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Nitric Oxide Synthase Complexed with Dystrophin and Absent from Skeletal Muscle Sarcolemma in Duchenne Muscular Dystrophy

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Summary

Nitric oxide (NO) is synthesized in skeletal muscle by neuronal-type NO synthase (nNOS), which is localized to sarcolemma of fast-twitch fibers. Synthesis of NO in active muscle opposes contractile force. We show that nNOS partitions with skeletal muscle membranes owing to association of nNOS with dystrophin, the protein mutated in Duchenne muscular dystrophy (DMD). The dystrophin complex interacts with an N-terminal domain of nNOS that contains a GLGF motif. *mdx* mice and humans with DMD evince a selective loss of nNOS protein and catalytic activity from muscle membranes, demonstrating a novel role for dystrophin in localizing a signaling enzyme to the myocyte sarcolemma. Aberrant regulation of nNOS may contribute to preferential degeneration of fast-twitch muscle fibers in DMD.

Introduction

Nitric oxide (NO) is a major endogenous mediator involved in diverse developmental and physiological processes (for reviews see Bredt and Snyder, 1994a; Marletta, 1993; Moncada and Higgs, 1993; Nathan and Xie, 1994). Because NO is a short-lived free radical, regulation of signaling occurs largely at the level of NO biosynthesis. Three mammalian NO synthase (NOS) genes have been identified, and each forms NO from the guanidine nitrogen of L-arginine in a unique cytochrome P-450-type reaction that consumes reduced nicotinamide adenine dinucleotide phosphate (NADPH). Endothelial and neuronal NOS enzymes (eNOS and nNOS, or types I and III, respectively) are discretely expressed in specific tissues and rapidly transduce signaling events in a calcium-dependent manner. eNOS activity accounts for endothelium-dependent blood vessel relaxation, while nNOS occurs discretely in a variety of cell types, including neurons, epithelial cells, mesangial cells, and skeletal muscle cells. Physiological actions for nNOS have been best characterized in the peripheral nervous system, where NO functions as a nonadrenergic–noncholinergic transmitter in numerous pathways, including the gastrointestinal and urogenital tracts. In contrast with these calcium-dependent enzymes, a largely inducible calcium-independent NOS (iNOS, or type II) is expressed at highest levels in immunologically activated cells. NO formed in this pathway functions in a nonspecific immune response that mediates certain cytotoxic and bacteriocidal actions of activated macrophages (Hibbs et al., 1987).

In addition to controlling diverse cellular processes, NO also participates in certain pathophysiological conditions. Deregulation of nNOS in brain is associated with glutamate receptor overactivity and contributes to neuronal damage in animal models of stroke (Dawson et al., 1992). Mutant mice lacking nNOS are relatively resistant to neuronal damage following focal cerebral ischemia (Huang et al., 1994). iNOS activity contributes to cellular damage in a variety of immunological disorders such as rheumatoid arthritis and septic shock.

Maintenance of physiological NO signaling in the face of this potential toxicity requires tight regulation of NOS at numerous points. In addition to transcriptional control, NOS proteins are all regulated by calmodulin (Bredt and Snyder, 1990; Cho et al., 1992a), which links NO formation to increases in cellular calcium. A more complex level of regulation is reflected by targeting of NOS proteins to intracellular membranes. This subcellular targeting restricts NO signaling to specific targets within a limited microenvironment while minimizing aberrant toxic pathways. Membrane association of eNOS is mediated by two fatty acid modifications (Robinson et al., 1995). Cotranslational N-terminal myristoylation of eNOS is necessary for membrane association. In addition, eNOS is posttranslationally palmitoylated by a dynamic process regulated by a specific agonist, bradykinin. nNOS, which lacks consensus sequences for fatty acid modification, also occurs largely in particulate fractions (Hecker et al., 1994). The majority of nNOS immunoreactivity in neurons is associated with rough endoplasmic reticulum and specialized electron-dense synaptic membrane structures, while in skeletal muscle, nNOS is associated with the sarcolemma (Kobzik et al., 1994).

Understanding mechanisms for targeting of nNOS within cells will help identify pathways associated with NO signaling and clarify how disruption of this transduction machinery contributes to disease processes. The regular and defined structure of skeletal muscle makes it an ideal tissue in which to address these questions. Furthermore, recent studies have identified a major role for NO in skeletal muscle. nNOS is expressed at higher levels in human skeletal muscle than in human brain (Nakane et al., 1993). In mature skeletal muscle, nNOS is enriched in fast-twitch muscle fibers, where NO opposes contractile force (Kobzik et al., 1994). Additionally, NO produced by cultured myocytes facilitates myoblast fusion (Lee et al., 1994) and mediates activity-dependent synaptic suppression in myocyte neuronal cocultures (Wang et al., 1995).

These physiological actions of NO in muscle are facilitated by restriction of nNOS protein to the sarcolemmal membrane (Kobzik et al., 1994). The sarcolemma of skeletal muscle is a complex structure reinforced by an actin-containing cytoskeleton. In addition to ubiquitous

structural elements such as spectrin, skeletal muscle sarcolemma contains a unique network formed around dystrophin and related proteins (Ervasti and Campbell, 1993). Dystrophin was originally identified by positional cloning as the gene product mutated in Duchenne muscular dystrophy (DMD) (Monaco et al., 1986). Subsequent studies have identified a family of intracellular and transmembrane glycoproteins in a dystrophin-associated complex that links the extracellular matrix with the actin-based cytoskeleton. Recent studies indicate a major role for this complex in neuromuscular development and disease. α-Dystroglycan, an extracellular glycoprotein linked to dystrophin, serves as a physiologic receptor for agrin, which mediates clustering of acetylcholine receptors (Campanelli et al., 1994; Gee et al., 1994). On the other hand, disruption of dystrophin or other proteins in this complex results in muscular dystrophy in both humans and animals (Campbell, 1995). Despite these data, it not known how the dystrophin complex mediates signal transduction, nor is it clear why disruption leads to muscle disease. Indeed, none of the previously identified dystrophin-associated proteins have known catalytic activities.

In this report we demonstrate that sarcolemmal nNOS is complexed with dystrophin in skeletal muscle. The dystrophin complex interacts with the N-terminus of nNOS, which contains a motif of previously unknown function present in a family of cytoskeletal-associated proteins including syntrophins, known binding partners of dystrophin. In human DMD and *mdx* mice, which lack dystrophin, nNOS is absent from the sarcolemma and accumulates in the cytosol. This derangement of nNOS is specific for dystrophin abnormalities, as nNOS disposition is unaffected in other muscular diseases. These observations provide molecular evidence for a specific intracellular signaling molecule linked to the dystrophin-associated complex and suggest roles for NO in processes of neuromuscular development and disease associated with this complex.

Results

nNOS but Not eNOS Is Present in Cytoskeletal Extracts from Skeletal Muscle

Previous work indicates that the majority of nNOS in human and rat skeletal muscle occurs in membrane fractions (Nakane et al., 1993). We similarly find that ~80% of the total nNOS protein in mouse skeletal muscle associates with the particulate fractions (data not shown). To understand mechanisms for membrane association of nNOS, we characterized the extractability of nNOS from mouse quadriceps and found that nNOS is anchored both to microsomal membranes and to cytoskeleton (Figure 1A). Thus, the majority of nNOS protein remained membrane associated following extensive washing of skeletal muscle heavy microsomes (Mitchell et al., 1983) with 0.5 M NaCl. Solubilization of washed membranes with 0.5% Triton X-100 released about half of this particulate nNOS, with the remainder in an insoluble cytoskeletal pellet (Figure 1A). By contrast, eNOS, which is membrane associated

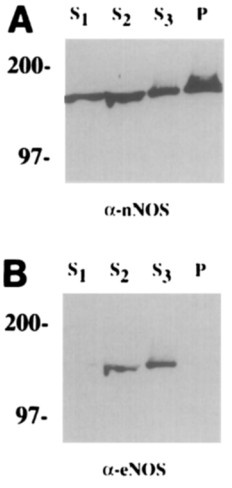

Figure 1. Differential Extractability of nNOS and eNOS in Skeletal Muscle Homogenates

(A) Western blotting indicates that significant nNOS remains in an insoluble pellet (P) following sequential extraction of mouse quadricep homogenates with 100 mM NaCl (S_1), 500 mM NaCl (S_2), and 0.5% Triton X-100 (S_3). nNOS is present in each of these fractions.

(B) eNOS occurs only in membrane-associated fractions. Probing the same blot with an eNOS monoclonal antibody indicates that eNOS is completely extracted by 500 mM NaCl (S_2) and 0.5% Triton X-100 (S_3). eNOS is absent from cytosol (S_1) and the insoluble pellet (P). After subcellular fractionation and 7.5% SDS–PAGE (100 μg protein per lane), nNOS and eNOS were sequentially detected by protein immunoblot. Positions of molecular size markers are indicated in kilodaltons.

owing to N-terminal myristoylation, was quantitatively solubilized from these same preparations by Triton X-100 (Figure 1B).

This differential fractionation suggested that unique determinants in nNOS anchor this isoform to the skeletal muscle cytoskeleton. The amino acid sequence of nNOS contains a 230 amino acid N-terminal domain that is not present in eNOS (Figure 2A). Beyond this extended N-terminus of nNOS, the two proteins share >60% sequence identity. Similar enzymatic activities of eNOS and nNOS suggest that the unique N-terminus of nNOS may not be required for catalytic activity. To analyze the role of the extended N-terminal region of nNOS, we constructed a deletion mutant, nNOSΔ1–226, lacking the first 226 amino acids. Expression vectors containing full-length nNOS and nNOSΔ1–226 were transiently transfected into COS cells. NOS catalytic activity was measured in tissue homogenates 3 days following transfection. Kinetic characteristics of NOS activity for the nNOSΔ1–226 mutant was essentially indistinguishable from the full-length isoform. Both constructs displayed similar V_{max} and K_m for arginine as well as regulation by calcium/calmodulin (Figures 2C and 2D).

Rather than regulating catalytic activity, we reasoned that the N-terminal domain of nNOS may instead target nNOS to skeletal muscle sarcolemma. Within this domain, nNOS contains a 66 amino acid motif that bears homology to a heterogeneous family of signaling enzymes that share the property of being localized to specialized cell–cell junc-

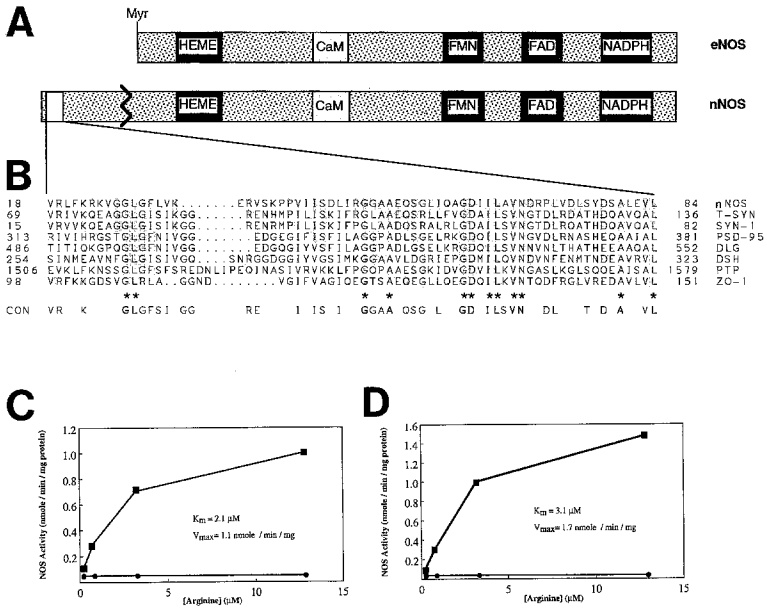

Figure 2. The Extended N-Terminus of nNOS Contains a GLGF Domain That Is Not Required for Enzyme Activity

(A) Schematic alignment of cofactor-binding domains of eNOS and nNOS, indicating N-terminal myristoylation (Myr) of eNOS and extended N-terminus of nNOS. The jagged line indicates the region deleted for the nNOSΔ1–226 mutant. Abbreviations not defined in text: CaM, calmodulin; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide.

(B) Alignment of the GLGF domain of nNOS with syntrophins and a family of other cytoskeletal-associated proteins. Abbreviations of proteins not defined in text: T-SYN and SYN-1, syntrophins; DLG, dlg-1; DSH, disheveled; PTP, protein-tyrosine phosphatase.

(C and D) COS cells were transfected with 10 μg of expression vector using a cytomegalovirus promoter to drive expression of either full-length nNOS (C) or the truncation mutant nNOSΔ1–226 (D). NOS activity was measured in cell homogenates 3 days following transfection in the presence of either 200 μM free calcium (closed squares) or 2 mM EDTA (closed circles). Kinetic constants (V_{max} and K_m) were calculated by Scatchard plot analysis. Data are means of triplicate determinations that varied by <10%. This experiment was replicated twice with similar results.

tions (Figure 2B). Proteins containing this motif, which is named GLGF for a conserved tetrapeptide (Cho et al., 1992b), include dlg-1, the product of the *lethal discs large* tumor suppressor gene that localizes to the undercoat of the septate junction in Drosophila (Bryant and Woods, 1992); *disheveled*, a gene required for planar cell polarity in Drosophila; PSD-95, a brain-specific protein that localizes to the postsynaptic density in mammals (Cho et al., 1992b); ZO-1, a peripheral membrane protein that localizes to tight junctions (zona occludens) of epithelial and endothelial cells (Willott et al., 1993); and certain protein-tyrosine phosphatases such as PTP1E, which are thought to localize at the junction between the plasma membrane and the cytoskeleton. Interestingly, we and others (Ponting and Phillips, 1995) noted homology to syntrophins, a family of recently cloned dystrophin-binding proteins, which colocalize with NOS beneath the sarcolemmal membrane of skeletal muscle (Adams et al., 1993; Ahn et al., 1994). In fact, syntrophins are more closely related to NOS in this domain than any other known gene (Figure 2B), suggesting a possible role for dystrophin in regulating the sarcolemmal localization of nNOS.

Association of nNOS and Dystrophin in Skeletal Muscle Extracts

We evaluated possible association of nNOS with dystrophin by means of succinylated wheat germ agglutinin (sWGA)–Sepharose affinity chromatography. nNOS, which lacks glycosylation sites, is not expected to adhere to a wheat germ column, while dystrophin, which is bound to a glycoprotein complex, is routinely purified by this technique (Ervasti et al., 1991). To ensure specificity of this association, we conducted parallel experiments with *mdx* mice that specifically lack dystrophin owing to a nonsense mutation (Sicinski et al., 1989), but that express nNOS at near-normal levels. Quadriceps from wild-type and *mdx* mice were homogenized and solubilized in buffer containing 0.2 M NaCl and 1% digitonin. Solubilized homogenates were applied to sWGA–Sepharose columns that were extensively washed with buffer containing 0.5 M NaCl and 0.5% Triton X-100. Tightly bound proteins were affinity eluted with 0.3 M N-acetyl-D-glucosamine (NAG). Western blot analysis reveals presence of nNOS in NAG eluates from wild-type but not *mdx* tissue (Figure 3A).

We also conducted analogous experiments and evaluated association of dystrophin with nNOS using a 2′,5′-ADP–agarose column, which tightly binds nNOS at its C-terminal NADPH-binding motif (Bredt and Snyder, 1990). Dystrophin has no nucleotide-binding site (Koenig et al., 1988) and would not be expected to adhere to a 2′,5′-ADP column. To ensure specificity of a potential nNOS–dystrophin interaction, we conducted parallel purifications from skeletal muscle of nNOS knockout mice, which are devoid of nNOS protein (Huang et al., 1993) yet express dystrophin at normal levels. Salt-washed heavy microsomes from wild-type and nNOS knockout mouse quadriceps were solubilized in 1% digitonin and allowed to adhere to 2′,5′-ADP–agarose columns. The columns were extensively washed with buffers containing 0.5 M NaCl and 0.5% Triton X-100. Tightly bound proteins were eluted with buffer containing 20 mM NADPH. Western blot analysis revealed presence of dystrophin in NADPH eluate fractions from wild-type but not nNOS knockout tissue (Figure 3B).

To evaluate directly binding of dystrophin-associated complexes to the N-terminal domain of nNOS, we coupled

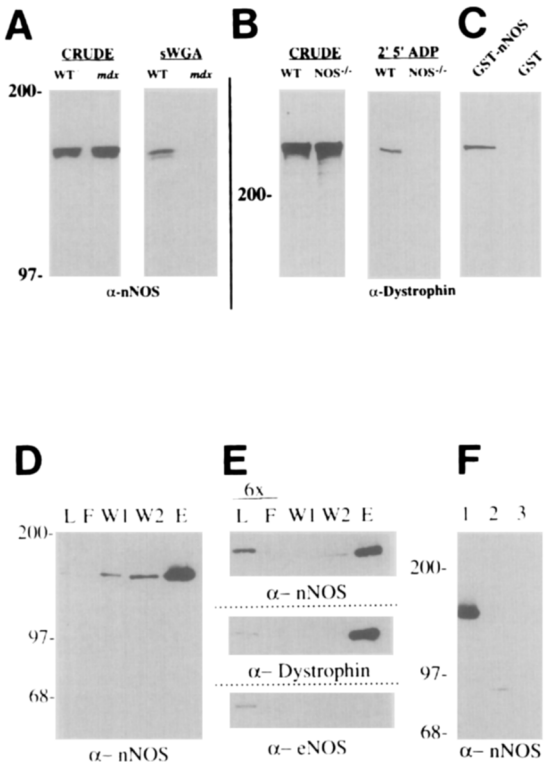

Figure 3. Association of nNOS and Dystrophin in Skeletal Muscle

(A and B) Dystrophin-associated glycoprotein complex and nNOS were purified by sWGA and 2′,5′-ADP–agarose chromatography, respectively, from wild-type (WT), *mdx*, and NOS knockout ($NOS^{-/-}$) skeletal muscle. For (A)–(C), protein homogenates (200 μg per lane) and aliquots of affinity eluates (150 μl per lane) were resolved on 7.5% (α-nNOS) or 6% (α-dystrophin) SDS gels and transferred to PVDF membranes, and immunoreactive bands were visualized by ECL.

(A) Western blotting indicates that total nNOS levels in crude extracts are similar in wild-type (WT) and *mdx* muscle. nNOS coelutes with dystrophin on an sWGA affinity column in muscle homogenates from wild-type mouse. nNOS does not adhere to an sWGA column in extracts from *mdx* mouse.

(B) Western blotting for dystrophin reveals that dystrophin levels are equivalent in crude samples from wild-type (WT) and nNOS knockout ($NOS^{-/-}$) skeletal muscle. Dystrophin coelutes with NOS on a 2′,5′-ADP affinity column in muscle homogenates from wild-type but not nNOS knockout mouse.

(C) Glutathione–Sepharose beads bound to GST or GST–nNOS(1–299) were incubated with solubilized skeletal muscle membranes. After extensive washing, the beads were eluted with 0.2% SDS and proteins were separated by SDS–PAGE, and retention of dystrophin protein was analyzed by Western blotting.

(D) Western blotting for nNOS from equally loaded (5 μg per lane) fractions from sWGA chromatography shows large enrichment of nNOS in NAG eluate fractions. Abbreviations: L, load; F, flowthrough; W1, 500 mM NaCl wash; W2, 500 mM NaCl and 0.5% Triton X-100 wash; E, 0.3 M NAG eluate.

(E) Reloading samples from sWGA column with 6-fold higher protein in load (L) and flowthrough (F) (21 μg per lane) than in wash (W1 and W2) and eluate (E) fractions (3.5 μg per lane) shows purification of nNOS and dystrophin, but not eNOS, by sWGA.

(F) Immunoprecipitation from NAG eluate fractions indicates that a monoclonal antibody to dystrophin (12 nM) potently precipitates nNOS (lane 1). Control experiments containing a monoclonal anti-Myc antibody (12 nM) or lacking any primary antibody (lanes 2 and 3) fail to precipitate nNOS.

a protein containing glutathione S-transferase (GST) fused to the first 299 amino acids of nNOS to glutathione beads. The GST–nNOS(1–299) beads and control GST beads were incubated with solubilized homogenates of mouse skeletal muscle. After extensive washing of the beads, bound proteins were eluted with sample buffer. Western blotting indicated that GST–nNOS(1–299) beads but not control beads retained dystrophin protein (Figure 3C). Other proteins evaluated, such as myosin, were retained in very small but equivalent amounts by both GST and GST–nNOS(1–299) beads (data not shown), indicating specificity of the association with dystrophin.

To quantitate enrichment of nNOS by sWGA chromatography, we conducted larger scale purifications from rat skeletal muscle tissue. Western blot analysis for nNOS in equally loaded fractions (5 μg per lane) from sWGA chromatography revealed a striking enrichment of nNOS in the NAG eluate (Figure 3D). nNOS was typically purified ~60-fold by sWGA chromatography; thus, equal nNOS immunoreactivity was detected in 30 μg of loaded fractions and 500 ng of NAG eluate fractions (data not shown). To evaluate the retention of nNOS by sWGA, we reloaded fractions from sWGA chromatography onto SDS gels with 6-fold more total protein in load and flowthrough lanes (21 μg) than in wash and eluate lanes (3.5 μg) (Figure 3E). Clearly, the majority of particulate nNOS adhered to the sWGA column as minimal nNOS is present in the flowthrough. We probed the blot shown in Figure 3E with antisera to dystrophin and found that essentially all the solubilized dystrophin adhered to the sWGA column and that dystrophin was enriched to a somewhat greater degree than nNOS by this procedure (~150-fold purification). The somewhat higher recovery of dystrophin appears to be due to slow dissociation of nNOS from the sWGA column (dystrophin complex) under stringent conditions, as evidenced by some "bleeding" of nNOS with the 500 mM NaCl and 0.5% Triton X-100 wash steps (Figure 3E). We also probed this same blot with an antibody to eNOS, which is similar to nNOS but lacks a GLGF domain. eNOS did not specifically adhere to the sWGA column, and only residual amounts of eNOS were found in NAG eluate fractions.

To determine whether nNOS associates with dystrophin complexes eluting from sWGA, we conducted immunoprecipitation experiments. NAG eluate samples were incubated for 1 hr with a monoclonal antibody to α-dystrophin, and immunocomplexes were pelleted with anti-mouse immunoglobulin G (IgG) linked to protein A–Sepharose. Western blot analysis revealed potent immunoprecipitation of nNOS with 2.0 μg/ml (12 nM) α-dystrophin. Control immunoprecipitations lacking the primary dystrophin antibody or containing an alternate monoclonal (anti-Myc, 12 nM; BABCO) did not precipitate detectable nNOS, demonstrating specificity of the interaction (Figure 3F).

nNOS Is Displaced from Sarcolemma and Accumulates in Cytosol of *mdx* Skeletal Muscle

The absence of dystrophin leads to a dramatic reduction of other dystrophin-associated proteins, including α- and

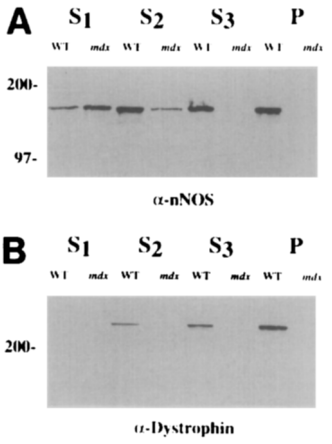

Figure 4. nNOS Is Displaced from Particulate Fractions of *mdx* Skeletal Muscle

(A) Subcellular fractions (S_1, S_2, S_3, and P) of mouse quadriceps from wild-type (WT) and *mdx* mice (age matched at 7–8 weeks) were prepared as described in Figure 1. Western blotting shows that nNOS in skeletal muscle of *mdx* mice is largely extracted from membranes with 100 mM NaCl (S_1) and is completely removed with 500 mM NaCl (S_2). In wild-type mice, the majority of nNOS remains membrane associated following the 500 mM NaCl wash. Of this remaining nNOS, approximately half is removed by a 0.5% Triton X-100 extraction (S_3) and half is present in an insoluble pellet (P).

(B) Dystrophin is enriched in detergent extract (S_3) and cytoskeletal pellet (P) fractions in wild-type mice (WT) and is absent from *mdx* muscle.

β-dystroglycan and syntrophins, in the sarcolemma of *mdx* mice (Ohlendieck and Campbell, 1991) and in patients with DMD (Ervasti et al., 1990). We therefore evaluated the distribution of nNOS in *mdx* mice. Overall nNOS levels and enzyme activity were modestly decreased (~80% of control levels) in skeletal muscle from *mdx* mice (data not shown). Subcellular analysis revealed that nNOS distributed with dystrophin in membrane-associated and cytoskeletal fractions from wild-type skeletal muscle (Figures 4A and 4B). However, in preparations from *mdx* mouse, nNOS was quantitatively solubilized from microsomal membranes washed with 0.5 M NaCl; no nNOS protein was detected in detergent extract or cytoskeletal fractions (Figure 4A).

NOS catalytic activity was also absent from particulate fractions of *mdx* skeletal muscle. As previously reported in rat (Kobzik et al., 1994), NOS activity was enriched in the particulate fraction of mouse skeletal muscle homogenates (Table 1). NOS activity in the soluble fraction of *mdx* skeletal muscle occurred at levels 75% greater than wild type, but NOS activity was not detectable in the particulate fraction from *mdx* muscle. In brain, NOS-specific activity was nearly equivalent in soluble and particulate fractions. This distribution was unchanged in *mdx* brain, suggesting that proteins other than dystrophin anchor nNOS to neuronal membranes.

Immunohistochemical studies further identified an absence of sarcolemmal nNOS in *mdx* skeletal muscle. Using a polyclonal antiserum, we found that nNOS immunofluorescence was restricted to the sarcolemma of a subset of skeletal muscle fibers (Figure 5), which were previously noted to be fast twitch (Kobzik et al., 1994). However, nNOS immunoreactivity was absent from the sarcolemma of *mdx* muscle (Figure 5). To determine whether this derangement of nNOS was specific for dystrophin abnormalities, we evaluated the distribution of nNOS in *dy* mice, which have severe muscular dystrophy due to absence of an extracellular matrix protein, merosin, but have a normal distribution of dystrophin at the sarcolemma (Sunada et al., 1994). We found that nNOS is present normally at the sarcolemma of *dy* dystrophic mice (Figure 5).

Table 1. NOS Activity in Extracts from Wild-Type and *mdx* Mouse Tissues

NOS Activity	Wild Type[a]	*mdx*[a]
Skeletal muscle		
Soluble fraction	33.3	58.3
Particulate fraction	252	<0.5
Brain		
Soluble fraction	1020	1060
Particulate fraction	1080	1210

Particulate and soluble fractions of quadriceps skeletal muscle from wild-type and *mdx* mice were prepared and assayed for NOS activity as described in Experimental Procedures. Data are means of triplicate determinations that varied by <10%. This experiment was repeated three times with similar results.

[a] In counts per minute per milligram of protein.

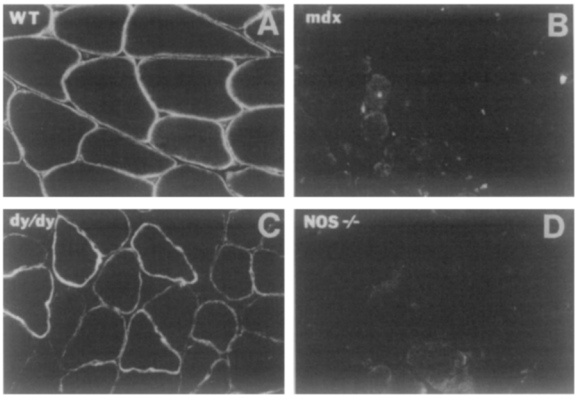

Figure 5. nNOS Is Selectively Absent from Sarcolemma of *mdx* Skeletal Muscle

Immunofluorescent staining for nNOS in quadriceps of wild-type, *mdx*, *dy*, and nNOS knockout mouse was performed using an affinity-purified polyclonal antibody. Cryostat muscle sections from wild-type, *mdx*, *dy*, and nNOS knockout mouse were stained under identical conditions using an affinity-purified nNOS antiserum and an FITC-linked secondary antibody.

(A and B) nNOS immunostaining is present at the surface membranes of skeletal muscle fibers from wild-type (WT) mouse (A), but is absent from *mdx* mouse (B) skeletal muscle sarcolemma.

(C) Homozygous dystrophic *dy* mice display normal sarcolemmal nNOS labeling of intact fibers.

(D) Skeletal muscle from NOS knockout ($NOS^{-/-}$) mouse is entirely devoid of immunostaining.

Magnification, 188×.

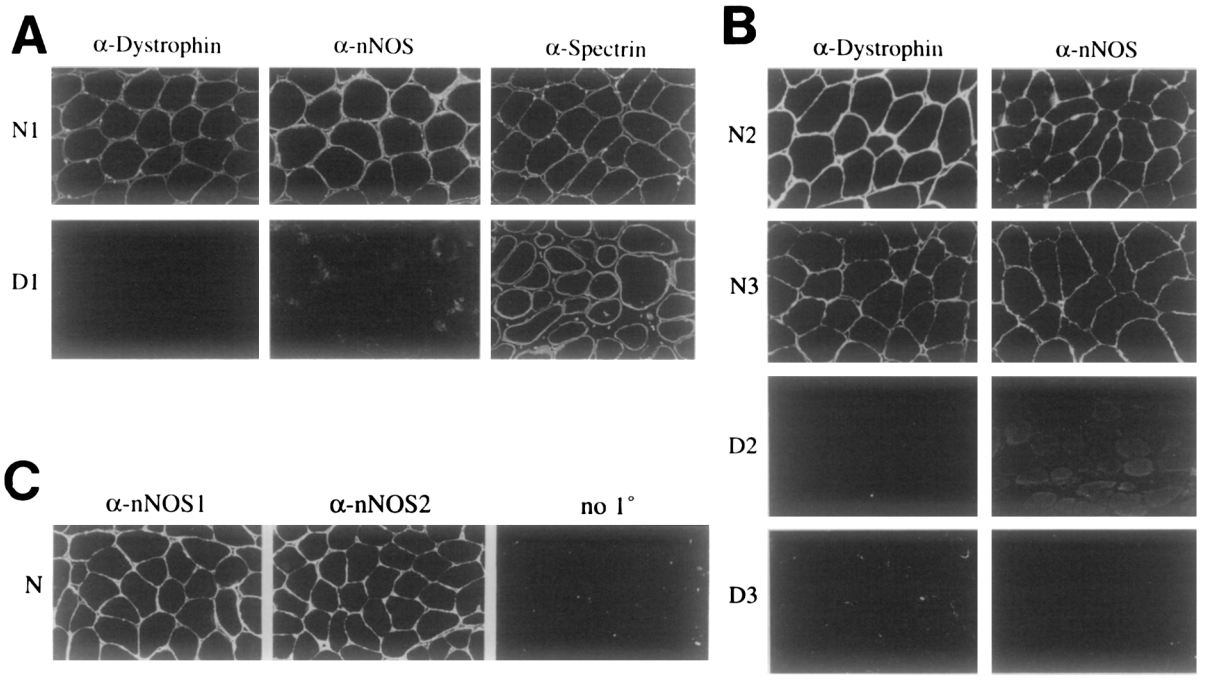

Figure 6. nNOS Is Absent from Sarcolemma of DMD Muscle Fibers

(A) Skeletal muscle cryosections of normal (N1) or DMD (D1) skeletal muscle were immunostained with antibodies to dystrophin, nNOS, and spectrin.

(B) Representative muscle sections from two additional normal patients (N2 and N3) and two DMD cases (D2 and D3) were labeled with antibodies to dystrophin and nNOS.

(C) Control experiments using two independently generated nNOS antisera showed similar staining of human tissues. No immunofluorescence was detected in the absence of primary (1°) antibody.

Magnification, 122×.

We conducted several control experiments to ensure for specificity of the immunofluorescence. No specific labeling was present in muscle tissues incubated with preimmune serum or without primary antiserum (data not shown). Most importantly, labeling was not present in transgenic mice containing a targeted mutation (Huang et al., 1993) of the nNOS gene (Figure 5).

nNOS Is Absent from Skeletal Muscle Sarcolemma in DMD

In addition to animal models, we evaluated the localization of nNOS in human muscle tissues. A total of 20 human tissues were evaluated, including 13 normal specimens and seven from patients with DMD. Skeletal muscle cross sections were processed for immunofluorescence for nNOS, dystrophin, and spectrin. All normal specimens showed colocalization of nNOS, dystrophin, and spectrin beneath the sarcolemma of muscle fibers (Figures 6A and 6B), indicating that nNOS shares a similar distribution in both human and rodent skeletal muscle. In all seven biopsies from patients with DMD, the disruption of dystrophin resulted in absence of nNOS staining of sarcolemma (Figures 6A and 6B). Normal sarcolemmal labeling for spectrin confirmed that this structural cytoskeleton was not disrupted in the DMD tissues (Figure 6A). Two independently raised nNOS antibodies (see Experimental Procedures) yielded similar immunofluorescent staining patterns, and no specific labeling was found in muscle sections incubated without primary antibody (Figure 6C).

Absence of dystrophin in DMD results in disruption of the dystrophin-associated glycoprotein complex and a dramatic reduction in overall levels of certain dystrophin-associated proteins in muscle. To evaluate total nNOS levels in skeletal muscle tissues from human DMD, we conducted Western blot analysis. Strikingly, we found that nNOS levels are dramatically reduced in human dystrophic muscle (Figure 7A). Densitometric scanning of nNOS immunoreactive bands in equally loaded Western blots revealed ~75% decrease of nNOS in DMD tissues. Western blots were also probed with an antibody to dystrophin that was essentially absent from all cases of DMD examined (Figure 7B). Immunoblotting for spectrin confirmed that similar amounts of protein were loaded in all cases and that the structural cytoskeleton was intact (Figure 7C).

Discussion

This study defines a role for the dystrophin complex in tethering nNOS to skeletal muscle sarcolemma. nNOS was originally characterized from brain cytosol that facilitated biochemical isolation of the enzyme (Bredt and Snyder, 1990). Subsequent studies indicate that the majority of nNOS activity and immunoreactivity are present in the particulate fraction of tissue extracts (Hecker et al., 1994;

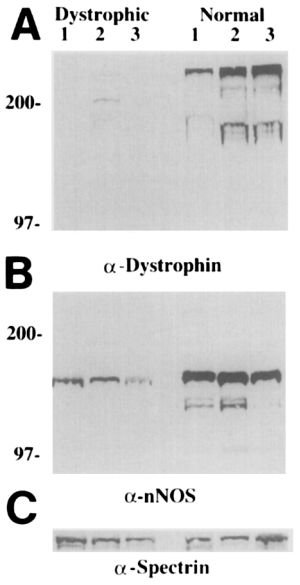

Figure 7. nNOS Levels Are Diminished in DMD Skeletal Muscle

Skeletal muscle tissue homogenates from three cases of DMD and three normal muscle biopsies were resolved by SDS–PAGE.

(A) Immunoblot analysis confirms that dystrophin is essentially absent from DMD muscle.

(B) nNOS levels are significantly decreased in all three cases of DMD.

(C) Spectrin levels are nearly equal in normal and DMD biopsies.

Kobzik et al., 1994). Thus, >60% of NOS activity is associated with the particulate fraction of cortical extracts (Hecker et al., 1994), and >85% of NOS immunoreactivity in monkey visual cortex is identified as axonal or dendritic profiles (Aoki et al., 1993). Membrane association of eNOS is mediated by fatty acid modifications; however, particulate nNOS in skeletal muscle and brain (J. E. B. and D. S. B., unpublished data) is not solubilized by detergent extraction, implying association of nNOS with the cytoskeleton. Our finding that nNOS remains membrane associated in brain of *mdx* mice implies that distinct nNOS anchoring complexes likely exist in other tissues. Possible candidates in brain include complexes formed about dystrophin-related proteins, utrophin (Matsumura et al., 1992), and the 87 kDa postsynaptic protein identified from Torpedo electric organ (Wagner et al., 1993), as well as homologs of protein 4.1, such as NF-2, which have been shown to bind GLGF domains in vitro (Marfatia et al., 1994).

The GLGF domain is a recently identified protein motif that is present in a heterogeneous family of enzymes. This observation has motivated suggestions that GLGF domains may regulate enzyme activities or, alternatively, that this motif may function as a protein–protein interaction domain (Cho et al., 1992b). We find that deletion of the GLGF domain of nNOS does not alter NOS catalytic activity in transfected cells. On the other hand, we find that Sepharose beads linked to a 299 amino acid fusion protein containing the GLGF domain in nNOS selectively retain dystrophin from skeletal muscle extracts, indicating that this domain is capable of interacting with the dystrophin-associated complex. The presence of GLGF domains in a diverse group of signaling enzymes suggests that some of these proteins may also interact with dystrophin complexes and that the family of dystrophin-associated proteins may far exceed the structural glycoprotein complex that occurs stoichiometrically with dystrophin in skeletal muscle.

The association of nNOS with the dystrophin–glycoprotein complex sheds light on protein interactions involved in maintaining the complex. Numerous data establish that the cysteine-rich and C-terminal domains of dystrophin are necessary and sufficient to establish the glycoprotein complex (Ahn and Kunkel, 1995; Campbell, 1995; Suzuki et al., 1994). The region(s) of dystrophin that interacts with syntrophin remains uncertain. The GLGF domain of syntrophin occurs near the N-terminus of the protein, analogous to the location of the GLGF domain in nNOS. In vitro binding assays show that the C-terminal half of syntrophins interacts with a protein fragment encoded by two C-terminal exons of dystrophin, exons 73 and 74 (Ahn and Kunkel, 1995; Suzuki et al., 1995; Yang et al., 1995). However, an *mdx* mouse containing a transgene for a truncated dystrophin protein lacking exons 71–74 fully restores syntrophins to dystrophin-associated complexes in skeletal muscle (Rafael et al., 1994). Together with the results of this study, these data suggest that the C-terminal half of syntrophins interacts with protein encoded by exons 73 and 74 of dystrophin and that the GLGF domains of syntrophins and nNOS interact with a distinct region of dystrophin or perhaps with a distinct protein of the dystrophin complex.

These results suggest potential roles for nNOS in neuromuscular signaling and disease associated with dystrophin. The dystrophin-associated glycoprotein complex has been proposed to mediate neurite-induced clustering of acetylcholine receptors via binding of α-dystroglycan to agrin (Campanelli et al., 1994; Gee et al., 1994), a neuron-derived component of the extracellular matrix (Nitkin et al., 1987). Association of nNOS (and potentially other enzymes containing a GLGF motif [Figure 2]) with dystrophin completes the link between the extracellular matrix and intracellular signal-transducing enzymes. NO, which modulates synaptic connectivity (Wu et al., 1994) and synaptic efficacy (Schuman and Madison, 1994) in brain, also serves as a retrograde messenger at neuromuscular synapses and mediates presynaptic suppression, a process that prevents polyneuronal innervation of muscle (Wang et al., 1995). Targeting of nNOS to dystrophin, which occurs at extrajunctional sarcolemma, may help eliminate formation of extrajunctional synapses (Sealock et al., 1991).

Aberrant translocation of nNOS from sarcolemma to cytosol in DMD and *mdx* muscle has implications for the pathogenesis of muscular dystrophy. We are currently evaluating nNOS distribution in a variety of muscle diseases and dystrophies. In preliminary studies we find that nNOS occurs normally at the sarcolemma in human neurogenic muscle atrophy, central core disease, and severe childhood autosomal recessive muscular dystrophy

(D. S. C., K. A., J. E. B., and D. S. B., unpublished data), suggesting specificity of the defect of nNOS in DMD. Whereas nNOS accumulates in the muscle cytosol of *mdx* mice, we find that overall nNOS levels in DMD tissue are significantly decreased compared with normal human muscle. Reasons for this peculiar difference are not clear. *mdx* mice lack skeletal muscle dystrophin, yet display a considerably more mild phenotype than human DMD patients (Bulfield et al., 1984). It is therefore possible that disease progression in DMD causes transcriptional downregulation of nNOS expression. This seems unlikely because we find that other animal and human muscle disorders are not associated with changes in nNOS levels. Alternatively, nNOS may be less stable in the cytosol of human skeletal muscle and would therefore be more rapidly degraded in DMD. Derangement of NO metabolism and reaction of NO with superoxide are responsible for tissue damage in certain diseases, including autoimmune (Nathan and Xie, 1994) and neurodegenerative processes (Dawson et al., 1992; Huang et al., 1994). Free radical oxygen intermediates, which occur at high levels in skeletal muscle (Reid et al., 1992a, 1992b), are known to contribute to cytotoxic damage in various muscle diseases, including DMD (Davison et al., 1988). Altered regulation of nNOS in dystrophic muscle may augment the toxic interaction of NO and superoxide and contribute to myofiber necrosis. Fast-twitch muscle fibers are preferentially affected in DMD. However, dystrophin and previously identified components of the dystrophin-associated complex are equally distributed between fast- and slow-twitch fibers (Ohlendieck et al., 1991). The selective enrichment of nNOS in fast-twitch muscle fibers (Kobzik et al., 1994) could help explain the preferential degeneration of this fiber type seen in DMD (Webster et al., 1988). Selective modulation of skelelal muscle NO levels may offer a novel therapeutic modality for DMD.

Experimental Procedures

Tissue Extraction and Western Blot Analysis

Mouse quadriceps skeletal muscle was homogenized in 10 vol (w/v) of buffer A (25 mM Tris–HCl [pH 7.4], 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF), and heavy microsomes were prepared by a standard protocol with minor modifications (Mitchell et al., 1983). Nuclei were pelleted by centrifugation at 1000 × g. The supernatant was then centrifuged at 20,000 × g, yielding supernatant S_1. The resulting heavy microsomal pellet was resuspended in buffer A containing 500 mM NaCl, incubated for 30 min at 4°C with agitation, and centrifuged at 15,000 × g, yielding supernatant S_2. This resulting pellet was resuspended in buffer A containing 500 mM NaCl plus 0.5% Triton X-100, incubated for 30 min at 4°C with agitation, and centrifuged at 15,000 × g, yielding supernatant S_3 and a final pellet, P.

Tissue extracts were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (7.5% polyacrylamide), and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore). Membranes were incubated overnight with primary antisera (bNOS, 1:250, Transduction Laboratories; eNOS, 1:250, Transduction Laboratories; dystrophin, 1:100, Novacastra Laboratories; spectrin, 1:100, Novacastra Laboratories) diluted in Tris–HCl-buffered saline containing 3% bovine serum albumin. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL) according to the specifications of the manufacturer (Amersham).

Affinity Chromatography

For sWGA affinity chromatography, mouse quadriceps from wild-type and *mdx* mice were homogenized and solubilized in 10 vol of buffer B (50 mM Tris–HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA, 1 mM PMSF) containing 1% digitonin. Solubilized membranes (4 mg) from wild-type and *mdx* mice were circulated for 1 hr with 250 μl of sWGA–agarose (Vector Labs) at 4°C. Columns were washed sequentially with 5 ml of buffer B containing 0.1% digitonin, buffer B containing 0.1% digitonin and 500 mM NaCl, and buffer B containing 500 mM NaCl and 0.5% Triton X-100. Columns were affinity eluted with 1 ml of buffer B containing 0.3 M NAG with 0.1% digitonin. Chromatography of rat skeletal muscle followed a similar procedure, except 20 mg of solubilized membranes was loaded onto 1 ml of sWGA that was washed with 10 ml of buffers containing 500 mM NaCl and Triton X-100 and eluted with 3 ml of 0.3 M NAG.

For 2′,5′-ADP affinity chromatography, mouse quadriceps from wild-type and nNOS knockout mice were homogenized in 10 vol (w/v) of buffer B, and heavy microsomes were prepared and solubilized in buffer B containing 1% digitonin. Solubilized membranes (4 mg) from wild-type and nNOS knockout mice were applied to 150 μl columns of 2′,5′-ADP–agarose (Sigma). Columns were sequentially washed with 5 ml of buffer B containing 0.1% digitonin, 1 ml of buffer B containing 500 mM NaCl and 0.1% digitonin, and 1 ml of buffer B containing 500 mM NaCl and 0.5% Triton X-100. Columns were affinity eluted with 1 ml of buffer B containing 20 mM NADPH and 0.1% digitonin.

GST-Fusion Proteins

A GST–nNOS(1–299) construct was generated by cloning sequences encoding the first 299 amino acids of rat brain NOS into the EcoRI site of the pGEX-2T vector. GST-fusion proteins were expressed in Escherichia coli and purified on glutathione–Sepharose beads according to the specifications of the manufacturer (Pharmacia; Smith and Johnson, 1988). Solubilized skeletal muscle membranes (2 mg) were incubated with control (GST) or GST–nNOS(1–299) beads for 1 hr. Beads were washed with buffer containing 0.5% Triton X-100 plus 300 mM NaCl, and proteins were eluted with 150 μl of loading buffer.

Dystrophin Immunoprecipitation

Monoclonal antibodies (2 μg) to dystrophin or Myc epitope (BABCO) were added to 1 ml aliquots of NAG eluate (15 μg), and samples were incubated on ice for 1 hr. Rabbit anti-mouse IgG (10 μg) (Cappel) was then added, and after 30 min, 50 μl of protein A–Sepharose was used to precipitate antibodies. Protein A pellets were washed three times with buffer containing 200 mM NaCl and 0.1% Triton X-100. Immunoprecipitated proteins were denatured with loading buffer and resolved by SDS–PAGE.

Antibodies, Immunohistochemistry, and Immunoblotting

Unfixed skeletal muscle samples were flash frozen in liquid nitrogen–cooled isopentane, sectioned on a cryostat (10 μm), and melted directly onto glass slides. Sections were then postfixed in 2% paraformaldehyde–phosphate-buffered saline (PBS). Tissues were blocked in PBS containing 1% normal goat serum. Monoclonal antibodies to dystrophin (1:200, Sigma), nNOS (1:100, Transduction Laboratories), spectrin (1:50, Novacastra Laboratories), or a polyclonal nNOS antibody (1:250; Kobzik et al., 1994) were applied to sections overnight at 4°C. For indirect immunofluorescence, secondary goat anti-rabbit fluorescence isothiocyanate (FITC) or donkey anti-mouse Cy-3 conjugated antibodies were used according to the specifications of the manufacturer (1:200, Jackson Laboratories).

Mammalian Cell Transfections

nNOS cDNAs were cloned into the mammalian expression vector pcDNA-3 (Invitrogen). Monkey COS cells were grown in culture medium consisting of DMEM (GIBCO BRL) supplemented with 10% fetal bovine serum. Cells were plated in 10 cm dishes at a density of 2 × 10^4 per square centimeter and transfected the following day using calcium phosphate as previously described (Bredt et al., 1991). Cells were washed with PBS 3 days following transfection, harvested in 2 ml of buffer containing 25 mM Tris–HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, and disrupted using a polytron.

NOS Catalytic Assays

Quadriceps skeletal muscle from wild-type and *mdx* mouse were homogenized in 10 vol of buffer containing 25 mM Tris–HCl (pH 7.4),

1 mM EDTA, 1 mM EGTA, and 0.1 M NaCl. The homogenate was centrifuged at 20,000 × g, yielding the soluble fraction. The pellet was extracted in the same buffer containing 0.5 M NaCl and centrifuged at 20,000 × g, yielding the particulate fraction. Aliquots from these fractions were assayed in 125 μl reactions containing 100,000 cpm of [^{3}H]arginine (60 Ci/mmol), 1 mM NADPH, 400 μM free calcium, 1 μM calmodulin, 3 μM each of tetrahydrobiopterin, FAD, and FMN. After incubation for 25 min at 22°C, assays were terminated with 4 ml of H_2O. Samples were applied to 0.5 ml Dowex AG50WX-8 (Na^+ form) columns. [^{3}H]citrulline was quantified by liquid scintillation spectroscopy of the 4 ml flowthrough. Crude homogenates of transfected COS cells were assayed using an identical procedure.

Characterization of Human Tissues

Human tissues were obtained from the pathology department at University of California, San Francisco. Clinical diagnosis of DMD was made on the basis of onset and progression of disease, the presence of creatine kinase in serum, and histologic study of the biopsied muscles. Tissues were snap frozen in liquid nitrogen–cooled isopentane. For Western blotting, cryostat sections were collected into plastic tubes and sonicated in buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF. Crude protein samples (200 μg per lane) were resolved by SDS–PAGE and analyzed by Western blotting as described above. Immunohistochemistry was performed on 10 μm human tissue samples as described above. To ensure specificity, we performed immunofluorescent nNOS staining using two independently raised antisera. The first antiserum (α-nNOS1) reacts only with determinants in the N-terminal domain of nNOS (Bredt and Snyder, 1994b), while the second (α-nNOS2) reacts only with the C-terminal region (Transduction Laboratories). Unless otherwise noted, all histologic sections were labeled with α-nNOS1.

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