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Effect of Carbon Monoxide on Dopamine and Glutamate Uptake and cGMP Levels in Rat Brain

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After the recognition of nitric oxide (NO) as a messenger molecule in the nervous system, carbon monoxide (CO) has received attention with similar properties. The present study aims to elucidate the effects of CO on synaptosomal dopamine (^{3}H-DA) and glutamate (^{3}H-Glu) uptake and on cGMP levels; possible interaction between NO and CO systems was also evaluated. Our results provide evidence for the inhibition of DA and Glu uptake by CO in a time-, dose-, and temperature-dependent manner in rat striatum and hippocampus, respectively; the inhibition observed was sexually dimorphic with more pronounced effects in females. Basal cGMP levels were higher in female rats than males in the striatum and exogenous CO increased striatal cGMP levels only in males; no effect of CO was observed in the hippocampus. *In vivo* nitric oxide synthase (NOS) inhibition increased DA and Glu uptake; however, CO was still effective in inhibiting uptake following NOS inhibiton. Taken together, these findings suggest a role for CO in trans-synaptic regulation through modulation of DA and Glu transporters and of cGMP levels; the effect on cGMP levels is independent of NOS activity and appears to be sexually dimorphic and region specific.
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INTRODUCTION

Carbon monoxide (CO) is endogenously produced by the microsomal enzyme heme oxygenase (HO; EC 1. 14.99.3), which cleaves heme to CO, biliverdin, and iron. Molecular characterization of HO revealed the existence of three distinct forms of HO designated as HO-1, HO-2, and HO-3 (Kutty and Maines, 1981, 1982; Maines, 1988, 1997; Galbraith, 1999; Johnson and Johnson, 2000). The most striking difference between HO-1 and HO-2 is that HO-1 is a heat shock protein (HSP32) and inducible by a variety of agents, whereas HO-2 is constitutively expressed. Body distribution of HO-1 and HO-2 are also different: high levels of HO-1 activity are seen in spleen and liver, and of HO-2 in brain and testis (Maines, 1988, 1997; Ewing and Maines, 1997). HO-3 is a recently identified isoform and appeares to regulate heme-dependent genes (Magnusson *et al*, 2000). CO, like nitric oxide (NO), binds to the heme moeity of soluble guanylate cyclase (sGC) (Brune *et al*, 1990; Verma *et al*, 1993; Ingi *et al*, 1996a; Snyder *et al*, 1998) and in cultured olfactory neurons HO inhibitors reduce cGMP levels (Ingi *et al*, 1996b). Cytochrome *c* oxidase, the terminal enzyme in the mitochondrial electron transport chain, is another potential target for the actions of CO in the cell (Piantadosi, 1996).

Several studies indicate the colocalization of HO-2 and nitric oxide synthase (NOS) in various neural and vascular tissues (Maines, 1997; Juckett *et al*, 1998; Snyder *et al*, 1998). CO binds to the heme moeity of NOS and changes the effects of NO depending on the concentration. Furthermore, higher concentrations of CO have been shown to increase peroxynitrite levels and induce oxidative stress (Ischiropoulos *et al*, 1996; Thom *et al*, 1997). Although direct evidence is missing, an interaction between the HO/CO and NOS/NO systems is plausible.

Neurotransmitter transporters, or reuptake carriers, terminate the action of released neurotransmitter by reuptake and play an important role in the regulation of synaptic transmission in neurons. While short-term modulation of transporters involves a direct effect, changes in gene expression are required for long-term modulation. Dopamine transporter (DAT) is closely related to serotonin and norepinephrine transporters and together they form a subfamily within the large family of Na^+/Cl^--dependent transporters. DAT, a membrane-bound glycoprotein, is localized presynaptically and controls dopamine (DA) levels in the synaptic cleft by mediating uptake of released DA (Kuhar, 1997; Kuhar *et al*, 1999; Pogun, 1997). DAT plays a critical role in the regulation of cognitive functions and locomotor activity and it is a target for psychostimulant drugs including cocaine and amphetamine (Kuhar, 1997; Kuhar *et al*, 1999; Pogun, 1997). Glutamate (Glu) is a major

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excitatory amino-acid (EAA) neurotransmitter in the brain and high-affinity glutamate transporters (GLUT) are thought to be essential for terminating synaptic transmission as well as for maintaining the extracellular Glu concentration below toxic levels (Tanaka, 2000).

The involvement of NO in a type of retrograde uptake regulation has been suggested. Pogun *et al* (1994a, b) have demonstrated the inhibition of DA, Glu, and 5-HT uptake by NO in a dose-, time-, and temperature-dependent manner in synaptosomes prepared from the rat brain. In accordance with these *in vitro* findings, Koylu *et al* have shown an increase in DA and Glu uptake following systemic administration of N_G Nitro-L-arginine (L-NA) (Koylu *et al*, 1998).

Since CO shares some of the chemical and biological properties of NO, it may also act as a neuromodulator in synaptic regulation. The aim of this study was to evaluate the effect of CO treatment on DA and Glu uptake in synaptosomes prepared from male and female rat brains. In order to examine the effect of exogenously applied CO on NOS and guanylate cyclase, and to elucidate the possible mediation of cGMP and/or NO in the effects of CO on uptake, we also determined cGMP levels and the stable end products of NO ($NO_2^- + NO_3^-$). To test the interaction between CO and NO, *ex vivo* uptake experiments were performed, with or without CO, in rats treated with systemic L-NA injections to inhibit NOS *in vivo*. Since the major aim of the study was to study the effect of CO on DA and Glu transport, we chose to do the assays in brain regions with high transporter expression, namely striatum for DA and hippocampus for Glu uptake.

EXPERIMENTAL PROCEDURES

Animals

A total of 34 male and eight female adult Sprague–Dawley rats (250 ± 40 g), maintained on a 12 : 12 h light : dark cycle with food and water provided *ad libitum*, were used for the assays. The protocol employed was approved by the Institutional Ethics Committee.

Chemicals

[^{3}H]-Glu and [^{3}H]-DA were purchased from Amersham. Nitrate reductase was obtained from Boehringer Mannheim. All other chemicals were obtained from Sigma.

In Vivo NOS Inhibition

Rats were injected with L-NA (50 mg/kg, i.p.) or saline and decapitated after 30 min (Koylu *et al*, 1998; Forman *et al*, 1998; Yilmaz *et al*, 2000). Corpus striata and hippocampi of control and NOS-inhibited rats were used for *ex vivo* DA and Glu uptake experiments, respectively.

Tissue Preparation

Rats were decapitated, brains rapidly removed and dissected (hippocampus and corpus striatum) on ice. [^{3}H]-Glu and [^{3}H]-DA uptake assays were carried out as previously described (Pogun *et al*, 1994a, b). Synaptosomes were prepared in 0.32 M sucrose and a P2 pellet was obtained by centrifugation. The synaptosomes were resuspended in sucrose (15 mg original wet weight/ml) and aliquots were used in uptake experiments; the remaining tissue suspensions were stored at −30°C for cGMP and $NO_2^- + NO_3^-$ determinations. To assess whether sex differences exist in the effect of CO on [^{3}H]-Glu and [^{3}H]-DA uptake, synaptosomes prepared from female rat brains were also used.

Uptake Assays

Uptake assays were carried out in modified Krebs–Ringer phosphate buffer (126 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl, 1.4 mM $MgSO_4$, 16.0 mM Na phosphate, 2 mg/ml dextrose, and 0.2 mg/ml ascorbic acid). Blank values were obtained by adding 1 mM nomifensin and 10 mM Glu for DA and Glu uptake, respectively. CO (99.99%) was bubbled into synaptosomes for 1, 5, or 10 min at 37°C. Control and CO-bubbled synaptosomes were preincubated at 30°C for 5 min. Incubation with either [^{3}H]-Glu or [^{3}H]-DA was carried out at 30°C for 3 min. The assay was stopped by the addition of ice-cold 0.32 M sucrose and the synaptosomes were collected by rapid filtration through Whatmann GF/C filters. All determinations were performed in triplicates. Radioactivity in the filters was measured by liquid scintillation spectrometry (Pogun *et al*, 1994a, b).

cGMP Assay

cGMP levels in synaptosome suspensions were measured by radioimmunoassay kit (Amersham). Samples were deproteinized by adding acidic ethanol (1 ml 1 N HCl/100 ml ethanol) and allowed to stand 10 min at room temperature before centrifuging. cGMP levels were expressed as pmol/g wet weight.

$NO_2^- + NO_3^-$ Assay

To evaluate the effects of CO treatment on NO levels, total $NO_2^- + NO_3^-$ levels were determined in synaptosomes prepared from rat brains. Total $NO_2^- + NO_3^-$ levels were measured based on the reduction of nitrate to nitrite by nitrate reductase (EC 1.6.6.2) from Aspergillus sp. in the presence of NADPH. NO_2^- levels were determined spectrophotometrically by the Griess reaction (Giovannoni *et al*, 1997; Taskiran *et al*, 1997). Sodium nitrate solutions were used for standard measurements. Total $NO_2^- + NO_3^-$ levels were expressed as μmol/g wet weight.

Statistical Evaluation

Results are given as the average of three values for each experiment and expressed as percent of control. SPPS Inc. V6.1 statistical package program was used for all statistical evaluations. Data were initially analyzed by multifactorial or one-way analyses of variance (ANOVA). *Post hoc* Bonferonni tests were applied for group comparisons. The difference between various treatments and control (100%) values were determined by *t*-tests.

RESULTS

Effect of Exogenous CO on [^{3}H]-DA and [^{3}H]-Glu Uptake

CO treatment (1, 5, and 10 min) caused a time-dependent inhibition of both DA and Glu uptake (Figure 1). The

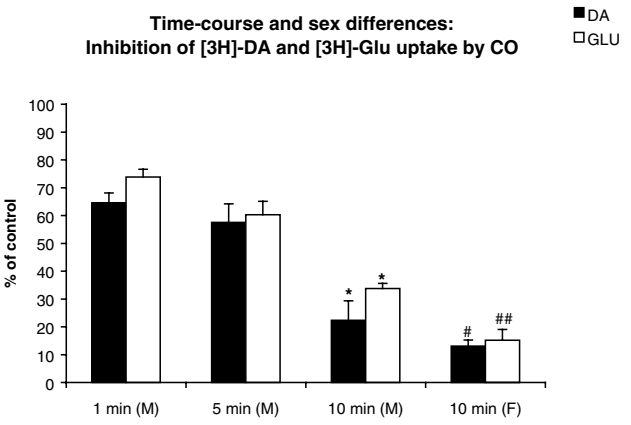

Figure 1 Time course of inhibition by CO treatment of [^{3}H]-DA and [^{3}H]-Glu uptake into synaptosomes in male rats and the sex difference observed after 10 min of CO exposure. Data are mean ± SEM percent of control. M: male, F: female (*different from 1 and 5 min exposure, male rats $p<0.0001$; different from male rats after 10 min exposure, $^{\#}p<0.05$, $^{\#\#}p<0.005$).

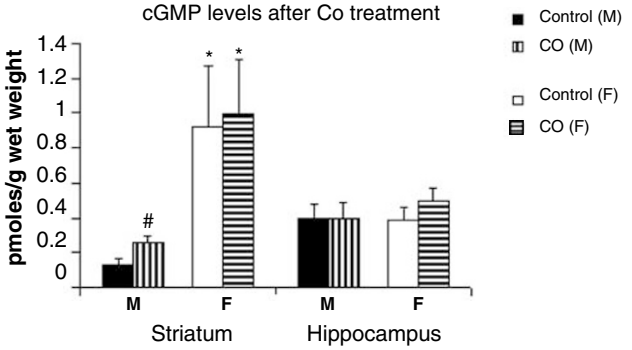

Figure 2 Striatal and hippocampal cGMP levels in female and male rats after CO treatment. Data are mean ± SEM percent of control. M: male, F: female (striatum: $^{\#}$different from control, $p<0.05$; *different from male control and male CO-treated groups, $p<0.005$).

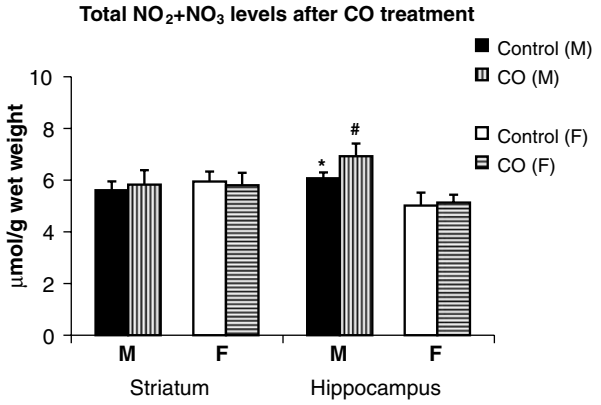

Figure 3 Striatal and hippocampal nitrate + nitrite levels in female and male rats after CO treatment. Data are mean ± SEM percent of control. M: male, F: female (hippocampus: *different from female control, $p<0.05$; $^{\#}$different from female CO-treated group, $p<0.01$).

difference between the groups was $F_{(2,19)}=17{,}81$, $p<0.0001$ for DA and $F_{(2,19)}=40{,}29$, $p<0.0001$ for Glu. The maximum effect of CO was observed at 10 min, where inhibition was 77.66% for DA and 66.28% for Glu ($p<0.0001$).

Sex Difference in [^{3}H]-DA and [^{3}H]-Glu Uptake after CO Treatment

In females both [^{3}H]-DA and [^{3}H]-Glu uptake were significantly decreased after CO treatment (86.95 and 84.83%, for DA and Glu, respectively, $p<0.001$) (Figure 1). Two-way ANOVA of [^{3}H]-DA and [^{3}H]-Glu uptake, with sex (male *vs* female) and CO treatment (CO present or absent) as factors, revealed a significant main effect of sex ($F_{(1,28)}=10{,}39$, $p<0.005$). Inhibition of [^{3}H]-DA and [^{3}H]-Glu uptake was more pronounced in females than in males ($p<0.05$ and $p<0.005$, for DA and Glu, respectively).

cGMP Levels after CO Treatment

There was a regionally specific and significant sex difference with regard to cGMP levels in both control and CO-treated preparations. Two-way ANOVA was employed with sex (male *vs* female) and treatment (CO present or absent) as the factors and cGMP levels as the dependent variable. ANOVA results revealed a significant main effect of sex ($F_{(1,28)}=10{,}39$, $p<0.005$) in the corpus striatum: cGMP levels were significantly higher in female rats than in male rats in both control and CO-treated groups ($p<0.05$) (Figure 2). Furthermore, CO treatment elevated the cGMP levels in males but not in females in this region ($p<0.05$). However, there was no difference between the groups in the hippocampus and no significant effect of CO treatment was observed (Figure 2).

Total $NO_2^- + NO_3^-$ Levels after CO Treatment

There was no significant effect of CO treatment on total $NO_2^- + NO_3^-$ levels in both corpus striatum and hippocampus (Figure 3), as assessed by the two-way ANOVA, with sex (male *vs* female) and treatment (CO present or absent) as the factors and total $NO_2^- + NO_3^-$ levels as the dependent variable. On the other hand, there was a significant main effect of sex in the hippocampus ($F_{(1,28)}=12{,}67$, $p<0.001$). NO metabolites were significantly higher in males than in females in both controls and CO-treated groups ($p<0.05$ and $p<0.01$, respectively) (Figure 3).

Effect of CO Treatment on [^{3}H]-DA and [^{3}H]-Glu Uptake after *In Vivo* NOS Inhibition

L-NA treatment increased both [^{3}H]-DA and [^{3}H]-Glu uptake compared to controls (104.37 and 108.87%, respectively) (Figure 4). One-way ANOVA and *post hoc* Bonferonni test revealed significant differences between the groups ($F_{(2,20)}=38{,}11$, $p<0.0001$; $F_{(2,20)}=46{,}48$, $p<0.0001$, for DA and Glu, respectively). CO treatment, with or without *in vivo* NOS inhibition, significantly decreased [^{3}H]-DA and [^{3}H]-Glu uptake compared to L-NA-treated group only ($p<0.0001$) (Figure 4). There was no interaction between *in vivo* L-NA treatment and *ex vivo* CO application, regarding DA and Glu uptake.

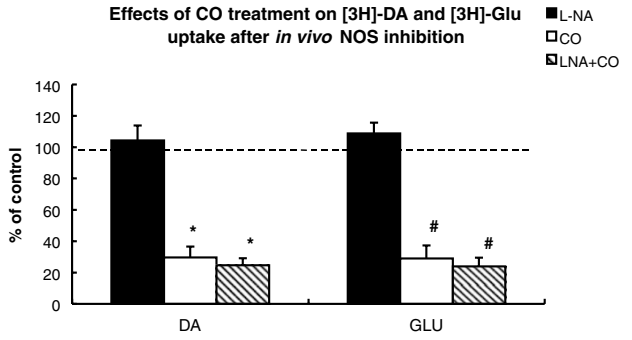

Figure 4 Effects of CO treatment on [^{3}H]-DA and [^{3}H]-Glu uptake following *in vivo* NOS inhibition (*different from *in vivo* NOS-inhibited group for [^{3}H]-DA, $^{\#}$different from *in vivo* NOS-inhibited group for [^{3}H]-Glu, $p<0.0001$).

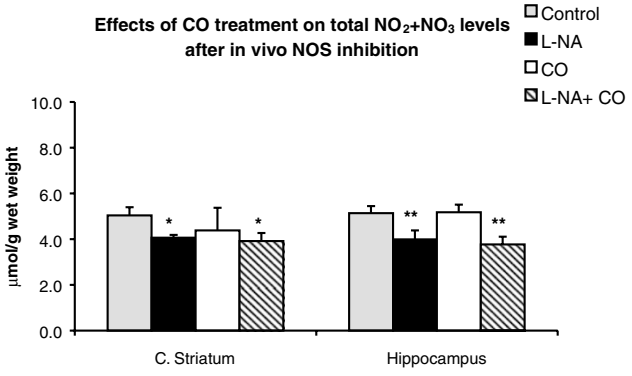

Figure 5 Effects of CO treatment on total nitrate and nitrite levels after *in vivo* NOS inhibition (different from control, *$p<0.05$ for c.striatum and **$p<0.001$ for hippocampus).

Total $NO_2^- + NO_3^-$ Levels after CO Treatment of *In Vivo* NOS-Inhibited Rats

The total $NO_2^- + NO_3^-$ levels after CO treatment in rats were evaluated with two-way ANOVA in the corpus striatum and hippocampus. L-NA and CO treatments (present or absent) were taken as factors and total $NO_2^- + NO_3^-$ levels as the dependent variable. Total $NO_2^- + NO_3^-$ levels were significantly lower in L-NA-treated groups than in controls ($F_{(3,32)} = 4{,}12$, $p<0.05$ for corpus striatum and $F_{(3,32)} = 13{,}83$, $p<0.001$ for hippocampus) (Figure 5). There was no significant effect of CO treatment alone.

DISCUSSION

Reuptake is the major mechanism that regulates synaptic levels of some neurotransmitters such as DA and Glu. The modulation of DA and Glu transporters not only influences the efficiency of synaptic transmission, but also mediates in neurotoxic processes. For example, while disturbed reuptake of Glu may underline some neurological disorders such as amyotrophic lateral sclerosis (ALS), inhibition of Glu uptake may also facilitate learning and memory processes (Rothstein, 1995). On the other hand, regulating dopaminergic neurotransmission by reuptake may affect reward systems or motor control with substantial influences in addictive or locomotor behavior, respectively (Taskiran *et al*, 2000).

Exposure to hypoxia and ischemic conditions increase extracellular levels of monoamine neurotransmitters in brain, and accumulation of DA and noradrenalin (NA) may influence the development of neuronal death during ischemia (Akiyama *et al*, 1991; Hiramatsu *et al*, 1996). Hiramatsu *et al* (1994) have reported significantly elevated levels of DA in striatal dialysates after CO exposure, while the levels of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) decreased.

The most significant finding of the present study is the time-dependent inhibition of both DA and Glu uptake by CO treatment. The maximum inhibition was observed at 10 min of CO treatment (Figure 1); the inhibition was also temperature dependent and was not observed at 0°C (data not shown). In agreement with our findings, Pogun *et al* have shown time-, temperature- and dose-dependent inhibition of DA and Glu uptake by the NO generator sodium nitroprusside (SNP) in synaptosomes prepared from rat brain, in a regionally selective manner (Pogun *et al*, 1994a, b; Pogun and Kuhar, 1994). DA and Glu uptake mechanism is an ATP-dependent process, and hypoxia causes ATP depletion within 5 min (Hansen, 1985). Indeed, CO binds to cytochrome *c* oxidase in the brain, resulting in a decreased rate of mitochondrial energy production (Piantadosi *et al*, 1995). Although the mechanism is not documented yet, one possible explanation of the inhibition of transport by CO may be reduced ATP production.

Sex is an important factor that influences neurotransmitter sytems, and the vulnerability of the male and female brain to ischemia and neuropathological conditions vary (Ferris *et al*, 1995; Meyer *et al*, 1998). In the present study, CO decreased DA and Glu uptake into synaptosomes prepared from both female and male rat brains. However, the inhibition was higher in female than in male rats (Figure 1). This finding suggests greater sensitivity of female rats to CO toxicity or to transporter modulation than males. However, CO toxicity may have other effects beyond transporter modulation, which influence vulnerability. A recent case report suggests differential susceptibility of the male and female brain to CO poisoning: A married couple was exposed CO and 1 month later only the husband developed Parkinsonism, a common neurological sequela of CO poisoning; the initial white matter damage studied by MRS was more severe in the husband than in the wife (Sohn *et al*, 2000).

Like NO, CO binds to the iron of the heme moiety in sGC to activate the enzyme. HO-sGC colocalization has been demonstrated in many brain regions and this colocalization is more pronounced than that of NOS-sGC. However, despite this high colocalization, the activation of sGC by CO is only about 1% of the activation of sGC by NO (Burstyn *et al*, 1995; Stone and Marletta, 1994). Furthermore, while the effect of CO on cGMP induction is reported in the cerebellum, no significant elevation in cGMP formation was observed in cortical regions (Laitinen *et al*, 1997). Although the activation of isolated sGC with CO is much weaker than with NO, CO can be a powerful activator in stimulating cGMP in the presence of an indazole; Friebe *et al* (1998) have shown a 100-fold increase in the affinity of

CO to sGC in the presence of YC-1, a benzylindazole derivative. On the other hand, there are studies demonstrating that CO may have effects independent of cGMP. For example, in a patch clamp study, Wang *et al* (1997) have shown that exogenous CO application can activate the 238pSK$_{Ca}$ channel independent of cGMP. Similarly, Kaide *et al* (2001) demonstrated the cGMP-independent activation of the 105pSK$_{Ca}$ channel by CO. These results provide evidence that other routes may be involved in CO action than cGMP.

Recent observations have suggested that NO can function as a very effective and short-lived stimulator of cGMP production, while CO produces long-term effects on cGMP levels because of its chemical stability (Zhuo *et al*, 1993). In our study, exogenous CO application had an effect only in the striatum and only in the male rats: of the two brain regions studied, CO increased cGMP levels in the striatum, but not in the hippocampus (Figure 2). In female rats, CO was without any effect on cGMP levels in either brain region. Basal cGMP levels were significantly higher in the striatum of female rats than males, while no sex difference was observed in the hippocampus. Palmon *et al* (1998) reported sex differences in basal cGMP levels, with higher levels in females. Furthermore, a dose-dependent increase in cGMP levels by estrogen was observed, however most significantly in the hippocampus.

Several studies have reported the interactions between CO- and NO-generating systems (Zhuo *et al*, 1993; Maines, 1997; Snyder *et al*, 1998). Since NOS is a hemoprotein, CO is able to bind to the existing NOS and inactivate the molecule. Conversely, NO could modulate HO-2 activity through free radical attack on −SH groups on HO-2 (Verma *et al*, 1993; Maines, 1997; Juckett *et al*, 1998). Ischiropoulos *et al* (1996) have found that CO exposure leads to the perivascular accumulation of nitrotyrosine, a marker of peroxynitrite ($OONO^-$) production. Furthermore, Thorup *et al* (1999) have shown that picomolar concentrations of CO, in renal resistance arteries from rats, facilitate the release of NO from a large intracellular pool, whereas micromolar concentrations of CO inhibit NOS activity and NO generation. Our results, in accordance with our previous findings, showed that while $NO_2^- + NO_3^-$ levels were higher in the male hippocampus than the female (Taskiran *et al*, 1997), CO treatment had no effect on total $NO_2^- + NO_3^-$ levels both in the male and female rat brain. Although this finding is not consistent with some of the previous studies mentioned above, it may be explained by the observation of Stamler and Piantadosi (1996). According to their hypothesis, CO may competitively displace NO by binding to heme irons in proteins and induce $ONOO^-$ production by disrupting mitochondrial electron transport. The study by Stamler and Piantadosi suggests that CO may be replacing NO and thereby causing peroxynitrite formation without affecting NOS. In the endothelium, CO may be competing for the intracellular binding sites of NO (hemoproteins) to increase steady-state NO levels and peroxynitrite without altering arginine transport or NOS activity (Thom *et al*, 1997, 1999). As Cary and Marletta (2001) discuss in a recent review, extensive research is needed before we can reach a conclusion about CO as a signaling molecule and its interactions with the nitrergic systems.

In our previous study, we have reported the facilitation of DA and Glu uptake in *in vivo* NOS-inhibited rats (Koylu *et al*, 1998). The present study confirmed our results. L-NA treatment increased the DA and Glu uptake compared to control levels. CO treatment with or without sytemic L-NA injection inhibited both DA and Glu uptake suggesting that CO action is not dependent on the NO system. Although CO and NO seem to share similar biological properties, they can regulate the transporter functions through different mechanisms.

In conclusion, the present study provides evidence for the inhibition of DA and Glu reuptake in a time-, dose-, and temperature-dependent manner in the rat striatum and hippocampus. The inhibition observed is sexually dimorphic and independent of the NOS activity. These results suggest that CO may act as a modulator of synaptic transmission.

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