Mol Neurobiol (2011) 43:41–66
DOI 10.1007/s12035-010-8159-8

NO Orchestrates the Loss of Synaptic Boutons from Adult "Sick" Motoneurons: Modeling a Molecular Mechanism

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Received: 20 October 2010 / Accepted: 2 December 2010 / Published online: 29 December 2010

Abstract Synapse elimination is the main factor responsible for the cognitive decline accompanying many of the neuropathological conditions affecting humans. Synaptic stripping of motoneurons is also a common hallmark of several motor pathologies. Therefore, knowledge of the molecular basis underlying this plastic process is of central interest for the development of new therapeutic tools. Recent advances from our group highlight the role of nitric oxide (NO) as a key molecule triggering synapse loss in two models of motor pathologies. De novo expression of the neuronal isoform of NO synthase (nNOS) in motoneurons commonly occurs in response to the physical injury of a motor nerve and in the course of amyotrophic lateral sclerosis. In both conditions, this event precedes synaptic withdrawal from motoneurons. Strikingly, nNOS-synthesized NO is "necessary" and "sufficient" to induce synaptic detachment from motoneurons. The mechanism involves a paracrine/retrograde action of NO on presynaptic structures, initiating a downstream signaling cascade that includes sequential activation of (1) soluble guanylyl cyclase, (2) cyclic guanosine monophosphate-dependent protein kinase, and (3) RhoA/Rho kinase (ROCK) signaling. Finally, ROCK activation promotes phosphorylation of regulatory myosin light chain, which leads to myosin activation and actomyosin contraction. This latter event presumably contributes to the contractile force to produce ending axon retraction. Several findings support that this mechanism may operate in the most prevalent neurodegenerative diseases.

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Keywords Nitric oxide synthase · Peripheral neuropathy · Amyotrophic lateral sclerosis · SOD1^{G93A} mice · Rho kinase · Protein kinase G · Soluble guanylyl cyclase · Myosin light chain · Neurodegenerative diseases

Introduction

Synaptic plasticity is an essential property of the adult brain which is thought to be the basis of cognitive processes such as learning and memory. Driven by experience, the synaptic array of neurons undergoes a balanced structural and functional remodeling which is involved in the achievement and/or improvement of a range of sensory motor tasks, from basic to very complex. Imbalance in this dynamic process may substantially contribute to the cognitive decline, behavioral impairment, and/or motor disturbances accompanying numerous neuropathological conditions. Cognitive alterations correlate better with synapse loss than with neuronal death in patients and/or animal models of Alzheimer's (AD; [1–3]), Parkinson's (PD; [4, 5]), Huntington's (HD; [6]) diseases, multiple sclerosis (MS; [7]), and HIV dementia [8] to quote the most prevalent examples. Somatic motoneurons also undergo synaptic stripping in the course of diverse motor pathologies, such as amyotrophic lateral sclerosis (ALS; [9–11]), lower motor neuron disease [11, 12], Werdnig–Hoffmann disease [11] and various types of peripheral neuropathies [11, 13–17]. Therefore, the ratio between elimination of existing synapses and the generation of new ones is clearly biased toward elimination, which emerges as a common hallmark of an extensive number of neuropathological events. However, net synapse loss does not necessarily involve neuropathological actions but can be neuroprotective against excitotoxic damage in some cases, as we will discuss later. In these pathological

conditions, synapse loss could represent the outcome of either a deficient production of synaptotrophic signals or the synthesis of synaptotoxins from targeted neurons and/or surrounding glia, or a combination of both phenomena. Knowledge of the molecular basis underlying this event is of fundamental interest for the understanding of pathology progression and development of new therapeutic tools.

Over the last decade, our group has investigated the possible role of the gas nitric oxide (NO) as a synaptotoxic molecule. Our initial hypothesis was supported by studies demonstrating that upregulation and/or de novo expression of NO synthase (NOS) is a widespread feature of many neuropathological states. For example, a dysregulated NOS expression has been detected in motoneurons and reactive astrocytes in ALS [18–20], in neurons and/or glial cells in AD [21–23] and PD [24], in the striatum of an HD model [25, 26], in MS [27–30], and in HIV dementia [28, 31, 32]. Upregulation of NOS in motoneurons, which are normally lacking in this enzyme, also occurs after a broad spectrum of acquired peripheral neuropathies (reviewed in [33]). Strikingly, NO participates in synaptic plastic phenomena such as long-term potentiation and depression [34] as well as in synaptogenesis and projection refinement during development [35, 36]. Furthermore, NO is a physiological mediator in sensory motor integration by regulating synaptic function [37–41]. Collectively, these previous findings motivated our initial working hypothesis: *NO could be a feasible synaptotoxin mediating synaptic loss from pathological neurons.*

Our group has focused its efforts on this issue using two well-characterized models of motoneuron pathologies: a traumatic lesion of a motor nerve and a mouse model of ALS. The aim of this manuscript is to review the recent advances on the role of NO in the process of synapse loss that occurs over the course of these two motor disturbances in adulthood. A feasible molecular mechanism by which some synapses, but not their neighbors, are NO-sensitive and become detached from motoneurons in pathologic conditions is also proposed. Finally, the possible ubiquity of this synaptotoxic action of NO in other neuropathological states is discussed.

Adult Motoneurons Undergo Synaptic Stripping in Pathological States

Acquired Peripheral Neuropathy: Motoneuron Axotomy

Physical injury to a nerve is the most frequently studied model of acquired peripheral neuropathy. Interruption of the bidirectional trophic communication between motoneuron and myocytes by axonal damage reduces the motoneuron response to the incoming synaptic drive, concomitantly with a marked decrease in the number and strength of synaptic inputs [13–15, 42–44]. Three types of traumatic injury inflicted to a peripheral nerve have been studied: crush, transection, and avulsion. Nerve crushing is a model of axonotmesis [45] in which a complete recovery is usually possible even in humans [46]. Crushing causes complete axotomy followed by target reinnervation and axonal maturation [44, 45]; unlike nerve transection or avulsion, it preserves the endoneural tube and the continuity of the basal lamina, providing neurotrophic support and a physical guide for the growing proximal axonal ends [33, 44]. As a result, a crushed motor nerve requires less time for muscle reinnervation [44]. In contrast, nerve transection disrupts axons and the endoneural tube. In this case, partial functional recovery only occurs when stumps of transected nerves are surgically joined to guide regenerating axons into the distal nerve stump [33].

Axotomized motoneurons usually undergo a massive loss of afferent inputs that correlates with reduced firing activity and attenuated synaptic responses. Rearrangement of synaptic contacts on axotomized motoneurons after several types of injuries inflicted upon different nerves and species has been widely studied by immunohistochemical and ultrastructural approaches [13–15, 17, 47–71]. Synaptic stripping from the motoneuron membrane surface is more pronounced at soma than at dendrites [59]. These changes generally occur within a week after injury and persist for a period that varies depending on lesion severity. Alterations in the firing properties and pre-synaptic structures are usually transient, recovering once muscle reinnervation is effective [13, 16, 58, 72].

Reversible Nerve Lesion

Motoneuron axotomy by nerve crushing offers an accessible and reliable lesion model to study the molecular mechanisms underlying synaptic dynamics (elimination and formation) at the CNS. In the adult rat, XIIth nerve crushing allows a regenerative response which leads to an almost full muscle reinnervation and functional recovery as soon as 1 month post-lesion [13, 14, 44]. At 2 months follow-up, a higher degree of functional and anatomical recovery is achieved after sciatic nerve crushing than after transection and surgical repair [73]. Crushing of either XIIth [13, 14] or sciatic [51] nerves in the rat induced a decrease in the density of the synaptic marker synaptophysin (syn) in the neuropil of motor nuclei. This paralleled with a reduction in the frequency of syn-immunoreactive (syn-ir) puncta apposed to hypoglossal motoneurons (HMNs) at 7 but not at 4 days after injury [14, 15]. Nerve crushing is therefore

a successful model of peripheral neuropathy with the objective of inducing central synaptic alterations at short-term post-injury.

Synaptic disturbances could be the outcome of an impairment of the pre-synaptic machinery rather than the actual withdrawal of boutons. Ultrastructural analysis conclusively showed synaptic detachment from the perikarya of several injured motoneuron pools 1 week or later after nerve injury in rodents [47–49, 51] and in the cat [50]. Accordingly, 4 days after intercostal 7th and 9th nerve crushing, no reduction occurred in the frequency of synaptic boutons on motoneurons, although a widening of the synaptic cleft between synaptic terminals and the soma was observed [50]. These findings suggest that after nerve crushing, the first signs of terminal detachment occur on the 4th day post-injury, while synaptic withdrawal is evident at 1 week and reached maximal values over the second week post-lesion [47–51]. Therefore, loss of syn-ir puncta correlated with actual synapse detachment, validating syn as a feasible marker to study synaptic rearrangements on axotomized motoneurons.

At the ultrastructural level, the synaptic boutons on motoneuron perikarya can be classified into four main morphological types as described by Conradi and colleagues [74]: S-type synapses with spherical synaptic vesicles, F/P-type synapses with flattened/pleomorphic vesicles, large C-type and M-type synapses with spherical vesicles, and a subsynaptic cistern in C-type (Fig. 1a). C- and M-type boutons are much less frequent on motoneurons than S-type or F/P-type terminals; therefore, they are excluded from analysis in most studies. Hence, we will focus mainly on S-type and F/P-type synapse alterations induced by nerve injury. Given the exclusive immunoreactivities of S terminals to glutamate and F/P boutons to glycine and/or GABA [75], it has become widely accepted that S-type and F/P-type boutons are excitatory and inhibitory, respectively [76–78]. A finding of interest is that crushing of cranial [47] or spinal [50] nerves caused the selective elimination of excitatory S-type, but not F-type, boutons. A selective loss of excitatory puncta apposed to axotomized HMNs was confirmed by immunolabeling for the vesicular glutamate (VGLUT2, excitatory puncta) and GABA (VGAT; inhibitory puncta) transporters [15] (Fig. 1b). In this case, it is possible that a preferential withdrawal of excitatory boutons from the insulted motoneurons may have a neuroprotective role, partially buffering the impact of excitotoxic agents. These

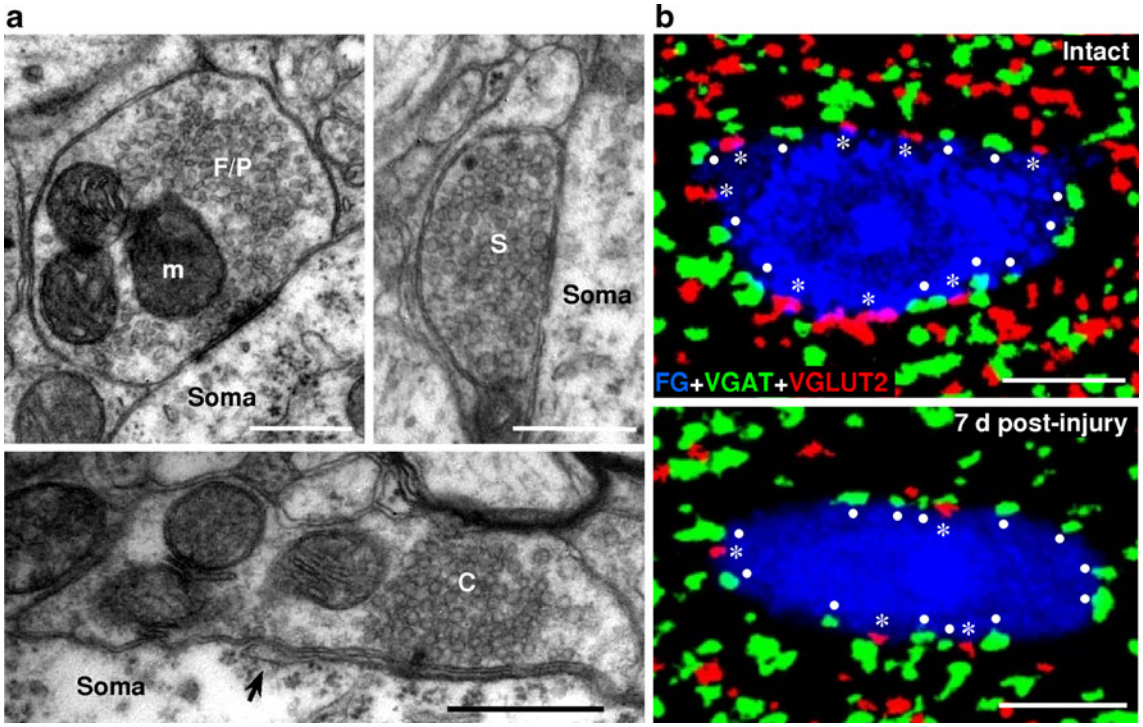


Fig. 1 Major types of synaptic terminals apposed to hypoglossal motoneurons. **a** Illustrative examples of synaptic boutons attached to the plasma membrane of a HMN with flattened/pleomorphic (F/P-type; inhibitory) or spherical (S-type; excitatory) synaptic vesicles. A large C-type bouton (*bottom*) with spherical vesicles and a subsynaptic cistern (*arrow*) is also shown. **b** VGAT-immunoreactive (*circle*; inhibitory) and VGLUT2-immunoreactive (*asterisk*; excitatory) puncta around FluoroGold (*FG*)-backlabeled HMNs obtained from intact animals and 7 days after XIIth nerve crushing treated daily with the steroisomer-inactive D-NAME. Note the reduction in VGLUT2-positive puncta around the insulted motoneuron. *Scale bars*: **a** 0.5 μm; **b** 10 μm. Reprinted from Sunico et al. [15]. Copyright 2010 by the Society for Neuroscience

findings suggest a higher sensitivity of excitatory synapses to synaptotoxins and/or synaptotrophic deficits than the inhibitory ones. A differential molecular array of both types of synapse could make S-type more vulnerable for stripping than F/P-type boutons.

Severe Nerve Lesions

Nerve transection, avulsion, or intramedullar disruption of motor axons represent mostly severe and irreversible nerve lesions which induce an extensive reduction in the synaptic coverage of insulted motoneurons [17, 48, 50–71, 79, 80]. Comparatively, XIIth or intercostal nerve transection evoked a more pronounced synaptic stripping of motoneurons than the crushing of the same nerves in cats [50, 80]. These data suggest a relationship between the magnitude of bouton loss and the severity of the trauma, at least in this species. However, apparently contradictory results have been reported on this issue, which could simply reflect variations in the response of different motoneuron pools, species, or even the species strain used as models. In the cat, a decrease (ranging from −50% to −75%) in the density of axosomatic synaptic endings was observed after transection of cranial [52] or spinal [50, 57–59] nerves. In many of these studies, excitatory and inhibitory synapses were differentially affected. Thus, while axotomy of abducens or intercostal motoneurons mainly induced loss of F/P-type boutons [50, 52, 57], the lesion of lumbosacral motoneurons affected both S-type and F/P-type boutons without a clear preferential affectation [59]. Note that crushing of intercostal nerves, performed by the same researchers in the cat, induced a preferential loss of S-type boutons [50]. This dismisses the possibility that variations in the experimental protocols and/or manipulation performed by different researchers could explain the disparity in the results.

In rodents, transection or avulsion of either cranial or spinal nerves [17, 48, 51, 53–56, 60–69, 79] is also accompanied by a reduction in the synaptic coverage on insulted motoneurons. In general, nerve transection in rats [17, 53, 55, 60, 79] seems to induce a greater reduction of motoneuron synaptic coverage (ranging −50% to −80%) than in mice (ranging −40% to −50%) [62–65]. This could be a sign of species differences, or factors related to the longer distance between the soma and site of axotomy, between studies. Species differences are highlighted by the fact that, at 7 days after Xth nerve sectioning, syn-ir puncta apposed on the surface of injured dorsal motoneurons of the vagus was reduced ~70% in Wistar rats but remained similar to controls in C57BL/6 mice [55]. This is corroborated by observations after performance of reversible lesions: XIIth nerve crushing in B6SJL mice failed to modify VGLUT2- and VGAT-ir puncta array apposed to HMNs 7 days after injury (Sunico and Moreno-López, unpublished observations). Finally, sciatic nerve transection at the obturator tendon level evoked different degrees of syn-ir puncta loss apposed to motoneurons depending on the mouse strain, being more noticeable in A/J and Balb/cJ (−50% to −60%) mice than in C57BL/6J mice [62, 63]. As happened after XIIth nerve crushing in rats, transection of this same nerve induced a preferential withdrawal of S-type boutons without apparent affectation of F/P-type terminals [17]. However, sciatic nerve sectioning, ventral roots avulsion, or intramedullar axotomy were followed by the elimination of both excitatory and inhibitory inputs in a similar proportion [59, 62–64, 67] or slightly biased to a higher S-type synapse loss [63, 66, 70, 71].

In summary, a well-known hallmark of the retrograde degeneration of axotomized motoneurons is the extensive detachment of synapses from the perikarya. Therefore, traumatic lesion of a motor nerve is a model of induced motoneuron pathology which can be appropriately used to study the molecular mechanisms underpinning synaptic remodeling in the CNS in adulthood. A cautious selection of the type of lesion, the motor system, and animal model could allow us to investigate differential synaptotrophin dependence of excitatory and inhibitory synapses as well as to isolate/identify bouton-specific synaptotoxins.

Amyotrophic Lateral Sclerosis

ALS, also known as Lou Gehrig's disease, is an adult-onset neurodegenerative disease belonging to a group of fatal human disorders characterized by the progressive degeneration and death of motor neurons in the spinal cord, brainstem, and motor cortex. ALS is the most frequent of the diseases that affect motoneurons and their connections to skeletal muscles, leading to weakness and finally paralysis. Patients usually die from respiratory failure within 3–5 years from the onset of symptoms, although ~10% of ALS patients live for 10 years or more. In the majority of ALS cases (90–95%), the disease occurs randomly with no apparent genetic link and no clearly associated risk factors (sporadic ALS; sALS). About 5–10% of the remaining ALS cases are inherited in a dominant form (familial ALS; fALS). Among them, ~20% have been related to missense mutations in the gene that encodes the enzyme superoxide dismutase 1 (SOD1) [81]. In several transgenic mouse models, overexpression of the mutated human SOD1 gene develops phenotypic and pathological signs similar to ALS in humans (reviewed in [82–84]). Research on transgenic mouse models of mutated SOD1 is contributing clues about the feasible causes of motoneuron death in ALS. The $SOD1^{G93A}$ transgenic mouse is the most widely used model of fALS. This strain overexpresses the human mutant cytosolic Cu/Zn SOD1 possessing a glycine to alanine substitution at the 93rd position (G93A) [85]. These mice, expressing ~20

copies of the transgene, develop an ALS-like disease with initial symptoms that include fine tremor or shaking and weakness in the hind limbs at 90 days postnatal (P90), leading to severe hindlimb paralysis at ~120 days of age [85–94].

The main challenge facing present research in ALS is to discover pivotal molecular mechanisms involved in motoneuron death. Glutamatergic-mediated excitotoxicity is one of the most studied mechanisms contributing to motoneuron degeneration in ALS. Excitotoxicity arises from excessive Ca^{2+} influx into motoneurons, which have a low Ca^{2+}-buffering capacity (reviewed in [83, 95–98]). Possible involvement of excitotoxicity in ALS gains credence from the clinical effectiveness of riluzole, an anti-glutamatergic agent with anti-excitotoxic properties [99]. Glutamatergic synaptic inputs evoke Ca^{2+} entry through NMDA and GluR2 subunit-deficient AMPA receptors [100]. The selective loss of the astroglial glutamate transporter EAAT2/GLT1, observed in both murine models [101, 102] and ALS patients [103, 104], has been proposed as a mechanism giving rise to deficient clearance and subsequent accumulation of glutamate [96]. Another, not mutually exclusive, mechanism documented in spinal cord motoneurons of $SOD1^{G93A}$ mice [105] is an increase in the number of Ca^{2+}-permeable AMPA receptors. Additionally, the imbalance between inhibitory and excitatory synaptic drive to motoneurons could be a key event for motoneuron degeneration in ALS [94, 106–109]. Changes in the synaptic array that result in an excitatory signaling gain and/or an inhibitory loss on motoneurons will increase Ca^{2+} influx and depolarization, favoring excitotoxicity. This could be a critical event in ALS progression, signaling molecular events underlying synaptic alterations as feasible targets for the development of neuroprotective strategies. Surprisingly, reorganization of pre-synaptic inputs onto motoneurons in ALS has not been hitherto the subject of much consideration.

ALS Patients

Immunohistochemical investigation of pre-synaptic protein expression has revealed that synaptic terminal degeneration is a pathological hallmark in human ALS progression. A decrease in the syn-ir density and puncta in the anterior horn neuropil at cervical [110, 111], thoracic [110], and lumbar [110–112] levels and in the hypoglossal nucleus (HN) [110] has been observed in ALS patients. This reduction correlated with the degree of degeneration of anterior horn cells [113, 114]. In some cases, there was a complete loss of syn-ir puncta in the anterior horn neuropil at cervical and lumbar levels [111]. This correlates with a decrease in syn-ir dots, up to the extreme of total absence, on proximal dendrites, and on the surface of chromatolytic motoneurons [110, 111, 113, 114]. Studies examining the pre-synaptic marker synapsin 1 contributed similar results [112]. Synapse elimination occurred even in patients who had no upper motor neuron and corticospinal tract impairment [12, 114]. This study suggests that synapse loss is not the consequence of motor cortical neuronal death and also supports a dying-back progression of disease from inferior motoneurons to motor cortical neurons. Strikingly, synaptic puncta on the surface of contemporary normal-appearing motoneurons were either preserved [113–115] or reduced [110, 111] in ALS cases. Ultrastructural analysis of axosomatic synapses revealed that synaptic abnormalities in anterior horn chromatolytic neurons were much more severe than in morphologically normal cells in patients with ALS [116]. This was accompanied by a reduction in the number of synapses on axon hillocks and perikarya of normal-appearing anterior horn neurons, which was more pronounced in neurons with central chromatolysis [9, 117]. These data point to the hypothesis that synaptic loss is a step preceding motoneuron death. Preventing this pathological event might slow down disease progression, signaling molecular partners involved in this degenerative process as feasible therapeutic targets in ALS.

Unfortunately little is known about how this synaptic reorganization could influence motoneuron physiology in the course of disease. Clarifying the nature of lost boutons will contribute information about whether synaptic changes in ALS may represent a form of homeostatic compensatory mechanism, protecting motoneurons from excitotoxic death, or, on the contrary, contribute to disease progression. In this way, expression analysis of the vesicular acetylcholine transporter on surviving neurons showed a loss of cholinergic inputs on lower motoneurons as an early event in cell death in sALS [115]. Functional studies, using transcranial magnetic stimulation of the motor cortex, showed an increase in the excitatory postsynaptic potentials recorded from the contralateral extensor digitorum communis muscle, which authors attribute to a marked loss of inhibition on corticomotoneurons in patients with ALS [118]. PET scanner studies [119] and in situ hybridization [120] revealed a decrease in both the density of the $GABA_A$ receptor and the expression of $GABA_A$ receptor subunit mRNA, respectively, in the motor cortex of ALS patients. Whether motoneurons actually undergo loss of the inhibitory coverage, facilitating excitotoxic events in ALS patients, is an unsolved puzzle that merits attention.

The $SOD1^{G93A}$ Mouse Model of ALS

The $SOD1^{G93A}$ mouse model of fALS has contributed specific information about synaptic alterations in the progression of this disease. In pre-symptomatic transgenic mice (P56–P70), the synaptic markers synaptotagmin 1 and

syn were almost intact in the neuropil and around motoneurons [94, 109, 121, 122]. A progressive decrease in the expression of these synaptic markers in the neuropil and, especially, around motoneurons was, however, observed in early-symptomatic $SOD1^{G93A}$ mice (P84–P91) at brainstem [94] and spinal cord [109, 121, 122] levels. Synaptic alterations were accentuated in older animals [109, 121, 122]. No motoneuron loss was observed until P100 [123]. In this sense, HMNs seem pathologically less affected than lumbar motoneurons taken from the same animal. However, the reduction in the linear density of syn-ir puncta around HMNs [94] was of a higher magnitude (−33% vs. −14%) than that measured in lumbar motoneurons [122] from 3-month-old $SOD1^{G93A}$ mice. Bouton loss on brainstem and spinal motoneurons was confirmed by ultrastructural analysis [94, 124]. Altogether, these findings strongly suggest that reduction in the number and density of boutons can occur before motoneuron loss in this genetic model of fALS.

Analysis of the excitatory and inhibitory nature of the lost inputs revealed a reduction in VGAT-ir puncta apposed to HMNs from early-symptomatic transgenic mice (P90) [94] and a gain in VGLUT2-ir [94] and VGLUT1-ir (unpublished results) puncta. Immunohistochemistry against the glycine transporter-2 (GlyT2) in the genetic model of ALS showed that glycinergic-bouton densities apposed to lumbar motoneurons were significantly lower at the same age. However, the density of synaptic terminals expressing the GABA-synthesizing enzyme glutamic acid decarboxylase 67 contacting these motoneurons remained unchanged [125]. Therefore, in the HN, the reduction of VGAT expression [94], which labels both glycinergic and GABAergic boutons [126], could be a consequence of the exclusive loss of glycinergic inputs. Reduction in the density of the vesicular inhibitory amino acid transporter was also observed at the lumbar level in older animals (P110–130) [109], supporting previous data on a selective loss of inhibitory inputs. In contrast, at this age, VGLUT2-positive puncta in apposition to motoneurons was found generally reduced in $SOD1^{G93A}$ mice [109]. Whether excitatory gain occurring at P90 in HMNs and the loss undergone by lumbar motoneurons at P110–130 is a consequence of age or the different embryological origins of motoneurons remains to be elucidated. In any case, this was concomitant with a decline in cholinergic pre-synaptic terminals around hypoglossal [87] and lumbar [109, 125] motoneurons at nearly the end point of disease (>P110). These observations were confirmed by ultrastructural analysis that reported a significant increase (52%) in the frequency of S-type and a reduction (−49%) in F/P-type boutons on transgenic HMNs at P90 [94] and a reduction of both types of boutons on older (P126) lumbar motoneurons [124].

These findings support the conclusion that the net loss of synapses on ALS motoneurons results in a lower ratio of inhibitory/excitatory inputs, which could be pivotal in the onset of the disease. Motoneuron disinhibition together with overexcitation could explain initial hyperreflexia and tremor in the transgenic mice [84, 92, 93] and exacerbation of spinal reflexes in ALS patients [84]. Based on these data, we can infer that synaptic rearrangement onto motoneurons in early-symptomatic $SOD1^{G93A}$ mice could act as a mechanism favoring disease progression rather than as a protective homeostatic response against excitotoxicity. This was supported by a recent paper describing genetic reduction of VGLUT2 protein levels as rescuing motoneurons at brainstem and lumbar spinal cord in transgenic mice [127].

In summary, the loss of motoneuron synaptic coverage represents a common phenomenon occurring after motor axon injury and also over the course of ALS progression both in humans and in the $SOD1^{G93A}$ mouse model. It would be very interesting to study whether the molecular substrates of this common event are similar for both of these motoneuron pathologies.

NO Sources in Motor Pathologies

NO is a short-life, bioactive free radical which, as a gaseous molecule, freely crosses plasma membrane, then supports both autocrine and paracrine actions. NO and L-citrulline are the products of hydroxylation of a guanidine nitrogen of L-arginine and subsequent oxidization of the N^{ω}-hydroxy-L-arginine intermediate by NOS. NOS is a heme-containing enzyme that utilizes tetrahydrobiopterin (H_4B) as a redox cofactor; their electron transfer reactions are regulated by a Ca^{2+}-binding protein (calmodulin). NOS also needs NADPH as an electron donor and requires molecular oxygen to carry out the reaction (reviewed in [128]). In their active form, the known NOS enzymes form dimers in which each NOS monomer is associated with a calmodulin molecule. Three major isoforms of NOS have been identified, coded by different genes and differing in localization, regulation, catalytic properties, and inhibitor sensitivity (reviewed in [129]). The nNOS isoform was the first to be purified and cloned (also known as NOS-I) and is predominantly found in the neuronal tissue. Three splice variants have been identified for nNOS, named α, β, and γ [130]. nNOSα corresponds with that initially described which has a wide distribution in the mammalian brain. Through their specialized postsynaptic density-95/disks large/zona occludens-1 domains, nNOSα, but not nNOSβ and nNOSγ, can physically associate with postsynaptic density protein-95 (PSD-95). PSD-95 in turn binds to motifs in the C-terminus of NMDA receptor NR2 subunits

[131]. These molecular interactions may provide the mechanistic basis for a functional coupling between Ca^{2+} influx through NMDA receptors and NO production [132]. The iNOS isoform (also named as NOS-II) is inducible in a wide range of cells and tissues, including activated macrophages and in pathological states, astroglia, and microglia in the CNS. This isotype is Ca^{2+}-independent and always catalytically active when expressed. However, as described below, iNOS is not the only isoform induced in pathological conditions. Finally, eNOS (or NOS-III) is the primary isoform found in vascular endothelial cells.

Most effects of NO are mediated by stimulation of a transition metal-containing protein, soluble guanylyl cyclase (sGC) leading to increases in intracellular cyclic guanosine monophosphate (cGMP) levels in target structures. Subsequent activation of protein kinase G (PKG) is probably the most widespread action of cGMP leading to the regulation of phosphorylation levels in effector proteins. Another NO signal transduction pathway entails the formation of nitrosothiol adducts in specific cysteine residues (or S-nitrosylation), mediating a regulatory post-translational mechanism of protein function. However, S-nitrosylation reactions in vivo are limited and occur mainly in pathological states under a changed redox environment. Transduction mechanisms for NO have been recently reviewed [133]. NO generated by nNOS and eNOS at low concentrations, in a Ca^{2+}-dependent manner, acts as a molecular messenger in multiple signal transduction pathways, whereas high concentrations of NO produced by iNOS result in cytotoxicity [16]. Overexpression of eNOS or nNOS in pathological conditions could yield toxic NO levels. Physiological NO concentration that played a role in neurotransmission and vasodilation ranges from ≤100 pM to ~5 nM [134]. Pathological NO levels can reach concentrations up to 4 μM after cerebral ischemia [135]; NO at pathological levels has been implicated in the pathogenesis of stroke, demyelination, and other neurodegenerative diseases.

In the pathological motoneuron, potential synaptotoxins should be diffusible molecules forming links between presynaptic and postsynaptic structures, probably, but not exclusively, synthesized in the insulted motoneuron, to signal the detachment of its afferent synaptic terminals and preventing the formation of new synapses. Several properties make NO a suitable candidate for the role of a mediator in synaptic remodeling at the CNS: (a) NO can freely cross cell membranes, thus acting as a three-dimensional (or volume) messenger; (b) neuronal NO modulates synaptic activity by regulating neurotransmitter release, an effect that requires activation of sGC in the target synapses [41]; (c) NO participates in synaptic plastic phenomena such as long-term potentiation and depression [34] as well as in synaptogenesis and projection refinement during development [35, 36]; and (d) in ALS [18–20] and after a broad spectrum of insults to nerves, motoneurons upregulate n/iNOS [33].

NOS Expression Timing and NO Sources in Acquired Peripheral Neuropathy

NOS expression is upregulated at three critical locations (injured motoneuron, insulted nerve, and denervated muscle) after injury of a peripheral motor nerve. The timing of expression and location of NOS isoforms after peripheral nerve injury has been the subject of a recent review [33] and will be briefly described here to support a feasible relationship between the time course and degree of nNOS upregulation and the magnitude of central synapse loss (modeled in Fig. 2a).

The three major NOS isoforms are concomitantly upregulated after traumatic lesion of a motor nerve. At the peripheral level, nNOS accumulates in the growing motor axons, eNOS is overexpressed in *vasa nervorum* in the distal stump and around the injury site, and iNOS is also expressed by the recruited macrophages and phagocytic Schwann cells [33]. Upregulation of nNOS, but not iNOS [14], takes place at the soma of brainstem and spinal motoneurons, which are normally lacking in this enzyme, after peripheral compression, crush, transection, and avulsion injuries in various species [14, 136–148]. However, eNOS induction has not been reported in injured motoneurons. On the contrary, after nerve transection, eNOS immunoreactivity was only detected in endothelial cells and even induced a reduction in eNOS mRNA levels at P7 in the lumbar spinal cord [149]. Besides, motoneuron apoptosis was not altered after nerve avulsion in eNOS knockouts compared to wild-type mice [145]. All these data strongly support that nNOS is the major NO source responsible for central synaptic disturbances after motor nerve injury.

In our recent review [33], we modeled the pattern and time course of nNOS expression after nerve injury, taking into account different motor systems, lesions, and species. Briefly, nNOS expression in injured motoneurons begins at day 3 post-injury; the level of expression and number of nitrergic motoneurons progressively rises, peaking between days 7 (crushing) and 28 (avulsion) depending on type of lesion, and then gradually returns to control values. Therefore, time-dependent increase in nNOS expression after motor nerve injury is expected to be paralleled by a progressive enhancement in NO synthesis at the somatodendritic portion of the injured motoneuron. It is interesting to remember here that the frequency of synaptic boutons on motoneurons was unaltered at 4 days after crushing of XIIth [15] or intercostal 7th and 9th [50] nerves and reached

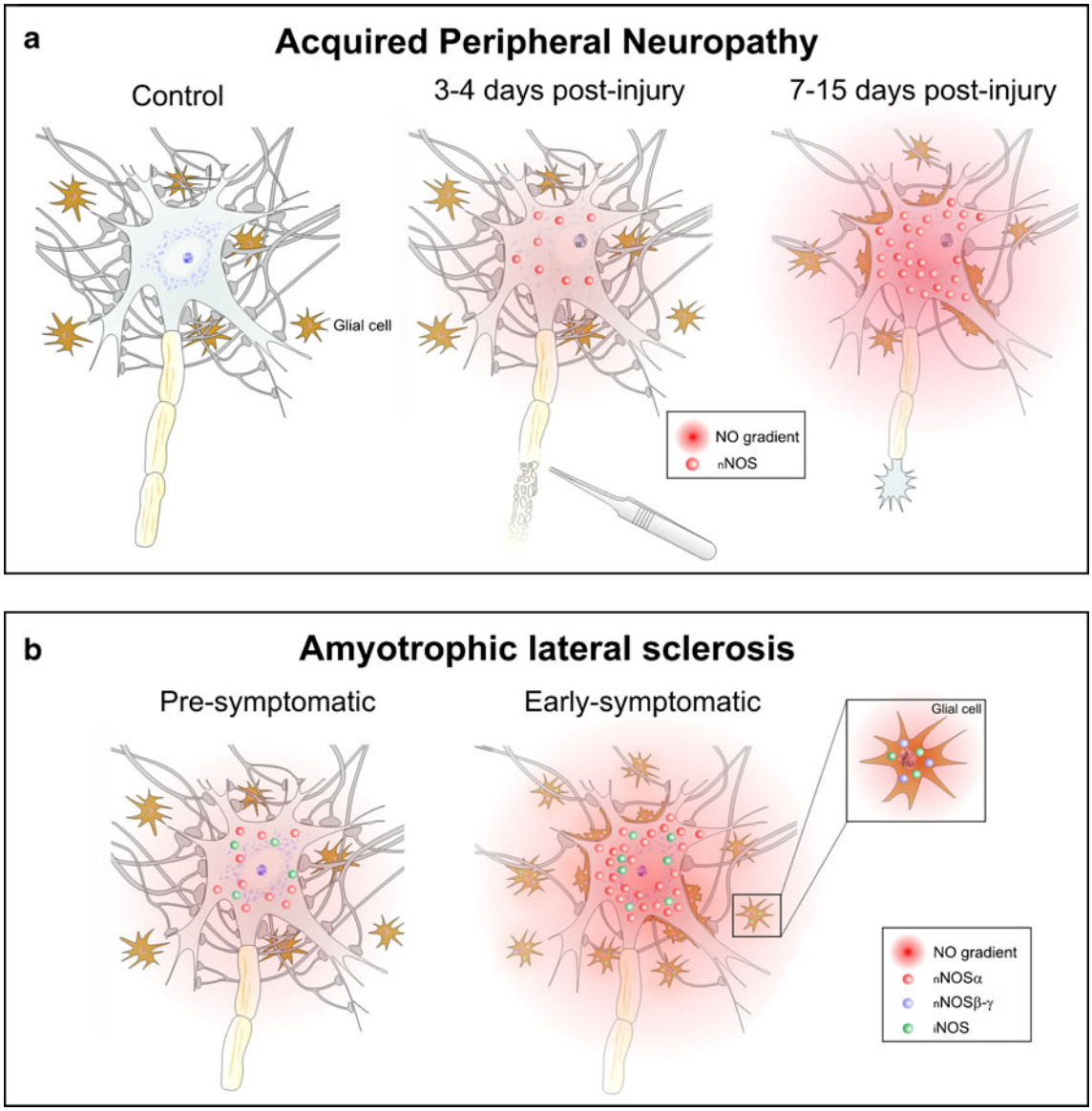


Fig. 2 Modeled time courses of NOS expression and synapse loss in motoneuron pathologies. **a** Acquired peripheral neuropathy induced by physical nerve injury induces upregulation of the neuronal isoform of NOS (nNOS) in motoneurons, which are physiologically lacking in this enzyme (*left drawing*). nNOS expression in injured motoneurons is already detected at day 3–4 post-injury; however, at this time the synaptic coverage of motoneurons is still unaltered (*middle drawing*). The level of expression of nNOS and number of nitrergic motoneurons progressively rise, peaking at 7–28 days after nerve injury (*right drawing*), depending on type of lesion. Activity-dependent nNOS activation creates a concentration gradient of NO at and around the injured motoneuron which increases in a time-dependent way after motor nerve injury. NO in turn can act autocrinely on the insulted motoneuron and/or paracrinely on neighboring structures. Nerve injury also evokes synapse loss, reaching maximal values between 7 and 15 days after damage. Then nNOS expression is a pathological process that precedes synaptic withdrawal after motoneuron axotomy, supporting a temporal relationship between the two events. **b** In the course of ALS, both isoforms, Ca^{2+}-dependent nNOS and constitutively active iNOS, are upregulated in motoneurons and glial cells. In the transgenic $SOD1^{G93A}$ mouse model, upregulation of nNOSα and to a lesser extent iNOS in motoneurons begins at pre-symptomatic (*left drawing*) and increases at early-symptomatic (*right drawing*) stages. A delayed upregulation of nNOS$\beta-\gamma$ and iNOS occurs in glial cells in early-symptomatic mice. Furthermore, motoneurons suffer a reduction in their synaptic coverage in the transition from the pre-symptomatic to early-symptomatic $SOD1^{G93A}$ mice. However, nNOS activity increased significantly in pre-symptomatic transgenic mice whereas iNOS activity is increased when early symptoms appear. Thus, nNOSα seems to be the major NO source time-related with the synaptic loss undergone by transgenic motoneurons over this period. However, delayed upregulation of nNOS$\beta-\gamma$ and iNOS could contribute in the additional synapse loss occurring at later stages of disease in transgenic mice. For details, see the text

maximal reduction at the 14th day post-lesion [47–51] (Fig. 2a). A comparison of the time course of the two alterations (NOS expression vs. synapse loss) indicates that upregulation of nNOS is a pathological event that precedes synapse loss (Fig. 2a). Strikingly, in the rat, the percentage of nitrergic motoneurons relative to the total pool depended on type of lesion, with the higher percentages being observed after more severe lesions. Analysis of NADPH-diaphorase histochemistry to detect NOS expression revealed that a maximum of 24–27% of the total pool of motoneurons presented nitrergic attributes after nerve crushing [14, 140, 147], while in more severe injuries the proportion increased: 48–60% after transection [140–142] and 80–98% after avulsion [136, 138, 140, 148]. It is remarkable that in the rat the reduction in syn-ir density in motor nuclei after nerve crushing (−9% to −33%; [13, 14, 51]) was less extensive than after transection (−60% to −68%; [55, 60]) or avulsion (−70%; [67, 69]). Taken together, it is reasonable to hypothesize a causal relationship between NOS expression, NO synthesis, and synapse impairment undergone by axotomized motoneurons.

NOS Expression Timing and NO Sources in ALS

ALS Patients

Free radical production underlined by excessive NO formation has been implicated in the pathogenesis of several neurodegenerative conditions including sALS and fALS [150, 151]. Glutamate-mediated stimulation evokes excessive Ca^{2+} influx and NO production, and surplus NO is oxidized, by combination with the anion superoxide (O_2^-), to a highly reactive anion, peroxynitrite ($ONOO^-$). Peroxynitrite causes DNA damage and inhibits mitochondrial function, leading to neuronal damage [152]. Peroxynitrite is in turn metabolized to the end-stable products nitrite (NO_2^-) and nitrate (NO_3^-). There is evidence that the sum of nitrites and nitrates as end-products of NO increases in the cerebrospinal fluid (CSF) [153–156] and in the serum [157] of sALS patients, suggesting overproduction of NO. This could be partially explained by the reported reduction in the CSF of asymmetrical dimethylarginine [158], a naturally occurring NOS inhibitor in the nervous system [159]. Taking into account that ex vivo measurement of nitrite and nitrate accurately correlates with the activity of NOS in the brain [160], the pronounced increase found in ALS patients, together with the reduction in endogenous NOS inhibitors, may also suggest a role for NO in the pathogenesis of this disease.

Initial approximations for the study of NO sources in ALS cases used an autoradiographic method employing [^{3}H]nitro-L-arginine, a potent inhibitor of the enzyme, as the binding ligand [161, 162]. The reported density of binding was higher in the ventral horn of ALS tissue compared to the corresponding region in the control tissue, suggesting that one or more upregulated isoforms of NOS in motor regions may contribute to the neurodegenerative alterations characterizing ALS. Immunohistochemical data provided evidence for the upregulation of both nNOS and iNOS in motoneurons and reactive astrocytes of ALS patients [18–20, 163, 164]. In sALS patients, most normal-appearing anterior horn neurons did not show iNOS immunoreactivity; however, many of the degenerating neurons displayed iNOS expression. Reactive astrocytes in the anterior horn were more intensely immunostained for iNOS than were the controls [164]. For all ALS patients, whether showing mild, moderate, or severe loss of anterior horn neurons, the mean number of nNOS-positive neurons was significantly larger than that found in the controls [20, 163]. Interestingly, nNOS-ir puncta apposed on anterior horn neurons were also positive for syn, indicating an increased pre-synaptic source of NO in the pathogenesis of ALS. Pathological neurons frequently demonstrated more intense nNOS-ir on the surface of the neurons than did normal-appearing neurons [20]. Glial cells in ALS spinal cord gray matter, most of them being reactive astrocytes, also showed a significant increase in nNOS staining, which paralleled the degree of gliosis [18]. A parallel study confirmed these findings and revealed that the spliced variants nNOSβ and nNOSγ, but not nNOSα, were upregulated in reactive astrocytes in sALS and fALS [19]. Taken together, these outcomes indicate that upregulation of the nNOS isoform occurs prior to iNOS in motoneurons and astrocytes in ALS progression and suggest that iNOS may have a more relevant role at the end-stage of disease.

The SOD1^{G93A} Mouse Model of ALS

HPLC analyses of citrulline, as an indicator of NO production, in the CSF from the SOD1^{G93A} mice indirectly revealed that the levels of NO are also significantly increased in the early-symptomatic genetic murine model of fALS [165]. The pattern of NOS expression, mainly the neuronal and inducible isoforms, has been studied in transgenic SOD1^{G93A} mice. However, controversy remains about what cell type upregulates NOS and which isoform is upregulated throughout disease progression. Differential NOS sources and expression time courses have been attributed to the number of expressed mutant SOD1^{G93A} copies in animal models [166] and/or the specificity of nNOS antibodies used [167]. Some of the work [167–169] was performed in SOD1^{G93A} mice expressing a low copy number (~10 copies; SOD1$^{G93A\text{-}low}$) of the transgene, with later onset and slower disease progression than in transgenic mice expressing a high copy number (~20 copies;

SOD1$^{G93A\text{-}high}$) of the mutant SOD1^{G93A} [90, 166, 170–172]. Relative to the iNOS isoform, the time course of its upregulation and gliosis in spinal cord paralleled that of motor neuronal loss in transgenic SOD1$^{G93A\text{-}high}$ mice. Two studies have shown the presence of numerous highly iNOS-ir cells with the appearance of glial cells, but not in neurons, at early-symptomatic and end stages of disease, but not in asymptomatic mice [170, 171]. On the other hand, the presence of iNOS mRNA in the spinal cord and brainstem motor regions has been reported using RT-PCR in SOD1^{G93A} mice and increased in pre-symptomatic (<P70) and early-symptomatic (P70–P91) stages [166, 170]. Western blotting and immunoprecipitation studies showed that iNOS protein levels in mitochondrial-enriched membrane fractions of spinal cord were increased at pre-symptomatic stages of disease [166]. This was corroborated by biochemical studies showing that iNOS activity in the spinal cord also increased at early-symptomatic stages of disease [166, 170]. Increased iNOS-ir occurred specifically in spinal and brainstem motoneurons at the pre-symptomatic and early-symptomatic stages of disease and then later (P105–P126) in cells appearing as microglia and astrocytes [90, 166]. In SOD1$^{G93A\text{-}low}$ mice, iNOS upregulation was observed in anterior horn neurons and their neuronal processes starting at late pre-symptomatic stage (~P196). At the symptomatic stage (~P224) and especially at the end-stage (~P245) of disease, the iNOS-ir was frequently observed in both chromatolytic and normal-appearing ventral horn neurons and also in reactive astrocytes [168]. Additionally, it has been shown that either selective iNOS inhibitors [166] or genetic iNOS knockdown [90] significantly prolonged survival of SOD1$^{G93A\text{-}high}$ mice. Thus, iNOS has a role in the pathobiology of ALS in this severe mouse model. Conversely, survival of SOD1$^{G93A\text{-}low}$ mice is unaffected by iNOS gene deletion [173], suggesting that contribution of iNOS to the disease mechanism probably differs between SOD1$^{G93A\text{-}high}$ and SOD1$^{G93A\text{-}low}$ mice.

Relative to expression of the neuronal isotype, even more controversial outcomes have been reported. The first study, focused on nNOS expression in SOD1$^{G93A\text{-}high}$ mice, reported intensely stained nNOS-ir astrocytes in the spinal cord and brainstem from symptomatic transgenic mice [172]. However, another group detected no nNOS immunostaining in cells other than neurons in the spinal cord of early-symptomatic or end-stage transgenic mice, in which several spinal cord neurons, as well as their surrounding neuropil, showed more intense nNOS-ir compared with control mice [170]. Biochemical approaches revealed that nNOS activity increased in the mitochondrial-enriched membrane compartment at the pre-symptomatic but not at late stages of disease [166]. In SOD1$^{G93A\text{-}low}$ mice, nNOS-ir motoneurons were more numerous and more intensely stained in transgenic than in control mice, particularly at pre-symptomatic stages. Glial elements also exhibited nNOS-ir, but only in the early-symptomatic and end stages of the disease [169]. The time course of nNOS expression in transgenic mice was similar to that reported previously for iNOS by the same group [168]. nNOS-positive anterior horn neurons began to increase at late pre-symptomatic and were more prominent in early-symptomatic mice, including chromatolytic neurons and many immunoreactive astrocytes at the end-stage of disease [167].

The nNOS antibody used in [170] will only recognize the full-length and most abundant nNOSα form but not the truncated and less abundant spliced variants β and γ because they lack some amino acids [130]. Conversely, the antibodies against nNOS used in [167] and [172] were directed against a conserved region of the three nNOS forms [130]. This could indicate that nNOSα is indeed specific to neurons, whereas nNOSβ and nNOSγ are upregulated in glial cells under pathological conditions that course with gliosis [170]. Supporting data on the nNOS involvement comes from in vivo experiments in which the highly specific nNOS inhibitor AR-R 17,477 prolonged the survival of both SOD1$^{G93A\text{-}high}$ and SOD1$^{G93A\text{-}low}$ mice [174]. Strikingly, SOD1$^{G93A\text{-}low}$ mice without nNOSα do not have prolonged survival, although Ca^{2+}-dependent NOS catalytic activity, which was fully inhibited by AR-R 17,477, was still detected [174], indicating that these mice still produce catalytically active β and γ isoforms of nNOS.

Altogether, the data obtained from patients and genetic murine models allow the modeling of a molecular scenario in the course of ALS in which both isoforms—nNOS, Ca^{2+}-dependent and therefore modulated by input activity, and iNOS, constitutively active—are upregulated in glial cells and motoneurons (modeled in Fig. 2b). In ALS patients, nNOS seems to be upregulated prior to iNOS in motoneurons, whereas upregulation of β and γ, but not α, nNOS forms and iNOS occurs in glial cells. In the transgenic mouse model, upregulation of nNOSα and iNOS in motoneurons begins at pre-symptomatic and increases at early-symptomatic stages. However, a delayed upregulation of nNOSβ, nNOSγ, and iNOS occurs in glial cells as observed in early-symptomatic mice (Fig. 2b). Motoneurons suffer a reduction in their synaptic coverage in the transition from the pre-symptomatic to early-symptomatic SOD1^{G93A} mice [94, 122] (Fig. 2b). The finding that nNOS activity increased significantly in spinal cord subcellular fractions from pre-symptomatic transgenic mice [166], whereas iNOS activity is increased when early symptoms appear [166, 170], points to nNOSα as the major NO source time-related with the synaptic loss undergone by transgenic motoneurons during this period. Delayed upregulation of nNOSβ, nNOSγ, and iNOS could explain additional excitatory loss

undergone by lumbar motoneurons at later stages (P110–130) of disease in transgenic mice [109].

NO-Triggered Synapse Loss from Pathological Motoneurons

Having reviewed the evidence of a close relationship between the timing, cell types upregulating NOS, and synaptic coverage reduction suffered by motoneurons after physical injury of their axons or in the course of ALS disease, we turn now to our group's work that provides strong evidence for a causal connection between upregulated NO synthesis and pre-synaptic deterioration in the course of these types of motoneuron pathology [14, 15, 94, 175]. We used a model of acquired peripheral motor neuropathy in the adult rat induced by the traumatic crushing of the XIIth nerve that causes a well-characterized range of functional and synaptic impairments in the insulted HMNs [13, 14, 175]. This type of lesion increases the number of nNOS-positive cell bodies at day 3 after lesion, reaches a plateau between days 7 and 15, and if neuromuscular reconnection occurs will return to control values 1 month after crushing [14]. The changes in syn-ir density in the HN after nerve lesion were similar in time course but opposite to those obtained for nNOS expression. No significant alteration in the optical density of syn-ir was observed at 1 day after nerve crushing. However, at 7 and 15 days after injury, the optical density was reduced in the experimental side compared with the control side. This was accompanied by a decrease in the linear density of syn-ir puncta apposed to injured HMNs, which was the consequence of the selective loss of excitatory boutons as evidenced by ultrastructural (S-type bouton loss) [17] and immunohistochemical (VGLUT2-ir puncta loss) studies. Synaptic density in the crushed side was recovered to the control-like condition at 1 month after injury [13, 14].

The vast majority of neurons with direct afferent connections to HN originate from the reticular formation of the lower brainstem [176]. Injection of the horseradish peroxidase into rat HN retrogradely identified the origin of projections mainly from three regions: the reticular formation, the spinal V complex, and the nucleus of the solitary tract [177]. A high proportion (68%) of pre-HN neurons were VGLUT2-positive and a low proportion (8%) were GABAergic. In addition, the major source of VGLUT2-positive inputs to HN originates from the reticular formation—the parvocellular, the "intermediate" zone, and the dorsal subdivision of the medullar portion—and the nucleus of the solitary tract [178]. Functionally, these excitatory sources to the HN are implicated in a wide range of autonomic, regulatory, and sensorimotor processes, including a critical role in organizing rhythmic oromotor behavior [178]. Therefore, it is likely that reduction of the rhythmic autonomic inspiratory activity of HMNs induced by XIIth nerve crushing [13, 14, 175] was, to a large extent, due to the loss of part of the brainstem's excitatory afferents.

Chronic administration of the broad spectrum NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) to adult rats, beginning on the day of crushing, prevented both the reduction in syn-ir and VGLUT2-ir puncta apposed to injured HMNs and the decrease in chemosensory-mediated responsiveness of motoneurons to end-tidal CO_2 changes. On the contrary, L-NAME treatment of intact/control animals for 7 days did not alter the discharge pattern of HMNs relative to untreated rats, which makes it unlikely that the protective effect of L-NAME was explained by a pre-synaptic action on physiological NO sources. In contrast, administration of the inactive stereoisomer D-NAME, at the same dose, did not prevent injury-induced changes. Administration of the relatively specific nNOS inhibitor 7-nitroindazole (7-NI), but not the specific iNOS inhibitor aminoguanidine, was also protective against the changes in activity and synaptic coverage induced by nerve lesion [14, 15]. Electrical stimulation of the XIIth nerve proximal to the crushing site failed to bring about a compound muscle action potential in the genioglossus muscle 7 days post-injury in rats treated daily with 7-NI [44]. This outcome disregards the possibility that recovery was the result of an accelerated axonal regeneration and muscle reinnervation. Strikingly, microinjection of a neuron-specific LVV system for miRNA-mediated nNOS knockdown (LVV-miR-shRNA/nNOS) in the HN, 3 days prior to XIIth nerve crushing, to a large extent prevented injury-induced synaptic and functional changes in HMNs [175]. These results indicate that NO synthesized by de novo expressed nNOS in injured motoneurons is a necessary signal for the molecular processes that lead to withdrawal of excitatory synaptic terminals.

It seems clear that induced neuronal NO is necessary for synaptic stripping of injured motoneurons, but *is it indeed sufficient?* Given that nNOS is only one of the numerous proteins dysregulated after nerve damage [179], the actual role for nNOS is less clear within the complex, synergistic, and/or antagonistic actions of multiple dysregulated proteins. To scrutinize whether nNOS-synthesized NO is "sufficient" to induce synaptic withdrawal in motoneurons, we used two approaches: (a) injection into either the tongue or the HN of a replication-deficient recombinant adenoviral vector (AVV) directing the expression of nNOSα, under the control of the human cytomegalovirus (hCMV) promoter, to transduce HMNs [15, 175] and (b) in vitro incubations of brainstem slices from rat pups (P6–P9) with the long half-life NO donor (Z)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl) amino]-diazen-1-ium-1,2-diolate-NO (DETA/NO).

AVV-nNOS injection into the genioglossus muscle retrogradely transduced neonatal HMNs. By means of ratiometric real-time NO imaging approaches, we confirmed that transduced motoneurons generated NO gradients in brain parenchyma (space constant ~12.3 μm) in response to a glutamatergic stimulus. NO synthesis was prevented by adding to the bath the NOS inhibitor L-NAME [175]. Therefore, the AVV-expressed nNOS in HMNs was functional and able to synthesize NO in response to the activation of glutamate receptors. Co-transduction of adult HMNs with AVV-eGFP/AVV-nNOS induced a significant reduction in their synaptic coverage, as shown by immunohistochemistry and electron microscopy. Remarkably, transduction of adult motoneurons with eGFP plus nNOS induced a selective reduction in the linear density of VGLUT2-ir puncta and S-type boutons. Interestingly, the array of VGAT-ir puncta or F/P-type boutons apposed to HMNs was unaltered [15]. These data strongly support that nNOS upregulation in adult motoneurons is sufficient to induce the detachment of excitatory, but not inhibitory, synapses. Thus, nNOS transfection mimicked the effect of axonal injury on the synaptic coverage of HMNs [15, 17]. These observations were supported by functional studies. Unilateral AVV-nNOS microinjection in the HN of adult rats induced axotomy-like changes in HMNs such as alterations in axonal conduction properties and reduction in the responsiveness to synaptic drive. These changes were concomitant with a decrease in the linear density of syn-ir puncta apposed to transduced HMN perikarya. Functional alterations were fully prevented by chronic treatment with nNOS inhibitors. Synaptic and functional changes were also completely avoided by prior intranuclear injection of LVV-miR-shRNA/nNOS [175].

HMNs are characterized by an uninterrupted inspiratory activity in vivo mediated by activation of glutamatergic receptors [180]. Thus, it would be expected that this rhythmic pattern of afferent excitation would support a sustained production of NO, both in nNOS-transduced or injured motoneurons. In an attempt to mimic this microenvironment, brainstem slices containing the HN were incubated (~6 h) with a long half-life NONOate, DETA/NO. Following this long-term treatment, we observed that the linear density of syn-ir puncta and boutons apposed to DETA/NO-superfused motoneurons was reduced. This functionally correlated with a reduction in the excitatory postsynaptic potential (EPSP) evoked in HMNs by electrical stimulation of the ventrolateral reticular formation. Strikingly, synaptic coverage reduction was the consequence of the loss of both excitatory (VGLUT2+ and S-type) and inhibitory (VGAT+ and F/P-type) pre-synaptic terminals, even though the excitatory was more profoundly affected. However, the differential selectivity of NO for different types of synapses at neonatal and adult stages seems to be a property acquired during postnatal development, since injection of AVV-nNOS into the tip of the tongue of neonatal rats (P3) evoked a decrease in VGLUT2- and VGAT-ir puncta [15]. These data point to an age-dependent change in the vulnerability of synapses to NO. This notion is supported by the evidence that NO participates in developmental synaptic refinement [35, 36], and at neonatal stages, spinal and brainstem motoneurons transiently express nNOS that fully disappear before P21 [181, 182]. Therefore, it can be proposed that synaptic refinement of afferent inputs on HMNs during maturation could involve specific loss of NO-sensitive GABAergic inputs. It is remarkable that VGAT-ir, but not VGLUT2-ir, puncta are reduced in adulthood relative to neonatal stage [15]. Loss of inhibitory inputs, occurring during postnatal maturation, was similar to that induced by DETA/NO incubation in neonatal motoneurons. Accordingly, a high proportion of GABAergic synapses disappear from P8 to P14 in the nucleus of tractus solitarii [183, 184].

These results raise the following question: *why does additional reduction of the excitatory coverage occur in DETA/NO-incubated or nNOS-transduced HMNs?* To account for this effect, we propose a concentration-dependent NO sensitivity differential between excitatory and inhibitory synapses. A dissimilar sensitivity in the potentiation of glutamatergic EPSPs and GABAergic IPSPs to NO has been described in the nucleus of tractus solitarii, reaching levels of saturation near 15 nM NO [185]. This concentration is higher than the physiological NO concentration ranging from ≤0.1 to ~5 nM [134, 186], which in the rat cerebral tissue increases to 4 μM (pathological) after ischemia [135]. Then, under physiological NO concentrations such as those assumed to be reached during developmental maturation, a preferential loss of NO-sensitive inhibitory synapses could occur. In contrast, pathological NO concentrations, such as those achieved under incubation with 1 mM DETA/NO (~1 μM NO) [187], by hCMV-directed nNOS overexpression in motoneurons, or in pathological upregulation of nNOS after axonal injury, could induce additional effects on high-threshold NO-sensitive excitatory synapses. Nonetheless, the possibility of changes in pre-synaptic molecular composition during postnatal maturation lowering the NO threshold of excitatory inputs cannot be dismissed.

It seems clear so far that NO, synthesized by upregulated nNOS in adult axotomized motoneurons, is not only necessary but also sufficient to trigger the molecular cascade leading to synapse withdrawal from HMN perikarya. Nevertheless, as aforementioned, differential synaptic alterations were observed depending on the type of lesion, the embryological origin of motoneurons, and animal species. Whether NO is also mediating or triggering excitatory and inhibitory boutons loss in the other models

of acquired motor neuropathy merits consideration. In this context, our recent report using another model of motoneuron pathology, the SOD1$^{G93A\text{-}high}$ mouse model of fALS [94], offers supporting data. In this model, motoneurons suffer a reduction in their synaptic coverage in the transition from the pre-symptomatic (2-month-old) to early-symptomatic (3-month-old) stage as a result of inhibitory bouton loss and excitatory synapse gain. In this transgenic mouse model, NOS upregulation in motor regions begins at pre-symptomatic and increases at early-symptomatic stages. Accordingly, our data showed that in animals receiving the NOS inhibitor L-NAME in the drinking water from P60 to P90, the synaptic coverage SOD1$^{G93A\text{-}high}$ HMNs did not differ from the control condition. Besides, the excitatory synaptic gain was not avoided by NOS inhibition but the loss of inhibitory synapses was significantly prevented.

Altogether, these results strongly support that NO also mediates synapse loss suffered by motoneurons during the third month of life in SOD1$^{G93A\text{-}high}$ mice. Strikingly, whereas D-NAME treated SOD1$^{G93A\text{-}high}$ animals showed a significant reduction in the time-to-fall in the hanging wire test relative to the non-transgenic control littermates, no animal treated with the NOS inhibitor fell down before the cutoff time [94]. This suggests beneficial effects of NOS inhibition also at spinal level and in the beginning of the first motor symptoms in this mouse model.

Paradoxically, NO mediates selective loss of excitatory synaptic inputs in the acquired motor neuropathy animals but reduction in the inhibitory coverage of the same motoneuron pool from transgenic mice. Whether changes in synaptic NO sensitivity is underlined by interspecies differences, synaptic molecular changes induced by SOD1^{G93A} expression, or different NO sources remains unknown and suggests a research line to be pursued.

NO Partners in Synaptic Withdrawal from 'Sick' Motoneurons

Feasible BDNF Contribution in Autocrine NO/cGMP/PKG Signaling

Since not all synapses are lost around nNOS-expressing motoneurons in pathological conditions, characterization of the molecular partners acting downstream to NO in synaptic stripping could unmask why some boutons are signaled to "go away" and their neighboring synapses are not. Most effects of NO are mediated by stimulation of sGC, leading to an increase in intracellular cGMP levels in target structures. The fact that sGC is expressed in motoneurons and fibers in the HN, together with the finding that cGMP levels are enhanced in HMNs, fibers, and bouton-like structures after perfusing adult rats with a NO donor [39, 146], support the idea that upregulated NO acts throughout sGC activation. Synaptic-ending loss undergone by injured HMNs or by nNOS adenovirally transduced motoneurons was fully prevented by daily intraperitoneal administration of the sGC inhibitor 1H-[1, 2, 4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), begun at crushing or AVV-nNOS administration day. Synapse loss induced by long-term incubation of neonatal slices with DETA/NO was also prevented by co-incubation with ODQ [14, 15, 175]. Therefore, NO-induced synaptic detachment is mediated by a cGMP-dependent mechanism in in vitro and in vivo preparations as well as at neonatal and adult stages.

An important target molecule for cGMP is PKG, which can be a feasible downstream effector for NO/cGMP in synapse loss. In this way, the molecular machinery to support an sGC/PKG-mediated action of NO is expressed by motoneurons and surrounding fibers [146, 188]. This hypothesis gains support from previous studies that implicate cGMP/PKG in collapse responses of retinal and ganglionar growth cones induced by ephrin-B1 and semaphorin 3A, respectively [189, 190]. Co-incubation of slices with DETA/NO plus the specific PKG inhibitor Rp-8-*p*CPT-cGMPS prevented reduction in the synaptic coverage and evoked EPSPs on HMNs. Thus, NO-induced reduction in synaptic puncta apposed to motoneurons was mediated via sGC/PKG activation.

An autocrine NO/cGMP-mediated mechanism acting at the postsynaptic element of the synapse and involved in the excitatory synapse detachment from injured motoneurons was proposed in a previous review by our group [16]. The brain-derived neurotrophic factor (BDNF) is a key partner in this model, since injured motoneurons upregulate both nNOS and BDNF and express sGC and PKG, the main downstream NO-activated targets. BDNF is a target-derived synaptotrophin synthesized and released postsynaptically in an activity-dependent form that can act as a paracrine retrograde signal in synaptogenesis and refinement of afferent projections. Besides, BDNF is a bidirectional messenger acting at glutamatergic synapses and upregulated together with its receptor TrkB by motoneuron injury; it promotes axon arborization and synaptogenesis and modulates structural dynamics of excitatory synaptic endings. Interestingly, the NO/cGMP pathway has been reported to inhibit BDNF secretion [16]. NO/cGMP-induced downregulation of BDNF secretion is mediated by PKG in cultured hippocampal neurons [191]. Finally, NO-induced growth cone collapse and axon retraction of developing retinal axons by actin filament (F-actin) depolymerization can be blocked by pretreatment with BDNF, which stabilizes F-actin [192, 193].

Based on our findings and the assumption that synapse maintenance requires a continuous supply of target-derived trophic signals, we proposed a model by which autocrine-acting NO synthesized by upregulated nNOS in motoneurons can induce excitatory synaptic withdrawal in injured HMNs. BDNF-containing secretory vesicles are accumulated postsynaptically at or near to glutamatergic synaptic contacts. Ca^{2+}-dependent secretion of BDNF provides trophic support to boutons acting in a retrograde paracrine form on TrkB receptors located at the axon terminal. In turn, at the pre-synaptic element of synapse, BDNF signaling stabilizes the cytoskeleton to keep synaptic endings in place. Axonal injury induces multiple changes, including synthesis of new products or alteration of pre-existing molecules that can compromise synapse stability. De novo synthesis of NO after motor nerve injury inhibits BDNF secretion in a cGMP/PKG-dependent way, and BDNF-mediated stabilization of the cytoskeleton at the pre-synaptic terminal is then diminished or absent, favoring synapse destabilization. BDNF expression and/or its downstream signaling cascade is also altered in ALS patients which suggests that this molecular mechanism could also be at work in the motor disease [16].

Paracrine NO Action Is Necessary for Synaptic Loss

The autocrine NO/cGMP/PKG-mediated mechanism modulating paracrine BDNF action could participate in excitatory synaptic detachment. Alternatively, we have recently found conclusive experimental evidence of a paracrine action of NO that is essential to this degenerative process [15]. AVV-nNOS-transfected rat pups were treated daily with the NOS inhibitor L-NAME, beginning the day of adenoviral administration, to minimize NO synthesis by tranduced nNOS until slice extraction. In these slices, both the linear density of syn-ir puncta apposed to motoneurons and the amplitude of EPSPs evoked in HMNs were reduced after immersion for ~6 h in the incubation bath. However, under the continuous presence of L-NAME or the membrane-impermeable NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide [194] in the bath solution, the reductions in synaptic coverage and EPSPs were fully avoided. These data indicate that synaptic reorganization around nNOS-transfected HMNs resulted from NO synthesis during the incubation period after slice extraction, requiring a paracrine/retrograde action of NO on target structures. We have further shown that XIIth nerve injury in the adult rat enhanced S-nitrosylated cysteine-ir puncta in the neuropil of the HN that was prevented by nNOS inhibition [14]. These observations suggest that an increased S-nitrosylation of proteins occur in bouton-like structures and could represent a mechanism by which NO acts in a paracrine form on synaptic endings. In support of this, it has been found that exogenous NO, but not membrane-permeable analogs of cGMP, causes growth cone collapse in retinal ganglion cells in vitro [195] and NO collapses neuronal growth cones by a modification of cysteine residues on substrate proteins [196]. In particular, S-nitrosylation of microtubule-associated protein 1B mediates NO-induced axon retraction in vivo and in cultured cells in vitro [197]. Action of NO/cGMP has also been shown to mediate growth cone collapse in cultured dentate granule cells [198]. Although a cGMP- and/or S-nitrosylation-mediated paracrine action of NO from upregulated nNOS in injured motoneurons is necessary, whether it is sufficient remains elusive.

NO-Induced Synapse Loss Involves RhoA/ROCK, a Cytoskeleton Regulatory Signaling System

It seems obvious that NO-induced synaptic withdrawal might involve actin cytoskeleton reorganization at the synaptic ending. Axon retraction is the result of two mechanisms that are not mutually exclusive, depolymerization and reconfiguration, which lead to retraction of the axonal cytoskeleton. Inhibition of the microtubule motor dynein causes axon retraction, which often assumes a sinusoidal morphology containing a curved bundle of microtubules. Axon retraction evoked by dynein inhibition depends on the endogenous actomyosin contractility in axons and is regulated by motor proteins that generate forces on the cytoskeleton (reviewed in [199]). In this way, NO induces axon retraction by microtubule reconfiguration, resulting in the formation of sinusoidal bends in the microtubular array. The formation of curvatures in the axonal array is likely to be a general feature of force-mediated axon retraction [199].

A variety of proteins regulate coordinated actin cytoskeletal rearrangements in growth cones. Among them, the small Rho GTPase RhoA and its major effector Rho kinase, ROCK1 or ROCKβ and ROCK2 or ROCKα, the latter highly expressed in the brain, could be firm partners of NO-directed synapse elimination during neuropathological states. In various neuronal types in culture, RhoA/ROCK mediates dendrite and neurite retraction, preventing axon growth initiation [200]. Activities of ROCK and PKG mediate semaphorin 3A-induced growth cone collapse in sensory neurons [189]. Finally, a functional link between long-term action of the NO/cGMP/PKG cascade and increased RhoA/ROCK activity has been previously suggested in arterial smooth muscle cells [201], in the penis [202], and in hearts from diabetic rats [203]. In the short-term, PKG phosphorylates RhoA on Ser188 and inhibits RhoA-dependent functions but, at long term, NO/cGMP/PKG positively regulates RhoA expression, in part through stimulation of RhoA gene transcription [201, 204]. In addition, PKG-induced phosphorylation of Ser188 protects RhoA, particularly its active form, from ubiquitin-mediated

proteasomal degradation [205]. This latter effect could be responsible for the increased RhoA/ROCK activity at the pre-synaptic ending caused by long-term activation of the NO/cGMP/PKG cascade.

RhoA cycles between the inactive guanosine diphosphate (GDP)-bound form and the active guanosine triphosphate (GTP)-bound form. The cycling of RhoA is regulated by three sets of proteins, at least 85 guanine nucleotide exchange factors (GEFs), 80 GTPase-activating proteins (GAPs), and three guanine nucleotide dissociation inhibitors (GDIs). GEFs are activators for downstream signaling, GAPs inhibit downstream signaling, and GDIs form a complex with Rho GTPases to protect against GDP release (reviewed in [206]). Of interest is that the action of the three types of RhoA regulators can be modulated by phosphorylation (reviewed in [207]). Regarding the process of synapse loss, however, it is unknown whether NO/cGMP/PKG-mediated phosphorylation of GEFs, GAPs, and/or GDIs may be a mechanism by which NO regulates RhoA activity. Consistent with this idea, PKG has been reported to phosphorylate a GAP factor in platelets [208]. It is also possible that NO may directly or indirectly regulate the expression levels of RhoA modulators. For instance, RhoGEF expression in the corpus cavernosum differs in eNOS null mice compared to wild-type mice [209]. NO also mediates post-translational modifications by nitration of GDI-2 in animal models of spinal cord injury [210]. Therefore, whether regulation of RhoA activity in NO-induced synapse loss also involves modulation of GEF, GAP, and/or GDI function/expression is a theme of great interest that merits investigation.

Another participating candidate is myosin and particularly myosin II, which can regulate the interaction of microtubules and the actin cytoskeleton contributing to axon retraction. The primary function for myosin II in non-muscle cells is to generate contractile forces essential for cell integrity, migration, and cytokinesis. Myosin II is highly concentrated at the interface between the central portion of the growth cone and the actin-rich lamellipodia. Activity of this class of non-muscle myosin is required for severing-induced axon retraction in vitro [211]. High-resolution microscopic studies of actin and myosin in migrating keratocytes suggest a dynamic network contraction model. Bipolar myosin II fibers, when attached to the criss-cross F-actin fibers, cause contraction of the actin fiber, forcing them to form actin bundles [212]. This process could also contribute to the driving force for synaptic retraction.

Myosin II is composed of two heavy chains that harbor the motor domain, along with two essential and two regulatory light chains. The motor function of myosin is positively regulated by phosphorylation of the myosin regulatory light chains (p-MLC). The balanced actions of MLC kinase (MLCK) and MLC phosphatase (MLCP) determine the level of p-MLC [200]. In turn, ROCK can phosphorylate MLC, a proposed mechanism underlying actomyosin contraction and neurite outgrowth retraction [213, 214]. RhoA/ROCK activation results in MLC phosphorylation either by a direct action on MLC and/or in an indirect manner by an inhibitory phosphorylation of MLCP. It then induces actomyosin contraction and neurite outgrowth inhibition/retraction, disturbing spine formation and maintenance. By decreasing synaptic connectivity during development, this mechanism has been proposed to underlie mental retardation [214]. Extracellular signals that cause axon retraction operate in a RhoA-, MLCK-, and myosin II-dependent manner but do not alter growth cone F-actin content [215]. This suggests that axon retraction mediated by RhoA, MLCK, and myosin II activities is not dependent on net F-actin depolymerization. In addition, both MLCK and ROCK contribute to severing-induced axon retraction [211].

In Vitro Evidence from Neonatal Animals

We raised the hypothesis that NO/cGMP/PKG-induced synaptic withdrawal could be mediated by RhoA/ROCK signaling, directing changes in actin cytoskeleton and mediating neurite retraction by direct or indirect MLC phosphorylation [200, 216]. Accordingly, DETA/NO-induced reduction in the EPSPs evoked in HMNs (see above) was fully avoided by co-addition to the bath of a Rho inhibitor (i.e., exoenzyme C3 transferase) or either of the two specific ROCK inhibitors (i.e., Y27632 and H1152). ROCK inhibitors also prevented the synaptic coverage reduction induced by NO, although syn-ir puncta density around motoneurons was not modified by either of the ROCK inhibitors alone [15]. Immunoblot analysis revealed a time-dependent increase in the ratio of p-MLC to MLC in DETA/NO-incubated relative to artificial CSF-incubated HNs, reaching a maximal value 3 to 4 h after addition of the NO donor to the bath. This was concomitant with an increase in the number of syn-ir puncta co-localizing with p-MLC apposed to motoneurons, although syn-ir coverage remained unaffected. In addition, DETA/NO-induced enhancement in syn-ir and p-MLC co-localization was mimicked by a membrane-permeable analog of cGMP, 8-Br-cGMP. The 8-Br-cGMP effect was prevented by co-incubation with PKG or ROCK inhibitors. However, ROCK inhibitors per se did not modulate this parameter, thus minimizing the possibility of a compensatory effect of basal ROCK activity on MLC phosphorylation. Interestingly, ROCK and p-MLC co-localization increased in puncta-like structures around HMNs under DETA/NO treatment [15]. Therefore, NO/cGMP/PKG-induced synapse remodeling is dependent on RhoA/ROCK signaling,

involving phosphorylation of the ROCK substrate MLC before synapse withdrawal in vitro. This is supported by previous studies showing that cGMP/PKG together with ROCK and/or MLCK mediates growth cone collapse responses to ephrin-B1, ephrin-A5, and semaphorin 3A in cells from different origins [189, 190, 217]. The function of MLCK is likely to be mediated through MLC phosphorylation, leading to myosin activation and actomyosin contraction [200].

In Vivo Evidence from Adult Animals

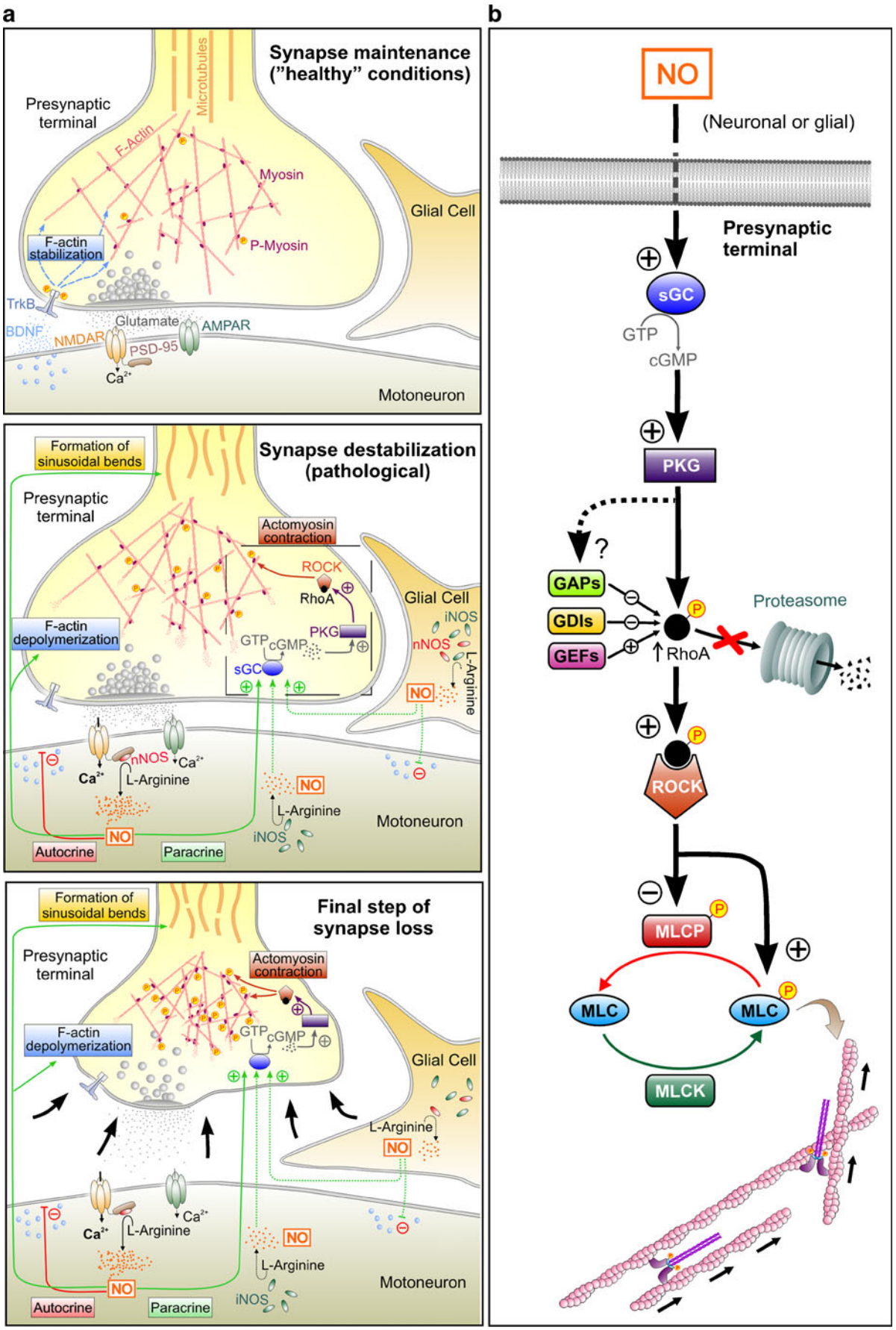
We found evidence that this mechanism is also working in pathological adult motoneurons. As mentioned below, XIIth nerve crushing induced both de novo expression of nNOS and a NO/cGMP-dependent synaptic loss on adult HMNs [14]. One week after crushing, animals treated daily with the inactive stereoisomer D-NAME had a reduction in the linear density of VGLUT2-ir, but not in VGAT-ir puncta apposed to injured HMNs. In contrast, chronic administration of the nNOS inhibitors L-NAME or 7-NI, or the sGC inhibitor, ODQ, avoided reduction in excitatory puncta. We next looked for glutamatergic inputs containing ROCK as feasible targets for the paracrine NO action. One week after unilateral nerve injury, in the intact (control) HN, ROCK-ir puncta of both isoforms, ROCKα and ROCKβ, were observed co-localizing or in close proximity to VGLUT2-ir puncta apposed to HMNs. This agrees with the regulatory role of ROCK in pre-synaptic cytoskeleton dynamics and microtubule organization [200]. We found a dramatic decrease in the number of VGLUT2-ir related with ROCK-ir puncta on the injured side. Furthermore, the number of p-MLC-ir puncta in close proximity to injured HMNs increased at 4 days after injury. At this same time point, the percentage of syn-ir co-localizing with p-MLC-ir puncta apposed to injured HMNs considerably increased. Remember that at this post-injury time (4 days), nNOS was already upregulated [14] but syn-ir reduction had not yet occurred [15]. These results suggest that, after motor nerve injury in adult rats, NO, through sGC, mediates the loss of most ROCK-containing excitatory inputs, likely by a mechanism involving previous increase of p-MLC in synaptic endings.

We also found evidence for the operation of this pathway in the SOD1^{G93A} mouse model of ALS. Immunoblot analysis of microdissected HNs revealed a significant increase in the ratio of p-MLC/MLC in pre-symptomatic animals [94]. Furthermore, an increase in syn-ir/p-MLC-ir puncta that preceded synapse loss was detected at this stage, as observed after traumatic XIIth nerve injury. To this age, synapse loss had not yet occurred on lumbar [122] and hypoglossal [94] motoneurons, although NOS upregulation had already begun [166–168]. Remarkably, ROCK is upregulated in the progression of the disease in late symptomatic transgenic mice [218], suggesting an increase in RhoA/ROCK signaling. All these findings argue for a NO-ROCK-pMLC-mediated synapse loss in the progression of the disease in SOD1^{G93A} mice as well. Although the two models of motoneuron pathology share common aspects, additional studies are needed to gain definitive insights on the widespread potential impact of this molecular mechanism.

Modeling a Feasible NO-Orchestrated Mechanism for Synaptic Loss in Motoneuron Pathology

Taking into account all collected data, we propose here a model by which de novo NO synthesis in injured HMNs directs synaptic detachment (Fig. 3). This could be extrapolated, at least in part, to other types of motoneurons

Fig. 3 Schematic model illustrating the contribution of NO to synapse elimination after axonal injury or in ALS. **a** Cartoon showing some of the molecular pathways implicated in synapse maintenance (*upper panel*) or destabilization (*middle and lower panel*) in motoneurons under normal or pathological conditions, respectively. The structural integrity of pre-synaptic terminals is maintained by an F-actin and microtubule-based cytoskeletal meshwork, which is stabilized by trophic signaling from the postsynaptic compartment. Among these trophic molecules is BDNF, which is released from the postsynaptic compartment in an activity-dependent form and binds to pre-synaptic TrkB receptors. Intracellular signaling cascades (*dashed light-blue arrows*) stimulated by TrkB activation eventually promote stabilization of F-actin cytoskeleton. Peripheral neuropathy, secondary to motor axon injury, and ALS, both evolve with the early induction of new sources of NO (see in Fig. 2). Under pathological conditions, NO synthesized by neurons and glial cells contributes to destabilization and detachment of pre-synaptic boutons acting via autocrine (*red arrows*; neuronal NO) and paracrine (*green arrows*; neuronal and glial NO) loops. At the postsynaptic compartment, NO inhibits BDNF secretion by a cGMP/PKG-dependent mechanism and promotes F-actin depolymerization. At the pre-synaptic side, NO induces bouton retraction through a combination of various effects on the cytoskeletal machinery which could potentially generate the mechanical force required to retract the axon. Firstly, NO favors F-actin depolymerization and evokes NO-mediated S-nitrosylation of microtubule-associated protein 1B leading to reconfiguration of microtubules into sinusoidal bundles along the axonal shaft. In addition, chronic production of NO indirectly stimulates contractility of the actomyosin network through a downstream signaling cascade involving activation of soluble guanylyl cyclase (*sGC*), cGMP-dependent protein kinase (*PKG*), and the RhoA/Rho kinase (*ROCK*) system (*area boxed in the middle panel*). This pathway is depicted in more detail in **b**. *Dotted lines* correspond to NO-triggered pathways that are exclusive of ALS, while *solid lines* denote the emergence of signaling cascades common to both ALS and motor neuropathies caused by traumatic axonal lesions. For simplicity, the components and details of some of these pathways have been omitted. Note that an excitatory terminal is illustrated. However, this degenerative event can also affect neighboring synapses of either nature whether they express the appropriated molecular array. **b** Schematic drawing of the molecular cascade leading to enhanced contractility of the pre-synaptic actomyosin system under pathological conditions (a detailed description of the interactions and components of this pathway is included in the text)


a
Synapse maintenance ("healthy" conditions)
Presynaptic terminal
Microtubules
F-Actin
Myosin
P-Myosin
Glial Cell
F-actin stabilization
TrkB
BDNF
Glutamate
AMPAR
NMDAR
PSD-95
Ca2+
Motoneuron
Synapse destabilization (pathological)
Formation of sinusoidal bends
Presynaptic terminal
Actomyosin contraction
ROCK
RhoA
Glial Cell
F-actin depolymerization
PKG
GTP
cGMP
sGC
iNOS
nNOS
L-Arginine
NO
Ca2+
L-Arginine
Motoneuron
Autocrine
Paracrine
Final step of synapse loss
Formation of sinusoidal bends
Presynaptic terminal
Actomyosin contraction
F-actin depolymerization
Glial Cell
Motoneuron
Autocrine
Paracrine
b
NO
(Neuronal or glial)
Presynaptic terminal
sGC
GTP
cGMP
PKG
?
GAPs
GDIs
GEFs
RhoA
Proteasome
ROCK
MLCP
MLC
MLCK

and pathological conditions if the required NO-sensitive molecular machinery is expressed in synaptic boutons. Upregulated nNOSα can anchor to NMDA receptors [131] at the injured motoneuron. This type of physical interaction can be the basis for coupling NO synthesis with Ca^{2+} influx through NMDA [132] and non-NMDA [219] receptors. Axonal injury of motoneurons increases NMDA expression and completely depletes the GluR2 subunit of AMPA receptors [220]; GluR2 prevents Ca^{2+} influx through them [100]. Thus, in impaired motoneurons, more sources are available to increase intracellular Ca^{2+} concentration, which in turn may facilitate NO production via Ca^{2+}/calmodulin-mediated nNOS activation, reaching higher concentrations than in physiological conditions. As a gas, pathological NO from sick motoneurons can act by autocrine and/or paracrine mechanisms. Autocrine-acting NO can stimulate sGC, leading to an increase in intracellular cGMP levels and PKG activation, which could reduce BDNF secretion [191] from the injured motoneuron and then impair its paracrine/retrograde synaptotrophic action on excitatory synapses through TrkB receptors. Reduction in the activity-dependent BDNF secretion can be harmful for cytoskeleton stability at the pre-synaptic terminal, by favoring paracrine NO-induced F-actin depolymerization [16]. Whether this postsynaptic action of the NO/cGMP/PKG cascade is necessary to induce synaptic detachment from injured motoneurons remains to be elucidated.

Additionally, a paracrine NO action is necessary to induce retraction of neighboring synapses if these have the appropriate molecular substrate. This could explain why not all synapses are lost in pathological conditions around nNOS-expressing motoneurons. This pathway involves paracrine intrasynaptic activation of the sGC/PKG pathway by NO. Subsequently, PKG could stimulate RhoA/ROCK signaling by preventing RhoA ubiquitin-mediated proteasomal degradation, particularly its active form, by phosphorylation of RhoA at Ser188 [205]. This explains why the mechanism works under long-term, but not short-term, NO action. In addition, we must consider the possibility that NO/cGMP/PKG pathway regulates the functionality and/or expression levels of the RhoA activity regulators—GEFs, GAPs, and GDIs [209–211]. The net effect of ROCK activation results in p-MLC either by a direct action on MLC and/or by inhibition of MLCP. p-MLC, as a regulatory factor, favors actomyosin contraction by interaction of myosin II fibers, with the criss-cross F-actin fibers forcing them to form actin bundles [212]. F-actin also undergoes retrograde transport and accumulates in the myosin II-enriched central portion, resulting in increased actomyosin contraction. This mechanism can be synergistic with other NO-mediated alterations that induce F-actin depolymerization or S-nitrosylation of microtubule-associated protein 1B, which participates in NO-induced axon retraction in vivo [197]. The latter could contribute to axon retraction by microtubule reconfiguration, leading to the formation of sinusoidal bends in the microtubular array. The formation of curvatures in the axonal array, together with actomyosin contraction, could be a general feature of force-mediated axon retraction (modeled in Fig. 3).

In ALS mice, three other variables must be added to the proposed model (Fig. 3): (a) upregulation of iNOS, an isoform that synthesizes NO in an activity-independent manner; (b) glial cells as additional NO sources; and (c) the increase in the GluR1/GluR2 ratio [105] and in the number of excitatory synapses [94]. These alterations, together with glutamate accumulation due to decreased clearance resulting from selective loss of the astroglial glutamate transporter EAAT2/GLT1 [96], would be expected to evoke higher intracellular motoneuron Ca^{2+} concentration in $SOD1^{G93A}$ mice than after nerve injury.

Therefore, synaptic endings expressing at least the sGC/PKG-RhoA/ROCK enzymatic machinery could be NO-targeted for withdrawal from "sick" motoneurons. This would explain why motoneurons from different embryonic origins or different species suffer loss of excitatory and/or inhibitory boutons.

The major histocompatibility complex of class I (MHC-I) has recently emerged as another feasible partner involved in NO-induced synaptic loss. MHC-I molecules are important for the synaptic withdrawal that physiologically occurs during development of the nervous system [221]. Interestingly, MHC-I molecules also were shown to be crucial for the selective maintenance of synapses during the synaptic removal process in axotomized motoneurons [65]. In this context, a link between NO synthesis and MHC-I in synaptic elimination has been recently reported. iNOS knockout mice displayed significantly reduced expression of MHC-I after nerve lesion and greater synaptic elimination after peripheral axotomy [222]. These initial findings open a promising research line for further studies that specifically focus on the role of NO as a regulator of MCH-I expression as well as their involvement as partners in regulating synaptic plasticity in motor pathologies.

Ubiquity of the NO-Orchestrated Mechanism for Synaptic Loss in the Most Prevalent Neurodegenerative Diseases: Signaling Possible Therapeutic Targets?

Synaptic stripping represents a common and early stage in the progression of several neurodegenerative diseases characterized in their later stages by extensive neuronal death. Synapse loss, rather than neuron death, correlates with cognitive decline in patients and/or animal models of

AD [1–3], PD [4, 5], HD [6], MS [7], and HIV dementia [8]. Interestingly, the pattern of neurodegeneration in the AD brain is consistent with a model in which synaptic scaling is the driving force for disease progression [2]. The relevance of synapse loss in AD progression is supported by studies revealing beneficial effects of exogenous application of synaptotrophic factors, such as cerebrolysin [223], in the treatment of mild AD patients [224]. A selective elimination of glutamatergic synapses on striatopallidal neurons has also been described in PD models [5], which provide a promising link with what takes place in adult injured HMNs. The first factor in the equation, n/iNOS, is also upregulated in neurons and/or glial cells in AD [21–23] and PD [24], in the striatum of a HD model [25, 26], and in MS [27–30] and HIV dementia [28, 31, 32], pointing to the appearance of new NO sources and/or higher levels of NO synthesis in the progression of these maladies. Given the ubiquity of the sGC/PKG in neurons throughout the brain [188, 225], it is more than probable that the NO/sGC/PKG cascade is overactive under neuropathological conditions. Several lines of evidence further support a causal relationship between NO production, synapse loss, and cognitive decline. Thus, pharmacological blockage of iNOS and treatment with telmisartan, which attenuates the Aβ-induced increase in expression of iNOS in the brain, either prevented or reduced cognitive decline in AD mouse models [226, 227]. Likewise, memory loss and $A\beta_{(1–40)}$ accumulation after chronic brain hypoperfusion in rats is preceded by an enhancement of hippocampal NO levels [228]. Relative to PD, administration of a relatively specific nNOS inhibitor attenuates spatial learning deficits and dopamine neuron loss, preventing disease progression in two models of experimentally induced parkinsonism [229, 230]. Finally, activation of constitutive NOS has been shown to occur before the development of cognitive deficits in a murine model of acquired HIV dementia complex, suggesting a temporal relationship between the two events [231].

Scattered findings obtained from different brain pathologies support that the mechanism underlying synapse loss described here could be a common hallmark to many neurodegenerative conditions. Relative to the BDNF-mediated destabilization of synapses, a recent study demonstrates that decreased BDNF levels are associated with impaired global cognitive functions as well as impairment in a specific component of memory in non-demented aging women [232]. Moreover, various reports describe decreases in BDNF in surviving neurons of hippocampus and certain neocortical regions in AD, in substantia nigra and striatum of PD brain (reviewed in [233]), in the caudate/putamen in HD patients [234], and in brain tissue of patients suffering from AIDS dementia [235]. Interestingly, upregulation of BDNF with a positive modulator of AMPA-type glutamate receptors rescues synaptic plasticity and memory in murine models of HD [236], while its downregulation resulted in earlier and more accentuated cognitive impairment [237]. A correlation between low BDNF production by peripheral blood mononuclear cells and poorer performances in cognitive tasks has also been reported in relapsing-remitting MS patients, suggesting a possible role of BDNF in cognitive impairment in MS [238].

Regarding the downstream partners, RhoA upregulation occurs in neurons surrounding amyloid plaques and an increase in phosphorylation of RhoA/ROCK substrates is involved in Aβ-induced inhibition of neurite outgrowth and synapse formation in a model of AD [239, 240]. RhoA expression decreased in synapses and increased in dystrophic neuritis in AβPP mice [241], which could be the consequence of RhoA-expressing bouton loss. In addition, ROCK inhibition improves rotarod performance and reduces huntingtin levels in a mouse model of HD [242]. RhoA upregulation has been observed in MS patients and rats with experimental autoimmune encephalomyelitis [243], and a Rho kinase inhibitor has been proposed as a new therapy in MS [244]. Finally, RhoA/ROCK-mediated MLC phosphorylation has been proposed to underlie mental retardation by actomyosin contraction and neurite outgrowth inhibition/retraction, decreasing synaptic connectivity during development [214].

Altogether, these evidences support the possibility that the action mechanism underlying synapse loss described here may be common to several neurodegenerative conditions. Further investigation of NO/cGMP/PKG-RhoA/ROCK-pMLC cascade involvement in the synaptic rearrangements occurring in these pathologies could lead to the identification of feasible therapeutic targets to avoid or delay cognitive impairments or even disease progression.

Acknowledgments This work was supported by grants from the Ministerio de Ciencia e Innovación, (SAF2008-01415) and the Consejería de Innovación, Ciencia y Empresa from the Junta de Andalucía (PAI2007-CTS-02606), both co-financed with FEDER funds and the Mútua Madrileña Foundation, Spain. We thank Elaine Lilly, Ph.D. (Writer's First Aid), for English language revision of this manuscript.

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