SUPPLEMENTARY MATERIALS

*Neutralizing a Surface Charge on the FMN Subdomain Increases the Activity of Neuronal Nitric Oxide Synthase by Enhancing the Oxygen Reactivity of the Enzyme Heme-Nitric oxide Complex **

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Materials and Methods for Redox Potentiometry-

Sample preparation and redox titrations were carried out in a glove box (Bell Technology) under nitrogen atmosphere with oxygen levels below 5 ppm as described previously (22). The E762N nNOSr protein concentration was 30–40 μM containing either EDTA (1 mM) or $CaCl_2$ (2 mM) + CaM (60–80 μM) in 40 mM EPPS buffer, pH 7.6, 10% Glycerol, and 150 mM NaCl. Absorption spectra were recorded in Cary 50 using a dip probe detector, and the potentials were monitored using Accumet AB15 coupled to a silver/silver chloride electrode saturated with 4 M KCl. Measurements were done in a custom-made glass beaker kept in a water bath at 15 ± 1 °C. A reductive titration was performed by stepwise addition of sodium dithionite, whereas an oxidative titration was done by adding potassium ferricyanide. The redox mediators with midpoint potentials in the range of flavin potentials used were 0.5–1 μM of phenazine methasulfate (+80 mV), indigo carmine (-125 mV), 2-hydroxy-1,4-naphthoquinone (-152 mV), anthraquinone-2,6-disulfide (-184 mV), anthraquinone-2-sulfonate (-225 mV), phenosafranin (-252 mV), safranin O (-280 mV), benzyl viologen (-348 mV), and methyl viologen (-443 mV). The electrode was calibrated against phenosafranin (-252 mV) and the potential of a 5 mM solution of ferricyanide/ferrocyanide in 0.1 M potassium phosphate (pH 7.0), at 25 °C (+425 mV). A correction factor of (+199 mV) was obtained, which is in good agreement with the reported potential of the electrode. Based on the temperature dependence of the electrode potential, a correction factor of (+209 mV) at 15 °C was used. The absorption changes at 457 nm and 600 nm were plotted with electrochemical potentials (mV). The midpoint potentials were calculated using the following four-electron Nernst equation (Equation S1), where *A* is the absorbance; *a–e* are the relative absorbance values contributed by the diflavin in each of five nondegenerate oxidation states; *E* is the observed system potential, and $E'_1 - E'_4$ are the four midpoint potentials, two for each flavin.

Equation S1:

$$A = \frac{\left(a10^{(2E-E'_1-E'_2)/57} + b10^{(E-E'_2)/57} + c + d10^{(E'_3-E)/57} + e10^{(E'_3+E'_4-2E)/57}\right)}{\left(1 + 10^{(2E-E'_1-E'_2)/57} + 10^{(E-E'_2)/57} + 10^{(E'_3-E)/57} + 10^{(E'_3+E'_4-2E)/57}\right)}$$

Table S1

Kinetic values for NOS proteins

Values of the k_r, k_{ox}, and k_d parameters were determined at 10 °C. k_r, rate of ferric heme reduction; k_d, dissociation rate of the ferric heme-NO complex; k_{ox}, rate of reaction between the ferrous heme-NO complex and approximately 140 μM O_2 (half air-saturated conditions).

Enzyme	Fe^{III} Heme Reduction (k_r, s^{-1})	Fe^{III} -NO Reduction ($k_{r'}$, s^{-1})	Fe^{II} -NO Oxidation (k_{ox}, s^{-1})	Fe^{III} -NO Dissociation (k_d, s^{-1})
nNOS	3.9-4.8	3.6	0.2	5.0
iNOS	1.0	1.0	3.0	2.0
eNOS	0.1	0.1	0.6	3.5

Table S2

Rates of ferrous-NO complex reaction with O_2 and ferric enzyme formation.

Anaerobic ferrous-NO enzymes were reacted with air-saturated solution in the stopped flow instrument at 10 °C. The rates of absorbance decrease at 436 nm and increase at 393 nm were used to determine ferrous-NO complex disappearance and ferric enzyme formation, respectively. Experiments were done in the presence of three different salt concentrations. The values are representative of two separate experiments.

Enzyme	With 50 mM NaCl		With 150 mM NaCl		With 250 mM NaCl	
	k_{436} (s^{-1})	k_{393} (s^{-1})	k_{436} (s^{-1})	k_{393} (s^{-1})	k_{436} (s^{-1})	k_{393} (s^{-1})
nNOS-wt	0.092 ± 0.008	0.089 ± 0.009	0.092 ± 0.005	0.09 ± 0.006	0.084 ± 0.008	0.09 ± 0.008
E762N Fl	0.145 ± 0.01	0.14 ± 0.012	0.15 ± 0.01	0.16 ± 0.011	0.155 ± 0.012	0.165 ± 0.015

Supplemental Figure S1

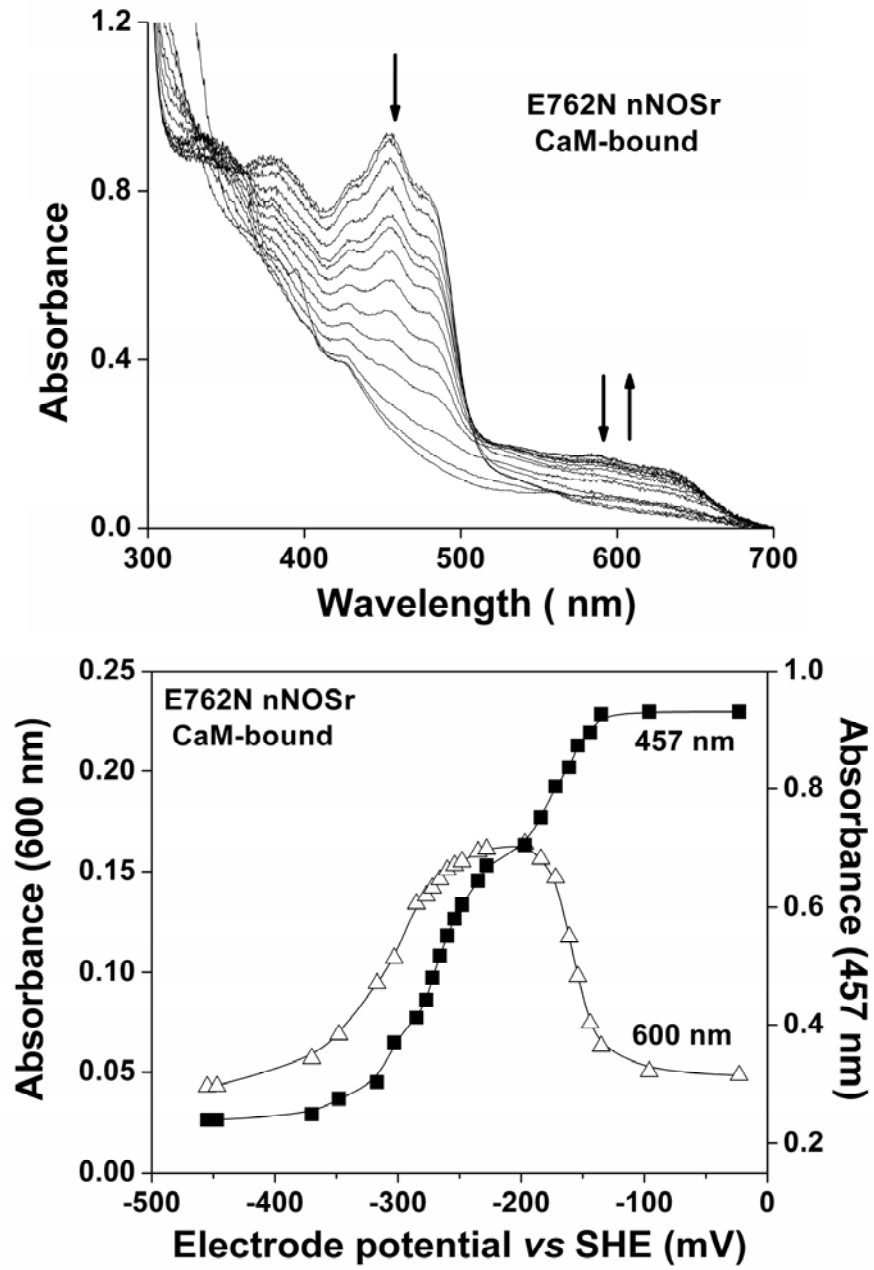

Fig. S1. Potentiometric titration of E762N nNOSr flavins in the presence of CaM at 15 °C. *upper panel,* representative set of visible spectra obtained during potentiometric titration of CaM-bound E762N nNOSr with sodium dithionite. *Lower panel,* plots of absorbance at 457 nm (*solid squares*) and at 600 nm (*open triangles*) *versus* the electrochemical potential (mV) for CaM-free E762N nNOSr along with the lines of best fit as calculated using the four electron Nernst equation described under "Experimental Procedures." Data are representative of at least two experiments.

Supplemental Figure S2

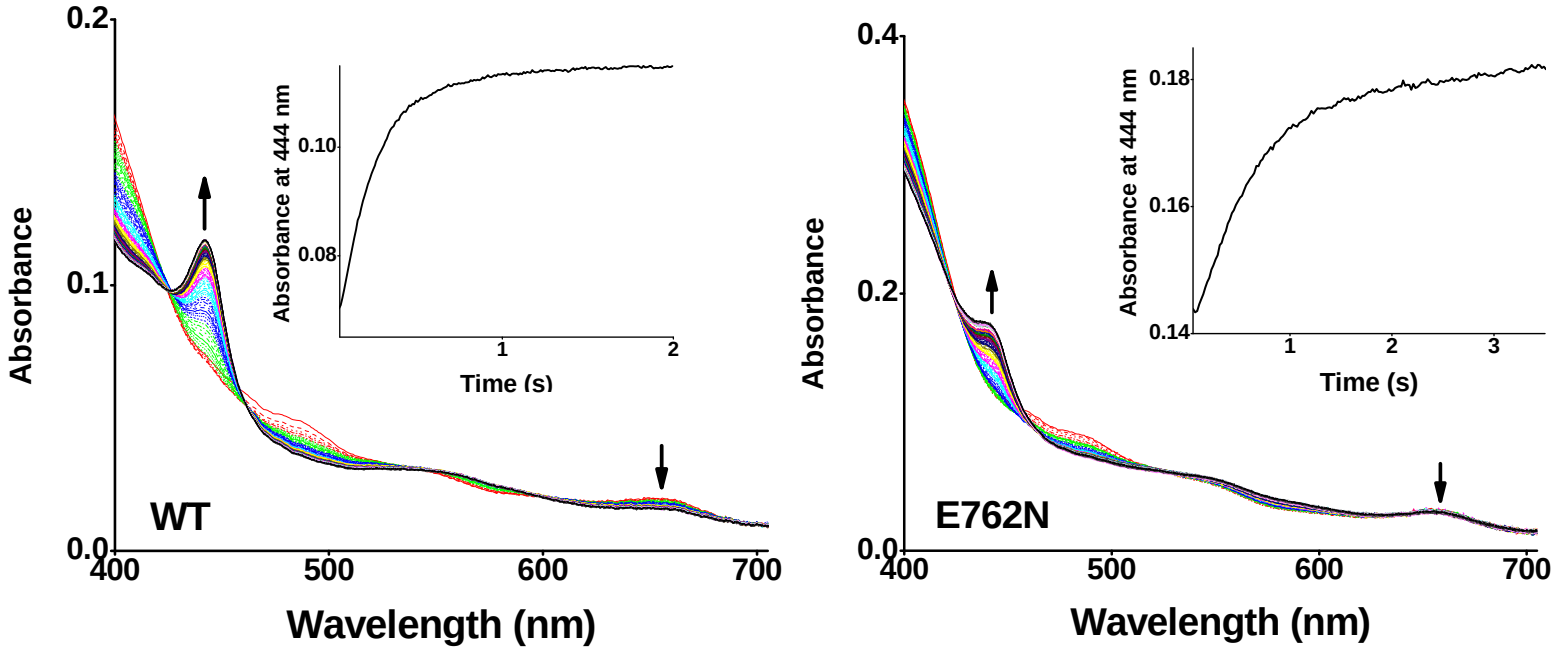

Fig. S2. ***Rate and Extent of Heme Reduction-*** The kinetics and extent of heme reduction were analyzed at 10 °C using a stopped-flow apparatus as described under "Materials and Methods". Ferric heme reduction was followed by formation of the ferrous heme-CO complex at 444 nm. Left panel shows the spectral traces for WT nNOS whereas the right panel shows spectral traces for E762N nNOS mutant. Kinetic traces are shown as insets.