# Elimination of Aggressive Behavior in Male Mice Lacking Endothelial Nitric Oxide Synthase

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Male mice with targeted deletion of the gene encoding the neuronal isoform of nitric oxide synthase (nNOS $^{-/-}$) display increased aggressive behavior compared with wild-type (WT) mice. Specific pharmacological inhibition of nNOS with 7-nitroindazole also augments aggressive behavior. We report here that male mice with targeted deletion of the gene encoding endothelial NOS (eNOS $^{-/-}$) display dramatic reductions in aggression. The effects are selective, because an extensive battery of behavioral tests reveals no other deficits. In the resident–intruder model of aggression, resident eNOS $^{-/-}$ males show virtually no aggression. Latency for aggression onset is 25–30 times longer in eNOS $^{-/-}$ males compared with WT males in the rare instances of aggressive behaviors. Similarly, a striking lack of aggression is noted in tests of aggression among groups of four mice monitored in neutral cages. Although eNOS $^{-/-}$ mice are hypertensive (~14 mmHg blood pressure elevation), hypertension does not appear responsible for the diminished aggression. Reduction of hypertension with hydralazine does not change the prevalence of aggression in eNOS $^{-/-}$ mice. Extensive examination of brains from eNOS $^{-/-}$ male mice reveals no obvious neural damage from chronic hypertension. *In situ* hybridization in WT animals reveals eNOS mRNA in the brain associated exclusively with blood vessels and no neuronal localizations. Accordingly, vascular eNOS in the brain appears capable of influencing behavior with considerable selectivity.

*Key words: aggression; hypertension; 7-nitroindazole; nitroarginine; nitric oxide synthase; endothelium*

Nitric oxide (NO) is formed by three distinct enzymes coded by different genes, neuronal NO synthase (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS) (Jaffrey and Snyder, 1995). Although nNOS is the primary neuronal form of the enzyme, there have been reports of eNOS in neurons (Dinerman et al., 1994; O'Dell et al., 1994; Doyle and Slater, 1997), and genetic manipulations of eNOS may influence hippocampal long-term potentiation (Son et al., 1996). Male mice with targeted deletion of the gene for nNOS (nNOS $^{-/-}$) (Nelson et al., 1995) and mice treated with 7-nitroindazole, an *in vivo* selective inhibitor of nNOS (Demas et al., 1997) display marked increases in aggressive and sexual behavior, The nNOS $^{-/-}$ mice also manifest defects in nocturnal balance coordination (Kriegsfeld et al., 1999). Initial studies of eNOS $^{-/-}$ mice revealed hypertension but grossly normal behavior (Huang et al., 1995; Sheseley et al., 1996).

We now report virtual elimination of aggressive behavior in male eNOS $^{-/-}$ mice, as well as improved fine motor coordination suggesting reciprocal behavioral roles for eNOS and nNOS. Our *in situ* hybridization studies reveal eNOS mRNA in blood vessels but not in neuronal populations in the brain.

## MATERIALS AND METHODS

*Animals and housing conditions.* The eNOS $^{-/-}$ mice were derived from animals originally developed by Huang et al. (1995). Controls included littermates of the eNOS $^{-/-}$ mice as well as C57/B16 mice that were bred in the same colony. Behavioral measures for the two groups did not differ so that they were combined for statistical analyses. All animals were 4–6 months of age (sexually mature) at the onset of behavioral testing. Animals were individually housed in polypropylene cages (27 × 17 × 12 cm) in a colony room with a 24 hr light/dark 16/8 photoperiod (lights on at 7 A.M. Eastern Standard Time). Ambient temperature was maintained at 20 ± 2°C, and relative humidity was maintained at 50 ± 5%. Food (Prolab 100; Agway, Syracuse, NY) and tap water were provided *ad libitum* throughout the experiment.

*Resident–intruder aggression.* An adult stimulus male mouse (i.e., intruder) was introduced into the home cage of either an experimental or control adult male mouse. Intruder mice were selected randomly from a pool of individually housed breeder males. These males tended not to attack but would respond aggressively if attacked. Intruder mice were marked on the tail with an indelible marker for purposes of identification. The bedding in the home cages was not changed for 10 d before behavioral testing. The latency to first aggressive encounter, the duration of each aggressive encounter, and the total number of aggressive encoun-

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ters initiated by the resident male were recorded. Aggression tests lasted 5 min and were conducted each day for 3 consecutive days between 3 and 5 P.M. A novel pairing of animals was made for each consecutive test, and intruder males were not used more than once per day.

*Grouped aggression in neutral arena.* Four adult wild-type (WT) or eNOS$^{-/-}$ mice were simultaneously introduced into a clear glass aquarium (38.5 × 26.5 × 30.7 cm). The floors of the aquarium were covered with 2–3 cm of fresh pine shavings. The latency to first aggressive encounter, the duration of each aggressive encounter, and the total number of aggressive encounters initiated by each male were recorded. Aggression tests lasted 15 min and were conducted in 1 d between 3 and 5 P.M.

*Sensorimotor tests.* A battery of sensorimotor tests was used to assess sensory function and motor coordination and balance (Nelson et al., 1995; Crawley and Paylor, 1997). A series of standardized tests to assess anxiety, including tests using an open field and elevated plus maze, was also conducted. Briefly, to assess open field activity, a mouse was placed in an open arena (1 m$^2$). An observer recorded the movement of the mouse during the testing period. The floor was marked off by 16 squares, and the number of squares crossed was counted. Also, the amount of time spent in the open field (inner squares) was compared with the time the mouse moved along the walls of the arena. This test was conducted for 10 min. Incidents of grooming and rearing and number of fecal boli produced were recorded. Mice were also placed in the center of an elevated plus maze with two open arms and two closed arms (67 × 5.5 cm). The closed arms contained a 15-cm-high, black-tinted Plexiglas panel and a 65-cm-long detachable roof. The maze was mounted 75 cm above the floor on a tripod. Choice behavior was observed for 5 min, and the number of visits to each arm and the time spent in each arm as well as in the central area were recorded. Incidents of grooming and rearing and number of fecal boli produced were also recorded.

In situ *hybridization.* In situ hybridization was conducted as previously described (Blackshaw and Snyder, 1997). The cRNA probe used corresponded to the region covering 2583–3499 bp of murine eNOS (Gnanapandithen et al., 1996). This technique uses a digoxigenin label so that resolution is comparable with immunohistochemistry and substantially better than with a radiolabeled probe. Control hybridization with sense probes provided negligible signals. Regions selected for analysis were those in which neuronal localizations of eNOS had been previously reported (Dinerman et al., 1994; O'Dell et al., 1994; Doyle and Slater, 1997). Sections (20–30) were selected at random for examination with the experimenter blinded to the identity of the mice, WT or eNOS$^{-/-}$.

## RESULTS

In routine handling of eNOS$^{-/-}$ mice we note marked docility. Thus, in contrast to WT mice, the eNOS$^{-/-}$ animals never bite their handlers and are much gentler in disposition. This contrasts markedly with nNOS$^{-/-}$ animals, which are extremely aggressive and routinely attack handlers and each other. In groups of animals, agonistic behavior is extremely rare among eNOS$^{-/-}$ male mice.

To examine this behavior in greater detail, we investigated intermale aggression using a resident–intruder model, an experimental model of territorial aggressive behavior (Thurmond, 1975). We focused our studies on males, because female mice typically do not display aggressive behavior in this context. We scored a variety of aggressive behaviors including wrestling, biting, and tail rattling (Fig. 1). The number ($t_{(22)}$ = 4.57; $p < 0.01$)
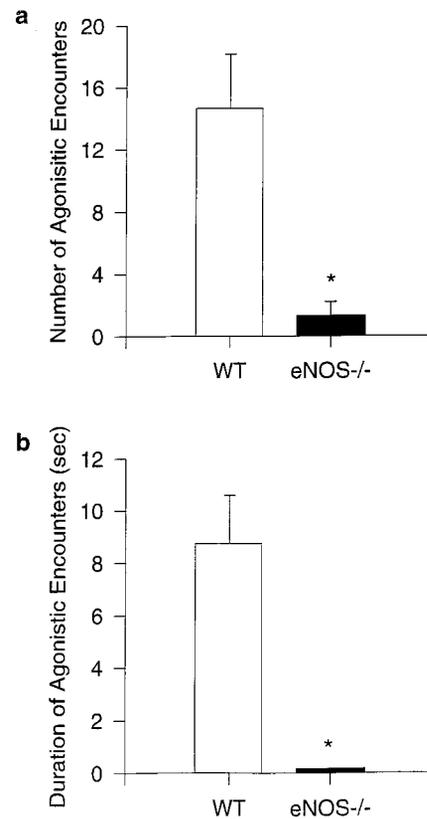
*Figure 1.* Aggressive behavior in WT ($n = 12$) or eNOS$^{-/-}$ ($n = 12$) mice in a resident–intruder model. *a*, Mean ± SEM number of aggressive encounters; *b*, mean ± SEM duration of aggressive encounters. Data were analyzed by $t$ test. *Statistically significant difference at $p < 0.001$.

of aggressive encounters and their duration ($t_{(22)}$ = 4.66; $p < 0.01$) are profoundly reduced in the eNOS$^{-/-}$ animals, whose values are not significantly different from zero. The latency to initiate an aggressive encounter is markedly increased for the very few aggressive encounters displayed by eNOS$^{-/-}$ animals ($p < 0.001$).

To evaluate nonterritorial aggression, we observed groups of WT or eNOS$^{-/-}$ males together in a large cage (Fig. 2). In this group test, as in the resident–intruder aggressive model, aggressive behavior is virtually eliminated in the eNOS$^{-/-}$ animals when monitored in terms of the number ($t_{(10)}$ = −5.093; $p < 0.001$) of aggressive encounters and their duration ($t_{(10)}$ = 0.558; $p > 0.05$). The latency ($t_{(10)}$ = 5.21; $p < 0.001$) for the limited number (3.00 ± 1.3) of attacks by the eNOS$^{-/-}$ mice is 25–30 times greater than for WT (60.5 ± 11) animals.

The eNOS$^{-/-}$ mice are derived from two parent strains, C57 and SV 129 (Huang et al., 1995). The depicted experiments (Figs. 1, 2) compared the mutant mice with WT C57 animals. In other experiments in the same paradigms we detect no difference in aggressive behavior between WT C57 and SV 129 mice or C57-SV 129 hybrid strains (data not shown), confirming our earlier observations (Nelson et al., 1995). Thus, the dramatic reduction of aggression in eNOS$^{-/-}$ mice does not merely reflect strain variations.

Because the blood pressure of eNOS$^{-/-}$ animals is ~14 mmHg higher than that of WT controls (Huang et al., 1995; Sheseley et al., 1996), we speculated that the decreased aggressive behavior might reflect hypertension. This seems unlikely, because in models of hypertension that are genetically determined (Potegal and Myers, 1989) or elicited by long-term treatment with sodium
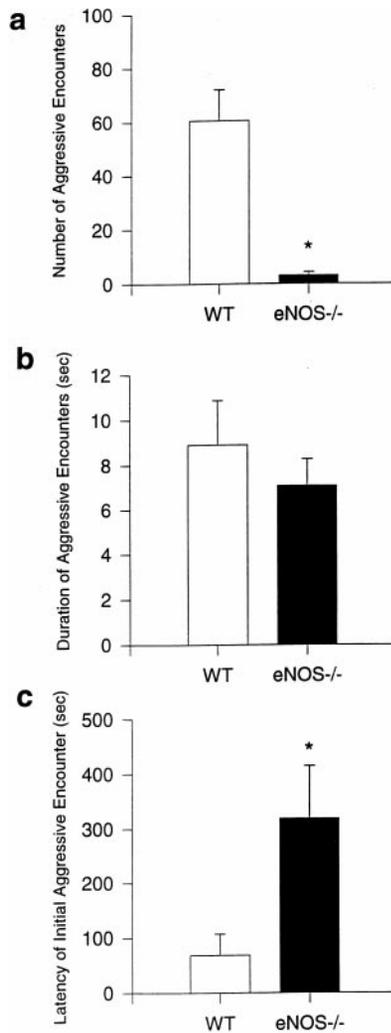
*Figure 2*. Aggressive behavior in WT or eNOS$^{-/-}$ mice in a grouped aggression model. Six groups of WT and six groups of eNOS$^{-/-}$ mice were evaluated, with four mice in each group. *a*, Mean $\pm$ SEM number of aggressive encounters; *b*, mean $\pm$ SEM duration of aggressive encounters; *c*, mean $\pm$ SEM latency to initial attack. Symbols, conventions, and statistical analysis are as in Figure 1.

chloride and corticosterone (Simon and Gandelman, 1978), aggressive behavior is generally increased; furthermore, there are no reports of decreased aggression associated with any form of hypertension in animals. To assess whether the eNOS$^{-/-}$ animals display hypertensive encephalopathy, we examined sealed sections of all regions of the brains of the mutant mice with Nissl and DAPI stains and found no morphological differences compared with WT mice (data not shown), similar to previous findings (Kantor et al., 1996; Son et al., 1996).

Although there is no practical way of reversing whatever effects are elicited by lifelong hypertension, we did make an effort to ascertain whether reducing blood pressure of the eNOS$^{-/-}$ animals would affect behavior. We treated the eNOS$^{-/-}$ animals with a regimen of hydralazine administration shown previously to render eNOS$^{-/-}$ animals normotensive (Huang et al., 1995). For 10 d eNOS$^{-/-}$ and WT mice received hydralazine in the drinking water (250 mg/l, resulting in ~1 mg/d). We examined aggressive behavior in the resident–intruder neutral arena and group aggression models. No change in aggressive behavior is evident in the behavioral tests (Fig. 3). eNOS$^{-/-}$ mice continue to show dra-
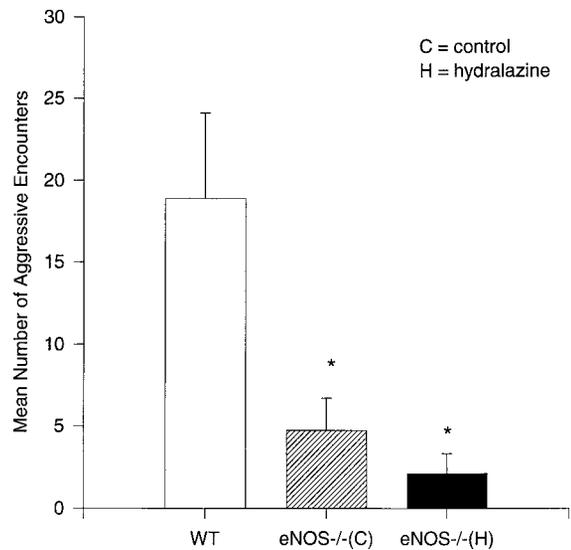


*Figure 3*. Hydralazine treatment does not alter aggressive behavior of eNOS$^{-/-}$ mice. Mice received hydralazine in their drinking water (1 mg/d) for 10 d. On the 10th day aggressive behavior was monitored in resident–intruder area and group aggression models. Numbers of animals in groups were as indicated in Figures 1 and 2. Hydralazine does not alter aggressive behavior of WT or eNOS$^{-/-}$ mice as analyzed by omnibus $F$ test.

matically reduced aggressive behavior relative to WT mice, and eNOS$^{-/-}$ mice rendered normotensive with hydralazine do not differ from eNOS$^{-/-}$ controls.

In addition to abnormalities in sexual and aggressive behavior in male nNOS$^{-/-}$ mice, apparent forelimb strength is decreased, as assessed by the latency to fall from a suspended wire (Nelson et al., 1995). By contrast, we observe a modest increase in forelimb strength in eNOS$^{-/-}$ animals (Table 1). Coordinated performance of eNOS$^{-/-}$ animals is also enhanced in terms of the ability to turn in a blind alley. Apparent alterations in fine motor coordination, such as the ability to turn in a blind alley, could conceivably reflect "emotional" disturbances such as anxiety. However, in anxiety models, such as the elevated plus maze, eNOS$^{-/-}$ animals do not differ from WT mice (Table 1). The

**Table 1. Performance of eNOS$^{-/-}$ and WT mice in sensorimotor tasks designed to test motor ability, agility, balance–coordination, sensory ability, and strength**

| Task | WT (sec) | | eNOS$^{-/-}$ (sec) | |
|---|---|---|---|---|
| Olfactory ability | 359 | $\pm$ 65 | 379 | $\pm$ 66 |
| Turning in an alley | 23.5 | $\pm$ 2.9 | 12.9 | $\pm$ 2.5* |
| Turning on an inclined screen | 8.58 | $\pm$ 2.1 | 6.83 | $\pm$ 1.1 |
| Initiation to walk | 27.5 | $\pm$ 7.3 | 18.6 | $\pm$ 5.1 |
| Balance on a 1 cm pole | 46.5 | $\pm$ 13 | 36.0 | $\pm$ 10 |
| Balance on a 2 cm plank | 115 | $\pm$ 3.5 | 104 | $\pm$ 10 |
| Forelimb strength | 22.9 | $\pm$ 5.4 | 70.8 | $\pm$ 14** |
| Elevated plus maximum | | | | |
| Duration (sec) | | | | |
| Closed arms | 156 | $\pm$ 19 | 128 | $\pm$ 30 |
| Central arena | 128 | $\pm$ 22 | 159 | $\pm$ 30 |
| Visits (no.) | | | | |
| Closed arms | 4.2 | $\pm$ 0.8 | 4.4 | $\pm$ 0.9 |
| Central arena | 5.7 | $\pm$ 1.4 | 5.9 | $\pm$ 1.5 |

Data are mean values $\pm$ SEM for groups of 12 mice.

*,** eNOS$^{-/-}$ differs from Wt; *$p < 0.02$; **$p < 0.001$.
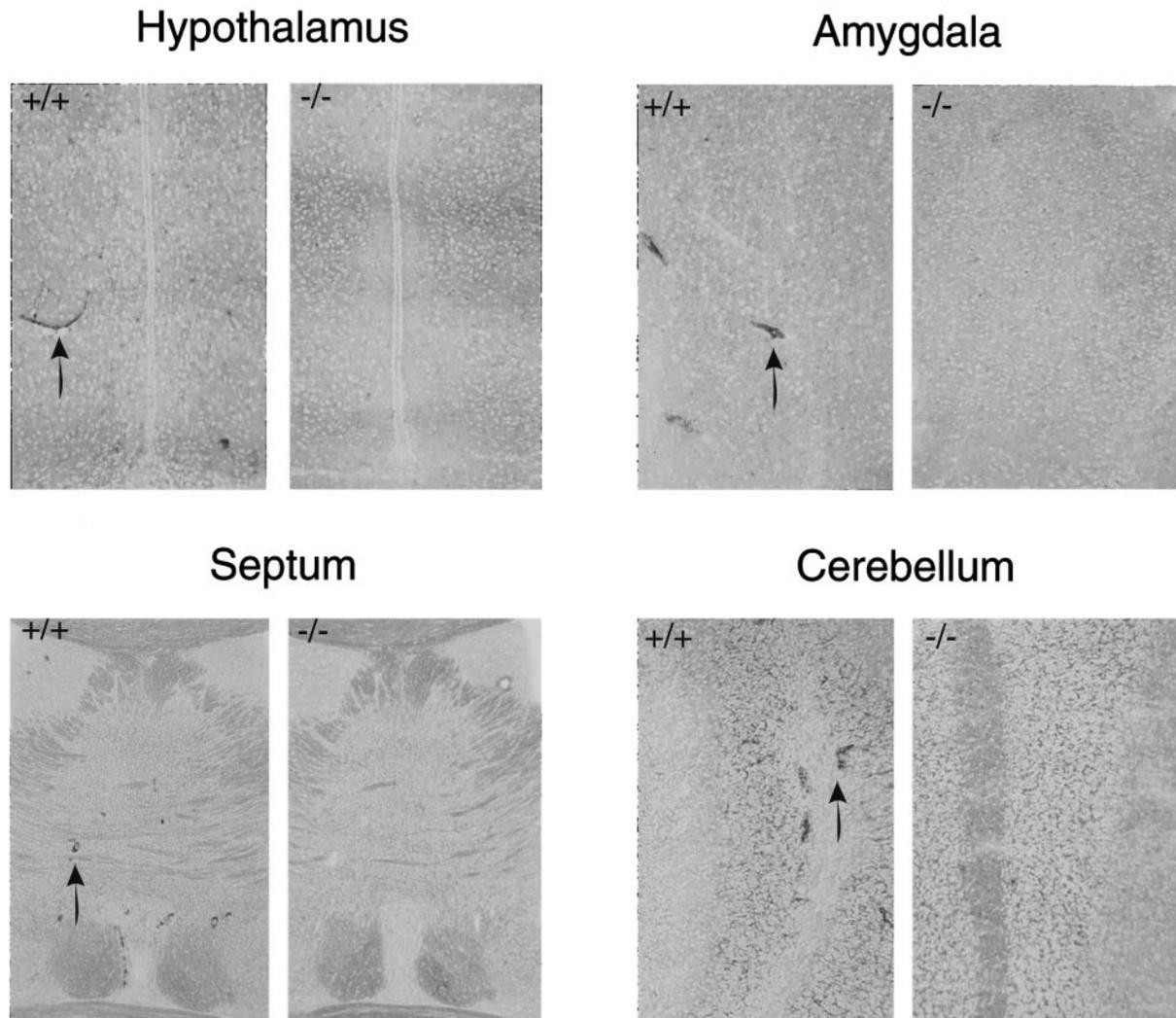
# eNOS expression in wildtype and eNOS knockout mice



*Figure 4.* *In situ* hybridization of endothelial nitric oxide synthase in selected brain regions of WT and eNOS $^{-/-}$ mice. Magnification, 100×, except for the septum, which is 50×. *Arrows* indicate selected eNOS-positive blood vessels. A slight background signal, which does not represent specific hybridization, is seen in white matter of the cerebellum and septum.

alterations in aggressive behavior, forelimb strength, and motor coordination do not seem to derive from generalized behavioral alterations, because most behaviors examined do not differentiate eNOS $^{-/-}$ mice from WT animals. Thus, we find no differences in the ability to initiate walking, olfactory ability, visual acuity, tactile behavior, balance, or body mass.

The pronounced alterations of aggressive behavior, as well as apparent forelimb strength and fine motor coordination, in eNOS $^{-/-}$ animals might be associated with alterations in putative neuronal populations using eNOS. eNOS is primarily localized to the endothelium of blood vessels throughout the body, including the brain. However, we (Dinerman et al., 1994; O'Dell et al., 1994) and others (Doyle and Slater, 1997) have reported putative eNOS by immunohistochemistry and NADPH diaphorase staining in some neuronal populations, whereas others have identified eNOS only in blood vessels in the brain (Seidel et al., 1997; Stanarius et al., 1997; Topel et al., 1998). Limitations of such immunohistochemical studies primarily involve the possibility of

cross-reactivity of eNOS antibodies with other forms of NOS or with unrelated antigens. NADPH diaphorase staining reflects oxidative enzyme activity using NADPH as an electron donor and so can involve enzymes other than NOS. *In situ* hybridization using appropriate probes can be much more selective. Because aggressive behavior is presumably mediated primarily through portions of the limbic system, we performed *in situ* hybridization focusing on the amygdala, the hypothalamus, and the septum, as well as the cerebellum (Fig. 4). The probe we used is a cRNA probe, which, under the high stringency conditions used, is highly specific for eNOS (see Materials and Methods). In all areas of the brain examined, we detect eNOS signal associated with blood vessels but fail to observe any signal in neurons.

## DISCUSSION

The most dramatic finding of this study is the virtual elimination of aggressive behavior in eNOS $^{-/-}$ animals. Additionally, the eNOS $^{-/-}$ mice display enhanced forelimb strength and apparent

fine motor coordination. It is noteworthy that these results are opposite to those observed in nNOS$^{-/-}$ animals that display extraordinary augmentation of aggressive behavior and defects in nocturnal balance coordination and forelimb strength (Nelson et al., 1995; Kriegsfeld et al., 1999).

The behavioral alterations we observe do not appear to reflect any type of nonspecific, general behavioral disruption. We evaluated an extensive sensorimotor repertoire and find no notable abnormalities in eNOS$^{-/-}$ animals. By direct observation, the gross appearance and overall behavior of the eNOS$^{-/-}$ animals do not differ from those of WT mice (Huang et al., 1995; Sheseley et al., 1996). The absence of any gross behavioral abnormalities is consistent with our failure to observe any neuroanatomical defect despite careful examination of multiple sections from many brain regions, similar to findings of others (Huang et al., 1995; Sheseley et al., 1996).

To ascertain the cellular substrate for any behavior associated with eNOS, we conducted *in situ* hybridization with a highly specific probe and observe label in blood vessels but not in neurons. In earlier studies using an antibody directed against eNOS peptide, we observed neuronal localizations (Dinerman et al., 1994; O'Dell, 1994). As controls, the earlier studies observed elimination of staining with preabsorption using the peptide and the absence of staining with preimmune serum. However, these types of controls do not rule out cross-reactivity with other proteins. Recent immunohistochemical studies using a number of selective eNOS antibodies reveal staining confined to blood vessels (Seidel et al., 1997; Stanarius et al., 1997; Topel et al., 1998). In our own recent studies we also observed immunoreactivity for eNOS restricted to blood vessels in the brain (S. Blackshaw, M. Eliasson, and S. H. Snyder, unpublished observations).

How might eNOS in blood vessels exert such selective influences on behavior? eNOS does not occur uniformly in all vessels. It tends to be most enriched in large and medium-size blood vessels, although there is some eNOS in small arterioles and veins, which lack smooth muscle (Catalan et al., 1996; Seidel et al., 1997; Stanarius et al., 1997; Topel et al., 1998). The selective regulation of behavior by NO derived from blood vessels is highlighted by the absence of observable morphological alterations in the brain, as well as the selectivity of the behavioral changes. Furthermore, the observation that behavior is improved in measures such as general motor coordination and forelimb strength argues against hypertensive encephalopathy as an explanation of the altered behaviors.

The apparent reciprocal regulation of behavior by nNOS and eNOS in the brain is puzzling, especially because one of the enzymes is neuronal and the other is associated with blood vessels. However, the fact that cerebral blood vessels of all sizes show extensive innervation (Kobayashi et al., 1985; MacKenzie and Scatton, 1987; Iadecola et al., 1993; Ruat et al., 1995) raises the possibility that NO derived from eNOS located in endothelial cells of the brain may diffuse to nearby neurons and influence their activity. This notion is supported by data demonstrating the importance of NO derived from eNOS in the control of inhibitory neurotransmitter release in mouse cerebral cortex (Kano et al., 1998).

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