Letter to Neuroscience

NOCTURNAL MOTOR COORDINATION DEFICITS IN NEURONAL NITRIC OXIDE SYNTHASE KNOCK-OUT MICE

L. J. KRIEGSFELD, M. J. L. ELIASSON, G. E. DEMAS, S. BLACKSHAW, T. M. DAWSON, R. J. NELSON* and S. H. NYDERS*

*Department of Psychology, The Johns Hopkins University, Baltimore, MD 21218, U.S.A.
Departments of †Neurology, ‡Pharmacology and Molecular Sciences, and §Psychiatry and Behavioral Sciences, The Johns Hopkins University School of Medicine, 725 N. Wolfe Street, Baltimore, MD 21205 (410), U.S.A.

Key words: balance, daily, rhythm, diurnal.

Nitric oxide is formed in the brain primarily by neurons containing neuronal nitric oxide synthase (nNOS), though some neurons may express endothelial NOS (eNOS), and inducible NOS (iNOS) only occurs in neurons following toxic stimuli. Mice with targeted disruption of nNOS (nNOS⁻) display distended stomachs with hypertrophied pyloric sphenoid reflecting loss of nNOS in myenteric plexus neurons. nNOS⁻ animals resist brain damage following middle cerebral artery occlusion consistent with evidence that excess release of nitric oxide mediates neurotoxicity in ischemic stroke.

Neuronal NOS⁻ mice have no grossly evident defects in locomotor activity, breeding long-term depression in the cerebellum, long-term potentiation in the hippocampus, and overall sensorimotor function. However, nNOS⁻ animals display excessive, inappropriate sexual behavior and dramatic increases in aggression. Because the cerebellum possesses the greatest levels of nNOS neurons in the brain, it was surprising that presumed cerebellar functions such as balance and coordination were grossly normal in nNOS⁻ mice. These previous studies were all conducted during the day (between 1400 and 1600, lights on at 0700). We now report striking, discrete abnormalities in balance and motor coordination in nNOS⁻ mice reflected selectively at night.

For wild-type (WT) and nNOS⁻ mice, overall wheel running activity is much greater at night than day, with no difference between the two groups (Table 1). Detailed circadian locomotor analysis (Fig. 1) shows WT and nNOS⁻ animals both initiating activity promptly at lights-out with the most intense locomotor activity during the initial portion of the dark phase. When the onset of the dark period is delayed 4 h, both WT and nNOS⁻ animals promptly accommodate. In constant light, nocturnal rodents typically display free-running cycles longer than 24 h with diminished locomotor activity. Both WT and nNOS⁻ animals manifest diminished activity under these conditions with lengthened periods. The mean periods in constant light are 24.9 h for WT and 25.0 h for nNOS⁻ animals.

Neuronal nitric oxide synthase (nNOS) is concentrated in the olfactory bulb with the accessory bulb possessing the highest density of nNOS in the brain. To assess olfaction, we monitored the latency to find a hidden cookie, which was modestly greater at night than during the day (Table 1). This may reflect increased feeding at night leading to lower motivation to seek food. WT and nNOS⁻ animals did not differ in olfactory performance in day or night. Forelimb strength assessed by the latency for mice to fall from a suspended wire, did not differ between WT and nNOS⁻ mice during the day or night (data not shown).

Balance/coordination was assessed by measuring the latency for mice to fall from a small pole or plank (Table 1). As the pole was narrower, this became a more difficult task. WT animals fell from the pole after about 15 and 55 s in day and night, respectively, indicating improved performance at night. In the plank experiment WT animals displayed latencies of about 20 and 80 s, respectively, in day and night. These findings are consistent with the well-known nocturnal activity patterns of rodents. Strikingly, the nNOS⁻ animals showed no improvement in their
Table 1. Behavioral and sensorimotor data for wild type and neuronal nitric oxide synthase \(-/-\) mice tested during either light (day) or dark (night) portion of the dark cycle

<table>
<thead>
<tr>
<th>Task</th>
<th>WT (Day)</th>
<th>WT (Night)</th>
<th>nNOS(^{-/-}) (Day)</th>
<th>nNOS(^{-/-}) (Night)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balance on a pole (Latency to Fall)</td>
<td>1.3(± 4.5)</td>
<td>54.6(± 16.0)*</td>
<td>17.8(± 2.2)</td>
<td>14.4(± 5.8)**</td>
</tr>
<tr>
<td>Balance on a plank (Latency to Fall)</td>
<td>21.4(± 5.8)</td>
<td>77.2(± 17.4)*</td>
<td>30.9(± 5.6)</td>
<td>23.6(± 6.0)**</td>
</tr>
<tr>
<td>Wheel running (Revolutions/h)</td>
<td>29.7(± 9.4)</td>
<td>477.8(± 92.9)*</td>
<td>10.1(± 6.0)</td>
<td>474.2(± 143.0)*</td>
</tr>
<tr>
<td>Olfactory ability (Latency to find a cookie)</td>
<td>267.3(± 34.6)</td>
<td>437.8(± 63.0)*</td>
<td>302.7(± 27.1)</td>
<td>403.0(± 58.6)</td>
</tr>
<tr>
<td>Visual placing score</td>
<td>1.0(± 0.0)</td>
<td>-</td>
<td>1.0(± 0.0)</td>
<td>-</td>
</tr>
</tbody>
</table>

WT and nNOS\(^{-}\) mice exhibit the same nocturnal increase in activity during the dark portion of the light:dark (LD) cycle. Mice were individually housed in polycarbonate cages equipped with an activity wheel. Wheel revolutions were monitored continuously by computer (Dataquest III; Mini Mitter Inc., Sunriver, OR). Cumulated counts were recorded every 10 min. Animals were maintained in an LD 14:10 photoperiod (lights on at 06.00) for three weeks to assess activity during entrainment to a LD cycle. nNOS\(^{-}\) mice display selective deficits in balance and coordination relative to WT mice when tested during the dark portion of the LD cycle. Mice were placed at the center of a 60-cm-long wooden bridge suspended 60 cm above a foam pillow. Mice were placed onto the pole with all four leg securely positioned onto the pole. Animals were tested in dim red light. Unsurpassable borders were placed on both ends of the pole to prevent the animals reaching safety. A foam pillow was placed 60 cm beneath the pole. The mice were tested using both a pole (2 cm in diameter; Test 1) and a plank (2 cm wide; Test 2). The latency to fall (up to 120 s) was recorded. Olfactory ability, as measured by the latency to find a cookie hidden beneath 3-6 cm of wooden shavings, did not differ between WT and nNOS mice when tested during either the light or dark portions of the LD cycle. nNOS\(^{-/-}\) mice did not exhibit any deficits in visual acuity (P>0.05). Each mouse was lowered slowly towards the edge of a table. A positive score was recorded if the animal extended its forepaws before touching the table on two or more of the three trials. * Differs from day values of the same genotype (P<0.05). ** Differs from WT mice tested during the dark portion of the LD cycle (P<0.05).

Fig. 1. Representative activity records of a WT and nNOS\(^{-}\) mouse. Light:dark (LD) bars depicted at the top of each record indicate initial photoperiodic conditions. Mice were individually housed in polycarbonate cages equipped with an activity wheel. Cumulated counts were recorded every 10 min. At the onset of the study animals were maintained in a LD 14:10 photoperiod (lights on at 06.00) for three weeks. Subsequently, behavior was “phase-delayed” by delaying the photoperiod by 4 h (LD 14:10; lights on at 10:00). Animals remained in this photoperiod for two weeks. Finally, animals were maintained in constant dim light (60 lux) for a three-week duration.

performance at night. Thus, compared to WT animals their latency at night in the pole test was 26% of the WT, while their latency at night in the plank test is 30% of the WT.

It is difficult to link individual behaviors to specific brain regions in rodents. The normal daytime balance/coordination in nNOS\(^{-}\) animals indicates that nocturnal deficits do not reflect gross neural disturbances. Abnormalities in motivation are likely not to be the explanation, as motivated olfactory behavior is normal in nNOS\(^{-}\) animals. Overall levels of sexual motivation, alertness, motor activity, and strength cannot explain the abnormalities, as a separate assessment of these behaviors revealed no disturbance in the nNOS\(^{-}\) animals. Deficits in balance/coordination at night are also unlikely to be the result of loss in visual acuity as a separate analysis of visual ability revealed no deficit in nNOS\(^{-/-}\) mice (Table 1). Thus, the balance/coordination deficits
most likely stem from disturbances of nitric oxide in the cerebellum or associated areas.

$
n$NOS occurs throughout the brain. Despite a fairly extensive evaluation of numerous behaviors,\textsuperscript{10} thus far only aggressive or sexual behavior, which is presumably associated with the limbic system, and balance/coordination, which is cerebellar-dependent, have been demonstrably disrupted in $n$NOS$^-$ mice. Might $n$NOS in the limbic system and the cerebellum respond to the targeted gene disruption differently than other brain areas? $n$NOS$^-$ mice were created by disrupting exon 2, which is absent in two alternatively spliced forms of nNOS termed $\beta$ and $\gamma$ that are retained in $n$NOS$^-$ mice and which account for a major portion of total $n$NOS in certain brain regions.\textsuperscript{2,6} We wondered whether $n$NOS might be differentially distributed in the cerebellum, limbic and other areas in the $n$NOS$^-$ mice. We compared expression of total $n$NOS in cerebellum, amygdala and corpus striatum of WT and $n$NOS$^-$ animals (Fig. 2). Although $n$NOS protein and mRNA have been previously characterized in $n$NOS$^-$ mice,\textsuperscript{6} this study provides a more detailed characterization in the cerebellum and amygdala. The corpus striatum of $n$NOS$^-$ mice displays only about a 50% loss in $n$NOS positive neuronal cells, as previously
noted. By contrast, cerebellar and amygdala staining is abolished in nNOS–/– mice. Thus, the behavioral abnormalities observed in nNOS–/– animals involve areas where gene disruption has depleted all enzyme protein. Conceivably, residual nNOS, which is prominent in areas such as the cerebral cortex and corpus striatum, protects nNOS–/– mice from more extensive abnormalities.

Because the cerebellum contains the highest density of nNOS in the brain, the lack of abnormal motor coordination in initial studies was surprising.10 Our present findings are the first indicating a role for nitric oxide, perhaps from the prominent cerebellar nNOS system, in regulating balance/coordination. The restriction of balance/coordination impairment to night-time may reflect a greater stress on cerebellar systems during the heightened nocturnal motor activity. Even though rodents are nocturnal animals, most behavioral studies, including learning, are conducted during the light period. Our findings emphasize the importance of examining diurnal variations, especially in gene knock-out research.

Acknowledgements—This Study was supported by USPHS Grant MH 57535 to R.J.N., a Gustavus and Louise Pfeiffer Scholarship to M.J.L.E., and USPHS Grant MH 18501 and Research Scientist Award DA-00074 to S.H.S.

REFERENCES


Fig. 2. (p. 313) Immunohistochemistry and in situ hybridization reveal selective retention of nNOS expression in nNOS–/– mice. Both nNOS protein (b) and mRNA (d) can be detected in the corpus striatum of nNOS–/– mice. The nNOS immunoreactivity in the corpus striatum is localized to perikarya (arrows) and lower in intensity compared to WT mice (a). The number of nNOS–/– immunoreactivity and nNOS mRNA-containing cells in the nNOS–/– mice is, however, comparable to WT mice (a–d), as previously reported. In contrast nNOS expression is undetectable in the cerebellum (f; h; n=5), and amygdala (i; j; n=5) of nNOS–/– mice. St, striatum; Cb, cerebellum; Am, amygdala; M, molecular-cell layer; G, granule-cell layer; f, f, nNOS–/– mice. (In situ plates 200 × ; immunohistochemistry plates 100 × ). Mice were perfused through the left ventricle with 37°C oxygenated Krebs-Henseleit buffer followed by 250 ml of 37°C 5% glutaraldehyde/0.5% paraformaldehyde containing 0.2% Na₂SO₄ in 0.1 M sodium phosphate (pH 7.4). Brains were postfixed in the same buffer for 2 h at room temperature, and cryoprotected for two days at 4°C in 50 mM sodium phosphate, pH 7.4/0.1 M NaCl/20% (vol/vol) glycerol. Brain sections (20-40 μm) were cut on a sliding microtome. Free-floating brain sections were reduced for 20 min with 0.5% NaBH₄ and 0.2% Na₂SO₄ in phosphate-buffered saline (PBS) (10 mM, pH 7.4/0.1M NaCl) to reduce background staining due to the glutaraldehyde fixation. After washing for 45 min at room temperature in PBS containing 0.2% Na₂SO₄, sections were blocked with 4% normal goat serum for 1 h in the presence of 0.2% Triton X-100, and incubated overnight at 4°C with the primary antiserum diluted in PBS containing 2% goat serum and 0.1% Triton X-100. A polyclonal antiserum to the C-terminal region of human NOS (resides 1419-1433) was kindly provided by Jeffrey Spangenberg (IncStar, Stillwater, MN) and used at a 1:15,000 dilution of crude serum. Immunoreactivity was visualized with the Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA) using 3,3′-diaminobenzidine–HCl as chromogen. The specificity of the antisera and the detection assay were tested by preabsorption with the antigen and omission of the primary antibody, respectively. Probes for digoxigenin in situ hybridization corresponding to residues 4196-5057. The sequence was subcloned into pBluescript and sense and antisense cRNA probes were generated by T3 and T7 RNA polymerases. For in situ hybridization, fresh-frozen 20-μm cryostat sections were cut onto Superfrost Plus slides (Fisher), allowed to air dry for 1-3 h, postfixed 5 min in 4% paraformaldehyde in PBS, washed 3 × 3 min in Tris-buffered saline (TBS), treated 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine pH 8.0, washed 3 × 3 min in TBS, prehybridized 2 h at room temperature in hybridization buffer (50% formamide, 5% standard saline citrate (SSC), 5 × Denhardt’s solution). 500 mg/ml sonicated herring sperm DNA, 250 mg/ml MRE (rRNA) without probe. Buffer (0.1 ml) containing 40 ng of cRNA probe was then added, covered with a siliconized coverslip, and hybridized at 65°C overnight. After coverslips were allowed to come off in 5 × SSC at 65°C, sections were then washed at 2 × 1h in 0.2 × SSC, 5 min at room temperature in 0.2 × SSC, 5 min at room temperature in TBS, blocked 1h at room temperature in 4%NGS/TBS at room temperature, incubated overnight at 4°C in 1:5000 dilution of anti-DIG Fab fragment (Boehringer) in 4%NGS/TBS. Sections were then washed 3 × 5 min in TBS, 5 min AP buffer (0.1 M Tris–Cl, pH 9.5, 0.1 M NaCl, 50 mM MgCl₂). Color was developed in AP buffer containing 3.375 mg/ml NBT (Boehringer), 3.5 mg/ml BCIP (Boehringer), and 0.24 mg/ml levamisole. Color reaction was carried out in the dark at room temperature for 24–72 h. Reaction was stopped in TE, and slides were coated with Aquapolymount (Polysciences).

Sections hybridized with identical amounts of sense cRNA in both cases yielded no specific signal.

(Accepted 2 October 1998)