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Hippocampal Long-Term Potentiation Is Normal in Heme Oxygenase-2 Mutant Mice

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Summary

We have generated mice deficient in HO-2, the major cerebral isoform of heme oxygenase, in order to assess the potential role of carbon monoxide as a retrograde messenger in hippocampal LTP. Cerebral HO catalytic activity was markedly reduced in the HO-2 mutant mice, yet no differences were found between wild types and mutants in gross neuroanatomical structure, in basal hippocampal synaptic transmission, or in the amount of potentiation produced by various LTP induction protocols. Furthermore, zinc protoporphyrin IX, an inhibitor of HO, had nearly identical inhibitory effects on LTP in wild-type and HO-2 mutant hippocampal slices. Our data indicate that carbon monoxide produced endogenously by HO is unlikely to be a neuromodulator required for LTP in the hippocampus.

Introduction

Long-term potentiation (LTP) is experimentally defined as a long-lasting enhancement of synaptic transmission that results from a strong high frequency presynaptic stimulus (Bliss and Lomo, 1973) or a low frequency presynaptic stimulus paired with postsynaptic depolarization (Kelso et al., 1986). It is well established by pharmacological studies that postsynaptic activation of the NMDA subtype of glutamate receptor and subsequent Ca^{2+} influx are required for the induction of LTP (Collingridge et al., 1983). In contrast, whether the expression of LTP occurs at either the pre- or postsynaptic site, or both, is still controversial (reviewed by Bliss and Collingridge, 1993; Manabe and Nicoll, 1994; Stevens and Wang, 1994). An example of a presynaptic cellular change underlying LTP would be an increase in glutamate release; a postsynaptic modification might involve an augmentation in postsynaptic responses to released transmitter. If there is indeed such a presynaptic component, there must correspondingly be some messenger that is synthesized postsynaptically but is released and has the ability to signal to the presynaptic terminal. The membrane-permeable molecule nitric oxide (NO), generated by nitric oxide synthase (NOS), is a candidate for this retrograde signal. Inhibitors of NOS can block hippocampal LTP. Also, hemoglobin, a molecule that binds NO and is confined to the extracellular space, will block LTP when added to hippocampal slices (O'Dell et al., 1991; Schuman and Madison, 1991). However, it has been difficult to determine conclusively a retrograde messenger role for NO in LTP, since NOS inhibitors are effective in reducing LTP only under certain experimental conditions (Williams et al., 1993), and LTP is normal in mice lacking a functional neuronal *NOS* gene (O'Dell et al., 1994).

Another candidate for the retrograde messenger is carbon monoxide (CO), which was initially investigated for neuromodulatory function because of its chemical similarities with NO. The sole enzyme known to produce CO stoichiometrically is microsomal heme oxygenase (HO), which metabolizes heme groups yielding biliverdin, free iron, and CO (reviewed by Maines, 1988). Separate genes encode two isoforms of HO. One of these isoforms, HO-2, is present throughout the brain, with high expression in hippocampal CA1 pyramidal cells (Verma et al., 1993). The other isoform, HO-1, is expressed mainly in peripheral organs and is detected only in scattered neurons of the CNS (Ewing et al., 1992; Ewing and Maines, 1993). Although relatively little is known about the neuromodulatory role of CO, there is evidence suggesting that CO may participate in olfactory response (Verma et al., 1993), smooth muscle relaxation (Utz and Ulrich, 1991; Farrugia et al., 1993), and modulation of Purkinje cell excitability (Nathanson et al., 1995). Recently, CO has been considered a candidate retrograde messenger in the hippocampus, based on experiments showing that metalloporphyrin inhibitors of HO, such as zinc protoporphyrin IX (ZnPP), are capable of blocking the induction and/or maintenance of LTP in hippocampal slices (Stevens and Wang, 1993; Zhuo et al., 1993). The ability of hemoglobin to inhibit LTP is consistent with this hypothesis since, hemoglobin sequesters CO in addition to NO. Moreover, CO application paired with weak tetanic stimulation can cause an enhancement of synaptic transmission in hippocampal slices that resembles LTP (Zhuo et al., 1993). Pharmacological evidence indicating that cGMP generation may be necessary for CA1 region LTP (Zhuo et al., 1994) is also in agreement with a retrograde messenger role for CO, since, like NO, CO can potently activate soluble guanylyl cyclase by binding to the heme group contained in the enzyme (Ignarro, 1989; Brune et al., 1990).

There remain significant challenges to the notion that CO participates in LTP. Metalloporphyrins may have inhibitory effects on HO-independent activities important for LTP. Studies have documented direct attenuation by ZnPP of guanylyl cyclase and NOS activity (Ignarro et al., 1984; Meffert et al., 1994), suggesting certain limitations in using such metalloporphyrins as reagents for investigating HO function. CO itself is a relatively stable molecule, in contrast with NO; this would appear to make temporally and locally controlled presynaptic activation more difficult.

Finally, since HO has not yet been found to be directly regulated by Ca^{2+} or any other components of the signal transduction pathway thought to be responsible for LTP, it is unclear how CO production could be coupled to changes in Ca^{2+} levels that are integral for the postsynaptic induction of LTP. However, Nathanson et al. (1995) did report increases in CO production from cerebellar slices following glutamate application.

To address further the role of CO as a signaling molecule in LTP or in other systems for which CO participation has been implicated, we have used gene targeting techniques to generate mice devoid of functional HO-2. In this study, these mice were used to examine hippocampal synaptic transmission and LTP.

Results

Generation of HO-2 Homozygous Mutant Mice

The *HO-2* targeting construct replaces a region of murine *HO-2* sequence that corresponds to rat *HO-2* exons 4 and 5 (McCoubrey and Maines, 1994) with a neomycin resistance cassette containing a *pgk* promoter (Figure 1A). This mutation removes ~80% of the coding sequence, including the putative membrane-spanning region. Following transfection of this construct into embryonic stem (ES) cells and G418 selection, Southern blot analysis was used to verify homologous recombination events. Approximately 8% of transfectants contained a targeted *HO-2* locus. Six male chimeras with the capacity to transmit the

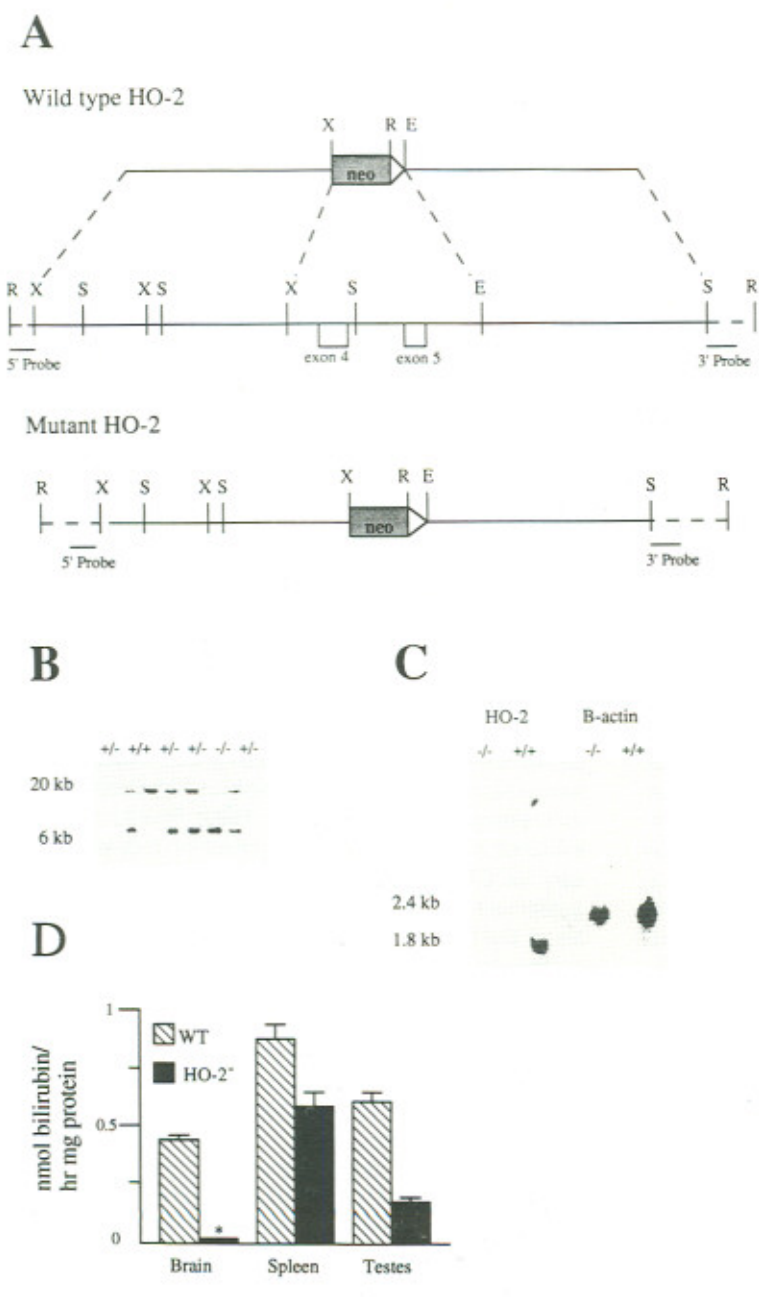

Figure 1. Targeted Disruption of the *HO-2* Gene

(A) *HO-2* genomic locus and targeting vector. A 3.4 kb region including exons corresponding to rat exons 4 and 5 was replaced with a *pgk–neo* cassette. The murine *HO-2* exon–intron structure has not yet been conclusively determined. The 5′ and 3′ probes used for screening ES cell clones and genotyping mice are shown. The 3′ probe hybridizes to a 20 kb EcoRV fragment containing the native *HO-2* gene and a 6 kb EcoRV fragment from the disrupted gene. E, EcoRI site; R, EcoRV; S, SpeI; X, XbaI.

(B) Southern blot analysis of EcoRV-digested tail DNA from ES cell–derived mice. The blot was hybridized with the 3′ *HO-2* probe. Genotypes of 1 HO-2 homozygous mutant mouse (−/−), 1 wild-type mouse (+/+), and 4 heterozygotes (+/−) are indicated.

(C) Northern blot analysis of total RNA from the brain of a wild-type (+/+) and a homozygous mutant (−/−) mouse. The blot was probed with a region of murine *HO-2* sequence contained in putative exon 4, which recognizes an mRNA in wild-type samples of ~1.8 kb, corresponding to the *HO-2* message. This band is absent in RNA samples from mutant mice. Similar results were obtained after probing with a full-length rat *HO-2* cDNA probe (data not shown). Afterward, blots were probed with a human B-actin cDNA fragment to control for amounts loaded.

(D) HO activity, as measured by bilirubin production with hemin substrate, in wild-type (WT) and mutant ($HO\text{-}2^-$) mice. Data are shown as mean ± SEM. Each value represents 8–10 assays done in two or three experimental trials. Asterisk indicates that no HO activity was detected in mutant brains.

targeted allele through the germline were obtained via blastocyst injection of two of these positive clones. Matings between heterozygous mice yielded expected Mendelian ratios; these offspring were typed using Southern blots (Figure 1B).

The absence of *HO-2* gene expression in homozygous mutant mice (referred to hereafter as "mutants") was confirmed by Northern blot analysis using adult brain RNA. A single band of ~1.8 kb was present in blots of RNA from wild-type mice and was missing from blots of RNA from mutants (Figure 1C). HO enzymatic assays revealed a lack of activity, measured indirectly by bilirubin production, in brains of mutant mice (Figure 1D). Although the assay used was not sensitive enough to detect very low levels of HO activity, this result confirms a previous report that found minimal cerebral contribution from the HO-1 isoform (Trakshel et al., 1988). RNA analysis further indicated no upregulation of *HO-1* mRNA in mutant brains (data not shown). HO activity was clearly reduced in the testes of mutant mice compared with wild-type mice, while the reduction was less pronounced in the mutant spleens (Figure 1D). These data are also consistent with previous work describing the contributions of each isoform to total HO activity in these tissues (Yoshida and Kikuchi, 1978; Trakshel et al., 1986).

Behavior and Anatomy of Mutants

HO-2-deficient mice are morphologically indistinguishable from their wild-type littermates throughout development and adulthood. The mutants are fertile and survive normally for at least 1 year. They appear to have typical feeding and grooming behavior, gait, and circadian rhythms. Histological examination of mutant brains at a gross level revealed no neuroanatomical differences from those of wild-type mice (data not shown). Several tissues other than brain were also examined histologically but displayed no mutation-linked abnormalities (data not shown). Additionally, no significant differences in major hematological parameters were noted between wild-type and mutant mice, including carboxyhemoglobin levels (data not shown).

Hippocampal Synaptic Transmission and LTP

In hippocampal slices from wild-type and mutant mice, we measured excitatory synaptic transmission in the CA1 region following Schaffer collateral stimulation. There was no significant difference seen in maximal field excitatory postsynaptic potentials (EPSPs; wild type [mean ± SEM]: 8.98 ± 0.27 mV [n = 10 mice, 59 slices]; mutant: 9.25 ± 0.30 mV [n = 10 mice, 64 slices]). We next analyzed paired-pulse facilitation, which is a short-lasting presynaptic form of synaptic plasticity characterized by an enhanced response to the second of a pair of stimulation pulses delivered in rapid succession. Paired-pulse facilitation was similar in wild-type and mutant mice over a range of 20–200 ms interstimulus intervals (n = 6 mice, 11 slices analyzed from each group; data not shown). Together, these results suggest that there is no gross irregularity of basal synaptic function in the mutant hippocampi, and they are consistent with reports showing that inhibitors of HO had no effect on baseline hippocampal synaptic transmission (Stevens and Wang, 1993; Zhuo et al., 1993; Meffert et al., 1994).

We used several different protocols to induce LTP in slices from wild-type and mutant animals. A high frequency tetanus protocol (two 100 Hz, 1 s trains) was initially used to induce a strong potentiation in hippocampal slices. There was no significant difference between the average LTP induced in wild-type slices (188.2% ± 7.1% of pretetanus baseline, measured at 60 min posttetanus; n = 6 mice, 13 slices) and that from mutant slices (212.0% ± 14.7%; n = 6 mice, 13 slices; Figure 2A). It remained possible that the tetanic stimulus used in these experiments was too powerful to allow for the detection of any subtle LTP differences between the two groups of mice. Therefore, we attempted to determine a protocol that might induce LTP in wild-type slices without meeting the threshold for LTP production in mutant slices. Protocols using 20, 10, or 5 Hz stimulation each produced a long-lasting potentiation in our experiments. Figures 2B–2D indicate that these weaker protocols did not reveal any significant differences in average LTP values between wild-type and mutant slices. From wild-type slices, protocols of 20, 10, and 5 Hz yielded LTP of 225.1% ± 13.3% (n = 4 mice, 10 slices), 154.8% ± 17.0% (n = 4 mice, 9 slices), and 115.2% ± 9.6% (n = 6 mice, 15 slices), respectively. Mutant slices had corresponding LTP values of 228.7% ± 16.3% (n = 4 mice, 9 slices), 164.2% ± 11.3% (n = 5 mice, 12 slices), and 125.2% ± 8.4% (n = 5 mice, 14 slices).

Finally, we measured CA1 LTP induced by presynaptic low frequency stimulation paired with intracellular postsynaptic depolarization (see Experimental Procedures). Similar LTP values were obtained from wild-type and mutant slices using this type of analysis as well (wild type: 274.3% ± 21.5% [n = 4 mice, 8 cells]; mutant: 279.6% ± 47.4% [n = 5 mice, 9 cells]; Figure 3). In summary, we observed normal amounts of LTP in mutant slices through use of a variety of LTP induction protocols, indicating that CO production by HO-2 is not required for LTP in the CA1 hippocampal region. On the contrary, the mutant LTP values were higher than wild-type values for each protocol used, though never by a statistically significant margin.

Effects of HO Inhibitor

Our results demonstrating essentially normal LTP in mutant mice do not support the findings of previous pharmacological studies, which indicated disruption of LTP by HO inhibitors (Stevens and Wang, 1993; Zhuo et al., 1993). To begin to address this issue, we tested the effects of 15 μM ZnPP on the induction of LTP using a single train of 100 Hz (1 s duration) in slices from wild-type and mutant mice. In wild-type slices, ZnPP application resulted in a significant reduction of LTP (control potentiation: 196.7% ± 26.3% [n = 5 mice, 11 slices]; ZnPP treatment: 122.9% ± 8.1% [n = 5 mice, 11 slices]; Figure 4A). Figure 4B indicates that 15 μM ZnPP had very similar effects on LTP in mutants (control: 191.6% ± 19.3% [n = 5 mice,

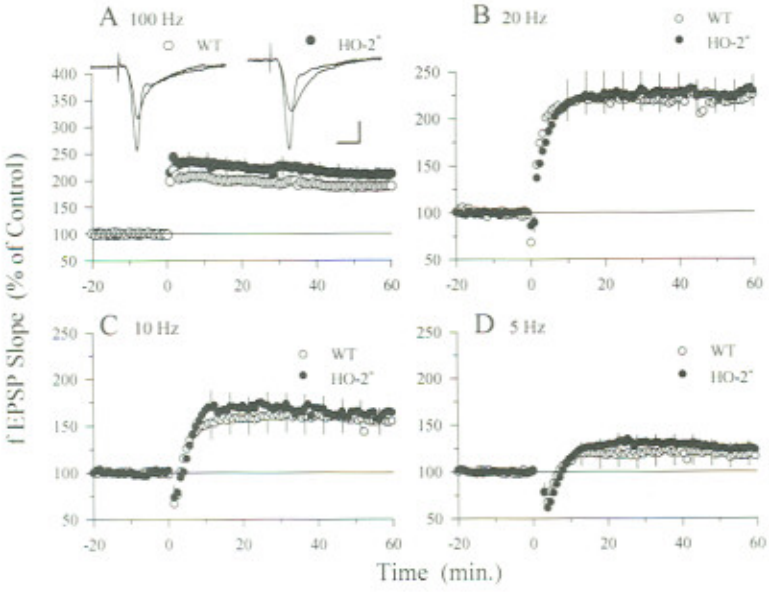

Figure 2. LTP Induced by Both High and Low Frequency Stimulation Protocols Is Normal in HO-2 Mutant Mice

(A) LTP was induced by two trains of high frequency stimulation (100 Hz, 1 s duration, 10 s intertrain interval) delivered at time 0. The amount of potentiation seen 60 min after high frequency stimulation in slices from mutant mice (HO-2$^-$; n = 6 mice, 13 slices) was not different from that observed in slices from wild-type mice (WT; n = 6 mice, 13 slices). Each point is the mean response (normalized to pretetanus baseline levels), and error bars (SEM) are shown every 5 min. Traces show field EPSPs recorded just before (smaller response) and 60 min after (larger response) high frequency stimulation. Bars, 2.0 mV and 5.0 ms. (B–D) Beginning at time 0, 900 pulses of 20 Hz (B), 10 Hz (C), or 5 Hz (D) stimulation were delivered. Following an initial depression, field EPSPs potentiated to different levels depending on the frequency of synaptic stimulation. At each frequency, the amount of LTP observed 60 min later was not significantly different in slices from mutant mice (HO-2$^-$) and wild-type controls (WT). Average data were obtained from the following: (B) wild type: n = 4 mice, 10 slices; mutant: n = 4 mice, 9 slices, (C) wild type: n = 4 mice, 9 slices; mutant: n = 5 mice, 12 slices, (D) wild type: n = 6 mice, 15 slices; mutant: n = 5 mice, 14 slices.

10 slices]; ZnPP: 123.7% ± 12.5% [n = 4 mice, 8 slices]). Thus, even though the major cerebral isoform of HO was absent from mutant hippocampal slices, ZnPP was able to block LTP to the same extent as in wild-type slices.

Discussion

CO, a product of HO activity, has been implicated as a neuromodulator and as a retrograde messenger involved in presynaptic contributions to potentiated synaptic transmission following the induction of LTP. In this study, we generated mice homozygous for a null mutation in the *HO-2* gene, which encodes the major isoform of HO present in the CNS. As expected, HO catalytic activity was undetectable in HO-2 mutant brains. The mutant mice exhibited no obvious abnormalities in appearance, and a systematic histological scan of mutant tissues revealed no anomalies. Moreover, based on maximal field EPSP amplitudes and paired-pulse facilitation, basal hippocampal synaptic transmission appeared normal in the mutants. Most importantly, we were unable to find any differences between wild-type and mutant mice in CA1 LTP produced by several different induction protocols. Even the weakest protocol yielding small LTP produced similar potentiation in wild-type and mutant slices. Our results clearly demonstrate that HO-2 is not required for LTP.

There are two likely reasons for the discrepancy between our observations of normal LTP in HO-2 mutant hippocampal slices and previous observations of disrupted LTP in metalloporphyrin-treated slices. First, as immunohistochemical staining has detected the HO-1 isoform in scarce, randomly distributed cells of the hippocampus (Ewing and Maines, 1993), it is possible that residual CO contribution from HO-1 is adequate for LTP. Second, as mentioned above and addressed by Meffert et al. (1994), the ability of metalloporphyrins to inhibit LTP in hippocampal slices might not be solely due to their effects on HO. Our observation that 15 μM ZnPP inhibits LTP in mutant slices addresses this issue. Importantly, previous experiments have demonstrated that HO-1 and HO-2 activities are equally inhibited by a wide range of ZnPP concentrations (Maines and Trakshel, 1992). Therefore, if only a minority of HO activity (<5% of wild-type levels) functions to accommodate LTP in the mutants, then a nonsaturating concentration of ZnPP should in theory have more pronounced effects on LTP in mutant slices, compared with its effects on LTP in wild-type slices that have normal levels of HO expression and activity. However, we observed that 15 μM ZnPP produces a nearly identical partial inhibition of LTP in mutant and wild-type slices, suggesting it is unlikely that the sustained normal LTP in the mutants is due to HO-1 activity. Accordingly, our results are most consistent with the notion that metalloporphyrins inhibit LTP via HO-independent mechanisms. Since the concentration of ZnPP used in our experiments does not significantly obstruct NOS activity in hippocampal slices (Meffert et al., 1994), the ability of ZnPP to inhibit LTP is probably not due to nonselective effects on NOS. However, because ZnPP also inhibits guanylyl cyclase activity (Ignarro, 1989; Luo and Vincent, 1994), nonselective effects on other components of the putative NO signaling pathway may account for the capacity of ZnPP to block LTP.

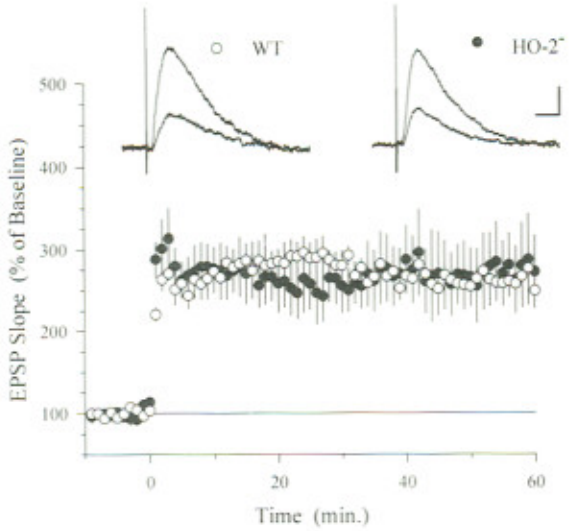

Figure 3. Intracellular LTP Induced by Pairing Protocol Is Normal in HO-2 Mutant Mice

Pairing postsynaptic depolarization with 40 presynaptic stimulation pulses (at time zero) produces similar amounts of LTP in CA1 pyramidal cells from mutant (HO-2⁻; n = 5 mice, 9 cells) and wild-type mice (WT; n = 4 mice, 8 cells). Traces show intracellular EPSPs recorded from CA1 pyramidal cells in slices from wild-type (left) and mutant (right) mice just before (smaller responses) and 60 min after (larger responses) inducing LTP. Bars, 5.0 mV and 10.0 ms.

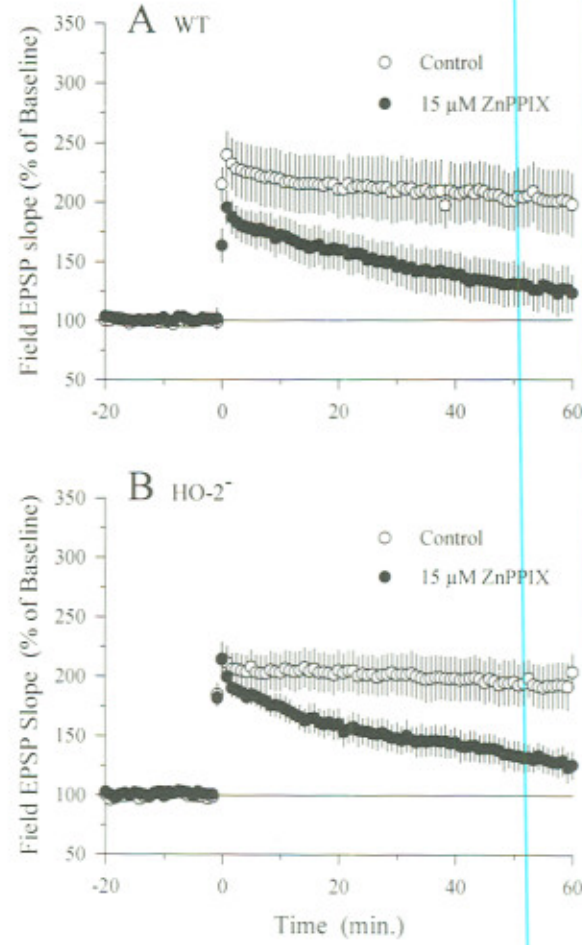

Figure 4. ZnPP Inhibits CA1 LTP in Slices from Wild-Type and HO-2 Mutant Mice

Slices were continuously bathed in ZnPP (beginning at least 30 min prior to high frequency stimulation), and LTP was induced with a single train of 100 Hz stimulation (1 s duration). In control experiments, this protocol produced similar amounts of LTP in slices from wild-type (WT; n = 5 mice, 11 slices; A) and mutant (HO-2⁻; n = 5 mice, 11 slices; B) mice. ZnPP produced a comparable, although incomplete, block of LTP in slices from wild-type (n = 5 mice, 11 slices; A) and mutant (n = 4 mice, 8 slices; B) mice.

In this light, the LTP-like enhancement of synaptic responses due to CO application observed in a previous study could best be explained by the idea that this type of potentiation either is not an LTP-related occurrence or is not representative of in vivo mechanisms. For instance, exogenous CO may stimulate guanylyl cyclase or other enzymes in slices and lead to potentiation, but no significant amount is generated endogenously by HO for this purpose. It might be possible to similarly explain other pharmacological results suggesting HO- and CO-related function in the olfactory system and cerebellum (Verma et al., 1993; Nathanson et al., 1995). However, because ZnPP does potently inhibit HO, it is logical to surmise that the putative undiscovered target of ZnPP-mediated LTP inhibition may not be involved in these pathways or expressed in the relevant tissues. HO-2-deficient mice should be appropriate models for testing these systems as well.

Although we interpret our data as being inconsistent with the notion that CO is a retrograde messenger in LTP, it is unreasonable to exclude CO based solely on analysis of HO-2 mutants. The examination of LTP in a strain of mice deficient in HO-1 or, ideally, in both HO isoforms would help resolve this issue. It remains imaginable that compensatory mechanisms such as increased production of other retrograde factors manifest themselves in the mutant background and sustain normal capacity for LTP in the absence of CO production. NO could be involved, as could other previously suggested retrograde signals, arachidonic acid and platelet-activating factor (Williams et al., 1989; Kato et al., 1994). An extensive pharmacological, biochemical, and/or genetic analysis would be necessary to discount this possibility.

Experimental Procedures

HO-2 Targeting Vector

The published rat *HO-2* cDNA sequence (Rotenberg and Maines, 1990) was utilized for synthesis of primers toward generating a mouse DNA probe by PCR amplification. This probe contained sequence corresponding to the final 68 amino acids of rat HO-2 protein. It was used to screen a λ EMBL3 library containing mouse 129/Sv strain genomic fragments, from which the *HO-2* gene was obtained. A 4.5 kb XbaI fragment and a 4.0 kb EcoRI–SpeI fragment were used as 5′ and 3′ arms of the construct, surrounding a 1.8 kb *pgk–neo* fragment in pBluescript KS(+). The construct was designed to remove a 3.5 kb XbaI–EcoRI fragment of mouse DNA containing intron sequences and coding sequence corresponding to rat HO-2 amino acids 67–315.

Targeting Experiments and Generation of HO-2 Mutant Mice

D3 ES cells derived from 129/Sv mice were grown on mitotically inactivated embryonic fibroblast feeder cells and electroporated with 50 μg of the NotI-linearized construct using a Bio-Rad Gene Pulser (800 V and 3 μF settings). G418 at 200 μg/ml was applied 24 hr later as selection for transfectants, and G418-resistant colonies were isolated on days 7 and 8 of selection. Genomic DNA for EcoRV restriction digests was isolated from ~300 colonies grown in 24-well plates. Southern blotting and hybridization with the 5′ external 0.8 kb probe or the 3′ external 2.0 kb probe (shown in Figure 1A) revealed 24 homologous recombinants. Six corresponding ES clones were used for injection into C57BL/6 blastocysts. Chimeric mice were generated as described (Bradley, 1987). Pseudopregnant C57BL/6 × DBA/2 (F1 generation) mice were used as blastocyst recipients. Mice heterozygous for the mutation were obtained by mating male chimeras with C57BL/6 females; these heterozygous mice were intercrossed to produce homozygous HO-2 mutants. Genotypes of mice were determined by Southern analysis of progeny tail DNA, as mentioned above (see Figure 1B). For all experments, C57BL/6 × 129/Sv wild-type and mutant mice were used.

RNA Analysis

Total RNA was isolated from brain by guanidine thiocyanate denaturation and cesium chloride gradient ultracentrifugation. Approximately 30 μg of RNA was electrophoresed in the presence of formaldehyde, Northern blotted onto Hybond-N nylon membrane (Amersham), and hybridized with the DNA probe used for isolating the *HO-2* genomic sequence. Afterward, blots were hybridized with a human B-actin cDNA probe to control for amounts loaded.

HO Enzymatic Activity

Pooled brains, spleens, or testes from wild-type or mutant mice were homogenized in 100 mM phosphate buffer (pH 7.4), and the 10,000 × g supernatant was immediately used for HO assays. Reactions with wild-type and mutant homogenates were set up in parallel. All experiments were performed under dim lighting. Reactions (3 ml) contained 3–5 mg of protein, 20 μM hemin, and 0.05% BSA in 100 mM phosphate buffer (pH 7.4). Reduced nicotinamide adenine dinucleotide phosphate (NADPH; 300 nmol) was added to initiate reactions; those without NADPH were considered to have zero activity. After 20 min at 37°C, reactions were stopped on ice and added to spectrophotometer cuvettes. Bilirubin production was calculated using the difference in absorbance between 468 and 520 nm. Bilirubin and hemin solutions were prepared as described previously (Sunderman et al., 1982).

Electrophysiology

Thick (400 μm) transverse slices of mouse hippocampus prepared using standard techniques were maintained in an interface-type recording chamber perfused (1–3 ml/min) with an oxygenated (95% O_2/5% CO_2) mouse artificial cerebrospinal fluid (ACSF; 124 mM NaCl, 4.4 mM KCl, 25 mM Na_2HCO_3, 1.0 mM Na_2PO_4, 1.2 mM $MgSO_4$, 2.0 mM $CaCl_2$, 10 mM glucose). All experiments were done at 30°C–31°C. Male mice 6–8 weeks old were used, and data collection and analysis were done in a blind fashion.

EPSPs elicited by stimulation of the Schaffer collateral/commisural fibers in CA1 were evoked using a bipolar nichrome wire stimulating electrode (stimulation rate = 0.02 Hz, 0.01–0.02 ms duration pulses) and were recorded using glass microelectrodes (5–15 MΩ, filled with ACSF) placed in stratum radiatum of the CA1 region of the hippocampus. A stimulation intensity sufficient to elicit field EPSPs that were ~50% of the maximal response was used throughout the experiment. In experiments examining the effects of ZnPP on LTP, Na_2PO_4 was omitted from the ACSF and $MgCl_2$ was substituted for $MgSO_4$ (see Meffert et al., 1994). ZnPP (Research Biochemicals) was first dissolved in 100% dimethyl sulfoxide (DMSO) and then diluted into aqueous base (0.002 N NaOH) prior to a final dilution into ACSF to give 15 μM ZnPP (final DMSO concentration was 0.05%).

Intracellular EPSPs were recorded from individual CA1 pyramidal cells using high resistance (60–120 MΩ) glass microelectrodes filled with 2.0 M CsCl. In these experiments, EPSPs were evoked every 15 s, and the ACSF contained 100 μM picrotoxin to block inhibitory synaptic transmission. To prevent spontaneous and evoked bursting, the CA3 region of the slice was removed and the concentrations of $CaCl_2$ and $MgSO_4$ were elevated to 4.0 mM each. LTP was induced by pairing 40 EPSPs (evoked at 1 Hz) with depolarization of the postsynaptic membrane potential to approximately −20 mV. Only cells with resting membrane potentials more negative than −60 mV and input resistances larger than 40 MΩ (measured with 0.2–0.3 nA hyperpolarizing current injections) were used. In these experiments, cells were hyperpolarized to between −80 and −90 mV by injecting hyperpolarizing current through the electrode to prevent potentiated EPSPs from eliciting action potentials. There were no differences between wild types and mutants in either resting membrane potentials (wild type [mean ± SEM]: −65.7 ± 3.5 mV [n = 4 mice, 8 cells]; mutant: −65.1 ± 4.0 mV [n = 5 mice, 9 cells]) or input resistances (wild type: 68.1 ± 19.6 MΩ; mutant: 63.9 ± 14.7 MΩ).

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