Absence Of Cerebellar Long-Term Depression in Mice Lacking Neuronal Nitric Oxide Synthase

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Abstract

Extensive pharmacological evidence suggests that nitric oxide (NO) is a crucial transmitter for cerebellar long-term depression (LTD), a long-lasting decrease in efficacy of the synapses from parallel fibers onto Purkinje neurons, triggered by coincident presynaptic activity and postsynaptic depolarization. We now show that LTD cannot be induced in Purkinje neurons under whole-cell patch clamp in cerebellar slices from young adult mice genetically lacking neuronal nitric oxide synthase (nNOS). This genetic evidence confirms the essentiality of NO and nNOS for LTD in young adult rodents. Surprisingly, LTD in cells from nNOS knockout mice cannot be rescued by photolytic uncaging of NO and cGMP inside Purkinje neurons, although such stimuli circumvent acute pharmacological inhibition of nNOS and soluble guanylate cyclase in normal rodents. Also slices from knockout mice show no deficit in cGMP elevation in response to exogenous NO. Therefore, prolonged absence of nNOS allows atrophy of the signaling pathway downstream of cGMP.

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Introduction

The gaseous messenger nitric oxide (NO) has recently been recognized as a modulatory neurotransmitter in the central nervous system (Madison 1993; Bredt and Snyder 1994). NO is made from arginine by the enzyme nitric oxide synthase (NOS). At least three isoforms of the enzyme are known, neuronal (nNOS), endothelial (eNOS) and inducible (iNOS). nNOS is particularly abundant in the cerebellum (Bredt et al. 1990), where NO is crucial for the induction of long-term depression (LTD), a reduction in efficacy of the synapses from parallel fibers (PFs) onto Purkinje cells (PCs). LTD is physiologically produced by simultaneous stimulation of PF and climbing fiber (CF) inputs to the PC. A rise in free Ca^{2+} concentration ($[Ca^{2+}]_i$) inside the PC is both sufficient and necessary to mediate the effect of the CF, because CF stimulation can be entirely replaced either by simple depolarization (Konnerth et al. 1992), which activates voltage-operated Ca^{2+} channels, or by photolytic uncaging of Ca^{2+} from a light-sensitive chelator (Lev-Ram et al. 1997). If $[Ca^{2+}]_i$ is buffered by chelators, then LTD cannot be elicited (Konnerth et al. 1992; Lev-Ram et al. 1995). Meanwhile NO production is both sufficient and necessary to mediate the effect of the PF, because photorelease of caged NO inside the PC completely replaces PF activity and synergizes with either depolarization or uncaged $[Ca^{2+}]_i$ to cause LTD (Lev-Ram et al. 1995, 1997). Inhibition of presynaptic NOS with L-nitroarginine, or trapping of NO by myoglobin either outside or in-

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side the PC, prevents LTD induction through PF activity, whereas introduction of L-nitroarginine directly into PCs had no effect. NO uncaged inside the PC circumvents NOS inhibition or extracellular myoglobin but not intracellular myoglobin, confirming that PF activity must generate NO outside the PC and that NO then has to diffuse into the PC to have its effect (Lev-Ram et al. 1995). The most likely molecular target of NO is soluble guanylate cyclase (sGC), which is very abundant in PCs and is the enzyme that synthesizes cGMP. Inhibition of sGC prevents PF activity or uncaged NO from participating in LTD induction, but such inhibition can be circumvented by uncaged cGMP, which synergizes with depolarization-induced $[Ca^{2+}]_i$ transients to elicit LTD. Therefore, cGMP is downstream of NO and is likewise necessary and sufficient for mediating the PF signal (Lev-Ram et al. 1997).

The previous dissection of the signals for LTD induction was accomplished by pharmacological means, including enzyme inhibitors, Ca^{2+} and NO traps, and caged compounds that release Ca^{2+}, NO, or cGMP upon flash photolysis. Specificity of pharmacological inhibition of NOS or sGC could be confirmed by rescuing the block by uncaging the downstream messenger NO or cGMP. A complementary approach to NO function would be genetic deletion of NOS or sGC. Although sGC knockouts have not yet been reported, mice lacking nNOS have been generated by homologous recombination (Huang et al. 1993), and the homozygotes are phenotypically healthy, but the males have been reported to be abnormally aggressive (Nelson et al. 1995). Likewise eNOS knockouts were also generated (Huang et al. 1995). Neither mouse line showed deficiencies in hippocampal long-term potentiation (LTP) or spatial learning; forms of plasticity that have also been proposed to require NO. However, deletion of both nNOS and eNOS does seem to prevent hippocampal LTP at least in apical dendrites, so the two isoforms seem to substitute for each other in the hippocampus (Son et al. 1996).

The only previous test of nNOS deletion on cerebellar neurons was by Linden et al. (1995), who cultured PCs from embryonic knockout mice and found no deficit in their ability to desensitize in response to simultaneous glutamate application and depolarization. This result confirmed their extensive previous data (including the lack of effect of NO donors or NOS inhibitors) showing that NO is not essential for LTD in cultured PCs (Linden and Connor 1992). However, the pharmacological tests already discussed show that LTD in cerebellar slices freshly prepared from young adult rodents is quite different from the desensitization in PCs isolated from much younger animals and maintained in culture for prolonged periods. Therefore, it was important to compare brain slices from young adult mice with and without nNOS for their ability to show LTD. If NO is as important as our previous results indicated, and if nNOS is the crucial isozyme responsible for NO production, then genetic deletion of nNOS should prevent LTD. We now show this prediction to be correct. Surprisingly, neither photoreleased NO nor uncaged cGMP, both combined with depolarization, could rescue LTD induction. The most likely explanation is that the absence of nNOS throughout development has allowed signaling components downstream of cGMP to atrophy.

Materials and Methods

The homozygous mice with targeted deletion of their nNOS gene have been described previously (Huang et al. 1993). Their appearance and behavior were at least superficially quite normal, and the males showed no sign of the previously reported aggressiveness (Nelson et al. 1995). Therefore, the absence of nNOS in the cerebellum was verified by immunocytochemistry (see below and Fig. 1).

ELECTROPHYSIOLOGY

Thin (300 μm) sagittal slices were cut with a Microslicer DSK-3000W (Dosaka EM Co., Japan) from the cerebellar vermis of mice aged 18–21 days. Synaptic currents in PCs were recorded in the whole-cell patch-clamp configuration (Hamill et al. 1981; Edwards et al. 1989). The cells were directly visualized through a 10× water immersion objective on an upright microscope (Axioplan, Zeiss). Tight-seal whole-cell recordings (seal resistance > 10 GΩ) were made with patch pipettes with 3–4 MΔ resistance and an Axopatch 200A (Axon Instruments) amplifier (holding potential, −70 mV). A 10-msec, 10-mV test depolarization preceded each PF stimulus to monitor the input resistance of the PC throughout the experiment, which was discarded if the resistance changed by >20%. The intracellular solution contained mM K-gluconate; 10 mM KCl; 10 mM K-HEPES; 1 mM

$MgCl_2$; 4 mM Na-ATP; 1 mM Na-GTP; 16 mM sucrose; (pH 7.2) osmolarity 300 Osm. Although these conditions are adequate for measuring excitatory postsynaptic currents (EPSCs), the voltage clamp is incapable of maintaining space clamp conditions in the dendrites and axon when spikes are generated. The external Ringer's solution contained 125 mM NaCl; 2.5 mM KCl; 2 mM $CaCl_2$; 1 mM $MgCl_2$; 1.25 mM NaH_2PO_4; 26 mM $NaHCO_3$; 25 mM glucose (pH 7.4); and 10 μM (−)-bicuculline methiodide (Research Biochemical International) to inhibit GABAergic synapses. Slices were continually perfused with Ringer's solution saturated with 95% O_2, 5% CO_2, during recording. All experiments were performed at room temperature (near 22°C).

Intracellular calcium increases were achieved by 50-msec step depolarizations to a level in which regenerative calcium spikes were induced in the out-of-clamp dendritic region. For PF stimulation a bipolar electrode was placed at the pial surface above and up to 100 μm on either side of the recorded PC. A 50-μsec pulse was delivered to stimulate the PF using an isolation unit (ISO-Flex, A.M.P.I., Israel). The timing of depolarization, PF stimulation, and shutter opening was orchestrated using a multichannel stimulator (Master-8, A.M.P.I., Israel). Voltage and current data were digitized and stored on a VCR recorder via a PCM2 A/D VCR adaptor (Medical System Corp., Greenvale, NY) and transferred via a PCMI2 interface to a personal computer for software extraction of synaptic current amplitudes.

ODQ (1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one) was purchased from Tocris Cookson Inc. (Sigma, St. Louis, MO). The spontaneous and caged NO donors $Et_2N(N_2O_2)Na$, CNO-4 dipotassium salt (Makings and Tsien 1994), and the caged cGMP CM-cGMP [guanosine 3′,5′-cyclic monophosphate, 4,5-bis(carboxymethoxy)-2-nitrobenzyl ester] (Lev-Ram et al. 1997) were synthesized in this laboratory. Caged compounds were introduced into the Purkinje neuron by inclusion in the patch pipette intracellular solution. For further details of the uncaging apparatus, see Lev-Ram et al. (1995, 1997).

cGMP IMMUNOASSAY

cGMP assays were performed on 300-μm-thick slices, which were incubated in oxygenated Ringer's solution throughout the experiment. The slices were preincubated with control Ringer, ODQ (8 μM), or IBMX (20 μM) for 10 min before stimulation with control Ringer, $Et_2N(N_2O_2)Na$ (100 μM), or glutamate (2 μM) for 3 min. At the end of the stimulation period the slices were dropped into liquid nitrogen. After all the slices were frozen, 1 ml of 5% TCA was added and the slices were sonicated for 5 min. Each slice was triturated and then transferred to a microcentrifuge tube and centrifuged in the cold for 5 min. The supernatant was collected and assayed for cGMP using an enzyme immunoassay kit (Cayman Chemical) following the procedure described in the kit instruction booklet. Each sample consisted of one or two slices, whose protein content was determined by the bicinchoninic acid method. The values of picomoles of cGMP/mg of protein were averaged for the two duplicate tissue samples that had been subjected to each set of preincubation and stimulus conditions, then normalized by the picomoles of cGMP/mg of protein for the samples with no inhibitors or stimulators. The resulting cGMP amplification factors were finally averaged for three separate experiments; the bars indicate the standard errors of the final averaging (Fig. 4).

IMMUNOHISTOCHEMISTRY

Mice were anesthetized with 4 ml/kg of body weight ketamine/Rompun and perfused using intracardiac catheterization. Perfusion with a balanced salt solution (135 mM NaCl, 14 mM $NaHCO_3$, 1.2 mM Na_2HPO_4, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$) at 35°C was followed by 4% formaldehyde (fresh from paraformaldehyde) in 0.1 M phosphate buffered saline (PBS) at pH 7.4 for 5 min. The cerebelli were removed and fixed an additional 1 hr, then sections 40–60 μm thick were cut with a vibratome (Lancer). The sections were permeabilized in a solution containing 0.1% Triton X-100, 1% normal goat serum, and 1% cold water fish gelatin (Sigma) in PBS for 30 min before incubation in affinity-purified anti-NOS antibody (Transductions Laboratory) at a dilution of 1:50 for 18 hr at 4°C. Sections were then washed in buffer and incubated in goat anti-rabbit IgG–FITC conjugate (Jackson ImmunoResearch) for 1 hr at 4°C. Following this, the sections were rinsed in PBS and mounted in Gelvatol. Confocal microscopy was performed using an MRC-1024 confocal microscope system (Bio-Rad) attached to an Axiovert 35M (Zeiss) using a 40× 1.3 N.A. objective. Excitation was 488 nm

from an argon ion laser. Digital images were printed using a Fujix Pictrography 3000 printer (Fuji).

Results

LOCALIZATION OF nNOS IMMUNOREACTIVITY IN CEREBELLAR SLICES FROM MUTANT AND CONTROL MICE

To confirm that the mutant mice are lacking nNOS, immunohistochemical localization of the enzyme was performed on cerebellar slices from knockout mice and C57Bl/6J as normal controls. nNOS immunoreactivity was present in the control C57Bl/6J cerebellum in non-PCs (Fig. 1A,B; see also Bredt et al. 1990) but was missing in the mutant as expected (Fig. 1C).

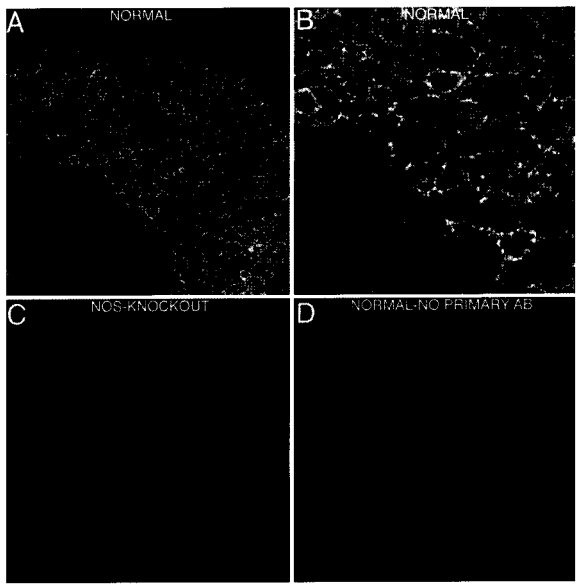

Figure 1: Localization in nNOS immunoreactivity in the cerebellum of normal and nNOS-deficient mice. nNOS immunoreactivity in normal (C57BL/6J) mice cerebellar vermis indicates the expected distribution in non-PCs (*A*). (*B*) A higher magnification of an area in *A*. (*C*) The lack of nNOS in the mutant mice. (*D*) No fluorescent labeling can be detected in the absence of the primary antibody. (*A,C,D*) 164 μm across; (*B*) 80 μm across.

LTD INDCUTION BY PF STIMULATION AND DEPOLARIZATION

LTD in acute cerebellar slices can be induced by stimulation of PFs synchronized with a 50-msec postsynaptic depolarization repeated at 1 Hz for 30 sec. Figure 2A demonstrates the effectiveness of this paradigm in causing LTD in six PCs from control mice. However, the same protocol (a) was completely ineffective in eight cells from nNOS knockout mice (Fig. 2C).

LTD INDUCTION BY PHOTORELEASED NO AND DEPOLARIZATION

If the only effect of genetic deletion of nNOS were the inability to generate NO, uncaged NO should bypass this deficit just as it does for L-nitroarginine block of NOS. Therefore, caged NO (CNO-4) (Makings and Tsien 1994; Lev-Ram et al. 1995) was included in the patch pipette (200 μM) and photolyzed in concert with a depolarization-induced calcium transient at 1 Hz for 30 sec. This protocol (b) by itself produced LTD in normal rats (Lev-Ram et al. 1995) and mice (Fig. 2B) but failed to induce LTD in any of the eight PCs from mutant mice (Fig. 2C). The inability to produce LTD was not confined to the particular dose of uncaged NO used in Figure 2C, because longer and shorter UV exposures to produce larger and smaller amounts of photoreleased NO were still ineffective in the mutant cells (data not shown). Thus, the lack of nNOS during development had presumably interfered with the expression, localization, or function of downstream elements in the signal transduction pathway.

LTD INDUCTION BY PHOTORELEASED cGMP AND DEPOLARIZATION

To assess whether the additional deficit in nNOS-deficient mice is upstream or downstream of cGMP, we asked whether LTD can be induced by photoreleased cGMP combined with depolarization. In slices from normal rats (Lev-Ram et al. 1997) and mice (Fig. 3B), this protocol reliably induced LTD. The same protocol failed to give LTD in nNOS-deficient mice (Fig. 3C). In five out of eight PCs, the EPSC amplitudes actually increased somewhat during and after the stimulation protocol, much like the behavior of normal rat PCs in which cGMP-dependent protein kinase was pharmacologically inhibited (Lev-Ram et al. 1997). Even in the three nNOS-deficient PCs in which the baseline remained flat, for example, the cell shown in Figure 3D, there was no sign of LTD. Therefore, the PCs from the mutant mice show a deficit in sensing cGMP, in addition to their lack of nNOS.

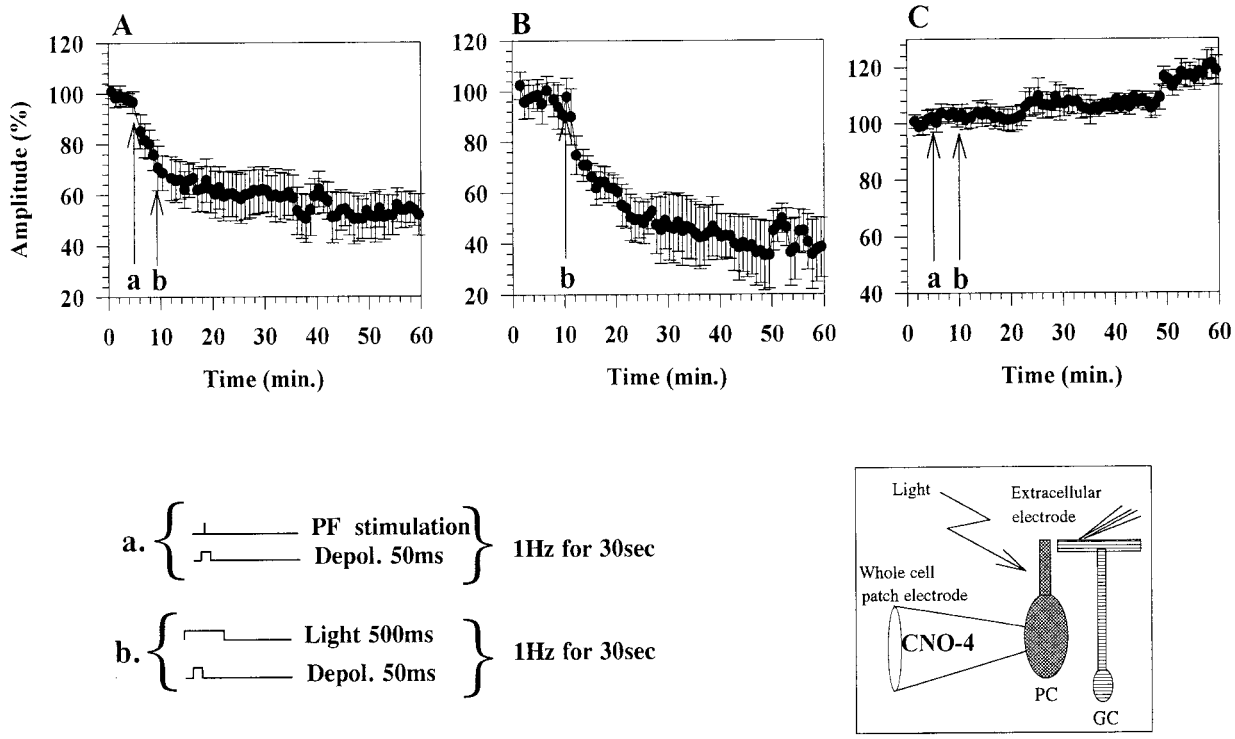

Figure 2: PF stimulation and uncaged NO in conjunction with depolarization elicit LTD in cells from normal mice (*A,B*) but not nNOS knockout mice (*C*). EPSCs in Purkinje neurons were monitored using whole-cell patch recording. Each trace is the average response from 6 control (*A*) 3 control (*B*), and 8 mutant (*C*) cells. Each point is an average of 10 EPSC peak amplitudes from consecutive PF stimuli delivered at 0.2 Hz, a test frequency too low to cause plasticity, normalized to the pretraining EPSC amplitude, averaged over all cells. Error bars represent the standard errors of each group. The patch pipette included 200 μM CNO-4 (caged NO). Simultaneous PF stimulation and depolarization at 1 Hz for 30 sec (protocol a), our standard for eliciting LTD, reduced the EPSC by 250 sec to 69 ± 7% of its value just before training, a depression significant at the $P = 0.005$ level by the one-tailed *t*-test. After photolytic release of NO by 200-msec flashes of 365 nm light (1 Hz for 30 sec) (protocol b), depression in the control group (*A*) continued to develop slightly; the EPSC 250 sec after protocol b averaged 60 ± 16% of pretraining, significant at $P = 0.007$. The EPSC amplitude in cells from nNOS-deficient animals was not depressed by either protocol. Mean amplitude after protocol a = 102 ± 4% of baseline. After protocol b the mean amplitude was 108 ± 5% of baseline.

GUANYLATE CYCLASE ACTIVITY IN THE nNOS KNOCKOUT

Because the PCs in the nNOS knockouts no longer have a normal electrophysiological response to cGMP, the above results do not show whether the genetic deletion has affected sGC or phosphodiesterase activity. We therefore sought to assay cGMP levels in response to exogenous NO and other modes of stimulation. Unfortunately, imaging of cGMP in analogy to cAMP imaging (Bacskai et al. 1993; Hempel et al. 1996) in individual living neurons has not yet been demonstrated. Therefore, a more traditional enzyme-linked immunoassay for cGMP was performed on entire cerebellar slices left unstimulated, directly stimulated with an exogenous spontaneous releaser of NO, $Et_2N(N_2O_2)Na$ (Maragosa et al. 1991; Makings and Tsien 1994), or indirectly stimulated by glutamate to produce NO. All three stimulation conditions were separately paired with no other additions or with ODQ, a relatively specific inhibitor of sGC, or a phosphodiesterase inhibitor, isobutylmethylxanthine (IBMX). Control and NO-stimulated slices from nNOS knockout mice displayed somewhat higher levels of cGMP than comparably treated slices from normal mice, although the differences between strains did not reach statistical significance. As expected, ODQ largely suppressed cGMP increases in both normal and mutant animals. These results suggest that sGC levels are at least the same if not higher in the nNOS knockout

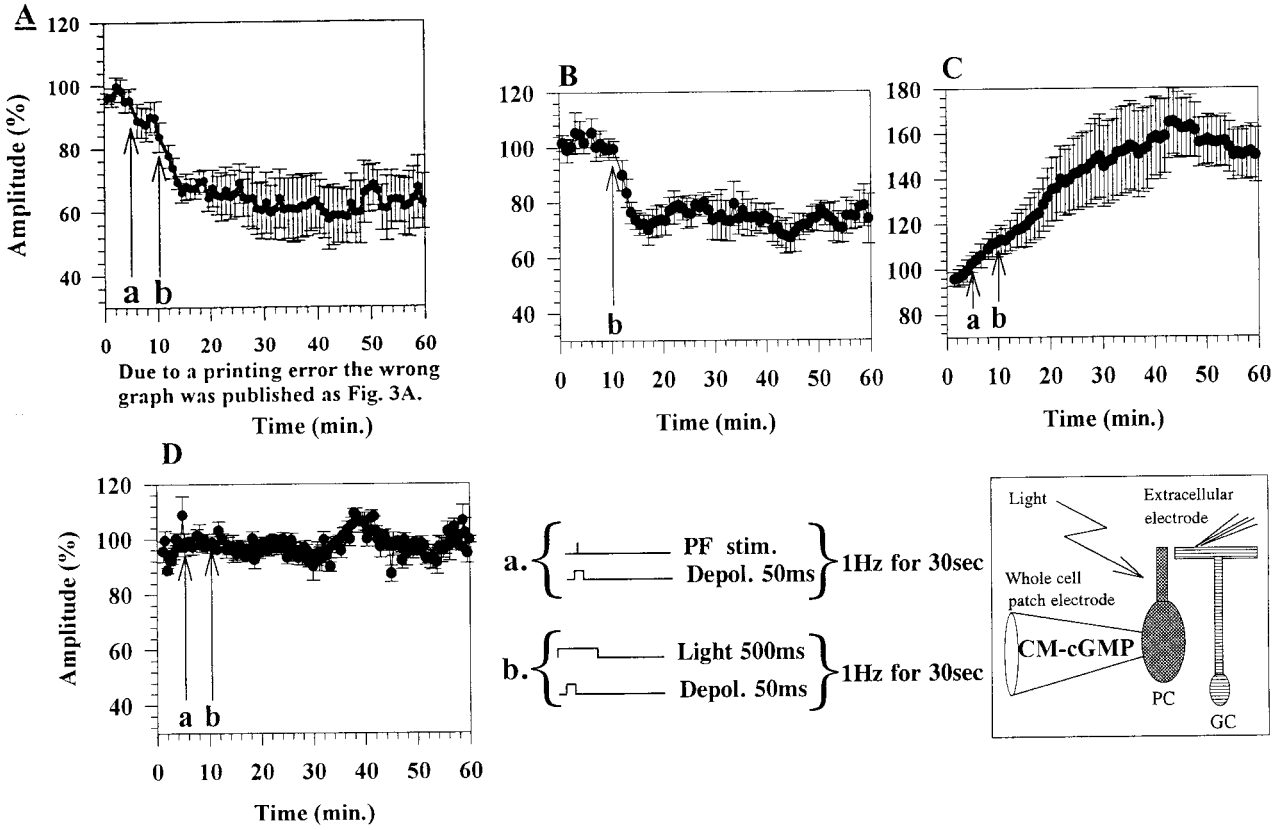

Figure 3: PF stimulation and uncaged cGMP in conjunction with depolarization elicit LTD in cells from normal mice (*B*) but not nNOS knockout mice (*C,D*). Procedures were the same as in Fig. 2 except that the patch pipette included 1 mM CM-cGMP (membrane-impermeant caged cGMP) instead of caged NO. Each trace is the average of 4 control (*A*) and 2 control (*B*), and eight mutant (*C*) cells. Panel *D* shows one of the 3 mutant cells that showed a flat prestimulus baseline. Simultaneous PF stimulation and depolarization at 1 Hz for 30 sec, a standard training protocol (a) for eliciting LTD, was effective in some cells in the control group and therefore the averaged trace indicates partial depression after protocol a was applied (mean EPSC 250 sec after protocol a was 70 ± 10% of baseline, $P = 0.03$). Photolytic release of cGMP by 500 msec flashes of 365 nm light (1 Hz for 30 sec) (protocol b) induced further depression in the control group (*A*) (mean 60 ± 13% of baseline, $P = 0.03$). Protocol b alone reduced the EPSC amplitude of two control cells to 76 ± 10% of the baseline level (*B*). The EPSC amplitude from nNOS-deficient animals (*C,D*) was not depressed by either protocol. Mean amplitude after protocol a = 113 ± 6% of baseline. After protocol b the mean amplitude was 149 ± 19% of baseline.

mice than in control mice. IBMX elevated the stimulated cGMP levels in slices from normal but not nNOS-deficient mice and thereby suppressed the differences between wild-type and mutant mice both in the basal state and with exogenous NO. These results suggest that the slightly high levels of cGMP in the knockout mice could be attributed to slower breakdown of cGMP in addition to (or rather than) increased production. IBMX unmasked substantial glutamate-stimulated production of cGMP in slices from normal mice, but slices from mutant mice failed to respond to glutamate even with IBMX present. This result is expected because glutamate stimulation causes $[Ca^{2+}]_i$ elevation to activate nNOS to produce NO to stimulate sGC. In the nNOS knockout mice, the missing nNOS should and did render glutamate challenge ineffective.

Discussion

The inability of Purkinje neurons in nNOS-deficient mice to show LTD is the first clear deficit in cellular synaptic plasticity to be revealed in these animals. LTP in the hippocampus had been tested previously in slices from the same line of nNOS knockout mice. LTP was reduced only slightly in comparison to control mice and was still blocked by NOS inhibitors. Because eNOS is expressed in hippocampal pyramidal neurons, it was possible that this isoform of the enzyme continues to gen-

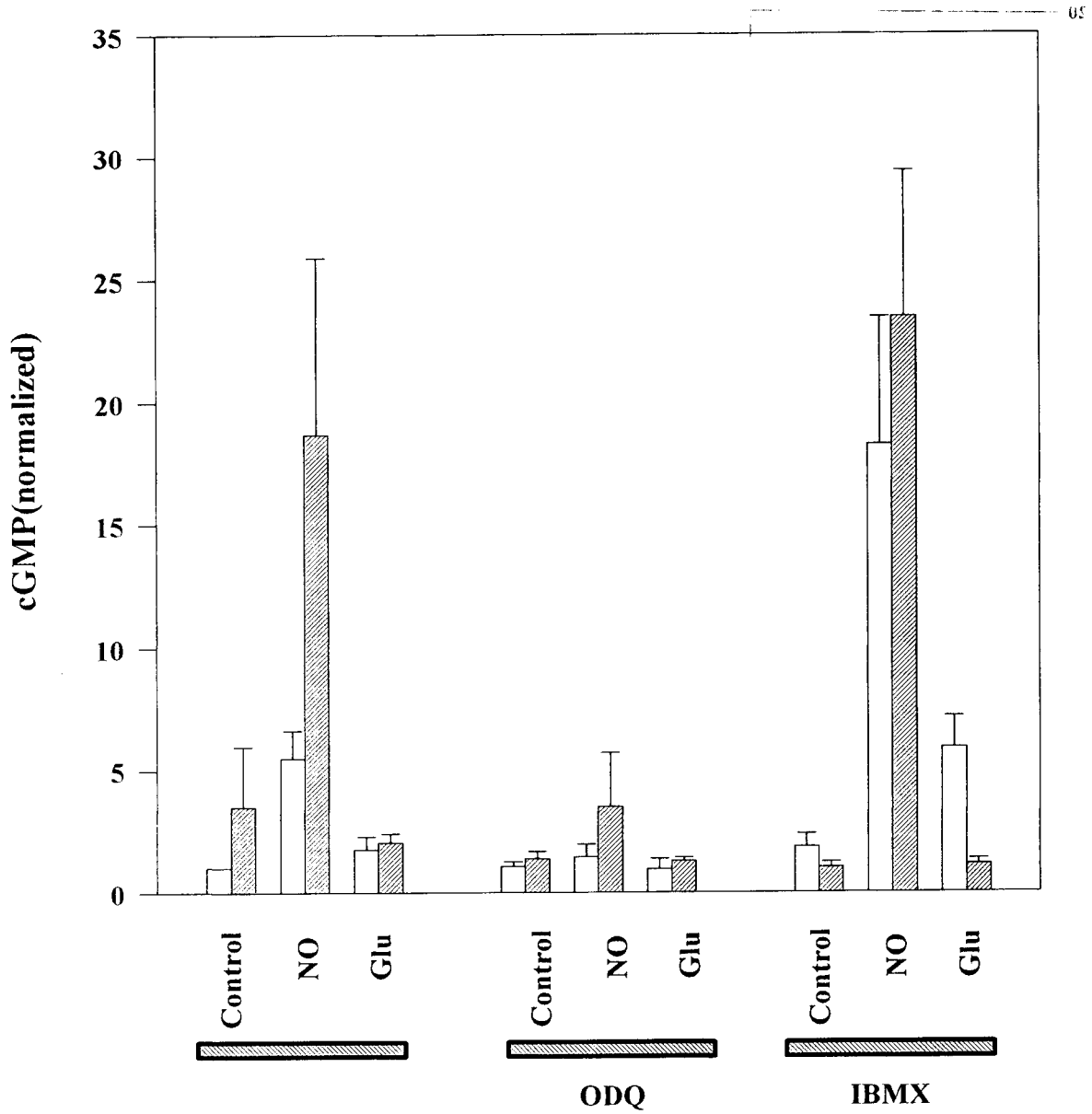

Figure 4: Comparison of total cGMP in acute slices of cerebellar vermis from normal mice (open bars) and mutant mice (shaded bars). cGMP was measured using an enzyme-linked immunoassay (EIA) in three independent experiments with standard error bars. Control, NO, and Glu indicate no stimulation or stimulation for 3 min with 100 μM of the NO donor $Et_2N(N_2O_2)Na$ or 2 μM glutamate, respectively. Preincubations with no inhibitor or for 10 min with 8 μM ODQ or 20 μM IBMX are grouped as indicated. For additional details, see Materials and Methods. Because the mutant cerebellar slices can generate cGMP as a response to the exogenous NO donor, reasonably normal levels of guanylate cyclase are expressed in the mutant mice despite the lack of nNOS.

erate NO during LTP induction (O'Dell et al. 1994). When both isoforms were knocked out, LTP on apical dendrites of hippocampal CA1 neurons was greatly reduced, and the remaining component could not be further blocked by NOS inhibitors (Son et al. 1996). Therefore, it was unclear whether eNOS might also compensate in the cerebellum for a lack of nNOS and permit normal LTD. However, our results (Fig. 2B,C) suggest that nNOS is essential for induction of LTD by PF activity plus depolarization. In contrast, in cultured cerebellar cells from immature animals, LTD induction is NO- and cGMP-independent, so PCs cultured from very young mice still desensitize their AMPA

receptors in response to paired glutamate application and depolarization regardless of whether or not the mice had nNOS (Linden et al. 1995). It remains unclear how and why cultured PCs should develop or unmask an independent biochemical cascade to respond to conjunctive extracellular and intracellular stimulation. If the absence of NO-delivering cells during culture simply promoted compensation by a NO-independent cascade, one might predict that the nNOS-knockout mice should behave like cultures, that is, show LTD that cannot be blocked by NOS inhibitors. Instead, the PCs in slices from young adult nNOS knockout mice seem incapable of LTD at least with our standard protocol of PF activation coincident with postsynaptic depolarization (Fig. 2). Surprisingly, this deficit could not be circumvented by the uncaging of NO or cGMP coincident with $[Ca^{2+}]_i$ elevation inside the Purkinje neurons (Figs. 2 and 3). In cells from normal rats, uncaged NO rescues LTD from inhibition by NOS inhibitors and NO buffers (Lev-Ram et al. 1995), whereas uncaged cGMP rescues LTD from inhibition of NO-stimulated guanylate cyclase (Lev-Ram et al. 1997). Therefore, the nNOS knockout mice are deficient at a step downstream from cGMP. An alternative formal possibility would be that the nNOS knockout mice are deficient in depolarization-induced $[Ca^{2+}]_i$ elevation, which is also required for NO or cGMP to induce LTD. We consider this alternative unlikely because the inward currents during the depolarization steps were indistinguishable between mutant and control mice. The normality of cGMP production in response to exogenous NO was verified by radioimmunoassay of cGMP production in slices from knockout and control mice, incubated with an NO donor (Fig. 4). Also, histochemistry of sGC appeared normal (results not shown). Therefore, the permanent absence of nNOS in the knockout mice caused a biochemical readjustment, an atrophy of responsivity to cGMP, rather than a loss of sGC or a compensatory mechanism to produce LTD by another pathway. At present we do not know precisely where the downstream blockade is. Perhaps cGMP-dependent protein kinase or its target(s) have been down-regulated, or inhibitors have been up-regulated.

NO also appears to mediate the cerebral blood flow increase during hypercapnia in normal mice, as NOS inhibitors block CO_2-induced increase in cerebral blood flow and the rise in cGMP caused by elevated CO_2. Continuous monitoring of arterial blood pressure, heart rate, and pial vessel diameter revealed that the nNOS knockout animals responded like normal mice to elevated CO_2 concentration, except that the response was not affected by NOS inhibitors. Thus, the cerebral circulatory response found a compensatory system not involving NO (Irikura et al. 1995). Another form of NO-dependent neuronal plasticity is nociception-induced long-term facilitation of excitability and synaptic efficacy in spinal and supraspinal neurons. This phenomenon as well seems normal in nNOS knockout mice (Crosby et al. 1995). On the other hand, cortical cultures from nNOS knockout mice demonstrated markedly attenuated NMDA-elicited neurotoxicity (Dawson et al. 1996). Thus there is no consistent pattern as to whether the genetic deletion produces compensation, or atrophy, or neither.

Although the nNOS knockout mice behave superficially normally in their cages, it is still possible that cerebellar-dependent learning is impaired. Therefore, it will be interesting to test these mice on behavioral challenges such as the eye-blink test and/or vestibulo-ocular reflexes. Unlike some other types of knockouts (Aiba et al. 1994), nNOS loss does not cause cerebellar ataxia or other signs of motor disorganization. Significant deficits in learning paradigms would provide further evidence that cellular LTD may play an important role in behavior, similar to what was found with knockouts of glial fibrillary acidic protein (Shibuki et al. 1996). If there were no significant difference between learning abilities in knockout and control mice, it would imply a redundant pathway, not measured by our cellular LTD paradigm, that would play a minor role in normal mice but would be able to take over gradually when the NO-cGMP pathway is missing.

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