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Preferential Conduction Block of Myelinated Axons by Nitric Oxide

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Abstract

Conduction block by nitric oxide was examined in myelinated and unmyelinated axons from both the CNS and PNS. In rat vagus nerves, mouse optic nerves at P12-P23, adult and developing mouse sciatic nerves, and mouse spinal cords, myelinated fibers were preferentially blocked reversibly by concentrations of nitric oxide similar to those encountered in inflammatory lesions. The possibility that these differences between myelinated and unmyelinated axons are due to the normal developmental substitution of Na⁺ channel subtype Na_v1.6 for Na_v1.2 at nodes of Ranvier was tested by repeating experiments on mice null for Na_v1.6. Results were unchanged in this mutant. In *Shiverer* optic nerve, which has only scattered regions with nodes of Ranvier, only the fastest component of the compound action potential was reduced. Nitric oxide was compared with 3 other methods of blocking conduction: low Na⁺, high K⁺ and TTX. In each of these 3 cases, unmyelinated axons lost conduction simultaneously with myelinated fibers. From changes in conduction velocity in myelinated axons as they were blocked, it was ascertained that nitric oxide acted most similarly to TTX. It is concluded that nitric oxide likely interacts with axonal Na⁺ channels through an intermediate that is associated with myelin.

Graphical Abstract

Nitric oxide, produced in inflammatory lesions of the CNS, blocks axonal conduction. Compound action potentials from postnatal day 16 mouse optic nerves contain components from both myelinated and unmyelinated axons. Nitric oxide reversibly blocks specifically the myelinated component, and this may be responsible for some deficits in multiple sclerosis.

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Keywords

inflammation; unmyelinated; ion channels; *Shiverer* (RRID:IMSR_JAX:001428)

Introduction

Nitric oxide (NO) is a highly reactive molecule with a complex chemistry that is involved in numerous normal and pathological processes. It is produced from L-arginine by a family of nitric oxide synthases: neuronal (nNOS), endothelial (eNOS), and inducible (iNOS). The latter is upregulated in microglia/macrophages and in astrocytes by inflammatory cytokines, resulting in significant increases in nitric oxide concentrations. Nitric oxide and its redox products react with proteins through nitration of tyrosine or S-nitrosylation of cysteine (Stamler et al. 1992b). It also activates guanylyl cyclase, raising levels of cGMP, a second messenger that regulates many cellular functions. There is growing evidence that nitric oxide plays a role in the pathology of multiple sclerosis and spinal cord injury, both of which include an inflammatory component (Smith and Lassmann 2002; Su et al. 2015). In one such role, it has been shown that nitric oxide blocks axonal conduction in a reversible manner (Redford et al. 1997; Shrager et al. 1998), and this has raised interest in developing therapeutic measures to prevent it (Smith and Lassmann 2002). In reading recent literature, we reviewed our earlier work (Shrager et al. 1998) and found a misinterpretation of one experiment. While most of that research was on rat sciatic nerves, we also recorded from vagus nerves, and found a large, fast component of the compound action potential (CAP) as well as a smaller, slower component. Both of these were blocked reversibly by nitric oxide, and we concluded that both myelinated and unmyelinated axons were therefore susceptible. However, when we re-examined these data we found that the conduction velocity of the smaller component was 5.9 m/s, much too fast to be attributed to unmyelinated axons, which typically conduct at 0.4 – 0.7 m/s at 37 °C. We therefore decided to re-examine the question of the interaction of nitric oxide with unmyelinated axons.

While extracellular signals recorded from myelinated fibers are in the range of 0.5 to 5 mV, and are readily detected, action potentials from unmyelinated fibers in adult nerves are typically only 0.01 to 0.05 mV, and must be extracted from background noise. This can be done with appropriate low noise amplifiers and signal averaging. While we recorded from a variety of adult tissues, we also examined developing nerves, in which the signals from unmyelinated (i.e., premyelinated) fibers are more robust. We applied nitric oxide through the use of a chemical donor, diethylamine NONOate (DEA NONOate). This compound was particularly useful for these experiments in part because a wide variety of controls and calculations have been done with it. Its half-life, releasing nitric oxide, is 16 minutes at 22-25 °C and 2 minutes at 37 °C, rates which we verified through use of the Greiss reagent (Green et al. 1982; Shrager et al. 1998). Correspondingly, its effects were minimal at 25 °C and only blocked when the temperature was raised to 37 °C, showing that the undissociated compound was without effect. Further, solutions were ineffective if allowed to fully dissociate into diethylamine and nitric oxide before applying to nerves (Shrager et al. 1998). (The half-life of nitric oxide in aqueous solutions is ~30 sec (Palmer et al. 1987).) Finally, solving the diffusion equation for a cylinder, and including the half-life of NO in tissue (~4

sec), we showed that at concentrations of the order used, DEA NONOate at 37 °C produces levels of nitric oxide in nerves that are similar to those that activated macrophages would produce in an inflammatory lesion (~1 μ M) (Shrager et al. 1998). Here we show that in both the CNS and PNS, in regions that contain both myelinated and unmyelinated axons, conduction in myelinated fibers is preferentially blocked by nitric oxide. This may provide a path toward development of a therapeutic approach that avoids a global reduction in nitric oxide, which can be deleterious.

Materials and Methods

Animals

All animals were housed in the University of Rochester vivarium, an AAALAC accredited facility. Animals were housed in sterile, individually ventilated cages with a 12:12 hour light cycle, and monitored regularly by vivarium staff. *Scn8a*^{tg/+} heterozygotes carrying an insertional mutation of *Scn8a* (Burgess et al. 1995; Kohrman et al. 1995; O'Brien and Meisler 2013) were a kind gift from Miriam Meisler and Julie Jones. Na_v1.6 null mice were generated from *Scn8a*^{tg/+} heterozygotes, bred in the Rochester vivarium under standard conditions. Tail biopsies taken at postnatal day 5 (P5) were shipped to the University of Michigan for genotyping by the Meisler-Jones laboratory. The wildtype *Scn8a* allele generates a 350 bp product using the forward primer CGGCT GCTGC TTCTA TAGGT GCTG and the reverse primer TCGAT GATGT ACTTC TCCAC GCTG. The mutant allele generates a 250 bp product using the forward primer GAGGG AGGGC TGAGG GTTGT AAGTC and the reverse primer CCATG GTGTC TGTTT GAGGT TGCTAG. PCR was carried out in a 25 μ l reaction volume containing Promega Green GoTaq buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.3 μ M concentration of each primer, and 1 unit of DNA polymerase (Promega GoTaq). An initial incubation for 3 min at 94°C is followed by 31 cycles of 30 sec at 94°C, 1 min at 65°C and 1 min at 72°C, terminating with 6 min at 72°C. Products were visualized after electrophoresis on 2% agarose gels and staining with ethidium bromide. *Shiverer* mice (RRID:IMSR_JAX:001428) were a kind gift from Martha Windrem and Steven Goldman. All protocols were approved by the University Committee on Animal Research.

Chemicals

Artificial cerebrospinal fluid (ACSF) had the following composition: (mM) NaCl 125, KCl 2.5, CaCl₂ 2.5, MgCl₂ 1.3, NaH₂PO₄ 1.25, NaHCO₃ 25, D-glucose 25. A high K⁺ version was prepared with KCl 25 mM, NaCl 102.5 mM. A low Na⁺ solution had NaCl 0, Tetraethylammonium chloride 125 mM. In both of these modified solutions, the remaining components, and the osmolarity, were normal. Diethylamine NONOate was purchased from Cayman Chemical Company, Ann Arbor, MI. It was dissolved in ACSF at 0.8 – 1.3 mM immediately before use and held at 4 °C until it flowed into the chamber. Tetrodotoxin (TTX) with citrate buffer was purchased from Calbiochem, San Diego, CA, dissolved at 2×10^{-4} M in distilled water, and stored at -35 °C. TTX was diluted in ACSF immediately before use. All other chemicals were reagent grade.

Electrophysiology

Animals were euthanized by CO₂ asphyxiation. All tissues were derived from mice (wt or mutant) except for vagus nerves which were dissected from adult Lewis rats. Nerves or spinal cords were dissected and placed in ACSF bubbled with 95% O₂ / 5% CO₂ at room temperature for at least 1 hour, then transferred to a temperature controlled chamber with, oxygenated ACSF flowing at 1 ml/min. Optic, sciatic, and vagus nerves were drawn into glass pipet suction electrodes (filled with ACSF) at each end for stimulation and recording. In general, both nerves from each animal (optic, sciatic, or vagus) were used, and there were no exclusion criteria for analysis. The recording pipet resistance was measured before (17-20 K Ω) and after (29-34 K Ω) insertion of the nerve, and monitored continuously during the experiment. The ratio of this resistance during each sweep, divided by the resistance of the pipet alone allowed a normalization of the amplitude of the compound action potential (CAP) to our standard ratio of 1.7 (Fernandes et al. 2014; Stys et al. 1991). This corrects for any drift in the seal resistance during an experiment. Signals were fed to one input of an AC differential amplifier of our design. The second input came from a pipet electrode placed near the recording electrode. This served to subtract much of the stimulus artifact. For spinal cords the recording electrode was a glass pipet with a tip ID of 30 μ m, filled with ACSF, and pressed against the cord. The contact resistance was monitored and was generally stable, and no corrections were applied. For a stimulation electrode on spinal cords we used a bipolar Pt electrode, insulated except for the wire ends. In all cases stimuli were delivered by an optically isolated constant current unit (WPI, Sarasota FL) driven by the computer. Stimulus durations were 50 μ s unless otherwise stated. Amplitudes were adjusted to be supramaximal (~20% above that required for a maximal response). Stimulus currents were monitored by a linear optically coupled amplifier of our design. All signals were electronically low pass filtered with a cutoff of 10 kHz. In some cases, as noted below, records were later filtered further with a Gaussian algorithm. All solutions were bubbled with 95% O₂ / 5% CO₂, and all records were taken at 37 $\pm$ 0.5 $^{\circ}$ C.

Computational model

The model axon consists of 12 nodes and 11 internodes. It has been described in detail, including tables of all parameters (Hines and Shrager 1991; Shrager 1993). We tested axon diameters (inner diameters) from 0.3 to 4 μ m, corresponding to the rodent and human CNS range (Barazany et al. 2009; Liewald et al. 2014), and with g-ratios of 0.8 for diameters > 0.5 μ m and 0.7 for diameters $\leq$ 0.5 μ m (Chomiak and Hu 2009; Winters et al. 2011). Internodal lengths ranged from 126 μ m (0.3 μ m diameter) to 473 μ m (4 μ m diameter) (Murray and Blakemore 1980). The temperature was 37 $^{\circ}$ C. Stimulation was at node 1, and records were plotted at nodes 4, 6, 8, and 10.

Analysis

As noted in the text or in figures, in some cases data were clustered among animals of a few ages, i.e., P15-P18 optic nerves in *Scn8a^{tg/tg}* mice. Peaks were analyzed by fitting with a third order polynomial, taking the derivative, and calculating the extremum from the resulting quadratic equation. Fits were monitored on the oscilloscope screen. Signals from unmyelinated axons in the vagus nerve had multiple components and, after nitric oxide

exposure, summed with a slow, broad depolarization. They were analyzed by subtracting the slow signal, which was fitted with a fifth order polynomial, and calculating the variance, as described in the text. Comparisons were between 2 sets of data, and were by the paired Student's two tailed t test, with $P < 0.05$ required for significance. Error bars in graphs are $\pm$ SEM.

Results

Preferential block of myelinated axons by nitric oxide

We revisited the issue of a requirement for myelination in the original preparation, the rat vagus nerve. Right and left vagi were tested, and results were the same for both. Using a stimulus of 2-4 mA/ 60-80 μ sec results in a CAP containing two components with velocities > 1 m/s. There is a large, sharp peak (24 m/s) and a smaller, more diffuse peak (6.1 m/s) (Fig. 1A), similar to what was seen before (Shrager et al. 1998), and originating from myelinated axons. At longer times, there is a burst of biphasic signals with a typical conduction velocity of 0.5-0.7 m/s, and small amplitude (~ 50 μ V) (Fig. 1D). The latter represent action potentials from a diffuse set of unmyelinated axons, and are resolved by averaging 4-16 sweeps. Exposing these nerves to nitric oxide blocks both of the myelinated CAP components, as measured from their peaks (Fig. 1B). The signals from unmyelinated axons are not blocked, but they appear to sum with a slow, broad depolarization that may represent the development of an afterpotential (Fig. 1E). There was no unambiguous way to measure the amplitude of these biphasic signals directly. In order to quantify them it was necessary to subtract the broad "pedestal" that appeared following nitric oxide exposure. This was accomplished by fitting the data with a fifth-order polynomial (Fig. 1G), and subtracting the fit. The signals in the remaining sweep (Fig. 1H) were then quantified by calculating the variance over the region of strongest signals. In the presence of nitric oxide, the myelinated components of the CAP decreased by $97 \pm 1\%$ while the unmyelinated contribution decreased by $32 \pm 4\%$, a difference that was significant ($P = 0.003$, $n = 4$). All of these changes were readily reversed by washing with ACSF (Fig. 1C,F).

While results from vagus nerves were suggestive, we sought a more definitive way to examine a possible dependence on myelination for conduction block by nitric oxide, and also to test this in the CNS. Adult mouse optic nerves have very few unmyelinated axons, but during development it is possible to record robust signals from both classes of fibers. In particular, myelination begins at about P9, and by P12 small signals from myelinated fibers can be recorded (Fig. 2A). At P16 CAP components from myelinated and unmyelinated axons are about equal in amplitude (Fig. 2D), and by P23 the myelinated peaks dominate, while the signal from unmyelinated fibers can still be detected as a small biphasic trace late in the sweep (Fig. 2G). At each of these stages, nitric oxide blocks only the myelinated component of the CAP, leaving the unmyelinated contribution relatively unchanged (Fig 2B,E,H). Overall, in these developing nerves the myelinated peak declined $78 \pm 2\%$ and the unmyelinated peak actually increased slightly ($2 \pm 5\%$). (At P23 the latter is small and subject to error.) This difference was significant ($P < 0.001$, $n = 6$). Again, the conduction block was at least partially reversed by removal of the nitric oxide from the bath by flowing in ACSF (Fig. 2C,F,I).

Dependence on Na⁺ channel subtype

What is responsible for the difference in response to nitric oxide between myelinated and unmyelinated axons? Aside from the presence of myelin, these fibers express different Na⁺ channel subtypes. Myelinated axons express Na_v1.2 early in development, but by P15 the nodal Na⁺ channels have largely been replaced by Na_v1.6 (Boiko et al. 2001). In the CNS, unmyelinated axons express Na_v1.2 (Debanne et al. 2011; Gong et al. 1999; Westenbroek et al. 1989). Our experiments on developing optic nerve included a period when Na_v1.2 is the primary voltage dependent Na⁺ channel present in myelinated fibers, and yet nitric oxide blocked conduction. However, in order to test more stringently the idea that conduction block by nitric oxide is dependent on myelin, and independent of the channel type, we repeated these experiments on developing optic nerves from *Scn8a^{tg}/Scn8a^{tg}* mice null for Na_v1.6 (Burgess et al. 1995; Kohrman et al. 1995). At P16 the CAP was similar to that from wt animals (Fig. 2J), and conduction block by nitric oxide was again restricted to the myelinated component (Fig. 2K). Tested on optic nerves from P15-P18, the myelinated peak declined $67 \pm 5\%$, while the unmyelinated peak declined $8 \pm 5\%$ ($P < 0.001$, $n = 6$). Thus, the differential susceptibility of myelinated axons to nitric oxide is not dependent on the presence of Na_v1.6 at nodes of Ranvier.

We investigated these ideas further with recordings in the PNS. Fig. 3A-C shows CAPs from myelinated axons in the adult mouse sciatic nerve. Fig. 3D-F illustrates corresponding records from unmyelinated fibers in these nerves. The latter traces consisted of a primary biphasic signal that is only about 2% of the amplitude of that from myelinated axons, and that required signal averaging to improve the signal-to-noise ratio. Consistent with the previous experiments, nitric oxide blocked preferentially and reversibly the signals from myelinated fibers (Fig. 3B,C). As in the vagus nerve, a slow waveform associated with the unmyelinated action potentials develops, but the primary biphasic trace remains largely intact (Fig. 3E). Overall, the amplitude of the myelinated component decreased $89 \pm 2\%$, while the unmyelinated signal (biphasic portion) declined $6 \pm 11\%$ ($P = 0.03$, $n = 5$). These experiments were then repeated in sciatic nerves from *Scn8a^{tg}/Scn8a^{tg}* mice. Juvenile lethality in this mutant restricted experiments to P16-18, but, in the PNS, myelination is well-advanced by that age. The records in Fig. 3 G-L show that, as in wt adult nerves, block by nitric oxide is largely restricted to the myelinated component of the CAP. Myelinated decline, $89 \pm 1\%$; unmyelinated decline, $11 \pm 3\%$ ($P < 0.001$, $n = 5$). Thus, as in the CNS, conduction block by nitric oxide is dependent on myelination, but independent of the Na⁺ channel subtype.

Conduction in the spinal cord is highly sensitive to nitric oxide block

Inflammation plays an important role in secondary damage in spinal cord injury and we therefore examined a possible role of nitric oxide in inhibiting excitability in that region of the CNS. Unmyelinated fibers, while present in both dorsal and lateral columns, are concentrated in the lateral funiculus (Chung et al. 1985). We were able to record from myelinated axons in dorsal columns, but signals from unmyelinated fibers in that region were very rare. Dorsal column action potentials were readily blocked reversibly by nitric oxide (Fig. 4A-C). On the other hand, records from lateral columns generally included small signals with conduction velocities ~ 0.4 m/sec (Fig. 4D). In this case, nitric oxide readily

blocked the myelinated component ($96 \pm 2\%$), but the unmyelinated signal declined much less ($13 \pm 4\%$, $P < 0.001$, $n = 3$). While we did not conduct a thorough study, spinal cord myelinated axons were much more sensitive to nitric oxide than all of the other preparations tested. Applying the donor at $0.5 - 0.6$ mM, which was half the concentration used above, the myelinated CAP amplitude declined at more than double the rate of other preparations.

Conduction block in *Shiverer*, a dysmyelinated mutant

Shiverer mice are null for myelin basic protein, and in the optic nerve, axons are only irregularly ensheathed, lamellae are not uniformly compacted, and axoglial junctions are abnormal (Inoue et al. 1981; Rosenbluth 1980; Rosenbluth 1981). Na^+ channel clusters are sparse, but are sometimes found adjacent to Caspr positive paranodes. Rarely, nodal zones with a Na^+ channel cluster between two Caspr positive regions are found (Boiko et al. 2001; Rasband et al. 1999). Total Na^+ channel expression is elevated, but is restricted primarily to $\text{Na}_v1.2$ (Noebels et al. 1991; Westenbroek et al. 1992). These axons could therefore in principle provide a means to judge the level of myelination required for nitric oxide block of conduction. Fig. 5 shows results of these experiments. CAPs from optic nerves of 2-4 month old *Shiverer* mice had multiple components. In Fig. 5 A the three peaks had velocities of 1.0, 0.80, and 0.55 m/s. Exposure to nitric oxide resulted in a general slowing of the record, and a decline primarily in the fastest peak (Fig. 5 B,C). This was partially reversible on washing with ACSF (Fig. 5 D). From these results, it is likely that the only axons blocked were those with the most normal myelin, and containing occasional nodes. Records from *Shiverer* nerves varied in the shape of the CAP, and were therefore difficult to quantitate, but the specific decrease in the fastest component (often just a shoulder) was seen in 6 of the 9 nerves examined. In the 3 other nerves, the shape of the CAP did not allow an unambiguous determination of a myelinated component.

Sensitivity of conduction to alternative perturbations

Is the differential behavior of myelinated and unmyelinated axons unique to nitric oxide, or would one expect similar results with other means of blocking conduction? We tested this with three experimental approaches: high extracellular K^+ , low extracellular Na^+ , and tetrodotoxin (TTX). Each of these would be expected to block conduction, but by different mechanisms. Experiments were done on optic nerves from P14-15 mice, a preparation that provides robust CAP components from both myelinated and unmyelinated axons. In Fig. 6, each column includes at the top a sweep in normal ACSF, several records taken sequentially while the CAP declined, and (for the ion changes) reversibility on washing with normal ACSF. Effects of 200 nM TTX can be reversed somewhat by exhaustive washing, but this was not done routinely. Raising the extracellular concentration of K^+ leads to a depolarization of the resting potential, which alters the threshold for action potentials, and inactivates Na^+ channels. Results are shown in column A of Fig. 6. Both CAP components declined roughly together after switching to the high K^+ solution. Fig. 6, column B shows that after switching to low Na^+ medium, which would reduce the inward current through Na^+ channels during the upstroke of the action potential, again both peaks decreased in amplitude approximately in synchrony. Similar results were obtained in 200 nM TTX, which blocks Na^+ channels with a K_D of 2 nM (Fig. 6, column C). Thus, in all 3 of these perturbations, results were very different from those with nitric oxide. Conduction in

unmyelinated axons was blocked in close association with that in myelinated fibers. Results of these experiments are quantified in Fig. 7 below.

In order to compare results among all of these disparate experiments we calculated the ratio of the fractional decline in the CAP from unmyelinated axons, divided by the fractional decline in the CAP component from myelinated fibers. This parameter would be zero if myelinated axons were exclusively blocked, and would be 1 if both components were blocked equally. Results are shown in Fig. 7. The black bars are all from experiments with nitric oxide, and the gray bars are from trials with high K^+ , low Na^+ , or TTX. The small negative bar in the case of wt P12-P23 optic nerve resulted from a small *increase* in the CAP from unmyelinated axons following exposure to nitric oxide in 2 of the 6 nerves examined. In all 6 preparations nitric oxide preferentially blocked myelinated fibers, while in all 3 tests with other means of blocking conduction, the CAP decline was independent of myelination. In order to test significance we ran t tests on the data for nitric oxide on wt P12-P23 optic nerves versus each of the P14 optic nerve controls (high K^+ , low Na^+ , TTX). The significance shown in the figure ($P < 0.001$) was in the same category for each of the 3 tests. This procedure was then repeated for each of the 5 other nitric oxide experiments in Fig. 7. Again, P values were in the same category for all 3 tests on each of the 5 sets of data.

In order to explore a possible mechanism for the conduction block seen in myelinated fibers with nitric oxide, we measured velocity changes that accompanied the reduction in CAP amplitude. We compared these data with those from the 3 alternative means of blocking conduction. All experiments were done on P12-P16 wt mouse optic nerves. Fig. 8 plots the conduction velocity relative to that before application of the drug/ion change, as a function of the fraction of the CAP amplitude. The data from the nitric oxide experiments are repeated in all 3 scatter graphs. In Fig. 8A the nitric oxide data are compared with those from block with TTX. There is a gradual decline in velocity as the signals are blocked, but no obvious difference between nitric oxide and TTX. In Fig. 8B the comparison is with block by high K^+ . The latter points are scattered, with some indicating a steep decline in velocity, and others relatively little. This is likely to be a consequence of small differences in the initial state (e.g., resting potential) of the fibers. In Fig. 8C there is a clear difference between nitric oxide and low Na^+ . The decline in velocity with low Na^+ is much more pronounced than that with nitric oxide. In Fig. 8D we calculated by interpolation the fractional conduction velocity when the amplitude had declined to 0.3 its initial value. The results show that the difference between the nitric oxide and the low Na^+ data is significant. There are some limitations on our ability to interpret these experiments due to the variability of the high K^+ results. Within these limits, the closest comparison with nitric oxide is that with TTX, suggesting that an interaction with voltage dependent Na^+ channels may be the mechanistic cause of conduction block in myelinated axons.

Can action potentials bridge regions of Na^+ channel block by passive cable properties?

Inflammatory lesions in multiple sclerosis are limited in size. Could action potentials bridge such a region despite Na^+ channel block by nitric oxide, extending to unexposed zones through passive currents via the space constant? We have addressed this issue through the use of a computational model that has proven useful in reproducing electrophysiological

data under a wide variety of conditions (Hines and Shrager 1991; Poliak and Peles 2003; Shrager 1993). Fast Na⁺ channels in DRG neurons were blocked 83% by a NONOate (Renganathan et al. 2002) and we therefore decreased the nodal Na⁺ channel density by that percentage starting at node 5, and continuing for up to 3 more nodes. In axons with diameters > 1 μm, failure required 4 affected nodes. In the smaller axons, conduction block occurred with 2 or 3 affected nodes. An example of the calculated records is shown in Fig. 9. With less than the number of affected nodes required for failure, the lowered Na⁺ channel density caused significant delays in conduction, but signals bridged the affected area. Thus, nitric oxide exposure and Na⁺ channel block over a length typically 0.25 to 1.1 mm, and at most 1.4 mm, was sufficient to end conduction. In contrast, MRI lesions in relapsing-remitting multiple sclerosis cover the range 3 to 20 mm, with a peak in the histogram at 6 mm, and 2 to 15 mm with a peak at 4 mm in secondary progressive MS (Wang et al. 1997). Thus, exposure to nitric oxide within such a lesion could result in a failure of conduction.

Discussion

We showed that in a considerable variety of nerve fibers, in both the CNS and PNS, and at different developmental stages, reversible nitric oxide block of conduction preferentially acts on myelinated axons. There were some changes to the compound action potential from unmyelinated axons: conduction velocity was slowed and, in the PNS, the sharp biphasic signals were added to a graded positive potential, whose origin is unclear. Conduction block, however, was typically only about 10% of that in myelinated fibers. Exposure to nitric oxide for hours, coupled with sustained electrical stimulation, produces Wallerian degeneration in myelinated axons (Smith et al. 2001). It is possible that at higher levels of nitric oxide, or with extended periods of exposure, unmyelinated axons could be further affected, but this was not tested in the present series. The presence of compact myelin is not the sole difference between the two classes of axons. Of particular note, expression patterns of Na⁺ channel subtypes differ markedly. In the CNS, Na_v1.2 is present in unmyelinated axons (Debanne et al. 2011; Gong et al. 1999; Westenbroek et al. 1989), but Na_v1.6 is the primary isoform at nodes of Ranvier, displacing Na_v1.2 during development (Boiko et al. 2001; Caldwell et al. 2000; Tzoumaka et al. 2000). In dysmyelinated axons, e.g., *Shiverer*, Na_v1.2 remains and may be upregulated at high densities (Boiko et al. 2001; Noebels et al. 1991; Westenbroek et al. 1992). In the PNS, Na_v1.6 is again found at high density at nodes of Ranvier, and unmyelinated axons express Na_v1.7, Na_v1.8 and Na_v1.9 (Dib-Hajj et al. 2010; Renganathan et al. 2002). The differential sensitivity of axons to nitric oxide is not likely to be due to these distinctions. In the Na_v1.6 null mouse (*Scn8a*^{tg}) Na_v1.2 is not replaced at nodes, yet nitric oxide blocked conduction specifically in myelinated fibers in both the CNS and PNS. Nitric oxide blocks Na_v1.7, Na_v1.8 and Na_v1.9 in dissociated cells (Li et al. 1998; Renganathan et al. 2002), but it is unclear why it has relatively little effect on conduction in unmyelinated PNS axons. In axons, sensitivity to block by nitric oxide may depend directly on compact myelin, but it is also possible that linking of Na⁺ channels to cytoskeletal elements via ankyrinG at nodes renders them susceptible. Nitric oxide action in other physiological processes often involves intermediates (Stamler et al. 1992a; Stamler et al. 1997).

The preferential block of myelinated axons by nitric oxide was found in both the CNS and PNS. This helps to narrow the possible mechanisms involved. In the CNS, unmyelinated axons are not individually ensheathed by glial processes, though they may be separated into groups by astroglia (Peters et al. 1991). In the PNS, on the other hand, Schwann cells ensheath all axons, but myelinate only the larger fibers. If a biochemical intermediate in myelin participates in the action of nitric oxide in blocking conduction, then it appears to be expressed only when there are multiple lamellae of compact myelin, and not just close contact with glia. This is supported by the results from *Shiverer* mice, in which only the fastest component of the CAP was blocked. This presumably represents the few axons that have regions of compact lamellae and nodes with normal localization of Na⁺ channels and Caspr. These zones could be sporadic, but conduction block need only be at a single site. Are myelinated axons generally more susceptible to conduction block than unmyelinated fibers, i.e., do they have a lower safety factor? Our experiments with high K⁺, low Na⁺, and TTX suggest that is not the case since in all of these situations, both components of the CAP declined concurrently.

Several lines of evidence suggest that conduction block by nitric oxide occurs by modification of Na⁺ channels. Firstly, whole cell patch clamp of both aortic baroreceptor neurons (Li et al. 1998) and dorsal root ganglia neurons (Renganathan et al. 2002) showed that Na⁺ currents can be blocked by nitric oxide released from a NONOate. In the former case, inhibition of I_{Na} was not reversed by removal of the donor, but in the latter study it was. Secondly, in this work, the similarity of action of nitric oxide to that of TTX provides circumstantial evidence. Conduction velocity was slowed significantly more when extracellular Na⁺ concentrations were lowered than it was by nitric oxide or TTX. TTX (and presumably NO) would be expected to block individual Na⁺ channels, leaving unreacted channels to function normally. Lowering Na⁺, on the other hand, would reduce I_{Na} globally. It would also alter several transport processes, including Na⁺ - Ca²⁺ exchange. In some systems, nitric oxide functions by activating guanylyl cyclase to raise levels of cGMP, but in our earlier study, 8-Br-cGMP was ineffective in blocking conduction (Shrager et al. 1998), and it was also ineffective in inhibiting Na⁺ currents in neurons *in vitro* (Li et al. 1998; Renganathan et al. 2002).

The preferential block of conduction in myelinated axons by nitric oxide, at levels commensurate with inflammation, may be important in a number of clinical conditions, including multiple sclerosis and spinal cord injury. There have been numerous studies attempting to modulate symptoms and disease progression in animal models via inhibition of iNOS, but nitric oxide plays several roles in inflammation, some of them beneficial, and it is necessary to develop more specific interventions. The experiments reported here suggest that conduction block involves an indirect interaction with Na⁺ channels, perhaps with compact myelin, or with components associated with the localization of these channels at nodes of Ranvier. If this mechanism can be further defined, it might lead to new targets for therapies that avoid the more global inhibition of nitric oxide action, and specifically improve axonal function.

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Significance

Inflammation is an important pathological component of many neurological diseases, including multiple sclerosis and spinal cord injury. Inflammatory cells synthesize nitric oxide, which has important regulatory roles, but at the elevated concentrations seen in disease, blocks axonal conduction. As shown here, this block occurs preferentially in myelinated fibers. Global inhibition of the enzyme that catalyzes nitric oxide synthesis is not useful as therapy because it also prevents the positive roles. If the molecular mechanism responsible for myelin to mediate conduction block can be determined, it might provide a route for a more specific approach to prevent loss of neuronal function.

Fig. 1. Conduction block by nitric oxide in the vagus nerve

A-C: CAPs from myelinated axons. **A:** before addition of the donor. **B:** 14 minutes after adding DEA NONOate to the bath. **C:** after 7 minutes of washing with ACSF. **D-F:** Sweeps taken at the same time points, respectively, but at higher amplification and slower sampling rate, to record from unmyelinated axons. 4 sweeps at 1 Hz were averaged, and the result was filtered at 0.65 kHz. The gap in each record represents the stimulus artifact alone (**A-C**) or together with the signal from myelinated fibers (**D-F**). **G:** A portion of the record in **E** fitted with a fifth order polynomial. **H:** After subtraction of the fitted curve. The variance was measured over the time indicated by the horizontal line.

Fig. 2. Records from developing optic nerves

A-C: wt, P12. **D-F:** wt, P16 **G-I:** wt, P23 **J-L:** *Scn8a^{tg}/Scn8a^{tg}*(Na_v1.6 null), P16. In each row sweeps are: before drug, after exposure to nitric oxide, and after reversal by ACSF alone. Drug exposure/wash-out times (minutes) are (top to bottom): 15/75, 21/15, 17/25, 21/21.

Fig. 3. Nitric oxide block in sciatic nerve

A-F: wt adult sciatic nerve. **G-L:** *Scn8a^{tg}/Scn8a^{tg}* ($\text{Na}_v1.6$ null), P18 sciatic nerve. In each row sweeps are: before drug, after exposure to nitric oxide, and after reversal by ACSF alone. **A-C** and **G-I:** CAPs from myelinated axons. **D-F** and **J-L:** corresponding records from unmyelinated fibers. **D-F:** filtered 0.65 kHz. **J-L:** filtered 2.0 kHz. Drug exposure/wash-out times (minutes) are (top to bottom): 42/9, 19/18.

Fig. 4. Records from spinal cord

A-C: Sweeps before, after nitric oxide, and after ACSF, from wt mouse dorsal columns. The CAP is solely from myelinated fibers. **D-F:** Corresponding records from lateral columns.

The arrow in **D** points to a small signal from unmyelinated axons. **G-I:** Amplified records of the unmyelinated CAPs in **D-F**. Note that in recording from tissue (spinal cord, brain slices) with extracellular electrodes pressed on the surface and the reference electrode in the bath, depolarizations appear as downward deflections, opposite to that with suction electrodes.

Drug exposure/wash-out times (minutes) are (top to bottom): 4/12, 11/21.

Fig. 5. Results from *Shiverer* optic nerves (age 14 weeks)

A: Before nitric oxide. **B,C:** 8 and 15 minutes, respectively, after addition of DEA NONOate to the bath. **D:** After 10 minutes of perfusion with ACSF alone.

Fig. 6. Conduction block by TTX or by changes in ion concentrations

All records are from P15 wt mouse optic nerves. In each column, the top sweep is the CAP in normal ACSF ($K^+_o = 2.5$ mM, $Na^+_o = 151.25$ mM) before the indicated perturbation. The next several sweeps are taken in succession as changes develop. Column **A**: $K^+_o = 25$ mM, $Na^+_o = 128.75$ mM. Column **B**: $K^+_o = 2.5$ mM, $Na^+_o = 26.25$ mM, $TMA^+_o = 125$ mM. Column **C**: TTX = 200 nM. The bottom record in columns **A** and **B** shows reversal after perfusion with ACSF.

Fig. 7. Quantitation of results on CAP amplitude

The fractional decline in the CAP from unmyelinated axons divided by the fractional decline in the CAP from myelinated axons is plotted for each of the 6 protocols with nitric oxide (black bars) and the 3 protocols with alternative methods of conduction block (gray bars). These ratios were calculated when the myelinated decline was typically ~90%. Student's two-tailed t test was applied to each of the 6 nitric oxide groups *versus* each of the 3 alternative groups. The latter 3 *P* values were in the same category (* *P* < 0.05, ** *P* < 0.01, *** *P* < 0.001) in each of the 6 nitric oxide protocols, and so are indicated once for each group. Negative values occur if the unmyelinated CAP increases slightly in nitric oxide. Values > 1 occur if the decline in the CAP from unmyelinated axons exceeded that from myelinated axons.

Fig. 8. Changes in conduction velocity accompanying amplitude decline. Scatter graphs plot the fractional change in velocity *versus* the fractional amplitude

A: Nitric oxide compared with TTX. **B:** Nitric oxide compared with high K^+_o (25 mM). **C:** Nitric oxide compared with low Na^+_o (26.25 mM). **D:** Fractional velocity at a fractional amplitude of 0.3 (***) $P < 0.001$).

Fig. 9. Calculated action potentials for a model CNS fiber, axon diameter 1 #m
 Signals are shown for nodes 4, 6, 8, and 10 of a fiber with 12 nodes, stimulated at node 1. Altered nodes have their Na^+ channel density lowered by 83%. **A:** no nodes altered. **B:** node 5 altered. **C:** nodes 5 and 6 altered. **D:** nodes 5, 6, and 7 altered.