Prenatal Exposure to a Low Concentration of Carbon Monoxide Disrupts Hippocampal Long-Term Potentiation in Rat Offspring

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ABSTRACT

The aim of the present study was to investigate whether functional changes at CA3-CA1 synapses in the hippocampus could underlie learning and memory deficits produced in rat offspring by a prenatal exposure model simulating the carbon monoxide (CO) exposure observed in human cigarette smokers. Electrophysiological endpoints, including long-term potentiation, were examined in 15- to 30-day-old male rats whose mothers were exposed, from day 0 to day 20 of gestation, to 150 ppm of CO resulting in blood levels of carboxyhemoglobin comparable to those found in human cigarette smokers. Evoked field excitatory postsynaptic potentials were measured in the stratum radiatum in hippocampal slices. Results show that before tetanus, input/output functions, presynaptic volley, and paired-pulse facilitation were not affected in CO-exposed offspring, indicating that basal synaptic excitability and terminal Ca$^{2+}$ influx were not influenced by prenatal exposure to this gas. Conversely, evoked field excitatory postsynaptic potential potentiation in response to tetanization was reduced by about 23% and decayed rapidly to baseline values in slices from CO-exposed animals. No changes between and within groups were observed in paired-pulse facilitation after tetanus. The selective impairment of long-term potentiation expression exhibited by CO-exposed rats was paralleled by a significant decrease in heme-oxygenase 2 and neuronal nitric-oxide synthase in the hippocampus. No changes in either enzymatic activity were found in frontal cortex and cerebellum. These electrophysiological and biochemical alterations might account for cognitive deficits previously observed in rats exposed prenatally to CO. Our findings could have clinical implications for the offspring of mothers who smoke during pregnancy.

Carbon monoxide (CO) is an air pollutant and one of the most important constituents of cigarette smoke. The primary target for CO toxicity is the central nervous system, as shown by the impairment of ongoing behavior produced by low concentrations of inhaled gas. In particular, the developing brain is extremely vulnerable to chronic, relatively mild, reduction in oxygen availability induced by CO (Annau and Fechter, 1984; Di Giovanni et al., 1993). Moreover, recent clinical reports indicate that children born to women who smoked during pregnancy exhibited poorer performance on cognitive tasks, as well as an impaired intellectual development (Frydman, 1996; Fried et al., 1998).

On the basis of these evidences, experiments have been carried out to investigate whether the cognitive deficits produced by a prenatal exposure model simulating the CO exposure observed in human cigarette smokers (Mactutus and Fechter, 1984; Di Giovanni et al., 1993) could be related to alterations in long-term potentiation (LTP), the most intensively studied cellular and molecular model for learning and memory (Collingridge, 1992; Bliss and Collingridge, 1993; Nicoll and Malenka, 1995).

We have conducted an in vitro electrophysiological study in...
rat offspring (postnatal days 15–30) chronically exposed to 150 ppm of CO during gestation. The synaptic transmission between the Shaffer collateral terminals and hippocampal CA1 neurons, before and after tetanization, was investigated. In particular, we analyzed input/output (I/O) relationship, post-tetanic potentiation (PTP), short-term potentiation (STP), LTP, and paired-pulse facilitation (PPF), a short-lasting form of synaptic plasticity related to Ca\(^{2+}\)-mediated transmitter release (Katz and Miledi, 1968; Asztely et al., 1986).

CO is an endogenously generated gas that plays an important physiological role in the brain (Verma et al., 1993). In particular, it has been suggested that CO could be involved in the phenomenon of LTP. In fact, LTP maintenance (or expression) requires the increase in synaptic strength mediated by \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) and metabotropic-glutamate receptor activation (Bashir et al., 1993; Bortolotto and Collingridge, 1993; Bortolotto et al., 1999), as well as by the production of retrograde intercellular messengers, notably nitric oxide (NO) and CO itself (Hawkins et al., 1994; Medina and Izquierdo, 1995). Accordingly, when CO is applied to slices in association with a weak tetanic stimulation, unable per se to produce LTP, it induces a rapid and long-lasting enhancement of synaptic response in the CA1 region of the hippocampus (Zhuo et al., 1993).

Thus, the aim of a second series of experiments was to investigate whether prenatal exposure to a low concentration of CO could affect the activity of two enzymes in the hippocampus, NO synthase (NOS) and heme-oxygenase 2 (HO-2), regulating the production of NO and CO, respectively (Snyder et al., 1998). Because 96% of NOS activity in the hippocampus is due to the neuronal isoform (Huang et al., 1993), we focused our attention on neuronal (\(n\))NOS, although the importance of the endothelial NOS form in LTP formation has been recently stressed (Son et al., 1996; Wilson et al., 1999). Finally, \(n\)NOS and HO-2 activities in the frontal cortex and cerebellum were also evaluated.

**Materials and Methods**

**Animals and Exposure Conditions.** Rats were exposed to CO as previously described (Di Giovanni et al., 1993; Cagiano et al., 1998). Briefly, primiparous Wistar female rats weighing 250 to 280 g were housed in hermetic chambers (Alco Industries, Segrate, Milan, Italy) and exposed to either air (0 ppm of CO, control group) or 150 ppm of CO mixed with air from gestational day 0 (GD 0) to GD 20. Temperature was maintained at 20–22°C and light was on from 8:00 AM to 8:00 PM. Concentrations of CO in each chamber were continuously monitored by an infrared CO detector (CO11 M; Environment SA, Poissy, France) at a wavelength of 4.67 \(\mu\)m. The actual CO concentration deviated by less than 3% from the stated value.

Litters were reduced to a standard size of six male pups per litter (when possible) within 24 h after birth. Litters from the control group or CO-exposed group were then assigned to nonexposed mothers whose pups were born on the same day. Data were collected from only male pups whose mothers were exposed either to 0 ppm of CO or to CO (150 ppm) during pregnancy. One pup per litter from different litters per treatment group was used in both electrophysiological and biochemical experiments. Pups were weaned at 21 days of age.

**Dam Carboxyhemoglobin (HbCO).** Catheters were implanted in the abdominal aorta of separate groups of pregnant CO (0 and 150 ppm)-exposed rats under anesthesia (Equithesin, 3 ml/kg i.p.). Maternal HbCO was measured by a spectrophotometric method described by Rodkey et al. (1979). Briefly, blood samples (10 \(\mu\)l) were taken into a heparinized syringe, diluted about 1000-fold in a solution containing Na\(_2\)SO\(_4\) (2 mg/ml), and analyzed for their absorbance in the Soret region (390–440 nm) using a UV/visible spectrophotometer (Perkin-Elmer Co., Norwalk, CT). Measurements were performed on GD 10 and 20.

**Slices.** At postnatal days 15 to 30 (31–95 g b.w.), transverse hippocampal slices were prepared following standard methods (Mereu et al., 1991; Bortolotto and Collingridge, 1993). Briefly, after deep anesthesia with 4.0% halothane in \(O_2\) was established in rats, they were decapitated, and the brain was rapidly removed under chilled Ringer solution. Slices (350 \(\mu\)m thick) were cut with a vibroslicer (World Precision Instruments, Sarasota, FL) and incubated at room temperature (20 ± 2°C) for at least 60 min and then individually transferred to an interface chamber. Ringer medium contained (mM): NaCl (124), KCl (3.5), NaH\(_2\)PO\(_4\) (1.25), NaHCO\(_3\) (22), dextrose (10), MgCl\(_2\) (1), and CaCl\(_2\) (2). The solution was maintained at pH 7.4 by continuous bubbling with 5% \(CO_2\) in \(O_2\).

**Electrophysiology.** Field excitatory postsynaptic potentials (f-EPSPs) were recorded from stratum radiatum of CA1 pyramidal cells in response to monopolar stimuli (20-\(\mu\)s duration) delivered to the Schaffer collateral/commissural pathway via platinum electrodes. Recording electrodes were filled with the medium (2–4 M\(\Omega\)). Synaptic responses were sampled at 5 to 10 kHz. Acquisition and analysis were performed by a pCLAMP 5.5/Digidata 1200 system (Axon Instruments Inc., Foster City, CA). The evoked response was measured as the slope of its rising phase after the presynaptic volley. An I/O curve was constructed for each slice by plotting increasing single stimulus intensity [scan (range of increasing currents to contract the I/O curves); 5–100 \(\mu\)A] versus the evoked f-EPSP. This curve was used to assess synaptic excitability and to set the stimulus intensity to obtain a test f-EPSP of about 30 to 40% of the maximal response.

Test stimulation was always given as a pair of stimuli (50-ms interval) repeated at 0.05 Hz. Tetanization consisted of single, continuous stimulation at 100 Hz for 1.0 s using the same intensity as for test stimuli. PPF was given by the ratio of the second f-EPSP (S2) compared with the first (S1), i.e. S2/S1 × 100 (Katz and Miledi, 1968; Manabe et al., 1993; Schulz et al., 1995). Changes of PPF in the same trial were calculated as percent variation after LTP induction, compared with the PPF average during the 30-min pretetanus period. Responses were followed up to 90 min and considered potentiated if their slope was ≥20% of baseline. The three temporal phases of f-EPSP changes (PTP, STP, and LTP) were distinguished as previously indicated (Bliss and Collingridge, 1993; Bortolotto and Collingridge, 1993; Schulz and Fitzgibbons, 1997).

**Enzyme Measurements: nNOS Assay.** NOS activity was assayed according to the method of Bredt and Snyder (1998) with only minor modifications (Kitamura et al., 1995). Briefly, after rapid dissection, hippocampal frontocortical and cerebellar tissues were immediately frozen on dry ice and then stored at −70°C. Subsequently, tissues were thawed and homogenized (1:20, \(g/mL\)) with a Teflon-glass homogenizer in a 50 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM EDTA, 0.5 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride. The supernatant obtained after centrifugation at 48,000g for 30 min was used as the source for nNOS activity to be measured by the conversion of L-[\(^{14}\)C]arginine to L-[\(^{14}\)C]citrulline (and NO in an equimolar ratio). The cytosolic fraction (40 \(\mu\)l, 50–70 \(\mu\)g of protein) was incubated at 37°C for 15 min with 1 mM NADPH, 0.1 mM BH\(_4\), 2 mM CaCl\(_2\), 1 mM dithiothreitol, and 3 \(\mu\)M L-[\(^{13}\)C]arginine (L-[\(^{13}\)C]arginine, 303 Ci/mol specific activity, obtained from Amersham Corp., Little Chalfont, UK) in a total volume of 120 \(\mu\)l. Reactions were stopped by adding 500 \(\mu\)l of ice-cold 20 mM HEPES buffer (pH 5.5) containing 5 mM EDTA. Thereafter, 500 \(\mu\)l of each sample were applied to chromatography columns containing 1 ml of Dowex AG50W-X5 (\(H^+\) form, 200–400 mesh) equilibrated with 20 mM HEPES (pH 5.5). The newly synthesized L-[\(^{13}\)C]citrulline was specifically eluted with 1 ml of MilliQ water. Liquid scintillation
Use of Laboratory Animals” as adopted and promulgated by the Council of the European Communities (86/609/EEC, 92/65/EEC and Council Directive 2010/63/EU). The Declaration of Helsinki, and the “Guide for the Care and Use of Laboratory Animals” (National Research Council, 2011) were referenced as guidelines released by Italian Ministry of Health (D.L. 116/92), the Declaration of Helsinki, and the “Guide for the Care and Use of Laboratory Animals” (National Research Council, 2011). The experiments have been conducted in accordance with these guidelines. Animal Care.

Results

Reproduction Data. As observed previously (Di Giovanni et al., 1993), prenatal CO exposure did not affect dam weight gain, number of dams giving birth, length of pregnancy, litter size at birth, pup weight gain, or postnatal mortality (data not shown).

Dam HbCO. As shown in Table 1, exposure to CO produced a significant increase in maternal blood HbCO levels on both GD 10 and 20 (P < .0001). Moreover, HbCO levels in control rats were significantly increased on GD 20 with respect to those found on GD 10 (P < .001).

Synaptic Excitability. Changes in basal synaptic excitability were investigated by the analysis of I/O relationship and PPF before tetanization. The number of slices exhibiting PTP was also considered a measure of synaptic excitability.

Figure 1A illustrates the I/O function in control slices and in slices from CO-exposed rats (CO-slices). The similarity of the two curves indicates that the responsiveness of CA3-CA1 synapses to stimuli of increasing intensity did not change between the two groups. No significant modifications were found in the amplitude of presynaptic volley (Fig. 2).

Similarly, the measure of PPF before tetanization showed comparable values in control and CO-slices (Fig. 1B).

PTP was induced in both control and CO-slices, as evidenced by significant increases in the f-EPSP slope after the tetanization of Schaffer fibers (Figs. 2 and 3A). Moreover, the occurrence of slices showing PTP was similar, being 24/24 and 18/20 in control and CO-slices, respectively (Table 2). However, the average value of PTP was significantly reduced by prenatal exposure to CO, being 236.11 ± 22.63 and 182.05 ± 20.16% in control and CO-slices, respectively (P < .05; Fig. 3A).

Taken together, these results indicate that there was no evident alteration in basal synaptic function in CO-slices, except for a significant reduction of about 23% in the average of PTP (P < .05; Fig. 3A).

STP and LTP. In control slices, the decay of f-EPSP potentiation, after tetanization, followed the typical biphasic curve which is shown in Fig. 3A. Thus, in agreement with previous studies (Bliss and Collingridge, 1993; Bortolotto and Collingridge, 1993; Schulz and Fitzgibbons, 1997), the f-EPSP slope in control slices showed a first fast decremental phase lasting 15 to 30 min (STP), and then it slowly decayed over, at least, the observation time, i.e., 90 min (which was assumed as the minimum time for LTP maintenance or expression). Fitting analysis was done, and the estimated interception point at which the average curve asymptotically subsided to a value of +20%, with respect to the baseline,

Table 1: HbCO levels in pregnant rats exposed to CO

<table>
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<tr>
<th>ppm</th>
<th>CO</th>
<th>GD 10</th>
<th>GD 20</th>
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<tr>
<td>0</td>
<td>0.97 ± 0.02 (6)</td>
<td>1.62 ± 0.10* (5)</td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>14.42 ± 0.52** (6)</td>
<td>16.08 ± 0.88** (4)</td>
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* P < .001 vs. GD 10; ** P < .0001 vs. controls (Student’s t test with Bonferroni’s correction).
was at 226.25 ± 18.84 min after tetanus (Fig. 3B). In 23 of 24 tested slices from control rats, LTP was induced and maintained. In only one case did the f-EPSP slope decay below 20% of the baseline before 60 min. None of the control slices exhibited f-EPSP depression (Table 2).

Conversely, in CO-slices a rapid decline of f-EPSP enhancement was observed immediately after PTP (Fig. 3A). It was difficult to obtain a full LTP expression in these slices. Of 20 slices tested, 14 showed STP not followed by LTP. In only one slice did the potentiation remain above the value of 20% for more than 60 min (Table 2). Moreover, in five slices the f-EPSP was depressed (−24.33% ± 3.6) after an initial potentiation, which lasted 15 to 40 min (Table 2). On average, the measure of the f-EPSP slope in CO-slices returned to the initial (pretetanus) value of PPF, as recently suggested by Schulz et al. (1995). However, the analysis of our data showed that the correlation between initial PPF and LTP was below the significance level in both groups (control slices: 0.32; n = 24; P > .05; CO-slices: r = 0.28; n = 20; P > .05).

To compare STP in isolation from LTP in both groups, STP was normalized to the f-EPSP value measured 25 min after tetanus, as recently suggested by Schulz and Fitzgibbons (1997). The resulting curves are shown superimposed in Fig. 4C. The similarity of their shapes further indicates that CO-slices to remain higher than in control slices. As shown in Fig. 4C, the first point of the two curves after tetanization did show a significant reduction (P < .02). This reduction, which appears to be due to the small amount of glutamate-mediated PPF remaining in tetanized cells (Manabe et al., 1993), was, however, similar in both groups.

Furthermore, we also examined whether PPF in individual experiments might vary with LTP in inverse relation to the initial (pretetanus) value of PPF, as recently suggested by Schulz et al. (1995). However, the analysis of our data showed that the failure to replicate the results of Schulz et al. (1995) may depend on differences in experimental conditions such as the animal age (35–45 versus 15–30 days), strain (Sprague-Dawley versus Wistar), and, most relevantly, the fact that these authors measured LTP at saturation.

**nNOS and HO-2 Activities.** As depicted in Fig. 5, the prenatal exposure to CO provoked a significant (P < .05), although not impressive (−32 and −25%), decrease of nNOS and HO-2 activities, respectively. Conversely, both frontocortical and cerebellar enzyme activities of rats exposed to CO prenatally did not differ from those observed in control animals (data not shown).

**Discussion**

The present study demonstrates that hippocampal slices from rats exposed to a low concentration of CO during development exhibited an impaired ability to maintain LTP over time. It occurred in association with a modest, but signifi-
Fig. 3. Prenatal CO suppresses LTP maintenance. A, averages (mean ± S.E.) of f-EPSP slopes from control (○) and CO-exposed (●) rats. Values of f-EPSP slopes have been normalized to the pretetanus period. Each point represents the average ± S.E. of six consecutive responses taken every 5 min. Values of PTP were 236.11 ± 22.63 and 182.05 ± 20.16% in control and CO-slices, respectively (F < .05; Student’s t test). B, duration of LTP. Evoked f-EPSPs were considered potentiated until their slope was ≥20% with respect to baseline. For the control group, LTP duration was estimated by fitting analysis of the curve in A to calculate the interception point where this curve asymptotically subsided to a value of +20%. It occurred at 226.25 min after tetanus. On the contrary the curve describing the f-EPSP time course in CO-slices returned to +20% of baseline in 24.15 min. Bars represent the means ± S.E. obtained from 24 and 20 slices of control and CO-exposed rats, respectively. *P < .0001 versus controls (Student’s t test).

C, comparison of STP in isolation from LTP in the two groups. These curves were obtained normalizing the two curves in A at the +25-min value. This value was subtracted from all points in the interval between −15 and +25 min in each curve of A, as described by Schulz and Fitzgibbons (1997). The resulting curves were superimposed to show that the STP is similar in the two groups of slices.

that caused by a strong tetanus (Zhuo et al., 1993; Hawkins et al., 1994). The inactivation of HO by zinc protoporphyrin IX blocks LTP induction and reverts previously established LTP (Stevens and Wang, 1993; Hawkins et al., 1994; Medina and Izquierdo, 1995). Moreover, HO activity has been reported to increase in the hippocampus, but not in the neocortex or cerebellum, during step-down inhibitory training (Bernabeu et al., 1995). Furthermore, zinc protoporphyrin IX infusion in the hippocampus, but not in the amygdala, inhibits avoidance learning in rats (Fin et al., 1994). Interestingly, the latter effect is similar to that observed in rat offspring exposed to CO prenatally (Mactutus and Fechter, 1984; Di Giovanni et al., 1993).

The rationale of our study was to combine the above-mentioned evidences to further investigate the effects of CO administered during brain development. The complete and selective suppression of LTP maintenance found in CO-slices, long after the exposure cessation, suggests a persistent and complex mechanism.

The present impairment of LTP expression in the offspring of CO-exposed rats was accompanied by significant decreases (−32 and −25%, respectively) in both hippocampal nNOS and HO-2 activities. Postnatal enzyme alterations did not appear to involve additional brain regions. In fact, frontocortical and cerebellar enzyme activities, respectively, did not differ from control counterparts at either 30 or 90 days of age (data not presented), when both HO-2 and nNOS in the hippocampus were still markedly affected. Even though the present electrophysiological results have demonstrated that the prenatal CO exposure causes LTP disruption in the hippocampus, a region primarily involved in learning and memory processes, we cannot rule out the possibility that other brain areas could be affected also. Nevertheless, biochemical data support the hypothesis that alterations in the hippocampus could be responsible, in part, for cognitive deficits produced by prenatal exposure to CO. The involvement of NO (see Son et al., 1996, for references) and CO (Grundemar and Ny, 1997) in the induction of LTP has remained controversial, particularly on the basis of genetic “knockout” mice lacking the genes for HO-2 and NOS (Poss et al., 1995; Son et al., 1996; Wilson et al., 1999). However, the contemporaneous decreases in LTP expression and HO-2 and NOS activities
observed in CO-exposed offspring is consistent with the postulated role of both CO and NO in LTP (Hawkins et al., 1994). Irrespective of whether or not it has to deal with LTP, the impairment of both enzymes was not unexpected because of the strict correlation existing between colocalized NOS and HO-2 in neurons (Vincent et al., 1994), where the latter activity is needed as a possible defense against the toxic activity of excess newly formed NO (Ding et al., 1999).

It could be hypothesized that gestational CO-exposure might inactivate HO, possibly via an excess of reaction products. If HO activity is reduced, the sensitivity of metabotropic-glutamate receptor could be consequently decreased. Accordingly, HO antagonists actually block the effect of metabotropic-glutamate receptor stimulation (Glaum and Miller, 1993), which, in turn, is an important factor for LTP maintenance (Bashir et al., 1993; Bortolotto and Collingridge, 1993).

Moreover, the fact that PTP was reduced in slices from CO-exposed rats could be related to a decreased sensitivity of glutamate NMDA and AMPA receptors secondary to long-term hypoxia, as recently suggested by Pichiule et al. (1996).

We have also found that the average post-tetanus PPF did not change during LTP in either control or CO-slices. This datum is consistent with the majority of previous studies that have failed to detect any interaction of PPF with LTP in the CA1 region (Manabe et al., 1993; Manabe and Nicoll, 1994; Wu and Saggau, 1994; Schulz et al., 1995; Asztely et al., 1996).

PPF depends on the increase in presynaptic Ca\(^{2+}\)-mediated release by the second stimulus (Katz and Miledi, 1968; Wu and Saggau, 1994; Schulz et al., 1995), so that the PPF ratio results inversely related to the probability of transmitter release (Katz and Miledi, 1968; Manabe et al., 1993; Wu and Saggau, 1994; Asztely et al., 1996). Therefore, within the hypothesis that CO production in CO-slices would be inhibited, our inability to detect significant variation in post-tetanus PPF between control and CO-slices implies that the role of CO as retrograde messenger is unrelated to Ca\(^{2+}\) influx. This is in line with the indication that CO might enhance glutamate release by activating soluble guanylyl cyclase leading to increased formation of cGMP, which does not require Ca\(^{2+}\) influx (Heisler, 1986; Hawkins et al., 1994). More generally, it is possible that the presynaptic mechanism underlying LTP has to be sought downstream of terminal Ca\(^{2+}\) influx, as suggested by the results of Wu and Saggau (1994).

In conclusion, our findings suggest that learning and memory deficits induced in rat offspring by prenatal exposure to CO could stem, at least in part, from the disruption of the processes required for long-term synaptic storage, as reflected by the suppression of LTP maintenance, possibly via a negative action on the HO and NOS enzymes.

Because the alterations in hippocampal synaptic transmission have been produced by prenatal exposure to CO levels
resulting in maternal blood HbCO concentrations equivalent to those maintained by human cigarette smokers, the present data further point out the large risk that the smoking mother poses for her offspring.

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References


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