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# Reciprocal regulation of nitric oxide and glutamate in the nucleus tractus solitarii of rats

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**Abstract**

Nitric oxide (NO) and glutamate are both important mediators of the central cardiovascular regulation in the nucleus tractus solitarii. Our previous studies revealed that the central cardiovascular effects of NO in the nucleus tractus solitarii could be inhibited by glutamate receptor blockade. On the other hand, nitric oxide synthase (NOS) inhibitor attenuated the cardiovascular effects of glutamate. Thus, NO and glutamatergic systems appear to interact in central cardiovascular regulation. The present study examined whether NO and glutamate may affect each other's release/production in the nucleus tractus solitarii. A microdialysis probe was implanted into the nucleus tractus solitarii of male Sprague–Dawley rats, and the changes in the extracellular levels of glutamate and NO were determined by high performance liquid chromatography coupled with electrochemical detection and an NO analyzer, respectively. The results showed that NO solution elicited $>10$ fold increases in the extracellular level of glutamate, which returned to normal 60 min after the end of NO perfusion. The NO donor *N*-acetyl-penicillamine (SNAP) had an effect similar to NO solution. Furthermore, the glutamate level was reduced to 61% of basal value by perfusion with the NOS inhibitor, $N^G$-monomethyl-L-arginine (L-NMMA). When glutamate receptor agonist *N*-methyl-D-aspartic acid (NMDA) or α-amino-3-hydroxy-5-methylixoxazole-4-propionic acid (AMPA) was administered into the nucleus tractus solitarii, the extracellular NO level was increased by 70–100%, whereas glutamate receptor antagonists (MK-801 hydrogen maleate and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX)) did not alter the basal levels of NO. These results suggest that NO and glutamate may enhance each other's release/production in the nucleus tractus solitarii. This reciprocal regulation of NO and glutamate may be important in central cardiovascular control in the nucleus tractus solitarii. 

*Keywords:* Nucleus tractus solitarii; Nitric oxide; Glutamate; Microdialysis

## 1. Introduction

The nucleus tractus solitarii is located in dorsal medial part of the medulla. Neurons of the nucleus tractus solitarii project into or receive input from other regions of the central nervous system important to cardiovascular regulation. The nucleus tractus solitarii is also the principal site of termination of afferent fibers from arterial baroreceptors and plays an important role in cardiovascular regulation (Reis, 1984; Ruggiero et al., 1996). In the nucleus tractus solitarii, L-glutamate is a well-recognized neurotransmitter that mediates central cardiovascular regulation. Microinjection of L-glutamate into the nucleus tractus solitarii can decrease blood pressure and heart rate (Talman et al., 1980). On the other hand, blockade of glutamate receptors in the nucleus tractus solitarii can inhibit baroreflexes (Kubo and Kihara, 1991). Furthermore, glutamate was found to exist in the vagal afferents in the nucleus tractus solitarii and can be released following baroreceptor activation or when the nucleus tractus solitarii is exposed to a high potassium-depolarizing solution (Sykes et al., 1997; Lawrence and Jarrott, 1994). The evidence suggests that

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L-glutamate is an important neurotransmitter in the nucleus tractus solitarii mediating cardiovascular regulation.

In addition to L-glutamate, recent evidence suggests that nitric oxide (NO), a gas molecule synthesized from L-arginine by nitric oxide synthase (NOS), may be involved in the cardiovascular regulation by the nucleus tractus solitarii (Tseng et al., 1996). Two types of NOS were identified. The constitutive form of NOS includes the endothelial NOS and neuronal NOS. They are constitutively expressed and their activities are regulated by intracellular $Ca^{2+}$ concentration. The inducible form of NOS is induced by various stimuli and not regulated by $Ca^{2+}$ activity (Nathan and Xie, 1994). It was found that the NOS enzyme existed in the nucleus tractus solitarii neurons as well as in the afferent terminals within the nucleus tractus solitarii (Ruggiero et al., 1996; Vincent and Kimura, 1992). Studies using rat brainstem slices showed that L-arginine, a precursor of NO, increased nucleus tractus solitarii neuronal activities in a dose-dependent manner and these effects can be blocked by the NOS inhibitor, $N^G$-monomethyl-L-arginine (L-NMMA) (Tagawa et al., 1994). More recently, we have demonstrated that microinjection of L-arginine into the nucleus tractus solitarii caused dose-dependent depressor and bradycardic effects, and reduced renal sympathetic nerve activity. NOS inhibitor attenuated these effects (Tseng et al., 1996). We have also shown that the NO-induced cardiovascular changes in the nucleus tractus solitarii were mediated through a cGMP-dependent pathway (Lin et al., 1999a). These studies suggest that NO produced in the nucleus tractus solitarii may play an important role in the regulation of the cardiovascular system.

Glutamate may act through a variety of receptors including *N*-methyl-D-aspartic acid (NMDA) and kainate/α-amino-3-hydroxyl-5-methylixoxazole-4-propionic acid (AMPA) (non-NMDA) receptors (Foster and Fagg, 1984). Studies have shown that NO formation effectively mediates glutamate-induced cGMP accumulation (Bredt and Snyder, 1989) and NMDA-evoked neurotransmitter release in the brain (Montague et al., 1994). The critical link between NMDA receptor and NO generation appears to be $Ca^{2+}$, which permeates opened NMDA channels, leading to the formation of a $Ca^{2+}$/calmodulin complex that can activate intracellularly localized nNOS (Garthwaite and Boulton, 1995). Additionally, NO release is also involved in physiological processes associated with altered sensitivity of the AMPA receptor (Dev and Morris, 1994). On the other hand, administration of an NO donor into the dorsal medial medulla oblongata (close to the nucleus tractus solitarii) could increase release of glutamate which acts as a neurotransmitter for baroreflex. (Lawrence and Jarrott, 1993, 1994). Furthermore, we have recently reported that the cardiovascular effects of L-glutamate, NMDA, and AMPA in the nucleus tractus solitarii could be blocked by inhibition of NOS or soluble guanylyl cyclase activity in microinjection studies. Similarly, glutamate receptor blockades also attenuated the cardiovascular effects of NO in the nucleus tractus solitarii (Lin et al., 1999b; Lo et al., 1997). These observations suggested that NO and glutamate may have a reciprocal regulatory mechanism in the nucleus tractus solitarii. The present study was, therefore, designed to use an in vivo microdialysis technique to elucidate whether NO and glutamate have a reciprocal regulation of release in the nucleus tractus solitarii.

## 2. Methods

### 2.1. Experimental procedures

Male Sprague–Dawley rats (300 ± 50 g; National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) were obtained and housed in the animal room of the National Defense Medical Center, Taipei, Taiwan. The animals were kept on a 12-h on, 12-h off light cycle and allowed access to rat chow and water ad libitum.

Rats were anesthetized with urethane (1.0 g/kg i.p.) supplemented with 300 mg/kg if indicated. The preparation of animals, methods used in localizing the nucleus tractus solitarii, and preparation of microdialysis probes have been described previously (Lin et al., 1999c). Briefly, the animal was mounted on a stereotaxic frame (Kopf). The skull was exposed and a craniotomy was performed for the placement of a microdialysis probe into the nucleus tractus solitarii. The dialysis probe was secured in place and perfused continuously with an artificial cerebral spinal fluid (aCSF: 140 mM NaCl, 1.2 mM $CaCl_2$, 3.0 mM KCl, 1.0 mM $MgCl_2$, 40 μM ascorbic acid, pH 6.3) at a speed of 1.1 μl/min. The collection of dialysates (20 min per fraction) was started 3 h after the probe is implanted. Basal levels of glutamate or NO were defined as the average of the first three samples. After collection of the first three samples, various reagents were continuously perfused into the nucleus tractus solitarii for 40 min, after which aCSF was again perfused. The reagents used in the experiments included NO solution, NO donor (*N*-acetyl-penicillamine (SNAP)), NOS inhibitor (L-NMMA), ionotropic glutamate receptor agonists (NMDA and AMPA), and glutamate receptor antagonists MK-801 hydrogen maleate (MK-801) and 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). The dialysates were collected for another 120 min after the end of reagent perfusion. Following each experiment, the rat was perfused intracardially with normal saline and 4% paraformaldehyde. The placement of the microdialysis probe in the nucleus tractus solitarii was verified in sections stained with cresyl violet.

### 2.2. Preparation of NO solution

NO solution used in the present study that was freshly prepared on nitrogen gas was infused into the aCSF on the day of each experiment (Rauhala et al., 1998). Briefly, a

test tube containing 15 ml of aCSF was wrapped in aluminum foil, placed in ice and capped with a stopper, which allowed inflow and outflow of gas. The aCSF was bubbled with nitrogen for 20 min, and then by NO gas (1000 ppm, 100 ml/min) for 40 min. The NO gas was filtered through 5 M NaOH solution to remove nitrites and nitrates before use. The solution was sampled for NO content determination as described below. This solution had an NO concentration of about 600 μM. It was further diluted with aCSF to a concentration of 400 μM or 100 μM for experimental use.

### 2.3. Detection of excitatory amino acids

Separation and analysis of glutamate was performed by high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD). A programmable solvent delivery system (BAS 480) with an electrochemical detector (LC-4C/CC-5, BAS) was used and coupled to a refrigerated auto-microsampler. We used a ternary gradient version of a PM-80 pump including a touchpad controller, and a proportioning valve and mixer. Glutamate was separated with a precolumn derivative process using *o*-phthaldialdehyde/2-mercaptoethanol followed by a gradient elution with an on-line degasser. The amino acids were separated with a BAS amino acid II, MF-6199 column (100 × 3 mm, 3-μm particle). The solution of the mobile phase contained a mixture of (1) 90% 0.1 M acetate buffer, pH 6.0 and 10 % acetonitrile; and (2) 10% 0.1 M acetate buffer, pH 6.0 and 90% acetonitrile. The neuronal origin of glutamate measured by the microdialysis technique was previously verified by high $K^+$ solution stimulation (Lin et al., 1999c). The standard curves of glutamate and aspartate were obtained using a series of freshly-made L-glutamate and L-asparate solutions (0, 0.5, 1, 5 and 15 μM) before each measurement. The correlation between the above-mentioned concentrations and the integrated areas of their peaks was linear (Fig. 1A). The correlation coefficient was 0.985.

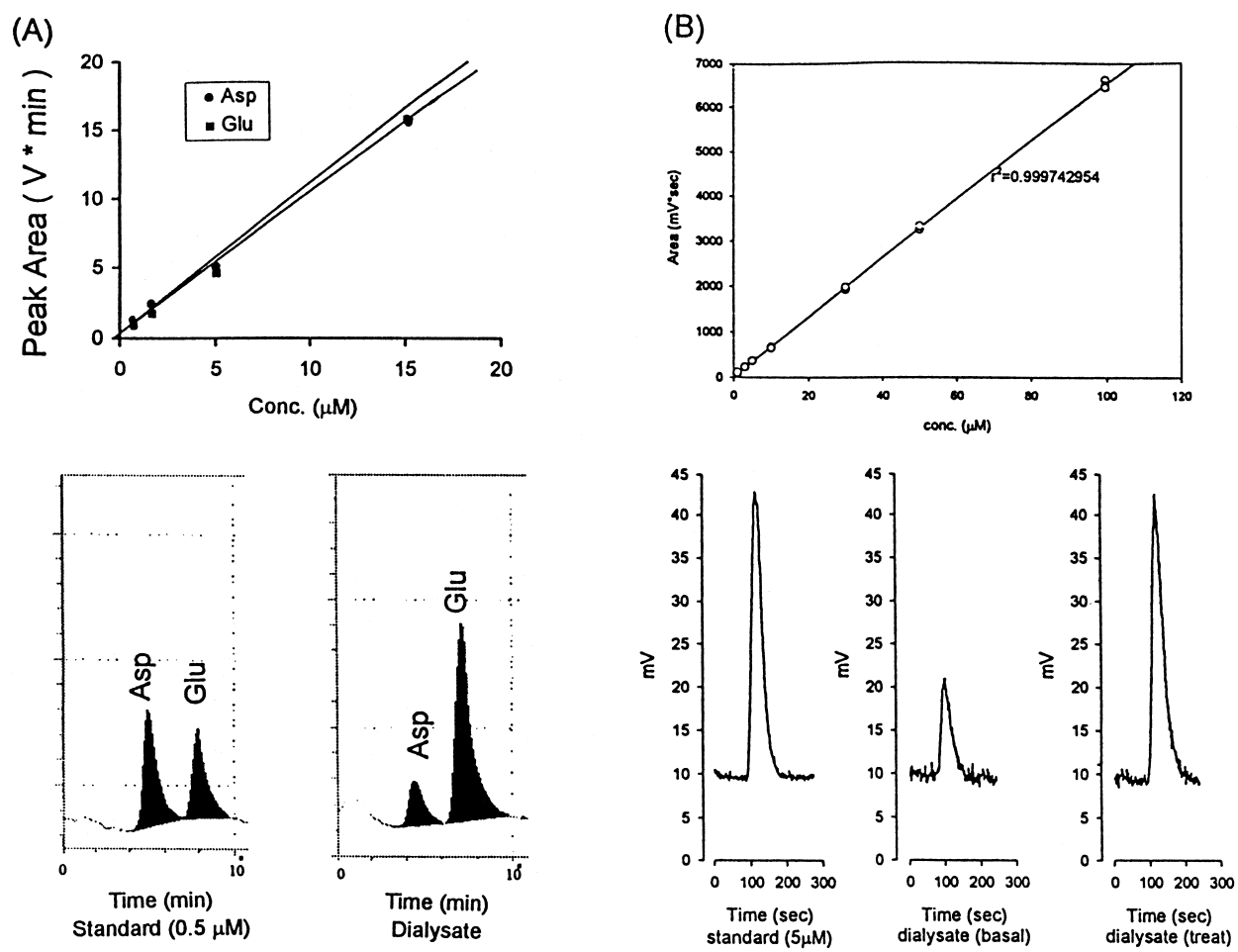


Fig. 1. Representative tracings showing peaks of excitatory amino acids and NO of standard solutions and dialysates. (A) The top panel shows the relationship between the concentrations of glutamate (Glu) and aspartate (Asp) standards and the integrated areas of the peaks. The bottom panels show the glutamate and aspartate responses after injections of standards (0.5 μM) and microdialysis dialysate. (B) The top panel shows the relationship between seven nitrite concentrations (three samples for one concentration) and the integrated areas of the peaks. The bottom panels show the NO responses after injections of standards (5 μM) and dialysates (basal and treated groups). The least-squares-fit regression line is $r^2 = 0.985$ for (A) and $r^2 = 0.999$ for (B).

### 2.4. Measurement of NO

NO content in the collected dialysates or prepared NO solution was determined by measuring NO and its oxidation products, nitrite/nitrate, using a chemiluminescence technique (NO-Analyzer 280, Sievers Research) as described previously (Archer, 1993; Lin et al., 1999c). Briefly, nitrite/nitrate in the dialysates were reduced to NO by a reducing agent, 0.1 M vanadium chloride in 8% HCL. The NO in turn reacts with $O_3$ to produce $^*NO_2$ (excited state). $^*NO$ will emit energy to become stable state $NO_2$, during which the emitted energy can be detected by the chemiluminescence technique. The standard curve of NO was obtained using a series of freshly-made sodium nitrite solutions (0, 1, 3, 5, 10, 30, 50 and 100 μM) before each measurement. The correlation between the above-mentioned nitrite concentrations and the integrated areas of their peaks was linear (Fig. 1B). The correlation coefficient was 0.999.

### 2.5. Data analysis

The levels of glutamate and NO were transformed as percentages of the basal value and expressed as mean ± S.E.M. Statistical analysis was performed using analysis of variance followed by a post hoc Dunnett's test. A $P$ value $< 0.05$ was considered significant.

## 3. Results

### 3.1. NO solution and no donor induced glutamate release

The basal level of extracellular glutamate in the nucleus tractus solitarii was 22.5 pmol/20 μl. The effect of NO on glutamate release in the nucleus tractus solitarii was evaluated in animals perfused with a NO solution through the dialysis probe. As noted in Fig. 2A, the level of glutamate in the control animals (perfused with aCSF only) remained at a relatively stable value. Perfusion with 100 μM NO solution caused a slight increase in the extracellular glutamate level (up to 214 ± 88% of basal value at time point 60 min), but this change did not reach statistical significance. At the 400 μM concentration, NO solution induced increases in the glutamate level after the initiation of NO perfusion and reached 1170 ± 230% of basal value ($P <$ 0.05, compared with control group). At the end of NO perfusion, the extracellular glutamate level returned to basal concentration.

The effect of NO on glutamate release was also investigated by using an NO donor, SNAP. We found that SNAP at the 1 mM concentration did not significantly affect the extracellular glutamate level. SNAP at 10 mM increased the glutamate level to 355 ± 45% ($P < 0.05$, compared with the control group), which rapidly reduced to basal level after cessation of SNAP perfusion. This pattern of changes in glutamate level was similar to that induced by NO solution (Fig. 2B).

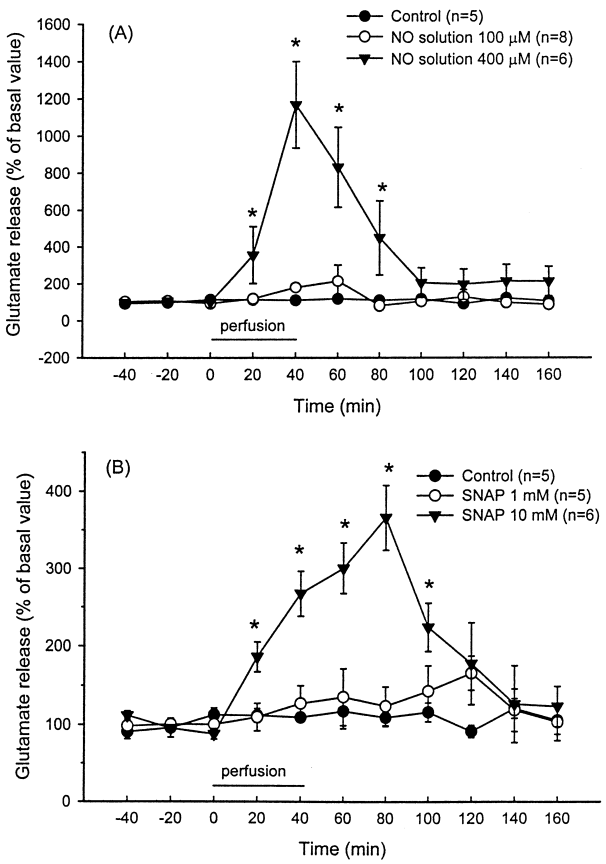


Fig. 2. Time course study of glutamate levels in nucleus tractus solitarii microdialysates from animals after 40 min of NO solution, NO donor (SNAP) or aCSF (control group) perfusion. (A) At 400 μM concentration, the NO solution induced dramatic increases in extracellular glutamate content, which peaked at the end of NO perfusion. (B) SNAP elicited increases in extracellular glutamate level at the concentration of 10 mM, but not 1 mM. Data are expressed as mean ± S.E.M. and $^*P < 0.05$ vs. control by Dunnett's test. Line indicates the perfusion time of drug administration.

### 3.2. L-NMMA reduced the basal level of glutamate

To evaluate whether endogenous NO could modulate glutamate release, the nucleus tractus solitarii region was perfused with an NOS inhibitor, L-NMMA. We found that L-NMMA affected the identification of the glutamate peak by HPLC. Therefore, the extracellular glutamate level could be determined only in dialysate fractions before and 40 min after L-NMMA perfusion. The results showed that 1 mM L-NMMA reduced the glutamate level to 61.4 ± 19.6% and 65.9 ± 9.9% at 40 and 60 min after cessation of L-NMMA perfusion, respectively ($P < 0.05$, compared with the control group). The decrease in glutamate level was reversible, and it gradually returned to basal level at the end of the experiment (after 120 min) (Fig. 3).

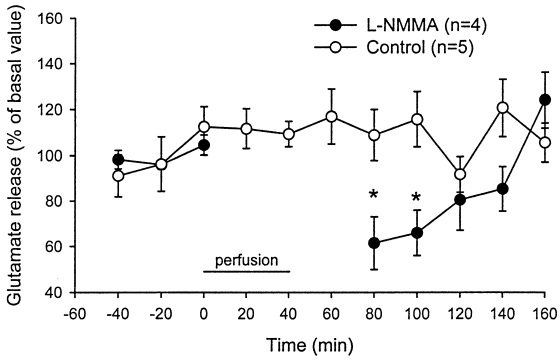


Fig. 3. Time course study of glutamate levels in nucleus tractus solitarii microdialysates from animals after 40 min of NO synthase inhibitor (L-NMMA) or aCSF perfusion. L-NMMA interferes with the detection of glutamate in the first three fractions (time points 20, 40 and 60 min) of the dialysates. The significant reduction of extracellular glutamate concentrations by L-NMMA was observed at time points 80 and 100 min of perfusion ($^{*}P < 0.05$, L-NMMA vs. control).

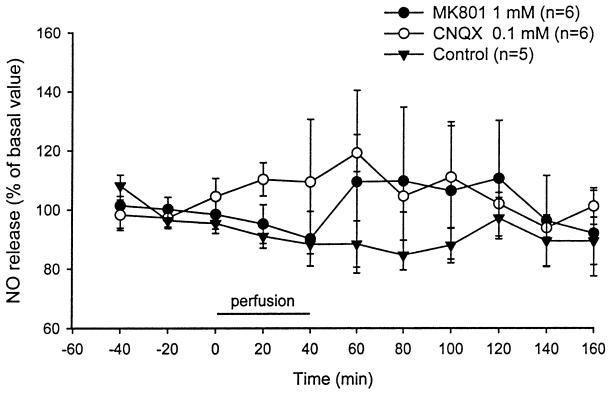


Fig. 5. Time course study of NO levels in nucleus tractus solitarii microdialysates from animals after 40 min perfusion with of NMDA and non-NMDA receptor antagonists (MK-801 or CNQX). Both MK-801 or CNQX did not significantly change the basal NO levels.

### 3.3. NMDA and AMPA induced NO production

The basal level of extracellular NO in the nucleus tractus solitarii was $1.8 \pm 0.3$ μM. The effects of glutamate receptor activation on NO production were examined by perfusing the nucleus tractus solitarii with ionotropic glutamate receptor agonists, NMDA and AMPA. As shown in Fig. 4, 40 min perfusion with 0.33 mM NMDA solution induced elevations in extracellular NO content, which reached a peak value of $197 \pm 12\%$ ($P < 0.05$, compared with the control group at 40 min). AMPA at 0.33 mM did not significantly alter extracellular NO content. At 1 mM concentration, AMPA induced a quick increase in NO level with a peak value of $180 \pm 28\%$ ($P < 0.05$, compared with the control group at 40 min).

### 3.4. Effects of MK-801 and CNQX on NO production

The role of glutamate receptor activity on NO production was also evaluated by perfusing the nucleus tractus solitarii with the NMDA receptor antagonist (MK-801) and non-NMDA receptor antagonist (CNQX). We observed that neither MK-801 (1 mM) nor CNQX (0.1 mM) altered the overall basal NO level in the nucleus tractus solitarii (Fig. 5).

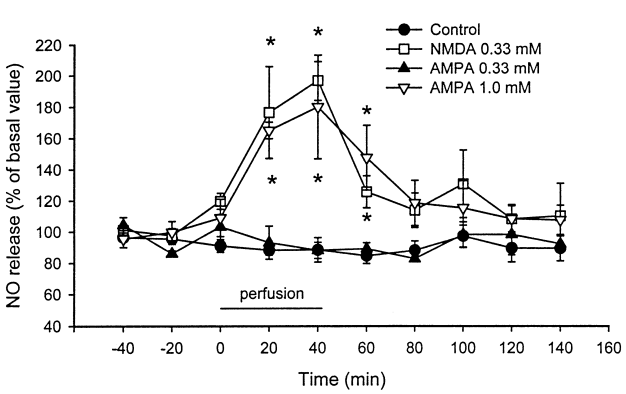


Fig. 4. Time course study of NO levels in nucleus tractus solitarii microdialysates from animals after 40 min of NMDA, AMPA or aCSF perfusion. Both 0.33 mM NMDA and 1 mM AMPA elicited increases in NO content ($^{*}P < 0.05$ vs. control).

## 4. Discussion

Pharmacological intervention has shown that the NO-generating and glutamatergic systems may regulate each other's physiological responses in the nucleus tractus solitarii in vivo (Garthwaite et al., 1988; Lin et al., 1999b). The current study provides direct evidence for this hypothesis. We found that NO administration via a microdialysis probe induced glutamate release, while NOS inhibition reduced the basal glutamate level. On the other hand, stimulation of the NMDA or AMPA subtype of the glutamate receptor enhanced NO production. These results agree with our hypothesis that a reciprocal regulatory mechanism of NO and glutamate exists in the nucleus tractus solitarii.

In this study, we have clearly seen that perfusion with either NO solution or SNAP through a microdialysis probe implanted in the nucleus tractus solitarii caused a prominent increase in glutamate release. This increase was readily reversible after cessation of the perfusion indicating that this is likely a physiological response of neurons to NO stimulation. These observations correlated with a previous study showing that perfusion with the NO donor SNAP induced glutamate release in the dorsal medial medulla oblongata (Lawrence and Jarrott, 1993). In addition to glutamate release subsequent to NO perfusion, we also observed that perfusion with L-NMMA, an NOS inhibitor, reduced the glutamate level to 61% of the basal value. These results indicate that exogenous as well as endogenous NO regulates glutamate release in the nucleus tractus solitarii.

In addition to the finding that NO causes glutamate release in the nucleus tractus solitarii, we also evaluated whether NMDA and non-NMDA glutamate receptors regulate NO production in the same brain region. It is known that activation of NMDA or non-NMDA glutamate receptors leads to $Ca^{2+}$ influx, which in turn activates $Ca^{2+}$/calmodulin-dependent NOS and increases NO production (Bredt and Snyder, 1989; Garthwaite, 1991). In the rat cerebellum, activation of NMDA or non-NMDA receptors has been shown to increase extracellular NO levels (Yamada and Nabeshima, 1997; Baltrons and Garcia, 1997; Bhardwaj et al., 1997). In agreement with these reports, we also found that both NMDA and AMPA induce NO production in the nucleus tractus solitarii. This notion is supported by our previous studies showing that glutamate-induced hemodynamic changes were inhibited by NOS inhibitors (Lin et al., 1999b; Lo et al., 1997). Furthermore, we tried to see if glutamate receptor activity regulates the tonic production of NO in the nucleus tractus solitarii. We found that both MK-801 and CNQX perfusion did not affect NO level in the nucleus tractus solitarii. Nonetheless, we have clearly shown that stimulation of either NMDA or AMPA receptors in the nucleus tractus solitarii leads to increased NO production.

It has been shown in many studies that NO can be released from postsynaptic neurons upon glutamate receptor activation and serves as an intercellular messenger acting on presynaptic neurons in a long term potentiation model (Garthwaite, 1991). Ogawa et al. (1995) have shown that NO can serve as a retrograde messenger enhancing glutamate release from presynaptic terminals in the nucleus tractus solitarii during hypoxia similar to the role of NO in the hippocampus. Our current study showed that both glutamate-mediated NO production and NO-mediated glutamate release mechanisms constitutively exist in the nucleus tractus solitarii in the physiological condition. Further, the present study suggests that a positive feedback loop between glutamate and NO is in constant operation in the nucleus tractus solitarii, since the inhibition of NOS decreased basal glutamate release.

In conclusion, the results showed that both glutamate-mediated NO production and NO-mediated glutamate release mechanisms exist in the nucleus tractus solitarii, and a reciprocal regulation of NO and glutamate may be important in central cardiovascular regulation.

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