Brain serotonin dysfunction accounts for aggression in male mice lacking neuronal nitric oxide synthase

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Genetically engineered mice with targeted disruption of the neuronal nitric oxide synthase (nNOS) gene established the inhibitory role of nitric oxide (NO) in male impulsive aggressive behavior. This was later confirmed by using selective nNOS inhibitors in male wild-type mice. The molecular mechanisms accounting for the aggressive behavior caused by the lack of neuronally derived NO is not known. Recent studies suggest that central serotonergic neuronal circuits and particularly 5-HT_{1A} and 5-HT_{1B} receptors play a prominent role in the regulation of aggression. Accordingly, we investigated whether the aggressiveness caused by the lack of nNOS might be because of alterations in serotonergic function. We now demonstrate that the excessive aggressiveness and impulsiveness of nNOS knockout mice is caused by selective decrements in serotonin (5-HT) turnover and deficient 5-HT_{1A} and 5-HT_{1B} receptor function in brain regions regulating emotion. These results indicate an important role for NO in normal brain 5-HT function and may have significant implications for the treatment of psychiatric disorders characterized by aggressiveness and impulsivity.

The gene encoding neuronal nitric oxide synthase (nNOS) was disrupted and inactivated by homologous recombination to clarify the functional role of nitric oxide (NO) as a messenger molecule in the nervous system (1). Mice lacking the gene for nNOS ($nNOS^{-/-}$) exhibit a variety of abnormalities including enlargement of the stomach (1), hypertrophied urinary bladders (2), reduced parasympathetic tone (3), and nocturnal motor deficits (4). In addition, male $nNOS^{-/-}$ mice display dramatic increases in aggressive and sexual behavior (5). Pharmacological treatment of male wild-type (WT) mice with selective nNOS inhibitor also evokes an increase in impulsive aggressive behavior to the levels displayed by $nNOS^{-/-}$ mice (6), indicating that the aggressive behavior is caused by loss of neuronally derived NO and not developmental abnormalities. nNOS is enriched throughout the limbic system (7, 8), an area important in emotional and agonistic behaviors and thus nNOS-derived NO is appropriately positioned to play a prominent role in regulating impulsive and aggressive behavior. Despite the evidence which implicates neuron-derived NO in the modulation of aggressive behavior, the molecular mechanisms by which NO regulate aggression are not known. Plasma androgen concentrations can affect the display of aggressive behavior in male mice, but testosterone concentrations in $nNOS^{-/-}$ and WT mice are similar (9).

Previous studies of the brain mechanisms underlying aggression reveal that central serotonergic neuronal circuits are prominent in the regulation of aggression. For instance, a negative correlation exists between serotonin (5-hydroxytryptamine; 5-HT) metabolites and impulsive aggressiveness in humans (10–12) and rodents (13, 14). Pharmacological manipulations that increase 5-HT neurotransmission decrease the severity of aggressiveness in humans (15) and reduce aggression in animal models (16, 17). Studies with selective 5-HT receptor agonists and genetically engineered mice strongly implicate the 5-HT_{1A}, 5-HT_{1B}, or both receptor subtypes in aggression (18–22). Here we investigate the relationship of 5-HT and NO in aggression and report that the excessive aggressiveness and impulsivity of male $nNOS^{-/-}$ mice is caused by selective decrements in 5-HT turnover and deficient 5-HT_{1A} and 5-HT_{1B} receptor function in brain regions regulating emotion.

Materials and Methods

Animals. Adult (3- to 5-month-old) male $nNOS^{-/-}$ and WT C57BL/6J mice from a breeding colony established at The Johns Hopkins University by using animals previously produced by homologous recombination (1) were singly housed in polycarbonate cages (28 × 17 × 12 cm) with ad libitum access to food (Prolab 2000; Agway, Syracuse, NY) and tap water. The $nNOS^{-/-}$ mice have been backcrossed to the C57BL/6J mice at least eight generations. Male young adult CD-1 mice (Charles River Breeding Laboratories) socially housed in groups of five per cage were used as intruders. All animals were maintained on a light/dark 16:8 photoperiod (lights on 700 h) with an ambient temperature of 22 ± 2°C and relative humidity of 50 ± 5% throughout the duration of the study. The behavioral tests as well as the decapitations were performed at the same period of the light cycle (1300–1800 h).

Drugs. The 5-HT precursor 5-hydroxy-DL-tryptophan ethyl ester hydrochloride (5-HTP) and the 5-HT synthesis inhibitor DL-*p*-chlorophenylalanine methyl ester hydrochloride (pCPA) were purchased from Sigma and dissolved in 0.9% saline solution. The 5-HT_{1A} agonist (±)-8-hydroxy-2-(di-*n*-propylamino)tetralin hydrobromide (8-OH-DPAT), purchased from Research Biochemicals (Natick, MA), was dissolved in 0.9% saline solution. The 5-HT_{1B} agonist 3-(1,2,5,6-tetrahydro-4-pyridyl)-5-propoxypirolo(3,2-*b*)pyridine (CP-94,253), a gift from Charles Pfizer (Groton, CT) was dissolved in 2.5% DMSO/2.5% Tween 20 in 0.9% saline solution. All drugs were made fresh on the day of use and injected in a volume of 1 ml/100 g of body weight. The HPLC standards 3-hydroxytyramine hydrochloride, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid, 3,4-dihydroxybenzylamine hydrobromide, and 5-HT were purchased from Sigma; 5-hydroxyindole-3-acetic acid (5-HIAA)

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Abbreviations: nNOS, neuronal nitric oxide synthase; 5-HT, serotonin (5-hydroxytryptamine); WT, wild type; 5-HTP, 5-hydroxy-DL-tryptophan ethyl ester; pCPA, DL-*p*-chlorophenylalanine methyl ester; 8-OH-DPAT, (±)-8-hydroxy-2-(di-*n*-propylamine)tetralin hydrobromide; CP-94,253, 3-(1,2,5,6-tetrahydro-4-pyridyl)-5-propoxypirolo(3,2-*b*)pyridine; 5-HIAA, 5-hydroxyindole-3-acetic acid.

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and norepinephrine bitartrate were purchased from Research Biochemicals.

Aggression Test (Resident–Intruder Paradigm). The home cages of resident mice (nNOS$^{-/-}$ or WT) were not changed during the last week of the isolation period of 3–4 weeks. A young adult stimulus male mouse (intruder) was introduced into the home cage of the resident, and the latency to first attack bite, the total number of attack bites, and the total duration of attack episodes (bursts of bites, sideways threats, and rough grooming) initiated by the resident male was recorded for 900 s in the first confrontation. Each resident mouse was used only once and the intruder mice were used only once a day. The test was videotaped overhead and scored by two observers unaware of either the genotype or experimental treatment. For the 5-HT$_{1A}$ and 5-HT$_{1B}$ agonist treatments, other WT or nNOS$^{-/-}$ isolated mice (residents) were previously exposed to an intruder each 3 days in a total of four confrontations of 15 min each. Previous studies have shown that the high variability of the initial confrontation is reduced after some encounters (23). Experimental tests were conducted at 3-day intervals with counterbalanced sequence of doses.

Open-Field Activity. Each mouse was placed in the center of an acrylic open arena (1 m^2) with the floor marked off in 42 squares. The frequency of locomotion (number of squares entered with all four paws) and rearing (number of times the mouse stood on its hindlimbs) were recorded during 300 s. The floor of the arena was washed with a 5% alcohol solution between each trial. Each trial was videotaped and scored by two observers uninformed of the experimental conditions.

Motor Coordination and Balance. Each mouse was placed in a separate lane of a rotarod (Economex, Columbus Instruments, Columbus, OH) on a 5-cm diameter rotating cylinder at 5 rpm. The performance was recorded as the mean latency to fall from the rod (up to 300 s) in three consecutive trials with a 5-min resting period between each trial.

HPLC Analyses. The nNOS$^{-/-}$ and WT mice were decapitated in random order and several brain structures were dissected as follows: hypothalamus, cerebellum, midbrain, hippocampus, amygdala, and cerebral cortex. They were immediately frozen in dry ice, and stored at −70°C until use. The tissues were thawed, weighed, and ultrasonically homogenized in 0.1 M cold perchloric acid/0.01% ascorbic acid and known amounts of 3,4-dihydroxybenzylamine hydrobromide as internal control. After centrifugation at 16,000 × g for 20 min at 4°C, the supernatants were filtered (0.45 μm) and injected (20 μl) onto the HPLC column [BAS (West Lafayette, IN) Biophase ODS 5 μm, 250 × 4.6 mm]. The mobile phase consisted of filtered 150 mM monochloroacetic acid/200 mg/liter sodium octyl sulfate/0.1 mM EDTA/4% acetonitrile/2.5% tetrahydrofuran (pH 3.0) and the flow rate was set at 1.5 ml/min. 5-HT, 5-HIAA, norepinephrine bitartrate, 3-hydroxytyramine hydrochloride, 4-hydroxy-3-methoxyphenylacetic acid, and 3,4-dihydroxyphenylacetic acid were electrochemically detected by using a working potential of 800 mV (BAS LC-4C amperometric detector with a glassy carbon electrode coupled to a Dynamax SD-200 system). The concentrations were calculated by reference to standard curves run daily.

5-HT Immunocytochemistry. Nine mice (four WT and five nNOS$^{-/-}$) were anesthetized with 400 mg/kg chloral hydrate and transcardiacally perfused with cold PBS (pH 7.4) solution followed by cold 4% paraformaldehyde. The brains were removed from the skull and postfixed for 4 h in the same cold fixative, and cryoprotected for 1 day in cold 20% sucrose in PBS and 1 day in cold 30% sucrose in PBS. The brains were frozen and cut at 30 μm (sagitally or coronally) on a freezing sliding microtome. The sections were incubated for 1 h in blocking solution (6% normal goat serum/0.2% Triton X-100/5% dry nonfat milk) and for 2 days with rabbit anti-5-HT antibody (1:10,000; Incstar, Stillwater, MN) in a cold room on a rotator. After washes in PBS, the sections were incubated for 40 min in biotinylated goat anti-rabbit secondary antibody (1:200), exposed to an avidin-biotin complex (Vector IgG Elite Kit), and processed in diaminobenzidine as a chromogen. The sections were mounted on subbed slides, allowed to dry overnight, and amplified by silver staining.

Pharmacological Manipulations of the 5-HT Turnover. Seventy mice (35 each genotype) were used for the 5-HT precursor studies. 5-HTP was administered i.p. in doses of 50 and 100 mg/kg 30 min before the tests. Fifty-two mice (26 each genotype) were used for the inhibition of the 5-HT synthesis. pCPA was injected i.p. in a single dose of 300 mg/kg 72 h before the aggression tests (1 × 300) or a regimen of daily injections of 300 mg/kg 24, 48, and 72 h before testing (3 × 300). The time point was selected based on previous studies that documented the peak of 5-HT decrease is obtained at 72 h after pCPA injection (24).

5-HT$_{1A}$ and 5-HT$_{1B}$ Agonists Dose Effects. The 5-HT$_{1A}$ agonist 8-OH-DPAT was administered s.c. in doses of 0.050, 0.075, and 0.100 mg/kg 20 min before the aggression test. The 5-HT$_{1B}$ agonist CP-94,253 was injected i.p. in doses of 2.5, 5.0, and 10.0 mg/kg 30 min before testing. Every animal (n = 8 WT and 8 nNOS$^{-/-}$ for 8-OH-DPAT and n = 8 WT and 9 nNOS$^{-/-}$ for CP-94,253) received each agonist dose once in a random order. One week after aggression tests, the same animals were evaluated in the open field and rotarod with the drugs and doses arbitrarily assigned.

Results

nNOS$^{-/-}$ Mice Have Selective Alterations in 5-HT Metabolism. To ascertain whether nNOS$^{-/-}$ mice have altered 5-HT metabolism, we compared 5-HT and its metabolite 5-HIAA levels in nNOS$^{-/-}$ and WT mice. Total tissue content of 5-HT is increased in the cerebral cortex (20.6%), hypothalamus (13.0%), hippocampus (14.2%), midbrain (16.1%), and cerebellum (40.4%) ($P < 0.05$ in each case) with no concomitant changes in 5-HIAA ($P > 0.05$) in nNOS$^{-/-}$ mice. The 5-HIAA/5-HT ratio, an indicator of 5-HT turnover, is reduced in the cortex (18.1%), hypothalamus (18%), midbrain (16.4%), and cerebellum (30.4%) of nNOS$^{-/-}$ mice ($P < 0.01$ for cerebellum and $P < 0.05$ for other areas; Fig. 1). Norepinephrine, 3-hydroxytyramine, 3,4-dihydroxyphenylacetic acid, and 4-hydroxy-3-methoxyphenylacetic acid are not altered in most brain areas studied, including cortex, hypothalamus, hippocampus, and midbrain ($P > 0.05$; data not shown). Thus, it is unlikely that alterations in monoamine oxidase account for the abnormalities in 5-HT in the nNOS$^{-/-}$ mice.

Augmentation of 5-HT Neurotransmission in nNOS$^{-/-}$ Mice Reduces Aggressive Behavior. To determine whether the alterations in 5-HT levels and turnover account for the aggressive behavior of nNOS$^{-/-}$ mice, we examined the effects of changing 5-HT levels with established pharmacological methods for altering 5-HT metabolism. Treatment of WT and nNOS$^{-/-}$ mice with the 5-HT precursor 5-HTP significantly increases 5-HT and 5-HIAA levels and dramatically increases 5-HT turnover. In conjunction with an increase in 5-HT levels and turnover, we observe a dramatic reduction in the aggressive behavior of nNOS$^{-/-}$ mice in the resident-intruder paradigm. As reported (5), the nNOS$^{-/-}$ mice are more aggressive than WT mice, exhibiting increased number and duration of attacks, as well as reduced latency for

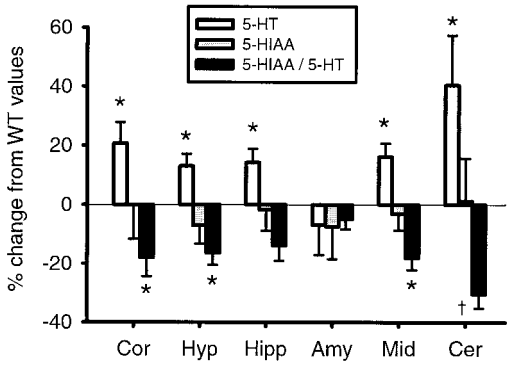

Fig. 1. 5-HT, 5-HIAA, and 5-HT turnover (5-HIAA/5-HT ratio) determinations by HPLC in cerebral cortex (Cor), hypothalamus (Hyp), hippocampus (Hipp), amygdala (Amy), midbrain (Mid), and cerebellum (Cer) of nNOS$^{-/-}$ mice as compared with WT. The levels of 5-HT, 5-HIAA (ng/mg tissue), and 5-HT turnover in WT mice are as follows (±SEM): Cor = 0.349 ± 0.012, 0.134 ± 0.005, 0.386 ± 0.018 (n = 6); Hyp = 1.200 ± 0.038, 0.683 ± 0.043, 0.569 ± 0.032 (n = 6); hipp = 0.614 ± 0.019, 0.351 ± 0.027, 0.571 ± 0.038 (n = 6); Amy = 0.807 ± 0.056, 0.364 ± 0.023, 0.470 ± 0.060 (n = 6); Mid = 0.949 ± 0.023, 0.557 ± 0.035, 0.586 ± 0.031 (n = 5); Cer = 0.093 ± 0.005, 0.100 ± 0.005, 1.094 ± 0.086 (n = 6), respectively. In nNOS$^{-/-}$ mice, n = 6 for Hyp, Hipp, Mid, and Cer; n = 5 for Cor and Amy. Data are percent change in relation to WT mice ± SEM; *, $P <$ 0.05 by Student's t test.

the first attack (comparison of WT and nNOS$^{-/-}$ mice treated with saline, $P < 0.01$ and $P < 0.05$; Fig. 2 *A* and *B*). 5-HTP treatment reduces the number and duration, and increases the

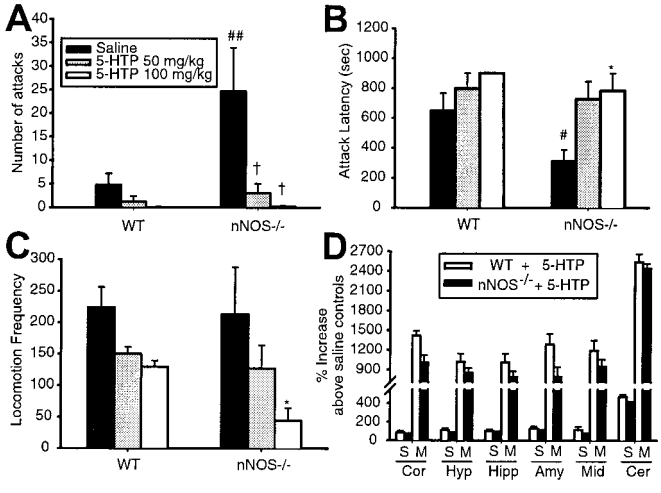

Fig. 2. Reduction in aggressive behavior and increase in 5-HT metabolism by the precursor 5-HTP. (*A* and *B*) Aggressive behavior as measured by the resident–intruder test 30 min after 0.9% saline solution, 50 mg/kg or 100 mg/kg 5-HTP i.p. injections in WT and nNOS$^{-/-}$ mice (n = 6 each group). (*C*) Locomotor activity in an open field 30 min after i.p. injections in different animals (saline and 5-HTP 100 mg/kg, n = 6 in each genotype; and 5-HTP 50 mg/kg, n = 5 in each genotype). *, $P < 0.05$; and †, $P < 0.01$ in relation to the saline group in the same genotype; and #, $P < 0.05$; and ##, $P < 0.01$ in relation to WT same treatment. Data are means ± SEM and were analyzed by using two-way ANOVA (genotype × treatment) with post hoc Tukey test. (*D*) HPLC determinations of 5-HT (S) and its metabolite 5-HIAA (M) in the cerebral cortex (Cor), hypothalamus (Hyp), hippocampus (Hipp), amygdala (Amy), midbrain (Mid), and cerebellum (Cer) of WT and nNOS$^{-/-}$ mice (n = 5 each genotype) 45 min after 100 mg/kg 5-HTP. Data are percent increase above saline groups in each genotype (n = 6 each; means ± SEM). The percent of turnover increase (5-HIAA/5-HT) ranged from 346% to 646% in these brain areas. All data in *D* are $P < 0.01$ in relation to saline controls (two-way ANOVA with post hoc Tukey test).

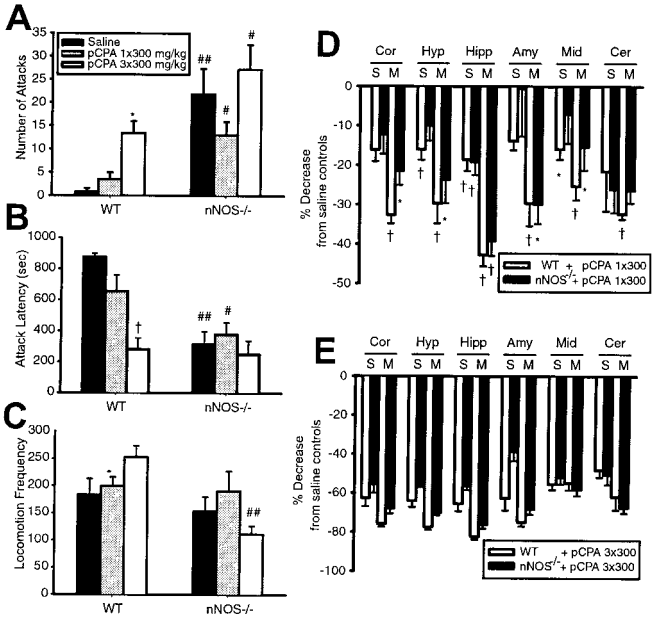

Fig. 3. Increase in aggressive behavior and 5-HT reduction by the 5-HT synthesis inhibitor pCPA. (*A* and *B*) Aggressive behavior as measured by the resident–intruder test 72 h after 0.9% saline solution (single injection, n = 5 for each genotype; three daily injections, n = 4 each genotype; group collectively named as saline because no differences are detected), single dose of 300 mg/kg pCPA (1 × 300, n = 10 each genotype) or three doses of 300 mg/kg pCPA (72, 48, and 24 h before tests, 3 × 300, n = 7 each genotype) i.p. injections in WT and nNOS$^{-/-}$ mice. (*C*) Locomotor activity in an open field 74 h after first i.p. injection (n = 6–10 in each group). *, $P < 0.05$; †, $P < 0.01$ in relation to saline in the same genotype; and #, $P < 0.05$; and ##, $P < 0.01$ in relation to WT same treatment. Data are means ± SEM and were analyzed by using two-way ANOVA (genotype × treatment) with post hoc Tukey test. (*D* and *E*) HPLC determinations of 5-HT (S) and its metabolite 5-HIAA (M) in the cerebral cortex (Cor), hypothalamus (Hyp), hippocampus (Hipp), amygdala (Amy), midbrain (Mid), and cerebellum (Cer) of WT and nNOS$^{-/-}$ mice 75 h after single i.p. injection of 300 mg/kg pCPA (*D*) and 3 × 300 mg/kg pCPA (*E*). Data are percent decrease from saline group in each genotype (means ± SEM, n = 5–6 mice each group). *, $P < 0.05$; and †, $P < 0.01$ in relation to saline in the same genotype. In *E*, all data are $P < 0.05$ or $P < 0.01$ in relation to saline controls (two-way ANOVA with post hoc Tukey test).

latency of attacks ($P < 0.05$; Fig. 2 *A* and *B*) in nNOS$^{-/-}$ mice, thus abolishing the aggressive phenotype. Reduction in aggressive behavior correlates with a striking increase in 5-HT turnover. The percentage of 5-HT increase was assessed in each brain area 45 min after 100 mg/kg 5-HTP. WT and nNOS$^{-/-}$ mice have equivalent levels of 5-HTP in each brain area ($P > 0.05$; data not shown) and 5-HT, 5-HIAA (Fig. 2*D*), and 5-HT turnover are dramatically increased in the cerebral cortex, hypothalamus, hippocampus, amygdala, midbrain, and cerebellum of both genotypes. Therefore, the aggressive phenotype of nNOS$^{-/-}$ mice can be eliminated in a dose-dependent manner by augmenting 5-HT neurotransmission in the brain.

Decrements in 5-HT Neurotransmission Enhances Aggressive Behavior. We next evaluated the effects of reducing 5-HT levels per turnover with pCPA, an inhibitor of the 5-HT-synthesizing enzyme tryptophan hydroxylase, on aggressive behavior in nNOS$^{-/-}$ and WT mice. A single i.p. administration of 300 mg/kg pCPA 72 h before behavioral testing fails to induce any alterations in aggression ($P > 0.05$; Fig. 3 *A* and *B*). However, this regimen produces only a partial reduction in 5-HT levels and turnover in some brain areas (Fig. 3*D*). A sequential regimen involving three daily injections of 300 mg/kg pCPA 72, 48, and 24 h before testing leads to a profound reduction of 5-HT levels

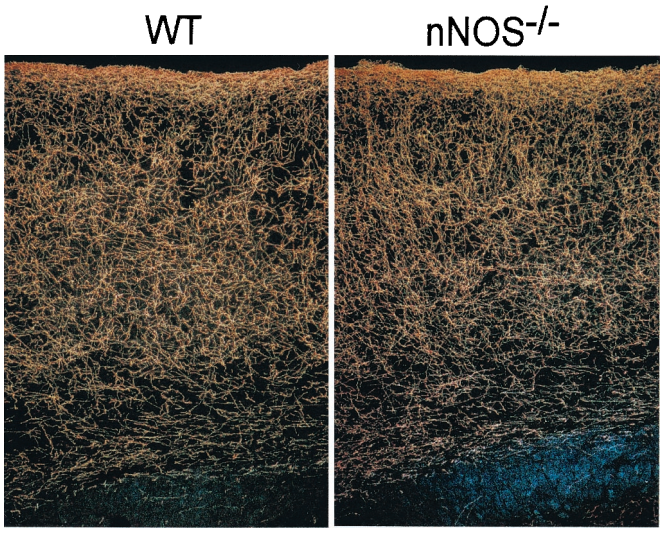

Fig. 4. Serotonergic innervations in the cerebral cortex of WT and nNOS$^{-/-}$ mice. Dark-field photomicrographs of 5-HT-immunoreactive axons in sagittal sections. (5× objective.) No differences are visualized in any brain area in sagittal or coronal sections of five nNOS$^{-/-}$ and four WT mice.

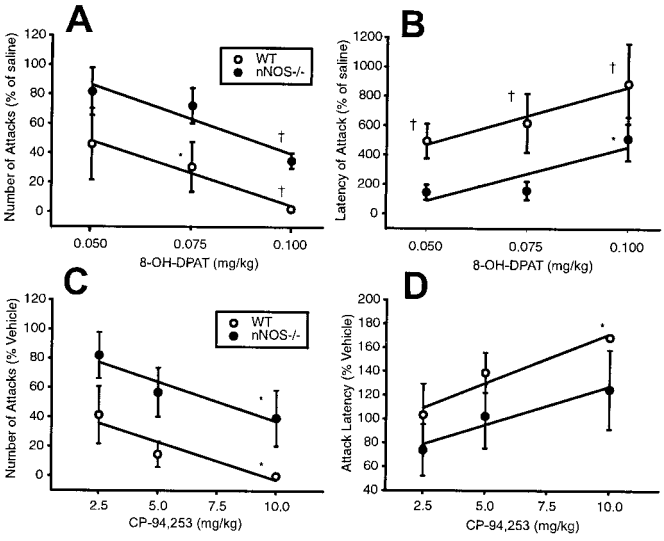

Fig. 5. (*A* and *B*) Aggressive behavior after increasing doses of the 5-HT$_{1A}$ agonist 8-OH-DPAT in WT and nNOS$^{-/-}$ mice ($n = 8$ each). Data are presented as percent change from saline groups in each genotype (means ± SEM) and a regression line. *, $P < 0.05$, and †, $P < 0.01$ in relation to saline control, two-way ANOVA (genotype × treatment) with post hoc Tukey test. The ED$_{50}$ in the reduction of number of attacks, calculated for each animal, is 0.019 ± 0.011 mg/kg for WT mice and 0.081 ± 0.007 mg/kg for nNOS$^{-/-}$ mice ($P < 0.01$, Student's *t* test). (*C* and *D*) Aggressive behavior after increasing doses of the 5-HT$_{1B}$ agonist CP-94,253 in WT and nNOS$^{-/-}$ mice ($n = 8$ WT and 9 nNOS$^{-/-}$ mice). Data are presented as percent change from vehicle groups in each genotype (means ± SEM) and a regression line. *, $P < 0.05$ in relation to vehicle in each genotype (two-way ANOVA with post hoc Tukey test). The ED$_{50}$ in the number of attacks, calculated for each animal, is 3.70 ± 1.21 mg/kg for WT mice and 6.76 ± 0.61 mg/kg for nNOS$^{-/-}$ mice ($P < 0.05$, Student's *t* test).

and turnover in all brain areas analyzed (Fig. 3*E*). Decrements in 5-HT neurotransmission are associated with an increase in number of attacks in WT but not in nNOS$^{-/-}$ mice ($P < 0.05$ and $P > 0.05$, respectively; Fig. 3*A*). The latency to first bite, an index of impulsivity, is reduced in WT mice with the highest dose of pCPA ($P < 0.01$), but latency is not reduced in nNOS$^{-/-}$ animals ($P > 0.05$; Fig. 3*B*). Thus, the effective reduction of 5-HT neurotransmission over a period of 3 days is able to induce aggressiveness in WT animals comparable to mice lacking nNOS.

Alterations in Aggressive Behavior Elicited by Changes in 5-HT Metabolism Are Not Associated with Alterations in Motor Function. To ensure that alterations in aggressive behavior induced by 5-HTP or pCPA are not caused by changes in motoric behavior, animals were evaluated in the open-field arena to assess exploration, locomotion, and rearing behaviors as well as balance and motor coordination on a rotarod. There is no significant alteration in rotarod performance and open-field behavior in WT and nNOS$^{-/-}$ mice after 5-HTP or pCPA treatment, except for an attenuation in horizontal locomotion in nNOS$^{-/-}$ mice treated with the highest dose of 5-HTP ($P < 0.05$; Fig. 2*C*). The repeated pCPA regimen does not change locomotor activity within each genotype ($P > 0.05$; Fig. 3*C*), but the total locomotion of nNOS$^{-/-}$ mice is reduced compared with WT ($P < 0.05$; Fig. 3*C*), despite the marked persistent increase in aggressiveness ($P < 0.05$; Fig. 3*A*). Taken together, these results suggest the alterations in aggressive behavior elicited by 5-HTP and pCPA are not associated with alterations in motor function.

Forebrain 5-HT Immunoreactivity Is Unaltered in nNOS$^{-/-}$ Mice. Mice lacking one copy of the brain-derived neurotrophic factor gene are aggressive and show age-related alterations in the disposition and density of serotonergic fibers and terminals (25). Accordingly, we examined the distribution and density of 5-HT terminals in nNOS$^{-/-}$ mice by immunocytochemistry (Fig. 4). There is no significant alteration in the density and pattern of 5-HT terminals in the nNOS$^{-/-}$ mice. Thus, the central 5-HT dysfunction in nNOS$^{-/-}$ mice is not caused by structural abnormalities in 5-HT axons/terminals.

5-HT$_{1A}$ and 5-HT$_{1B}$ Receptors Are Functionally Altered in nNOS$^{-/-}$ Mice. Abnormalities in 5-HT levels and/or turnover in nNOS$^{-/-}$ mice could lead to or reflect alterations in 5-HT receptor function. The 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors are candidates for modulating aggressive behaviors because agonists of these receptors have antiaggressive properties in rodents (19, 21, 26, 27), and mice lacking the 5-HT$_{1B}$ receptor are aggressive (22). To ascertain whether alterations in 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor function could contribute to the aggressive behavior of nNOS$^{-/-}$ mice, we examined the effects of selective 5-HT$_{1A,}$ and 5-HT$_{1B}$ agonists on the behavior of WT and nNOS$^{-/-}$ mice (Fig. 5). To compare the effects of the 5-HT agonists in WT vs. nNOS$^{-/-}$ mice, the level of aggressiveness in WT mice must be experimentally increased. Previous studies indicate that repeated exposures to the resident–intruder paradigm increase aggressive behavior (23). Accordingly, both WT and nNOS$^{-/-}$ mice were repeatedly exposed to the resident–intruder paradigm for 15 min every 3 days for a total of four exposures, which significantly increases the aggressive behavior of WT mice to levels near to the level observed in nNOS$^{-/-}$ mice, but does not change the aggressive behavior of the nNOS$^{-/-}$ mice (data not shown). The 5-HT$_{1A}$ agonist, 8-OH-DPAT, and the 5-HT$_{1B}$ agonist, CP-94,253, significantly decrease aggressive behavior in both WT and nNOS$^{-/-}$ mice by reducing the number and duration of attacks and increasing the latency to first attack (Fig. 5 *A*, *C*, *B*, and *D,* respectively). Although both 5-HT agonists reduce aggressive behavior in both WT and nNOS$^{-/-}$ mice, significantly higher concentrations of both agonists are required to reduce the aggressive behavior in the nNOS$^{-/-}$ mice. The ED$_{50}$ for the antiaggressive effect of 8-OH-DPAT and CP-94,253 for nNOS$^{-/-}$ mice is 4- and 1.8-fold higher, respectively, than the ED$_{50}$ for WT mice in number of attacks ($P < 0.01$ and $P < 0.05$, respectively; Fig. 5 *A* and *C*). We

controlled for potential changes in motoric function by assessing behavior in the open-field arena and rotarod in response to 0.1 mg/kg 8-OH-DPAT and 10.0 mg/kg CP-94,253—i.e., the highest doses used in the aggression tests. There is no significant effect of these compounds in locomotion, exploratory behavior, or motor coordination ($P > 0.05$; data not shown). Thus, the reduced sensitivity to the antiaggressive effects of 5-HT_{1A} and 5-HT_{1B} receptor agonists are likely to represent functional alterations of these receptors in the brains of nNOS$^{-/-}$ mice that lead to the dramatically aggressive phenotype.

Discussion

Here we report that the intense aggression observed in mice lacking neuronal-derived NO is caused by alterations in serotonergic signaling. Specifically, we note that 5-HT turnover in several brain regions is significantly reduced in male nNOS$^{-/-}$ mice with no detectable modifications in the dopamine or norepinephrine systems. Pharmacological treatment that increases central 5-HT neurotransmission decrease, in a dose-dependent manner, aggressive behavior in nNOS$^{-/-}$ mice. We can induce similar high levels of aggression after reducing 5-HT turnover in WT mice. Moreover, the alterations in 5-HT turnover do not reflect changes in the disposition and density of serotonergic fibers and terminals. Importantly, there is a hypofunction in the 5-HT_{1A} and 5-HT_{1B} receptors in nNOS$^{-/-}$ mice. Taken together, our results suggest that neuronal-derived NO is essential to the normal function of the central 5-HT system.

The linkage of NO with 5-HT function in aggression-related brain areas reveals novel molecular insights into the mechanisms of aggression. NO seems to play an important modulatory effect in the serotonergic system, especially in the 5-HT_{1A} and/or 5-HT_{1B} postsynaptic receptor function and the absence of NO leads to an increase in impulsive and aggressive behavior through alterations in serotonergic circuits. Other causes of aggressive and impulsive behavior may also lead to selective decrements in serotonergic function, and thus this may represent a final or major common pathway for aggressiveness and impulsivity. Although dysfunction in central 5-HT neurons may represent a final common pathway for the elaboration of aggressiveness and impulsivity, our data suggest a specific upstream mechanism involving nNOS/NO. These observations link two major pathways of neuronal signaling in one pathophysiological cascade and have significant implications for the treatment of psychiatric disorders characterized by aggressiveness and impulsivity.

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