points for each neuron was counted manually. The total dendrite length for each neuron was calculated using the NeuronJ tracing tool in ImageJ. The degeneration index (DI) was calculated by summing the lengths of breaks along the dendrites and dividing the sum by the total dendrite length for each neuron. Only dendrite breaks that were greater than 5 µm were measured and used to calculate the DI. Statistical significance was evaluated by comparing all groups ($n = 9-12$ neurons per genotype) using one-way analysis of variance (ANOVA) with Tukey's HSD *post hoc* analysis.

Results

Dendrites of class IV da neurons are largely resistant to hypoxic stress

We investigated the *in vivo* effects of prolonged hypoxia on dendrite morphology by examining class IV da sensory neurons of *Drosophila* larvae exposed to varying degrees of hypoxia. Class IV neurons are multi-dendritic sensory neurons of the peripheral nervous system (PNS) that elaborate highly branched dendritic arbors that effectively cover the entire larval body wall (Grueber et al., 2002). Class IV neurons are ideal for these studies because their dendrite arborization patterns are highly stereotyped and their dendritic arbors are largely confined within a two-dimensional space between the epidermis and body wall muscles (Han et al., 2012; Kim et al., 2012), allowing for immediate and direct live visualization of dendrite morphology without any artifacts associated with fixation. We used the class IV neuron-specific *ppk-Gal4* reporter driving expression of a green fluorescent protein (GFP)-tagged membrane marker (mCD8::GFP) to examine class IV neurons in wild-type third instar larvae [96-100 hrs after egg laying (AEL)] that were subjected to varying times (4, 8, and 14 hrs) of hypoxia (< 1.0% O₂). Despite prolonged hypoxia, GFP-expressing larvae remained viable under these conditions. Surprisingly, the dendritic arborization patterns of class IV neurons were largely unchanged in these larvae when compared with age-matched normoxia (21% O₂) controls (data not shown), and a slight, though statistically significant, reduction in the total number of terminal dendritic branches was only observed after 14 hrs of hypoxia exposure (Fig. 1A,D,G). However, the total dendrite length of class IV neurons in hypoxia-exposed larvae at 14 hrs did not differ statistically from normoxia controls (Fig. 1H). Furthermore, despite prolonged hypoxia exposure, class IV dendritic arbors remained intact and did not exhibit any apparent degeneration phenotypes (Fig. 1I), suggesting that these neurons possess an endogenous protection mechanism against hypoxic stress.

Nmnat functions endogenously to maintain dendritic integrity of class IV neurons under hypoxic stress

We previously found that Nmnat is cell-autonomously required for the proper maintenance of class IV dendrites and that it functions as a neuroprotective factor against progressive dendritic loss (Wen et al., 2011). The recent finding that Nmnat is upregulated in response to hypoxia and can mitigate the negative effects of oxidative stress on adult *Drosophila* lifespan (Ali et al., 2011) led us to investigate whether endogenous Nmnat serves a neuroprotective role in class IV neurons under hypoxia. Consistent with our previous results (Wen et al., 2011), we found that class IV neurons in larvae heterozygous for a loss-of-function mutation in *nmnat* (*nmnat*^{Δ4792}) exhibited significant reductions in both the total number of terminal dendritic branches (25.5%) and total dendrite length (13.7%) under normoxia when compared to wild-type controls (Fig. 1B,G,H). However, when *nmnat*^{Δ4792} heterozygous mutant larvae were placed under 14 hrs of hypoxia, the total number of terminal dendritic branches and the total dendrite length of class IV neurons were further reduced by 49.2% and 23.8%, respectively, when compared to class IV neurons of wild-type larvae under the same hypoxic conditions (Fig. 1E,G,H). In addition to reduced dendritic branching, class IV neurons in hypoxia-exposed *nmnat*^{Δ4792} heterozygotes exhibited extensive beading and fragmentation of the dendritic arbor (Fig. 1E). Both proximal and distal dendrites were affected as these neurons showed breaks at regular intervals along the entire lengths of the dendritic branches (Fig. 1E). We calculated the dendrite degeneration index (DI) for each genotype by summing the lengths of all breaks along the dendrites and dividing that sum by the total dendrite length. Whereas the DI for wild-type class IV neurons in hypoxia was 0.02, the DI for class IV neurons of hypoxia-exposed *nmnat*^{Δ4792} heterozygous mutants was 0.16 (Fig. 1I), with 90% of these neurons having a DI greater than 0.10 as opposed to 0% for wild-type.

We then determined whether Nmnat functions cell-autonomously to protect class IV neurons under hypoxia by using *ppk-Gal4* to express a wild-type Nmnat transgene specifically in class IV neurons of both wild-type and *nmnat*^{Δ4792} heterozygous mutant larvae. While overexpression of Nmnat did not significantly alter the dendritic properties of wild-type class IV neurons under normoxia or hypoxia (Fig. 1G,H), overexpression of Nmnat fully rescued the dendritic phenotypes of class IV neurons in *nmnat*^{Δ4792} heterozygotes under both normoxic and hypoxic conditions (Fig. 1C,F–I). Class IV-specific rescue restored total dendrite branching and dendrite lengths to that of wild-type controls and effectively prevented the degeneration of class IV dendrites under hypoxia (Fig. 1G–I). Collectively, these results demonstrate that endogenous Nmnat functions cell-autonomously to maintain the dendritic integrity of class IV neurons under hypoxic stress.

Overexpression of HSF and HIF-1α/Sima rescues hypoxia-induced dendrite degeneration in *nmnat* mutants

The *Drosophila* hypoxia-inducible factor 1α (HIF-1α) ortholog Similar (Sima) indirectly upregulates Nmnat expression in response to hypoxia through the induction of heat shock factor (HSF), which functions as a central regulator of *nmnat* transcription under stress conditions, and overexpression of either HSF or Sima has been shown to upregulate Nmnat in response to hypoxic stress (Ali et al., 2011). We therefore tested whether selective overexpression of HSF or Sima could rescue hypoxia-induced dendritic phenotypes of class IV neurons in *nmnat*^{Δ4792} heterozygous mutants. Overexpression of HSF or Sima had no effect on the development or maintenance of class IV dendrites in wild-type larvae under both normoxic and hypoxic conditions (Supplementary Fig. S1I–N). Although overexpression of HSF or Sima partially suppressed the loss of terminal dendritic branches observed in *nmnat*^{Δ4792} heterozygotes under normoxia, the total dendrite length remained unchanged in these mutants (Supplementary Fig. S1A–D,I–N). This may be explained by

moderate increases in *Nmnat* expression that are observed upon HSF or Sima overexpression in non-stress conditions (Ali et al., 2011). On the other hand, we found that overexpression of HSF or Sima fully rescued the dendrite regression and degeneration phenotypes of class IV neurons in hypoxia-exposed *nmnat*^{Δ4792} heterozygotes (Supplementary Fig. S1E–N), consistent with the ability of either transcription factor to significantly upregulate *Nmnat* expression upon exposure to stress (Ali et al., 2011). These data provide further evidence that hypoxia engages *Nmnat* signaling through transcriptional regulation mediated by Sima and HSF.

Impairment of autophagy suppresses hypoxia-induced dendrite degeneration in *nmnat* mutants

The susceptibility of class IV neurons in *nmnat*^{Δ4792} heterozygotes to hypoxia-induced dendritic damage suggests that these neurons demonstrate increased vulnerability to cellular degradation pathways that mediate dendrite degeneration. Autophagy can be induced by HI (Adhami et al., 2006; Carloni et al., 2008; Nitatori et al., 1995; Puyal et al., 2009; Rami et al., 2008; Wen et al., 2008; Zhu et al., 2005) and has been implicated to play a role in neurite degeneration (Knoferle et al., 2010; Wang et al., 2006; Yang et al., 2007), but whether autophagy promotes neuronal survival or destruction in this context remains unclear. We therefore investigated the role of autophagy in class IV neurons under hypoxia and tested whether genetically blocking autophagy could modify dendritic phenotypes associated with reduced *nmnat* function. We first sought to determine whether hypoxia induces autophagy in class IV neurons by using *ppk-Gal4* to selectively express well-established autophagy markers (mCherry-Atg8, GFP-Atg5, or GFP-huLC3) in these neurons (Rusten et al., 2004; Scott et al., 2004). However, expression of these markers either led to overexpression artifacts or was not reliably detected at the single-cell level (data not shown). We therefore tested the endogenous role of autophagy in hypoxia by examining class IV neurons in third-instar larvae homozygous for a loss-of-function *Autophagy-specific gene 1* (*Atg1*) allele (*Atg1*^{Δ3D}) (Scott et al., 2004). The *Atg1* gene encodes for a serine/threonine kinase that is important for the initiation of autophagy and is highly conserved among eukaryotes (Chen and Klionsky, 2011; Scott et al., 2007). We found that loss of *Atg1* had no discernible effect on the development of class IV dendrites in normoxic conditions when compared to wild-type controls (Fig. 2A,D,I–K). Furthermore, class IV neurons of hypoxia-exposed *Atg1*^{Δ3D} homozygous mutants did not exhibit any dendrite branching defects or degeneration phenotypes (Fig. 2E,H,I–K), suggesting that autophagy is dispensable for maintaining the dendritic integrity of these neurons under hypoxic stress. However, when we examined class IV neurons in *Atg1*^{Δ3D} homozygous mutants with only one functional copy of the *nmnat* gene, we found that loss of *Atg1* fully suppressed both the dendrite regression and degeneration phenotypes of *nmnat*^{Δ4792} heterozygotes under hypoxia (Fig. 2F,G,I–K). Loss of *Atg1* did not suppress the dendrite maintenance defects found in class IV neurons of *nmnat*^{Δ4792} heterozygotes under normoxia (Fig. 2B,C,I–K), pointing to separable neuroprotective roles for *Nmnat* in neurons under normoxia and hypoxia. These results further suggest that endogenous *Nmnat* serves to mitigate the detrimental effects of hypoxia-induced autophagy in these neurons.

In addition to its function in autophagy, *Atg1* has been implicated to play a role in neuronal vesicular transport and autophagy-independent signaling pathways (Akers et al., 2012). We therefore determined the role of other autophagy-related genes in hypoxia-induced dendrite degeneration by using *ppk-Gal4* to express RNAi transgenes directed against *Autophagy-specific genes* in class IV neurons (Scott et al., 2004). We first tested the role of *Autophagy-specific gene 5* (*Atg5*), which is required for the early stages of autophagosome formation (Xie and Klionsky, 2007). Similar to *Atg1*^{Δ3D} homozygous mutants, we found that RNAi knockdown of *Atg5* had no effect on the dendritic branching of class IV neurons under

normoxia or hypoxia when compared to wild-type controls (Fig. 3A,D,E,H,I–K), and furthermore, failed to rescue dendritic phenotypes associated with reduced *nmnat* function in normoxic conditions (Fig. 3B,C,I,J). However, expression of Atg5-RNAi in class IV neurons of *nmnat*^{Δ4792} heterozygotes completely suppressed the dendrite regression and degeneration phenotypes normally observed in these neurons under hypoxia (Fig. 3F,G,I–K). We further used *ppk-Gal4* to express RNAi transgenes directed against *Autophagy-specific genes 7* (*Atg7*) or *12* (*Atg12*), two autophagy-related genes that are also required for autophagosome formation (Xie and Klionsky, 2007), in class IV neurons of both wild-type and *nmnat*^{Δ4792} heterozygous larvae. We found that, similar to class IV-specific expression of Atg5-RNAi, knockdown of either *Atg7* or *Atg12* had no effect on the development or maintenance of class IV dendrites under normoxia, but effectively suppressed the dendrite degeneration phenotype of *nmnat*^{Δ4792} heterozygotes under hypoxia (Fig. 4). Taken together, these results suggest that autophagy is dispensable for dendrite maintenance under normoxia, but implicate a cell-autonomous role for autophagy in hypoxia-induced dendrite degeneration.

Hypoxia-induced dendrite degeneration in *nmnat* mutants occurs independently of caspase activity

While both autophagy and caspases are thought to contribute to autophagic cell death, recent studies provide evidence that autophagy can mediate cellular destruction independent of caspase activity (Ryoo and Baehrecke, 2010). We therefore determined whether caspases are important for autophagic degradation of class IV neurons under hypoxia by selectively expressing the effector caspase inhibitor p35 in class IV neurons of wild-type and *nmnat*^{Δ4792} heterozygotes. We found that the dendritic arborization patterns of class IV neurons expressing p35 were largely unaltered and that expression of p35 failed to suppress the dendrite degeneration phenotypes of class IV neurons of *nmnat*^{Δ4792} heterozygotes under hypoxia (Fig. 5). Although these data argue against caspase involvement, we cannot completely exclude the possibility that caspases are important for later events as it was not possible to determine whether dendrite fragments are cleared at later time points because of the reduced viability of larvae that are subjected to greater than 14 hrs of hypoxia. However, these results suggest that autophagy, and not caspases, mediate the initial events that lead to dendrite degeneration under hypoxia.

Increased autophagy induces dendrite degeneration under hypoxia

Our results suggest that hypoxia-induced autophagy causes dendrite degeneration in larvae with reduced *nmnat* function, but whether elevated levels of autophagy are sufficient to cause dendrite degeneration is unclear. Overexpression of Atg1 has been previously shown to induce high levels of autophagy in larval fat bodies, salivary glands, and neurons (Berry and Baehrecke, 2007; Scott et al., 2007; Shen and Ganetzky, 2009). When we overexpressed Atg1 in class IV neurons of wild-type larvae under normoxia, these neurons exhibited significant reductions in both total number of terminal dendritic branches (30.6%) and total dendrite length (29.7%), but did not exhibit any apparent dendrite degeneration phenotypes according to our criteria (Fig. 6A,D,I–K). While overexpression of Atg1 enhanced the dendritic regression phenotype of class IV neurons in *nmnat*^{Δ4792} heterozygotes under normoxia, inducing autophagy had no effect on the dendritic integrity of these mutants (Fig. 6B,C,I–K). When we overexpressed Atg1 in class IV neurons of wild-type larvae under hypoxia, we found that these neurons exhibited significant dendrite loss and extensive fragmentation of the dendritic arbor despite the presence of two functional copies of *nmnat* (Fig. 6H–K), suggesting overactivation of autophagy can counteract the protective effects of endogenous *Nmnat* in hypoxia. However, the extent of dendrite degeneration in Atg1-expressing neurons (44% of neurons with a DI > 0.10) was less than what was observed for

class IV neurons in *nmnat*^{Δ4792} heterozygotes under hypoxia (90% of neurons with a DI > 0.10).

We further found that overexpression of Atg1 in *nmnat*^{Δ4792} heterozygotes did not enhance dendrite degeneration phenotypes observed in these mutants (Fig. 6F,G,K), consistent with the possibility that *nmnat* and *Atg1* act in the same pathway. Although Atg1 overexpression did lead to a further reduction in total dendrite length of class IV neurons in *nmnat*^{Δ4792} heterozygotes under hypoxia, this did not differ statistically from class IV neurons overexpressing Atg1 in a wild-type background under the same hypoxic conditions (Fig. 6J).

We then tested whether overexpression of Nmnat could prevent the dendrite degeneration induced by Atg1 overexpression in class IV neurons under hypoxia. We found that overexpression of Nmnat did not suppress the loss of dendrites caused by Atg1 overexpression in class IV neurons under normoxia (Fig. 7A–C,G–I), and furthermore, failed to protect Atg1-overexpressing class IV neurons from hypoxia-induced dendrite degeneration (60% of neurons with a DI > 0.10) (Fig. 7D–F,G–I). Collectively, these data suggest that autophagy acts downstream of *nmnat* and that elevated levels of autophagy lead to dendritic damage of neurons under hypoxia.

Overexpression of ROS scavengers suppresses dendrite degeneration in hypoxia-exposed *nmnat* mutants

Reactive oxygen species (ROS) are generated in neurons in response to oxygen deprivation and reoxygenation (Abramov et al., 2007), and overproduction of ROS has been implicated to play a role in neuronal damage and cell death associated with ischemia/reperfusion (Abramov et al., 2007; Keller et al., 1998). Furthermore, ROS are potent activators of autophagy (Scherz-Shouval and Elazar, 2011). The previous finding that Nmnat can inhibit ROS accumulation (Press and Milbrandt, 2008), coupled with our data showing that Nmnat acts upstream of autophagy, together support the possibility that Nmnat may suppress autophagy by maintaining low levels of ROS under hypoxic conditions. We therefore asked whether increased levels of ROS contribute to the hypoxia-induced dendrite degeneration in *nmnat* mutants. To test this idea, we expressed the ROS scavenging enzymes Catalase (Cat) and Superoxide dismutase (SOD) in class IV neurons of both wild-type and *nmnat*^{Δ4792} heterozygous larvae. Although overexpression of Cat led to a reduction in terminal dendritic branching of class IV neurons in wild-type larvae under normoxia (Fig. 8A,D,I), the extent of dendritic regression in Cat-overexpressing neurons under hypoxia (13%) did not exceed that of wild-type controls (15%) (Fig. 8E,H,I), suggesting physiological levels of ROS may be important for dendrite development, but that the modest dendritic regression phenotype observed in hypoxia-exposed wild-type larvae is unlikely to be a consequence of increased ROS levels. On the other hand, overexpression of Cat did not enhance or suppress the dendrite maintenance defect of *nmnat*^{Δ4792} heterozygous mutants under normoxia (Fig. 8B,C,I,J), but partially suppressed the dendrite regression phenotype of class IV neurons in hypoxia-exposed *nmnat*^{Δ4792} heterozygotes, and fully rescued the dendrite fragmentation phenotype normally observed in these neurons under hypoxic conditions (Fig. 8E–K). Overexpression of SOD also rescued the dendrite fragmentation phenotype of hypoxia-exposed *nmnat*^{Δ4792} heterozygotes, though to a lesser extent than that observed with Cat overexpression (data not shown). These data suggest that *nmnat* mutants have elevated levels of ROS under hypoxia and that ROS signaling pathways contribute to the dendrite degeneration phenotype observed in these mutants.

Discussion

In this study, we found that dendrites of *Drosophila* sensory neurons are largely resistant to morphological changes in response to prolonged hypoxia and that Nmnat functions

endogenously to maintain the dendritic integrity of class IV neurons under hypoxic stress. We demonstrated that *nmnat* heterozygous mutants exhibit extensive fragmentation of class IV dendrites when exposed to hypoxia and that *nmnat* functions in a cell-autonomous manner to prevent hypoxia-induced dendrite degeneration. Dendrite degeneration in *nmnat* mutants is independent of the apoptotic machinery and can be suppressed by blocking autophagy, suggesting autophagy mediates cellular destruction independent of caspase activity in this context. Finally, our data suggest that Nmnat acts upstream of autophagy pathways, by possibly inhibiting ROS accumulation, to mitigate the damage incurred by dendrites in response to hypoxic stress.

Endogenous Nmnat protects dendrites against hypoxic stress

Dendrites of vertebrate neurons demonstrate a remarkable sensitivity to low oxygen conditions and are highly susceptible to rapid morphological changes even after brief periods of HI (Hori and Carpenter, 1994; Hsu and Buzsaki, 1993; Ikonomidou et al., 1989; Park et al., 1996). CA1 pyramidal neurons typically die several days after a transient ischemic episode (Kirino, 1982), but exhibit widespread beading of dendrites and breakdown of the dendritic cytoskeleton within hours after the ischemic event (Hori and Carpenter, 1994; Hsu and Buzsaki, 1993; Matesic and Lin, 1994). On the other hand, both *Drosophila* larvae and adults are exceptionally tolerant of hypoxic conditions and CNS neurons demonstrate complete recovery of evoked responses even after prolonged periods of anoxia (Haddad et al., 1997a; Haddad et al., 1997b; Wingrove and O'Farrell, 1999), suggesting *Drosophila* employ endogenous protection mechanisms to prevent hypoxia-induced changes in dendritic structure that consequently impair neuronal function. However, although understanding of the genetic basis of hypoxia tolerance in *Drosophila* has significantly progressed (Zhou et al., 2008), the mechanisms by which *Drosophila* neurons maintain their structural integrity upon hypoxia exposure are still relatively unknown.

Our finding that dendrites of class IV neurons are largely resistant to morphological changes upon hypoxia exposure led us to investigate the neuroprotective mechanisms that are endogenous to these neurons. From previous studies, we found that Nmnat is important for maintaining dendritic coverage of class IV neurons during larval development (Wen et al., 2011). While the enzymatic function of Nmnat in NAD biosynthesis is well known (Magni et al., 2004), Nmnat has garnered significant attention recently due to reports of neuroprotective effects stemming from its overexpression. Indeed, overexpression of Nmnat has been shown to delay injury-induced axonal degeneration (Coleman and Freeman, 2010), protect against neurotoxic insults in both vertebrate and *Drosophila* models of human neurodegenerative diseases (Ali et al., 2012; Ljungberg et al., 2012; Zhai et al., 2008), reduce the extent of neonatal HI-induced CNS injury (Verghese et al., 2011), and, from our previous work, protect against dendritic regression (Wen et al., 2011). Our finding that Nmnat is important for maintaining the dendritic integrity of class IV neurons under hypoxia provides further support of an evolutionarily conserved role for endogenous Nmnat in the maintenance of neuronal integrity (Gilley and Coleman, 2010; Zhai et al., 2006), and are consistent with recent reports of Nmnat's function as a stress response protein (Ali et al., 2011). *Drosophila* Nmnat is upregulated in the brain in response to hypoxia, and while reducing *nmnat* function was shown to severely compromise the survival of flies exposed to oxidative stress (Ali et al., 2011), the cytoprotective effects of Nmnat upregulation in hypoxia are largely unknown. Our studies here reveal that upregulation of Nmnat is important for maintaining the structural integrity of dendrites in neurons under hypoxia and further support the notion that a critical threshold of endogenous Nmnat is required for its proper protective function against cellular stress.

Autophagy as the major mode of hypoxia-induced dendrite degeneration

Our finding that impairment of autophagy suppresses hypoxia-induced dendrite degeneration in *nmnat* mutants implicates autophagy as a self-destructive mechanism in HI. Autophagy is well known to be induced in both neonatal and adult brain in response to HI (Adhami et al., 2006; Nitatori et al., 1995; Rami et al., 2008; Wen et al., 2008), but whether autophagy acts as a protective or destructive process remains controversial. While basal levels of autophagy are certainly required for neuronal survival (Hara et al., 2006; Komatsu et al., 2006), genetic and pharmacological evidence indicate that inhibiting autophagy may serve as an effective strategy to reduce HI-induced cellular damage (Koike et al., 2008; Puyal et al., 2009). Our study further argues against a neuroprotective role for autophagy in hypoxia as we found that class IV neurons lacking *Atg1* or expressing RNAi transgenes targeting *Atg5*, *Atg7*, or *Atg12* were largely unaffected when exposed to prolonged hypoxia. Our data are more consistent with a self-destructive role for autophagy in hypoxia as we found that blocking induction of autophagy or subsequent steps in autophagosome formation prevented the hypoxia-induced dendrite degeneration in *nmnat* mutants. The inability of p35 overexpression to block dendrite degeneration in *nmnat* mutants further suggests that this autophagic degradation occurs independently of caspase activity. Although autophagy and caspases are intimately linked in autophagic cell death, recent studies suggest that autophagy can also mediate cellular destruction in a caspase-independent manner (Denton et al., 2009).

It is interesting to note that induction of autophagy by overexpression of *Atg1* was not sufficient to cause fragmentation of class IV dendrites in either wild-type or *nmnat* mutant larvae under normoxia and that *Atg1* overexpression induced dendrite degeneration only in larvae exposed to hypoxic conditions, suggesting autophagy integrates with other stress response pathways to exert its effects (Kroemer et al., 2010). Furthermore, our finding that overexpression of *Atg1* can induce dendrite degeneration in hypoxia-exposed wild-type larvae despite the presence of two functional copies of *nmnat*, but does not enhance degeneration phenotypes in *nmnat* heterozygous mutants under hypoxia, suggests that *Nmnat* acts upstream of autophagy to mitigate its effects on the cell in response to hypoxia. This is consistent with our results showing that overexpression of *Nmnat* fails to suppress dendrite degeneration induced by *Atg1* expression. On the other hand, the inability of *Atg1* mutants to suppress the dendrite maintenance defect of *nmnat* heterozygotes under normoxia suggests that *Nmnat* protects against dendritic regression and hypoxia-induced degeneration through mechanistically distinct pathways. Furthermore, while loss of *Atg1* had no effect on the development or maintenance of class IV dendrites under normoxia, overexpression of *Atg1* significantly enhanced the dendrite regression phenotype of *nmnat* heterozygotes, providing further evidence of separable roles for *nmnat* and autophagy in normal dendrite development.

How might *Nmnat* protect against hypoxia-induced dendrite degeneration?

Although the neuroprotective effects of *Nmnat* are well documented, the mechanisms by which *Nmnat* achieves this protection remain particularly elusive. Our studies provide a novel link between *Nmnat* and autophagy and suggest that *Nmnat* mitigates the self-destructive effects of autophagy induced by hypoxic stress. However, what exactly is the role of *Nmnat* in this context? The enzymatic activity of *Nmnat* has been shown to be important for neuroprotection (Coleman and Freeman, 2010), but recent studies point to an additional chaperone function for *Nmnat* in this role (Ali et al., 2011; Ali et al., 2012; Zhai et al., 2008). Transient cerebral ischemia can cause aggregates of ubiquitinated proteins (Hu et al., 2000), and stress response chaperones such as Hsp70 have been shown to reduce cell death induced by oxygen glucose deprivation (Giffard et al., 2004). This is particularly interesting in light of the recent finding that *Nmnat* can suppress tau-induced neurodegeneration by promoting the ubiquitination and clearance of toxic tau species (Ali et

al., 2012). The recent identification of Nmnat as a stress response chaperone suggests that Nmnat may protect against hypoxia-induced cellular damage by preventing the aggregation of proteins. Although autophagy is a major mode of clearance of protein aggregates (Kirkin et al., 2009), we found that autophagy is largely dispensable for the maintenance of neuronal integrity under hypoxia. Autophagy may therefore be an adaptive response to increased protein aggregation in *nmnat* mutants under hypoxia leading to increased protein and organelle degradation eventually causing cellular self-destruction. This may further explain why we see dendrite degeneration only when autophagy is induced in hypoxia-exposed larvae.

Our studies further support the possibility that Nmnat protects against dendrite degeneration by maintaining low levels of ROS or by attenuating the deleterious effects of accumulated ROS induced by hypoxia. Our data are consistent with the finding that Nmnat can delay axonal degeneration in neurons treated with rotenone, which induces production of mitochondrial ROS, by preventing ROS accumulation in axons (Press and Milbrandt, 2008). The generation of ROS has been shown to be significantly increased in neurons in response to oxygen deprivation and plays a critical role in neuronal cell death (Abramov et al., 2007). Furthermore, overproduction of ROS is a common pathological feature of most age-related neurodegenerative disorders (Lin and Beal, 2006), as sustained high levels of ROS are known to cause mitochondrial dysfunction, cellular damage, and eventual cell death. In addition to its cytotoxic effects, ROS have also been shown to activate autophagy (Scherz-Shouval et al., 2007). While this is generally thought to promote cell survival in response to oxidative stress (Scherz-Shouval and Elazar, 2007), overactivation of autophagy may cause damage at the cellular level and may also lead to autophagic (type II) cell death. In this manner, Nmnat may function to gate ROS accumulation in response to hypoxia to moderate the levels of autophagy in the neuron.

In summary, our studies demonstrate a cytoprotective function for endogenous Nmnat in hypoxia and provide further evidence for its role as a stress response protein. Our studies further support the idea that targeting autophagy may serve as an effective strategy to reduce cellular damage associated with HI.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CNS	central nervous system
da	dendritic arborization
HI	hypoxia-ischemia
NAD	nicotinamide adenine dinucleotide
PNS	peripheral nervous system

ROS reactive oxygen species

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Figure 1. Nmnat protects against dendritic damage in hypoxia-exposed larvae

(A,B) Dendrite morphology of dorsal class IV ddaC neuron in a wild-type (A) and *nmnat*^{Δ4792} heterozygous mutant (*nmnat*^{+/+}) (B) third instar larva (110-114 hrs AEL) after exposure to 14 hrs of normoxia. (C) Selective overexpression of wild-type Nmnat in class IV neurons rescues the dendrite regression phenotype of *nmnat* heterozygous mutants under normoxia. (D,E) Dendrite morphology of ddaC neuron in a wild-type (D) and *nmnat* heterozygous mutant (E) third instar larva after exposure to 14 hrs of hypoxia (< 1.0% O₂). Class IV neurons of hypoxia-exposed *nmnat* heterozygotes show extensive breaks along the dendritic arbor. (F) Selective overexpression of wild-type Nmnat in class IV neurons rescues the dendrite degeneration phenotype of *nmnat* heterozygous mutants under hypoxia. (G–I) Quantification of total number of terminal dendritic branches (G) and total dendrite length (H) per 9.6 x 10⁴ μm² field (mean ± SD), and degeneration index (DI) (I) for ddaC neurons in wild type, *nmnat* heterozygous mutant (*nmnat*^{+/+}), *nmnat* heterozygous mutant background overexpressing Nmnat (*UAS-Nmnat*^{+/+}; *nmnat*^{+/+}), and wild-type background overexpressing Nmnat (*UAS-Nmnat*^{+/+}) under normoxia and hypoxia (* *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001). Statistical comparison of *UAS-Nmnat*^{+/+} with other groups not shown for simplification purposes. Dorsal is up and anterior is to the left in this and all subsequent figures. Scale bar, 50 μm.

Figure 2. Loss of *Atg1* suppresses hypoxia-induced dendrite degeneration in *nmnat* mutants (A,B) Dendrite morphology of class IV ddaC neuron in a wild-type (A) and *nmnat* heterozygous mutant (*nmnat/+*) (B) third instar larva after exposure to 14 hrs of normoxia. (C) Loss of *Atg1* fails to suppress the dendrite regression phenotype of class IV neurons in *nmnat* heterozygous mutants under normoxia. (D) Class IV dendritic arborization patterns are largely unaltered in *Atg1* homozygous mutants under normoxia. (E,F) Dendrite morphology of ddaC neuron in a wild-type (E) and *nmnat* heterozygous mutant (F) third instar larva after exposure to 14 hrs of hypoxia. (G) Loss of *Atg1* suppresses the dendrite regression and degeneration phenotypes of class IV neurons in *nmnat* heterozygous mutants under hypoxia. (H) Class IV dendritic arborization patterns are largely unaltered in *Atg1* homozygous mutants under hypoxia. (I–K) Quantification of total number of terminal dendritic branches (I), total dendrite length (J), and degeneration index (DI) (K) for ddaC neurons in wild type, *nmnat* heterozygous mutant (*nmnat/+*), *Atg1* homozygous mutant with loss of one copy of *nmnat* (*Atg1, nmnat/Atg1, +*), and *Atg1* homozygous mutant (*Atg1/Atg1*) under normoxia and hypoxia (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistical comparison of *Atg1/Atg1* with other groups not shown for simplification purposes. Scale bar, 50 μm.

Figure 3. Knockdown of *Atg5* suppresses hypoxia-induced dendrite degeneration in *nmnat* mutants

(A,B) Dendrite morphology of class IV ddaC neuron in a wild-type (A) and *nmnat* heterozygous mutant (*nmnat*^{+/+}) (B) third instar larva after exposure to 14 hrs of normoxia. (C) Cell-specific knockdown of *Atg5* fails to suppress the dendrite regression phenotype of class IV neurons in *nmnat* heterozygous mutants under normoxia. (D) Knockdown of *Atg5* has no effect on the development or maintenance of class IV neurons under normoxia. (E,F) Dendrite morphology of ddaC neuron in a wild-type (E) and *nmnat* heterozygous mutant (F) third instar larva after exposure to 14 hrs of hypoxia. (G) Cell-specific knockdown of *Atg5* suppresses the dendrite regression and degeneration phenotypes of class IV neurons in *nmnat* heterozygous mutants under hypoxia. (H) Knockdown of *Atg5* has no effect on the development or maintenance of class IV neurons under hypoxia. (I–K) Quantification of total number of terminal dendritic branches (I), total dendrite length (J), and degeneration index (DI) (K) for ddaC neurons in wild type, *nmnat* heterozygous mutant (*nmnat*^{+/+}), *nmnat* heterozygous mutant background expressing *Atg5*-RNAi (*UAS-Atg5-RNAi*^{+/+}; *nmnat*^{+/+}), and wild-type background expressing *Atg5*-RNAi (*UAS-Atg5-RNAi*^{+/+}) under normoxia and hypoxia (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistical comparison of *UAS-Atg5-RNAi*^{+/+} with other groups not shown for simplification purposes. Scale bar, 50 μm.

Figure 4. Knockdown of *Atg7* and *Atg12* suppresses hypoxia-induced dendrite degeneration in *nmnat* mutants

(A–B) Quantification of total number of terminal dendritic branches (A), total dendrite length (B), and degeneration index (DI) (C) for ddaC neurons in wild type, *nmnat* heterozygous mutant (*nmnat*^{+/+}), *nmnat* heterozygous mutant background expressing *Atg7*-RNAi (*UAS-Atg7-RNAi/+; nmnat*^{+/+}), and wild-type background expressing *Atg7*-RNAi (*UAS-Atg7-RNAi/+*) under normoxia and hypoxia (* *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001). Statistical comparison of *UAS-Atg7-RNAi/+* with other groups not shown for simplification purposes. (D–F) Quantification of total number of terminal dendritic branches (D), total dendrite length (E), and degeneration index (DI) (F) for ddaC neurons in wild type, *nmnat* heterozygous mutant (*nmnat*^{+/+}), *nmnat* heterozygous mutant background expressing *Atg12*-RNAi (*UAS-Atg12-RNAi/+; nmnat*^{+/+}), and wild-type background expressing *Atg12*-RNAi (*UAS-Atg12-RNAi/+*) under normoxia and hypoxia (* *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001). Statistical comparison of *UAS-Atg12-RNAi/+* with other groups not shown for simplification purposes.

Figure 5. Hypoxia-induced dendrite degeneration in *nmnat* mutants is independent of the apoptotic machinery

(A–B) Quantification of total number of terminal dendritic branches (A), total dendrite length (B), and degeneration index (DI) (C) for ddaC neurons in wild type, *nmnat* heterozygous mutant (*nmnat*^{+/+}), *nmnat* heterozygous mutant background expressing p35 (*UAS-p35*^{+/+}; *nmnat*^{+/+}), and wild-type background expressing p35 (*UAS-p35*^{+/+}) under normoxia and hypoxia (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistical comparison of *UAS-p35*^{+/+} with other groups not shown for simplification purposes.

Figure 6. Overexpression of Atg1 induces dendrite degeneration under hypoxia

(A,B) Dendrite morphology of class IV ddaC neuron in a wild-type (A) and *nmnat* heterozygous mutant (*nmnat*+/+) (B) third instar larva after exposure to 14 hrs of normoxia. (C) Overexpression of Atg1 enhances the dendrite regression phenotype of *nmnat* heterozygous mutants under normoxia. (D) Class IV neurons overexpressing Atg1 show reduced dendritic branching. (E,F) Dendrite morphology of ddaC neuron in a wild-type (E) and *nmnat* heterozygous mutant (F) third instar larva after exposure to 14 hrs of hypoxia. (G) Overexpression of Atg1 enhances dendritic regression, but not dendrite degeneration, phenotype of class IV neurons in *nmnat* heterozygous mutants under hypoxia. (H) Class IV neurons overexpressing Atg1 show reduced dendritic branching and extensive breaks along the arbors. (I-K) Quantification of total number of terminal dendritic branches (I), total dendrite length (J), and degeneration index (DI) (K) for ddaC neurons in wild type, *nmnat* heterozygous mutant (*nmnat*+/+), *nmnat* heterozygous mutant background overexpressing Atg1 (*UAS-Atg1*, *nmnat*+/+), and wild-type background overexpressing Atg1 (*UAS-Atg1*+/+) under normoxia and hypoxia (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistical comparison of *UAS-Atg1*+/+ shown only with wild-type controls (## $p < 0.01$, ### $p < 0.001$). Scale bar, 50 μm.

Figure 7. Overexpression of Nmnat fails to suppress dendritic phenotypes associated with Atg1 overexpression

(A,B) Dendrite morphology of class IV ddaC neuron in wild-type (A) or overexpressing Atg1 (B) under normoxia. (C) Overexpression of Nmnat fails to suppress the dendritic branching defect of class IV neurons overexpressing Atg1. (D,E) Dendrite morphology of class IV ddaC neuron in wild-type (D) or overexpressing Atg1 (E) under hypoxia. (F) Overexpression of Nmnat fails to suppress the dendritic branching and degeneration phenotype of class IV neurons overexpressing Atg1. (G–I) Quantification of total number of terminal dendritic branches (G), total dendrite length (H), and degeneration index (DI) (I) for ddaC neurons in wild type, wild-type background overexpressing Atg1 (*UAS-Atg1*), wild-type background overexpressing Nmnat and Atg1 (*UAS-Nmnat/+; UAS-Atg1/+*), and wild-type background overexpressing Nmnat (*UAS-Nmnat/+*) under normoxia and hypoxia (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistical comparison of *UAS-Nmnat/+* with other groups not shown for simplification purposes. Scale bar, 50 μm.

Figure 8. Overexpression of Catalase suppresses the dendrite fragmentation phenotype of hypoxia-exposed *nmnat* mutants
 (A,B) Dendrite morphology of class IV ddaC neuron in a wild-type (A) and *nmnat* heterozygous mutant (*nmnat*+/+) (B) third instar larva after exposure to 14 hrs of normoxia. (C) Overexpression of Catalase does not suppress the dendrite regression phenotype of class IV neurons in *nmnat* heterozygous mutants under normoxia. (D) Dendrite morphology of class IV ddaC neuron overexpressing Catalase under normoxia. (E,F) Dendrite morphology of ddaC neuron in a wild-type (E) and *nmnat* heterozygous mutant (F) third instar larva after exposure to 14 hrs of hypoxia. (G) Overexpression of Catalase suppresses the dendrite fragmentation phenotype of class IV neurons in *nmnat* heterozygous mutants under hypoxia. (H) Dendrite morphology of class IV ddaC neuron overexpressing Catalase under hypoxia. (I–K) Quantification of total number of terminal dendritic branches (I), total dendrite length (J), and degeneration index (DI) (K) for ddaC neurons in wild type, *nmnat* heterozygous mutant (*nmnat*+/+), *nmnat* heterozygous mutant background expressing Catalase (*UAS-Cat*+/+; *nmnat*+/+), and wild-type background expressing Catalase (*UAS-Cat*+/+) under normoxia and hypoxia (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Statistical comparison of *UAS-Cat*+/+ shown only with wild-type controls (# $p < 0.05$, ## $p < 0.01$). Scale bar, 50 μ m.