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*Conflicts of interest*

**The author discloses the following: Dr Pockros has received research grants from Human Genome Sciences, Genetech, BMS and Roche and consulting and/or speaking honorariums from Genetech, Roche, BMS, and Three Rivers Pharmaceuticals.**

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**0016-5085/$36.00**
**doi:10.1053/j.gastro.2010.08.015**

# Pathogenesis of Achalasia: Lessons From Mutant Mice

**See "Loss of lsc/p115-protein leads to neuronal hypoplasia in the esophagus and an achalasia-like phenotype in mice," by Zizer E, Beilke S, Bäuerle T, et al, on page 1344.**

The pathophysiology of esophageal achalasia is not well understood, although the primary defect in this disease is known to be impairment in nitrergic inhibitory neuromuscular transmission.[1] Findings in a variety of mutant mice are advancing our understanding of the molecular pathophysiology of achalasia. However, one of the major problems in this area of research has been the difficulty in recognizing early achalasia and inadequate characterization of the dilated esophagus as achalasia. In this issue of the GASTROENTEROLOGY, Zizer et al[2] report that deficiency of a guanine nucleotide exchange factor (GEF), Lsc, leads to achalasia (Lsc is murine analog of human p115 protein). Lsc causes activation of RhoA, which is a Ras GTPase. These studies reveal a key role of GEF-RhoA signaling in esophageal neural function.

Young Lsc-deficient mice have normal-appearing esophagi, but as they grow older, they develop progressive esophageal dilation. On histopathology, these mutant mice show decreases in muscle mass. Because Rho-GEF signaling is thought to be responsible for muscle tone and contraction, esophageal dilation could be assumed to be the result of esophageal smooth muscle weakness.[3] Commendably, the authors performed esophageal manometry and found that these mutants had loss of peristalsis and hypertension with impaired relaxation of the lower esophageal sphincter (LES), establishing that esophageal dilation in these mutant mice was due to achalasia associated with disruption of nitrergic inhibitory neurotransmission.[2]

Studies in mutant mice lacking neuronal nitric oxide synthase (nNOS)$\alpha$ (Nos1-null) initially established that nitric oxide (NO/NO$^{\cdot}$) was the main inhibitory neurotransmitter at the esophageal smooth muscle neuromuscular junctions and its loss led to achalasia (see Sivarao et al[4]). Nitrergic neurotransmission may also be disrupted at various levels in the cascade, including presynaptic, nitrergic neuron, junctional, and postjunctional levels

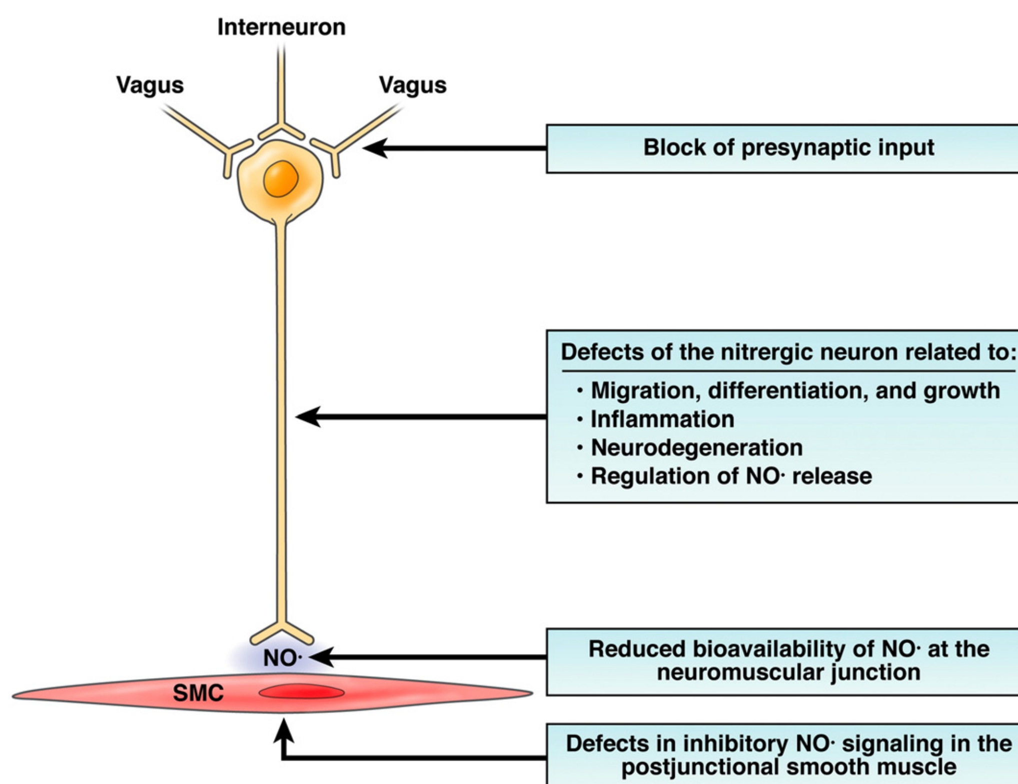


**Figure 1.** Sites at which nitrergic neurotransmission may be disrupted.

(Figure 1). Known genetic defects associated with achalasia in mice and humans are summarized in Table 1.

The pathophysiology of achalasia in Lsc deficiency is not clear. It could be due to disruption of the nitrergic neurons caused by deficient neural migration, differentiation, growth, neurodegeneration, inflammatory damage, or defective regulation of the enzyme nNOS$\alpha$. Enteric plexuses are derived from colonization of the gut from enteric neural crest–derived cells (ENCC). Lsc-null mice were found to have markedly reduced nitrergic and cholinergic myenteric neurons and glial cells as well as reduced expression of nerve growth factor (NGF). Therefore, it is possible that NGF deficiency in Lsc-null mice was responsible for the loss of enteric motor neurons. However, NGF is known to be particularly involved in sensory and sympathetic neurons.[5] Deficiency of NGF is not reported to be associated with gastrointestinal motor abnormalities. Loss of glial cells is also associated with loss of glial cell line–derived neurotrophic factor (GDNF), which may be responsible for impaired Ret-mediated migration of the ENCC to the esophagus. However, absence of GDNF or its receptors, GFR$\alpha$1 and Ret, leads to absence of enteric neurons beyond the stomach. The migration of ENCC into the esophagus occurs via a Ret-independent mechanism involving achaete-scute homolog 1, or achaete-scute complex homolog-like 1 (ASH-1; also known as mASH1). Absence of ASH-1 results in loss of myenteric ganglia in the esophagus. Ascl1-null mice die within 1 day of birth and have no milk in the stomach suggestive of achalasia.[6] The phenotype of Lsc deficiency resembles milder version of ASH-1 deficiency rather than GDNF deficiency. Moreover, gain of GDNF function in Spry2 (sprouty2)–null mice has been reported to cause esophageal dilation and achalasia with intestinal involvement associated with intestinal neuronal dysplasia. However, the diagnosis of achalasia and its pathogenesis in these mutant mice remains unresolved.[7]

Suppression of Lsc (GEF)-RhoA signaling may have direct effect on the esophageal myenteric plexus neurons. The interaction of fibronectin with $\alpha v\beta 3$ and $\alpha 5\beta 1$ integrins and heparin syndecan is thought to activate GEF-Rho signaling to affect focal adhesion molecules and polymerization of actin stress fibers that is necessary for cell migration.[8] However, the localization of the defect to the esophageal neurons is intriguing, but may be related to the participation of the GEF species other than Lsc in other tissues and organs. In any case, this study indicates the unique importance of Lsc in the development of myenteric plexus in the esophagus. However, this explanation is limited by the weak expression of Lsc in the neural tissue in the esophagus. Although weak expression of Lsc does not exclude its potentially important function, further studies are needed to address this issue. Further studies are also needed to understand why the Lsc-null mutant mice have hypertensive LES. LES hypertension in achalasia is usually ascribed to unopposed

**Table 1.** Genetic Defects Associated With Achalasia

| Site of defect/pathophysiology gene[a] | Function of gene product (Change) | Neuropathology of myenteric neurons | Achalasia/loss of nitrergic inhibition | Abnormality beyond esophagus | Human counterpart |
|---|---|---|---|---|---|
| Nitrergic neurons | | | | | |
| Commitment, migration, differentiation and growth | | | | | |
| Ascl1/Mash1 | TF (D) | Absent | + (?) | – | ? |
| Arhgef1/Lsc | GEF (D) | Reduced | + (M) | – | ? |
| Spry2 | Inh. RTK (D) | IND | ? (Dil) | + | ? |
| Nf1 | Inh. Ras sig (D) | GNM | + | + | NF-1 |
| Ret | RTK (I) | GNM | + | + | MEN B |
| Inflammatory loss of neurons | | | | | |
| Rassf1 | Cycle arrest (D) | Inflamm. & reduced | + | – | ? |
| Aaas | NPC (D) | | + | – | AAA-S |
| Neurodegeneration | | | | | |
| Atxn7 | CAG repeats (I) | Nuc. Incl. | + | + | ADCA |
| Snca | Inh. NTR (I) | Lewy body | + | | PD-LB |
| nNOSα regulation | | | | | |
| Nos1 | Source of NO (D) | Normal | + (M) | + | ? |
| Dlg4/PSD95 | NTR (D) | Normal (?) | + | ? | ? |
| Neuromuscular junction | | | | | |
| Disorganized junction | | | | | |
| Col19a1 | ECM, NTR (D) | Normal (?) | + (M) | ? | ? |
| Postjunctional | | | | | |
| Loss of NO- signaling in the smooth muscles | | | | | |
| Gucy1b3 | NO Receptor (D) | Normal | + (?) | + | ? |
| Prkg1/CGK1 | cGMP target (D) | Normal | ? | + | ? |
| Mrvi/Irag | Inh. IP3 (D) | Normal | ? | + | ? |

+(?), Achalasia is strongly suspected because the mutant infants have no milk in the stomach and die shortly after birth or characteristic barium swallow; ?(dil), dilation, not yet proven to be due to achalasia; +(M), proven manometrically; AAA, syndrome (Allgrove syndrome)—achalasia, alacrima, addisonian; ADCA, autosomal-dominant cerebellar ataxia (SCA-7: spinocerebellar ataxia type 7); Ascl, achaete-scute complex homolog 1; Arhgef1, Rho guanine nucleotide exchange factor 1; CAG, a trinucleotide; Col19a1, collagen, type XIX, alpha 1; (D), decrease in function; DLG4, disks large homolog4; ECM, extracellular matrix; GNM, ganglioneuromatosis; GUCY, guanylyl cyclase (I)—increase in function; IND, intestinal neuronal dysplasia; Inh, inhibition; LB, Lewy bodies—intracytoplasmic aggregates of synuclein and other proteins; Lsc, lethal of scute; Mash1, mammalian achaete-scute homolog 1; Mrvi1, murine retrovirus integration site 1; MEN B, multiple endocrine neoplasia; NF1, neurofibromin 1; NPC, nuclear pore complex; NTR, neurotransmitter release; PD LB, Parkinson's disease associated with Lewy bodies; Prkg1, protein kinase, cGMP-dependent, type I; PSD95, postsynaptic density protein 95; RTK, receptor tyrosine kinase SNCA—Synuclein, alpha; Spry2, Sprouty2; TF, transcription factor.
[a]Official symbol for the murine gene.

activity of the cholinergic neurons in the absence of the inhibitory neurons. However, this study suggests that LES hypertension in achalasia may be due to factors other than unopposed cholinergic activity.

Neurodegeneration of the myenteric plexus with nuclear inclusions in achalasia occurs in autosomal-dominant cerebellar (spinocerebellar) ataxia type-7 (ADCA or SCA-7) which is due to expanded glutamine repeats in the allele, Atxn7. The transgenic mice, *PrP-SCA,* have a prion promoter expression construct with inserted Ataxin-7 complimentary DNA with a pathogenic 92 glutamine (Q) long expansion. These mice exhibit features of SCA7. Although esophageal motor function has not been investigated in this model, these mutant mice show loss of nerve fibers in the myenteric plexus and neurodegeneration of nitrergic neurons in the intestines. Cholinergic neurons are intact, but show nuclear inclusions typical of this disease. The reason for the selective loss of nitrergic neurons is currently not known.[9] Eosinophilic cytoplasmic Lewy bodies and neural degeneration occur in some cases of Parkinson disease and in achalasia. The Lewy bodies are aggregates of synuclein with other proteins. Lewy body achalasia in mice with synuclein overexpression might be expected.[10]

Inflammatory damage to the nitrergic neurons is a common cause of achalasia. Viral infections have long been suspected as a cause of achalasia, but direct evidence for this has been lacking. However, a recent unexpected discovery of the presence of RNA of a bornavirus in the DNA of the parrots with proventricular dilation disease has enhanced the suspicion about the role of viruses in achalasia. This observation has sparked a major effort in identifying viral RNA and DNA in myenteric plexus of achalasia patients.[11] Viral infections seem to be noncytopathogenic, but viral antigens are displayed on the surface of the neurons that elicit a host T-cell–mediated

response and subsequent neuronal death. This mechanism of neural damage is similar to that described for Chagas disease. Inflammatory damage to the myenteric plexus owing to mutations in genes that regulate inflammatory molecules may occur in the absence of an invading organism. For example, achalasia has been associated with inflammatory myenteric damage in Rassf1a-null mice. Rassf1 gene (Ras Association family member 1) is a tumor suppressor gene that is effector for a Ras oncoprotein that normally suppresses inflammatory cytokines and nuclear factor-$\kappa$B pathway.[12] Deficiency of tumor suppressor genes may link cases of achalasia and cancer. In other cases, mutations may predispose the myenteric neurons to damage by oxidants. An example of this phenomenon is achalasia in Allgrove (AAA) syndrome. This syndrome is due to mutations or deletion of the AAAS (achalasia, alacrima, Addisonian syndrome gene). It has been suggested that its product, aladin, a nuclear transport protein, acts to protect the neurons from oxidative damage and its deficiency makes the neuron vulnerable to oxidative damage.[13]

Loss of nitrergic neurotransmission may also occur because of the down regulation of nNOS$\alpha$ in the myenteric nerves. Down-regulation of nNOS$\alpha$ function may occur at several levels, including its translation, post-translational modifications, intracellular targeting to the varicosity membrane, and associations with other enzymes and ion channels that provide its allosteric regulation, so that its gaseous product, NO· can serve as a neurotransmitter.[14] For example, vasoactive intestinal peptide deficiency may act to inhibit NO· neurotransmission.[15] Achalasia owing to down-regulation of nNOS$\alpha$ may reveal no histologic changes in the myenteric plexus. Achalasia owing to junctional defects causing impaired nitrergic neurotransmission include cases where the neurally released NO· is inactivated before it has a chance to act on the smooth muscles. NO· is produced and released de novo on demand and is a very labile chemical with short life span. Its integrity is highly regulated in the junctional space by its physical and chemical environment and a number of enzymes. Collagen 19a1-null mice show extensive extracellular matrix disorganization that may be responsible for the loss of nitrergic neurotransmission owing to the inactivation of normally released nitric oxide.[16] Most recently, collagen 19 has also been shown to be involved in extracellular–intracellular interactions and formation of normal nerve terminals in certain neurons, suggesting that disturbance of these mechanisms may contribute to the pathogenesis of achalasia in collagen 19 deficiency.[17] It has been suggested that deficiency of intramural interstitial cells of Cajal (ICC-IM) may be associated with achalasia because ICC-IM has been proposed to transduce neural NO· signals to smooth muscle. However, the role of ICC-IM in nitrergic neurotransmission is open to question and the c-kit deficient WWv mutant mice, showing loss of ICC-IM, are not associated with achalasia.[18]

Postjunctional defects in nitrergic signaling in the smooth muscles are mediated by NO sensitive guanylyl cyclase (NO-GC), cGMP kinase 1 (GK1), and IP3-receptor–associated GK1 substrate (MRVI1 or IRAG). Mice lacking NO-GC, GK1, or IRAG exhibit suppressed nitrergic neurotransmission.[19–21] The abnormalities were most marked in the NO-GC–lacking mice that also showed esophageal dilation.[20,21]

In conclusion, the phenotype of early achalasia is easily overlooked and the finding of a dilated esophagus requires functional studies to establish a diagnosis of achalasia. This is particularly important because impaired nitrergic neurotransmission may not correlate with the size of the myenteric plexus. Moreover, because of the incomplete knowledge of gene products that affect nitrergic neurotransmission in the esophagus, the finding of achalasia in many of the mutant mice has emerged as a surprise. Therefore, a careful search for the phenotype of achalasia in various relevant mutant mice, including constitutive and inducible tissue-specific gene knockouts, as well as genome wide analysis of gene expression, and any viral nucleic acid in normal and established cases of achalasia will no doubt further enhance our understanding and treatment options for this important disease.

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*Conflicts of interest*
**The authors disclose no conflicts.**

*Funding*
**Supported by grant DK062867 (NIDDK).**
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**0016-5085/$36.00**
**doi:10.1053/j.gastro.2010.08.013**

# New Cell Culture Models of Hepatitis C Virus Entry, Replication, and Virus Production

**See "Production of infectious hepatitis C virus in primary cultures of human adult hepatocytes," by Podevin P, Carpentier A, Pène V, et al, on page 1355; and "Hepatitis C virus infection of neuroepithelioma cell lines, by Fletcher NF, Yang JP, Farquhar MJ, et al, on page 1365.**

As readers of Gastroenterology know all too well, hepatitis C virus (HCV) is a major cause of acute and chronic liver disease, often leading to cirrhosis, hepatocellular carcinoma, and liver failure.[1] In addition, chronic HCV infection is frequently associated with extrahepatic sequelae such as lymphoproliferative disorders and neurologic defects. HCV is an enveloped, positive-strand RNA virus that infects hepatocytes, and possibly other cells in the body. HCV has been historically difficult to study because of the lack of efficient culture systems and small animal models of infection. Recently developed tools have enabled the biology of this virus to be studied in cell cultures of a transformed hepatoma line. However, host–pathogen interactions between HCV and bona fide hepatocytes are still poorly understood, and we know even less about the interaction of HCV with other host cell types. Two articles in this issue of Gastroenterology promise to help fill these gaps, reporting new cell culture models of HCV infection in primary hepatocytes and in neuroepithelioma cell lines.

The interaction between HCV and hepatocytes has been difficult to study because hepatocytes rapidly lose their differentiated phenotype in cell culture.[2] Recent in situ studies have shown that the virus persists in approximately 7%–20% of hepatocytes in chronically infected patients[3] and that HCV transiently induces and then actively down-regulates innate antiviral responses in newly infected hepatocytes.[4]

In the absence of facile systems to experimentally infect authentic hepatocytes, much of what we know about HCV biology has come from studies in the cultured hepatoma cell line Huh-7 with HCV replicons,[5,6] retroviral pseudoparticles that display the HCV glycoproteins (HCVpp),[7,8] or an unusually efficient genotype 2a virus that produces infec-