Neuropsychopharmacology (2004) 29, 1419–1425

www.neuropsychopharmacology.org

Antagonism by NOS Inhibition of the Behavioral Effects of Benzodiazepine and $GABA_A$ Receptor Agonists in the Mouse Elevated Plus-Maze

Geraldine S Elfline[1,2], Emily M Branda[1,3], Michael Babich[1,4] and Raymond M Quock*,[1]

[1]*Department of Biomedical Sciences, University of Illinois College of Medicine at Rockford, Rockford, Illinois, USA*

Earlier we implicated nitric oxide (NO) in mediation of the behavioral effects of benzodiazepines. Since benzodiazepines work through facilitation of GABAergic inhibitory neurotransmission, this study was designed to determine whether the direct-acting γ-aminobutyric $acid_A$ ($GABA_A$) receptor agonist THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) evokes behavioral effects similar to those of benzodiazepines and whether behavioral effects of THIP are also NO dependent. When challenged with either chlordiazepoxide or THIP in an elevated plus-maze paradigm, male NIH Swiss mice exhibited a dose-related increase in open-arm activity. The chlordiazepoxide-induced effects were sensitive to antagonism by a benzodiazepine antagonist, and the effects of THIP were blocked by a $GABA_A$ receptor antagonist. Pretreatment with the NO synthase (NOS) inhibitor L-N^G-nitro arginine antagonized the effects of both chlordiazepoxide and THIP; similar pretreatment with the D-isomer, D-N^G-nitro arginine, which is inactive as an NOS inhibitor, was without effect on chlordiazepoxide and THIP. These findings indicate that chlordiazepoxide and THIP evoke similar behavioral effects in mice in the elevated plus-maze through actions on different parts of the $GABA_A$ receptor, and that NO appears to play a key role in mediation of the behavioral effects of both chlordiazepoxide and THIP.
Neuropsychopharmacology (2004) **29,** 1419–1425, advance online publication, 11 May 2004; doi:10.1038/sj.npp.1300437

Keywords: benzodiazepines; $GABA_A$ receptors; nitric oxide; elevated plus-maze; anxiety

INTRODUCTION

The γ-aminobutyric $acid_A$ ($GABA_A$) receptor-chloride channel complex is a heteropentameric protein polymer comprised of homologous membrane-spanning glycoprotein subunits (Sieghart, 1995; Whiting *et al*, 1995). Benzodiazepines are positive allosteric modulators of GABAergic neurotransmission. The benzodiazepine binding site is sited in a cleft between the α_1 and γ_2 subunits and increases the affinity of chloride channel openings by GABA, which acts at its own distinct receptor site between the α_1 and β_2 subunits (Sigel, 2002). Thus, activation of the benzodiazepine site causes an increase in GABA inhibition.

A number of studies have established a relationship between activity at the $GABA_A$ receptor and regulation of anxiety. When tested in ethological models of experimental anxiety such as the elevated plus-maze, mice treated with direct-acting $GABA_A$ agonists like muscimol and baclofen respond with an anxiolytic-like behavior (Corbett *et al*, 1991; Nastiti *et al*, 1991). THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) is a potent and efficacious direct-acting agonist at $GABA_A$ receptors. It has been reported to produce anxiolytic effects in man (Krogsgaard-Larsen *et al*, 1997). A subsequent clinical study confirmed that THIP significantly reduced anxiety ratings on multiple measures but reported that anxiolytic doses also produced sedation and undesirable side effects (Hoehn-Saric, 1983). In animal studies, THIP partly reduced the behavioral effects of Rhesus monkeys treated with an anxiogenic β-carboline (Crawley *et al*, 1985). THIP also exhibited anxiolytic-like activity in rodents in the social interaction test and elevated plus-maze (Corbett *et al*, 1991). THIP also suppressed anxiety in a rodent model of premenstrual anxiety (Gulinello *et al*, 2003).

Previously we reported that the anxiolytic-like effects of chlordiazepoxide and nitrous oxide were antagonized by inhibition in production of nitric oxide (NO). If benzodiazepine-induced anxiolytic effects are mediated through the GABA receptor (Korpi *et al*, 2002) and if such benzodiazepine-induced effects can be blocked by inhibitors of nitric

*Correspondence: Dr RM Quock, Department of Pharmaceutical Sciences, College of Pharmacy, Washington State University, PO Box 646534, Pullman, WA 99164-6534, USA, Tel: 1 + 509-335-5545, Fax: 1 + 509-393-5902, E-mail: quockr@wsu.edu.
[2]Pierce High School Science Teacher Summer Research Fellow from Morrison High School, 643 Genesee Avenue, Morrison, IL 61270, USA
[3]Current address: Department of Psychology, University of Wisconsin–Milwaukee, PO Box 413, Milwaukee, WI 53201, USA
[4]Current address: ImmvaRx, 3701 J Street, Suite 106, Sacramento, CA 95816, USA
Received 22 September 2003; revised 21 January 2004; accepted 11 February 2004
Online publication: 16 February 2004 at http://www.acnp.org/citations/Npp02160403433/default.pdf

oxide synthase (NOS) (Quock and Nguyen, 1992; Li and Quock, 2001; Li *et al*, 2003b), then one would expect that the anxiolytic-like responses to a direct $GABA_A$ agonist should also be sensitive to antagonism by NOS inhibitors.

MATERIALS AND METHODS

Animals

Male NIH Swiss mice, 18–25 g, were purchased from Harlan Laboratories (Indianapolis, IN) and used in these experiments, which were reviewed and approved by the Biologic Resource Committee, the institutional animal care and use committee of the University of Illinois College of Medicine at Rockford, and were conducted in accordance with The Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Mice were housed five per cage in an ALAAAC-accredited animal facility. Food and water were available *ad libitum*. The facility was maintained on a 12-h light/dark cycle (lights on 0700–1900) under standard conditions ($22 \pm 1^{\circ}$C room temperature, 33% humidity). Mice were kept in the holding room for at least 4 days following arrival in the facility prior to use. Animals were used only once.

Elevated Plus-Maze

The elevated plus-maze was constructed from black Plexiglas® and consisted of two open arms (30 cm L $\times$ 5.5 cm W) and two enclosed arms (30 cm L $\times$ 5.5 cm W $\times$ 16 cm H) mounted at 90° to one another. The plus-maze was on top of Plexiglas® legs 24 cm in height. In this test, animals were initially individually placed in the central area of the elevated plus-maze facing an enclosed arm and then observed for a 5-min period. The number of entries into either open or enclosed arms of the maze was recorded as was the duration of exploratory activity on open and enclosed arms of the plus-maze.

Drugs

Drugs used in this research included the following: chlordiazepoxide hydrochloride (Sigma Chemical Company, St Louis, Missouri); CGS 8216 (2-phenylpyrazolo[4,3-c]quinoline-3[5H]-one, a gift from CIBA Geigy, Summit, New Jersey); THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol, also known as gaboxadol), SR-95531 (2-[3-carboxypropyl]-3-amino-6-[4-methoxyphenyl]pyridazinium bromide and L-NOARG (N^G-L-nitro arginine, all from Research Biochemicals International, Natick, Massachusetts); and D-NOARG (N^G-D-nitro arginine (Alexis Corporation, San Diego, California).

Chlordiazepoxide and THIP were freshly prepared in 0.9% physiological saline solution and administered intraperitoneally (i.p.) 30 min prior to testing in the elevated plus-maze. CGS 8216 was suspended in 0.3% Tween 80 (Sigma) in saline and administered subcutaneously (s.c.) 30 min prior to testing. L-NOARG and D-NOARG were similarly dissolved in physiological saline and administered s.c. 30 min prior to testing in the plus-maze. The dose of L-NOARG and D-NOARG was determined in preliminary dose–response experiments. Control animals received appropriate vehicle injections i.p. or s.c. The injection volume of drug challenges and pretreatments was 0.1 ml per 10 g body weight.

SR-95531 was administered by the intracerebroventricular (i.c.v.) route in a dose of 2.0 nmol 30 min prior to testing. The drug was delivered directly into the lateral cerebral ventricle of mice lightly anesthetized with halothane using a modification of the method of Haley and McCormick (1957). An incision was made in the scalp, and the skin was retracted to expose the calvarium. The injection was made using a 26-gauge microsyringe (Hamilton, Reno, Nevada) at a point on calvarium 2.0 mm lateral and 2.0 mm caudal to bregma to a depth of 2.5 mm from the skull surface. The i.c.v. microinjection technique was verified in preliminary experiments by microinjection of a dye marker and finding stained walls in the ipsilateral ventricular space.

Statistical Analysis of Data

The mean percent entries into open arms was calculated as follows:

$$\% = 100 \times \frac{\text{Number of entries into open arms}}{\text{Total number of entries into both open and closed arms}}$$

The mean percent entries into open arms was used as an index rather than the total number of entries into open arms in order to demonstrate that increased open arm exploratory activity reflected a specific anxiolytic drug effect and was not a component of a generalized increase in exploratory activity.

Similarly, the mean percent time spent on the open arms was determined as follows:

$$\% = 100 \times \frac{\text{Number of seconds spent on open arms}}{300 \text{ total sec(ie 5-min observation time)}}$$

Differences in behavioral indices between groups were analyzed by one- or two-way analysis of variance (ANOVAs) as appropriate; *post hoc* pair-wise comparisons were made with the Bonferroni multiple-comparison test (significance set at 0.05). Percent changes were arc–sine transformed before statistical analysis.

RESULTS

Figure 1 shows the effects of increasing doses of chlordiazepoxide and THIP on open-arm activity in the elevated plus-maze. Vehicle control animals showed 6.4 ± 3.5% entries into the open arms and 3.2 ± 1.7% total time spent in the open arms. Chlordiazepoxide produced dose-related increases in both the percent of total entries into open arms as well as the percent of total time spent on open arms of the elevated plus-maze, especially at the intermediate (8.0 mg/kg) and high (16 mg/kg) doses tested. THIP also produced dose-related increases in both the percent of total entries into open arms as well as the percent of total time spent on open arms of the elevated plus-maze, again at the

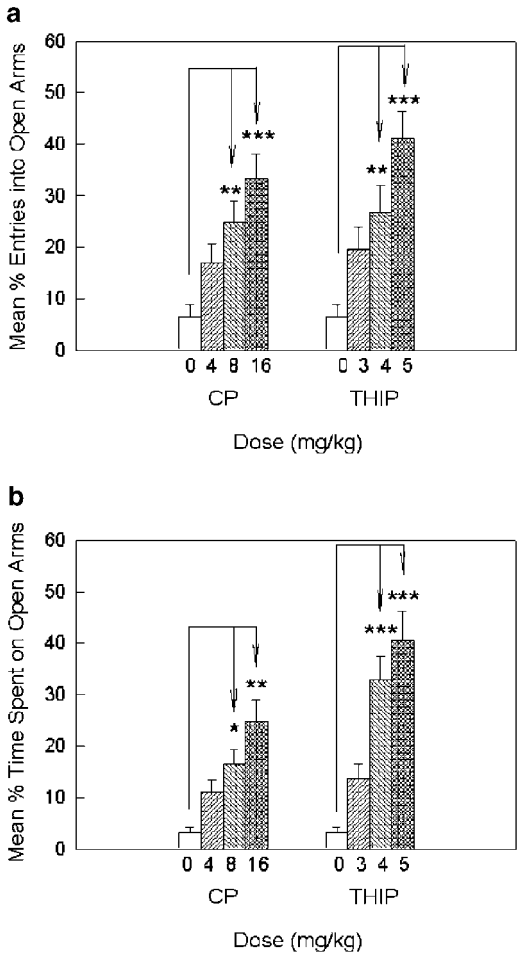

Figure 1 Influence of increasing doses of chlordiazepoxide (CP) and THIP on (a, top panel) the percent of total entries that were into open arms of the elevated plus-maze and (b, bottom panel) the percent of time spent on open arms of the plus-maze. The height of each bar represents the mean and each vertical line is the SEM of 20–26 mice per group. Significance of difference: *, $p<0.05$, **, $p<0.01$ and ***, $p<0.001$, compared to the vehicle control group (open bars).

intermediate (4.0 mg/kg) and high (5.0 mg/kg) doses that were tested. The dose–response relationship for THIP resembled that of chlordiazepoxide but within a far narrower dose range; the ceiling dose of THIP was 5.0 mg/kg, while that of chlordiazepoxide was 16 mg/kg. Higher doses tended to suppress open-arm activity, 10 mg/kg of THIP and 32 mg/kg of chlordiazepoxide producing total immobilization of animals (data not shown). Based on the dose–response experiments, standard challenge doses of chlordiazepoxide (8.0 mg/kg) and THIP (4.0 mg/kg) were adopted for the drug interaction experiments.

Figure 2 represents the influence of pretreatment with the benzodiazepine antagonist CGS 8216 on the behavioral effects induced by standard challenge doses of chlordiazepoxide and THIP in the elevated plus-maze. Chlordiazepoxide-induced increases in percent of total entries into open arms as well as percent of total time spent on open arms were both lowered, antagonized by CGS 8612, but only the reduction in percent of total entries was statistically significant. On the other hand, comparable behavioral effects induced by THIP were unaffected by pretreatment with CGS 8612.

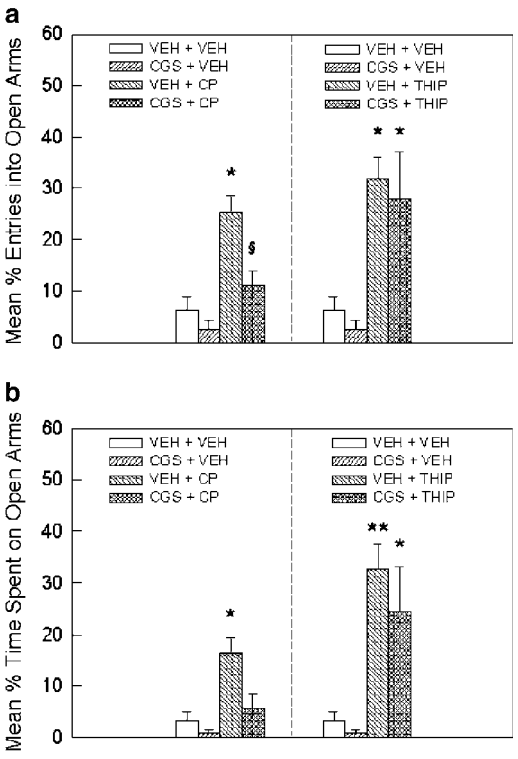

Figure 2 Influence of benzodiazepine antagonist CGS 8216 on the effects of standard challenge doses of CP (8.0 mg/kg) and THIP (4.0 mg/kg) on (a, top panel) the percent of total entries that were into open arms of the elevated plus-maze and (b, bottom panel) the percent of time spent on open arms of the plus-maze. The height of each bar represents the mean and each vertical line is the SEM of 20–26 mice per group. Significance of difference: *, $p<0.05$ and **, $p<0.01$, compared to the vehicle control group (open bars); and §, $p<0.05$, between the CP or THIP control group and the CGS + CP or THIP group (ie cross-hatched bars *vs* adjacent solid bars).

Figure 3 depicts the interaction between the $GABA_A$ receptor antagonist SR 99531, on the one hand, and chlordiazepoxide and THIP, on the other. SR 95531 significantly antagonized the increase in percent of total entries into open arms and percent of total time spent on open arms that was induced by THIP but not chlordiazepoxide. On the contrary, blockade of $GABA_A$ receptors appeared to enhance the anxiolytic-like behavioral effects of chlordiazepoxide in the elevated plus-maze.

Figure 4 demonstrates the influence of pretreatment with the NOS-inhibitor L-NOARG on the behavioral effects evoked by standard challenge doses of chlordiazepoxide (8.0 mg/kg) and THIP (4.0 mg/kg) in the elevated plus-maze. In preliminary experiments, different pretreatment doses of L-NOARG were administered to determine the minimum dose that would antagonize the behavioral effects of chlordiazepoxide and THIP (data not shown). At a dose of 0.45 mg/kg, L-NOARG alone produced no appreciable effect on either the percent of total entries into open arms or the percent of total time spent on open arms of the elevated plus-maze. This pretreatment with L-NOARG significantly lowered the chlordiazepoxide-induced increase in percent of total entries into open arms. The effect of chlordiazepoxide on percent of total time spent on open arms was also attenuated, but this reduction was not statistically

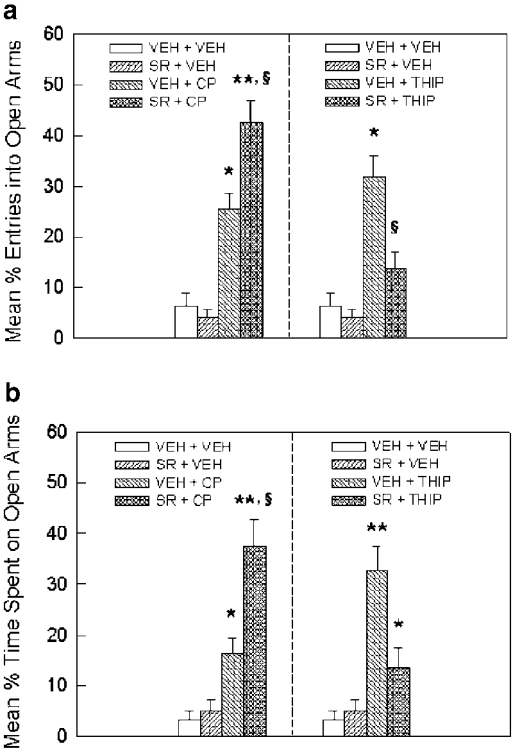

Figure 3 Influence of GABA$_A$ antagonist SR-95531 on the effects of standard challenge doses of CP (8.0 mg/kg) and THIP (4.0 mg/kg) on (a, top panel) the percent of total entries that were into open arms of the elevated plus-maze and (b, bottom panel) the percent of time spent on open arms of the plus-maze. The height of each bar represents the mean and each vertical line is the SEM of 20–26 mice per group. Significance of difference: *, $p<0.05$, and **, $p<0.01$, compared to the vehicle control group (open bars); and §, $p<0.05$, between the CP or THIP control group and the SR + CP or THIP group (ie cross-hatched bars *vs* adjacent solid bars).

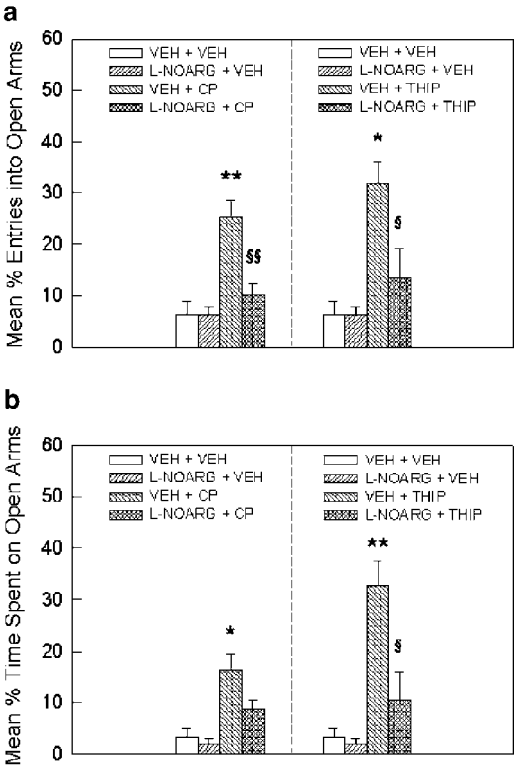

Figure 4 Influence of NOS inhibitor L-NOARG on the effects of standard challenge doses of CP (8.0 mg/kg) and THIP (4.0 mg/kg) on (a, top panel) the percent of total entries that were into open arms of the elevated plus-maze and (b, bottom panel) the percent of time spent on open arms of the plus-maze. The height of each bar represents the mean and each vertical line is the SEM of 20–26 mice per group. Significance of difference: *, $p<0.05$, and **, $p<0.01$, compared to the vehicle control group (open bars); and §, $p<0.05$, and §§, $p<0.01$, between the CP or THIP control group and the L-NOARG + CP or THIP group (ie cross-hatched bars *vs* adjacent solid bars).

significant. On the other hand, L-NOARG pretreatment significantly antagonized the effect of THIP on both indices of open-arm activity in the plus-maze. In contrast to L-NOARG, the inactive control drug D-NOARG failed to appreciably alter the effects of either chlordiazepoxide or THIP in the elevated plus-maze (Figure 5).

DISCUSSION

THIP, also known as gaboxadol, is a directly acting GABA$_A$ receptor agonist (Krogsgaard-Larsen *et al*, 2002) that has reported to produce anxiolytic drug effects in humans (Krogsgaard-Larsen *et al*, 1997). Clinical studies of the effects of THIP on human sleep patterns have led to the recently initiated Phase III clinical trials as a treatment for insomnia (H. Lundbeck A/S, a press release; http://www.pressi.com/int/release/68357.html; Mathias *et al*, 2001). The results of the present study show that increasing doses of THIP caused a dose-related increase in open-arm activity not unlike the effects of chlordiazepoxide. The anxiolytic-like effects of THIP were attenuated by the GABA$_A$ receptor antagonist SR-95531 but not the benzodiazepine antagonist CGS-8612. The anxiolytic-like effects of chlordiazepoxide were significantly antagonized by CGS-8216 but not SR-95531. In fact, there seemed to be an enhanced response to chlordiazepoxide in the presence of GABA$_A$ receptor blockade—an explanation of this heightened anxiolytic-like effect of chlordiazepoxide effect is not immediately evident. The behavioral effects of both THIP and chlordiazepoxide were sensitive to antagonism by pretreatment with L-NOARG but not D-NOARG. Since only the L-stereoisomer of NOARG can inhibit NOS enzyme activity, it is concluded that THIP and chlordiazepoxide share a common requirement for NO in the signaling mechanism downstream from the GABA$_A$ receptor.

These findings are consistent with other research from our laboratory regarding what appears to be a critical involvement of NO in mediating the anxiolytic-like behavioral effects of benzodiazepines in different animal models of experimental anxiety. In earlier research, the effects of chlordiazepoxide in the mouse elevated plus-maze were markedly antagonized by L-NOARG (N^G-L-nitro arginine) inhibition of NOS; this antagonism was stereoselectively reversed by treatment with L-arginine but not D-arginine (Quock and Nguyen, 1992). L-NOARG, which preferentially inhibits the neuronal and endothelial forms of NOS (Gross *et al*, 1990; Klatt *et al*, 1994), also blocked the ability of chlordiazepoxide to selectively reduce rearing activity in the mouse staircase test, in which rearing is considered an index of anxiety (Lake and Quock, 2001).

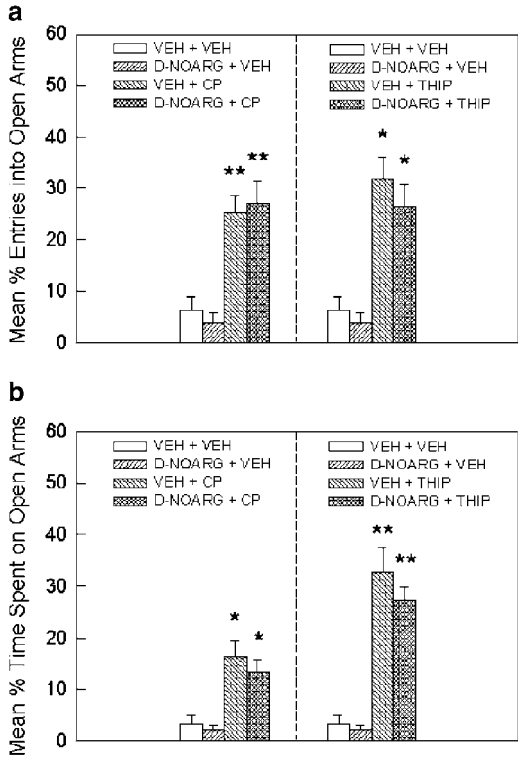

Figure 5 Influence of D-NOARG on the effects of standard challenge doses of CP (8.0 mg/kg) and THIP (4.0 mg/kg) on (a, top panel) the percent of total entries that were into open arms of the elevated plus-maze and (b, bottom panel) the percent of time spent on open arms of the plus-maze. The height of each bar represents the mean and each vertical line is the SEM of 20–26 mice per group. Significance of difference: *, $p<0.05$ and **, $p<0.01$, compared to the vehicle control group (open bars). There were no significant differences between the CP or THIP control group and the D-NOARG + CP or THIP group (ie cross-hatched bars *vs* adjacent solid bars).

Moreover, the effects of chlordiazepoxide and nitrous oxide—an anesthetic gas that reduces anxiety through direct or indirect activation of brain benzodiazepine receptors (Quock *et al*, 1992; Emmanouil *et al*, 1994)—in the light/dark exploration test were also antagonized by the neuronal-selective NOS inhibitors 7-nitroindazole (Li and Quock, 2001) and *S*-methyl-*l*-thiocitrulline (Li *et al*, 2003b) as well as an antisense oligodeoxynucleotide directed against neuronal NOS (Li *et al*, 2003a). Based on these findings, it was concluded that NO might play a key role in mediating the anxiolytic-like behavioral effects of chlordiazepoxide.

It should also be noted that THIP and chlordiazepoxide-induced anxiolytic effects in the elevated plus-maze were both antagonized by a dose of L-NOARG much lower than that used to inhibit NOS in our earlier investigations. Subcutaneous pretreatment with 10 mg/kg effectively antagonized the anxiolytic-like behaviors induced by chlordiazepoxide (Quock and Nguyen, 1992) and nitrous oxide (Caton *et al*, 1994) in the mouse elevated plus-maze. Preliminary to the present study, we conducted dose–response experiments of L-NOARG antagonism of chlordiazepoxide and discovered a threshold dose for antagonism of about 0.4 mg/kg. Accordingly, a dose of 0.45 mg/kg was adopted as the standard antagonist dose of L-NOARG for these experiments.

The scientific literature does contain evidence for interaction between GABA and NO, but the majority of such studies focus on NO modulation of the neuronal release of GABA (Wall, 2003; Li *et al*, 2004). However, a recent study demonstrated that activation of $GABA_A$ receptors by muscimol or activation of the benzodiazepine binding site by diazepam increases the population of nNOS-positive cells in the frontal and parietal areas of the developing cortex (Mantelas *et al*, 2003). In addition, activation of GABA receptors increased the count of brain-derived neurotophic factor (BDNF)- and phosphorylated cAMP-response element binding protein (CREB)-doubly positive cells. The increases in the above multiple immunoreactivities were sensitive to antagonism by nimodipine, which blocks L-type voltage-gated calcium channels. Two possible pathways have been postulated. GABA may directly induce nNOS expression as has been demonstrated in mouse cerebellar granule cell culture (Baader *et al*, 1997). Alternatively, stimulation of the $GABA_A$ receptor may increase expression of BDNF, which has been shown to upregulate NOS activity (Xiong *et al*, 1999). In either event, the induction of nNOS and BDNF expression appears to be mediated by the influx of calcium ions that causes phosporylation (activation) of CREB (Sasaki *et al*, 2000; Auger *et al*, 2001; Mantelas *et al*, 2003).

An analogous mechanism can be envisioned in brain regions where chlordiazepoxide and THIP appear to work to induce anxiolysis. Activation of $GABA_A$ receptors may result in activation of CREB which then directly or indirectly through BDNF induce expression of neuronal NOS. Such a mechanism that couples NOS to the $GABA_A$ receptor would explain why the anxiolytic effect of benzodiazepines would be NO dependent. However, there is controversy over the precise role of NO in the regulation of anxiety.

Based on our research, if NO mediates the anxiolytic effect of benzodiazepines and nitrous oxide, inhibition of NOS alone should be anxiogenic. A number of research groups have indeed reported that suppression of NO production can induce anxiogenic-like behavior in a variety of animal models of experimental anxiety (De Oliveira *et al*, 1997; Vale *et al*, 1998; Monzon *et al*, 2001; Pokk and Vali, 2002; Czech *et al*, 2003). That NO is involved in reduction of anxiety is supported by the observation that administration of the NO donor 3-morpholinosyndnonimine (SIN-1) evoked an anxiolytic-like response in mice in the light/dark exploration test (Li and Quock, 2002). Restraint stress induced an anxiogenic-like response in the elevated plus-maze that was suppressed in a diazepam-like manner by L-arginine and exacerbated by L-NAME (Masood *et al*, 2003). Diazepam among several psychotropic drugs increases levels of inducible NOS mRNA in rat brain, suggesting that its effects might be mediated by NO (Suzuki *et al*, 2002).

However, there is equally convincing evidence implicating exactly the opposite role of NO in anxiety, that is NO appears to be responsible for anxiogenesis. Systemic treatment with L-NAME, L-NOARG and 7-NI produced anxiolytic-like behavior in rats in the elevated plus-maze (Volke *et al*, 1995; Faria *et al*, 1997; de Oliveira *et al*, 1997; Dunn *et al*, 1998). Systemic 7-NI produced anxiolytic-like

responses in mice in the light/dark exploration test and elevated plus-maze (Volke *et al*, 1997). Microinjection of L-NAME and L-NOARG into the dorsolateral periaqueductal gray induced anxiolytic-like behavior in the rat elevated plus-maze, although higher doses produced anxiogenic-like responses (Guimarães *et al*, 1994).

In other research, the neuronal-selective NOS inhibitor 1-(2-trifluoromethylphenyl)-imidazole (TRIM) but not 7-NI produced an anxiolytic-like response in mice in the light/dark exploration test (Volke *et al*, 2003). Transient cerebral ischemia in mice causes anxiogenic-like activity in elevated plus-maze and social interaction test that was suppressed by nonselective L-NAME and I-NOS-selective S-ethylisothiourea (EIT) but not nNOS-selective 7-nitroindazole (Nakashima *et al*, 2003). Administration of L-arginine, presumed to increase NO production, abolished the anxiolytic-like response of rats to diazepam in the elevated plus-maze (Volke *et al*, 1998). There is also increased expression of neuronal NOS mRNA in stress-related brain regions, suggesting an increase in NO during stress (de Oliveira *et al*, 2000).

Why there is such disagreement in the relationship of NO to anxiety is the subject of much speculation and investigation. The differences in reported behavioral effects of NOS inhibitors have been attributed to a number of factors, including drugs, doses, experimental species, routes of administration, and models of experimental anxiety (see discussion by Li *et al*, 2003b). A significant finding of the present study is that THIP and chlordiazepoxide-induced anxiolytic-like effects were antagonized by pretreatment with 0.45 mg/kg L-NOARG. This is significant in light of the often high doses of NOS inhibitors used to evoke anxiolytic-like effects or provoke anxiogenic-like effects in other elevated plus-maze studies in mice, for example 20 and 40 mg/kg (Pokk and Vali, 2002), 20 and 40 mg/kg of L-NAME (Pokk and Vali, 2002), and 80–120 mg/kg 7-NI (Volke *et al*, 1997; Pokk and Vali, 2002). Drug dose may be one contributing factor in the incongruities of experimental findings with NOS inhibitors.

A better explanation for these discrepancies may very well lie in the multitude of functions served by NO in the central nervous system. NO may have biphasic influences (ie both excitatory and inhibitory) based on a complex interaction among glutamatergic, GABAergic, and other (eg opioid) neuronal systems, all of which are modulated by NO (Lovick and Key, 1996; Wang *et al*, 1997; Hall and Behbehani, 1998; Lin *et al*, 2000). This may account for the contradictory results that NO appears to both enhance and suppress not only anxiety but also other functions such as pain (Moore *et al*, 1993; McDonald *et al*, 1994), seizure activity (Buisson *et al*, 1993; De Sarro *et al*, 1993), and neurotoxicity (Contestabile *et al*, 2003). These findings are contributing to a more complete understanding of the role of NO in brain function, which would potentially have many ramifications for more effective treatment of not only anxiety but other brain dysfunctions as well.

ACKNOWLEDGEMENTS

This work was supported by NIH Grant DA-10343. Geraldine S Elfline from Morrison High School (Morrison, Illinois) was supported by a High School Science Teacher Summer Fellowship from Pierce Chemical Company (Rockford, Illinois). We are thankful to Dr Shuang Li (University of Washington, Seattle, Washington) for her assistance with the statistical analysis and graphics.

REFERENCES

Auger AP, Perrot-Sinal TS, McCarthy MM (2001). Excitatory *versus* inhibitory GABA as a divergence point in steroid-mediated sexual differentiation of the brain. *Proc Natl Acad Sci USA* **98**: 8059–8064.

Baader SL, Bucher S, Schilling K (1997). The developmental expression of neuronal nitric oxide synthase in cerebellar granule cells is sensitive to GABA and neurotrophins. *Dev Neurosci* **19**: 283–290.

Buisson A, Lakhmeche N, Verrecchia C, Plotkine M, Boulu RG (1993). Nitric oxide: an endogenous anticonvulsant substance. *NeuroReport* **4**: 444–446.

Caton PW, Tousman SA, Quock RM (1994). Involvement of nitric oxide in nitrous oxide anxiolysis in the elevated plus maze. *Pharmacol Biochem Behav* **48**: 689–692.

Contestabile A, Monti B, Contestabile A, Ciani E (2003). Brain nitric oxide and its dual role in neurodegeneration/neuroprotection: understanding molecular mechanisms to devise drug approaches. *Curr Med Chem* **10**: 2147–2174.

Corbett R, Fielding S, Cornfelt M, Dunn RW (1991). GABAmimetic agents display anxiolytic-like effects in the social interaction and elevated plus-maze procedures. *Psychopharmacol* **104**: 312–316.

Crawley JN, Ninan PT, Pickar D, Chrousos GP, Linnoila M, Skolnick P *et al* (1985). Neuropharmacological antagonism of the beta-carboline-induced 'anxiety' response in rhesus monkeys. *J Neurosci* **5**: 477–485.

Czech DA, Jacobson EB, LeSueur-Reed KT, Kazel MR (2003). Putative anxiety-liked effects of the nitric oxide synthase inhibitor L-NAME in three murine exploratory behavior models. *Pharmacol Biochem Behav* **75**: 741–748.

de Oliveira CL, Del Bel EA, Guimarães FS (1997). Effects of L-NOARG on plus-maze performance in rats. *Pharmacol Biochem Behav* **56**: 55–59.

de Oliveira RM, Del Bel EA, Mamede-Rosa ML, Padovan CM, Deakin JF, Guimarães FS (2000). Expression of neuronal nitric oxide synthase mRNA in stress-related brain areas after restraint in rats. *Neurosci Lett* **289**: 123–126.

De Sarro G, Di Paola ED, De Sarro A, Vidal MJ (1993). L-Arginine potentiates excitatory amino acid-induced seizures elicited in the deep prepiriform cortex. *Eur J Pharmacol* **230**: 151–158.

Dunn RW, Reed TA, Copeland PD, Frye CA (1998). The nitric oxide synthase inhibitor 7-nitroindazole displays enhanced anxiolytic efficacy without tolerance in rats following subchronic administration. *Neuropharmacology* **37**: 899–904.

Emmanouil DE, Johnson CH, Quock RM (1994). Nitrous oxide anxiolytic effect in mice in the elevated plus maze: mediation by benzodiazepine receptors. *Psychopharmacology* **115**: 167–172.

Faria MS, Muscara MN, Moreno Jr H, Teixeira SA, Dias HB, de Oliveira B *et al* (1997). Acute inhibition of nitric oxide synthesis induces anxiolysis in the plus maze. *Eur J Pharmacol* **323**: 37–43.

Gross SS, Stuehr DJ, Aisaka K, Jaffe EA, Levi R, Griffith OW (1990). Macrophage and endothelial cell nitric oxide synthesis: cell-type selective inhibition by N^G-aminoarginine, N^G-nitroarginine and N^G-methylarginine. *Biochem Biophys Res Commun* **170**: 96–103.

Guimarães FS, de Aguiar JC, Del Bel EA, Ballejo G (1994). Anxiolytic effect of nitric oxide synthase inhibitors microinjected into the dorsal central grey. *NeuroReport* **5**: 1929–1932.

Gulinello M, Gong QH, Smith SS (2003). Progesterone withdrawal increases the anxiolytic actions of gaboxadol: role of $\alpha4\beta\delta$ GABA$_A$ receptors. *NeuroReport* **14**: 43–46.

Haley TJ, McCormick WG (1957). Pharmacological effects produced by intracerebral injections of drugs in the conscious mouse. *Br J Pharmacol* **12**: 12–15.

Hall CW, Behbehani MM (1998). Synaptic effects of nitric oxide, enkephalinergic, GABAergic and glutaminergic networks of the rat periaqueductal gray. *Brain Res* **805**: 69–87.

Hoehn-Saric R (1983). Effects of THIP on chronic anxiety. *Psychopharmacol* **80**: 338–341.

Klatt P, Schmidt K, Brunner F, Mayer B (1994). Inhibitors of brain nitric oxide synthase. Binding kinetics, metabolism, and enzyme inactivation. *J Biol Chem* **269**: 1674–1680.

Korpi ER, Gründer G, Lüddens H (2002). Drug interactions at $GABA_A$ receptors. *Prog Neurobiol* **67**: 113–159.

Krogsgaard-Larsen P, Frolund B, Kristiansen U, Frydenvang K, Ebert B (1997). $GABA_A$ and $GABA_B$ receptor agonists, partial agonists, antagonists and modulators—design and therapeutic prospects. *Eur J Pharmaceut Sci* **5**: 355–384.

Krogsgaard-Larsen P, Frolund B, Liljefors T (2002). Specific $GABA_A$ agonists and partial agonists. *Chem Rec* **2**: 419–430.

Lake A, Quock RM (2001). Behavioral effects of benzodiazepines and $GABA_A$ receptor agonists in the mouse staircase test are dependent on nitric oxide. *Abst Pharmacol Vancouver 2001 Mtg*, p 89.

Li DP, Chen SR, Finnegan TF, Pan HL (2004). Signalling pathway of nitric oxide in synaptic GABA release in the rat paraventricular nucleus. *J Physiol* **554**: 100–110.

Li S, Dai Y, Quock RM (2003a). Antisense knockdown of neuronal nitric oxide synthase antagonizes nitrous-induced behavior. *Brain Res* **968**: 167–170.

Li S, Ohgami Y, Dai Y, Quock RM (2003b). Antagonism of nitrous oxide-induced behavior in mice by pharmacologic disruption of endogenous nitric oxide function. *Psychopharmacology* **166**: 366–372.

Li S, Quock RM (2001). Comparison of N_2O- and chlordiazepoxide-induced behaviors in the light/dark exploration test. *Pharmacol Biochem Behav* **68**: 789–796.

Li S, Quock RM (2002). Effects of a nitric oxide donor on behavior and interaction with nitrous oxide in the mouse light/dark exploration test. *Eur J Pharmacol* **447**: 75–78.

Lin HC, Kang BH, Wan FJ, Huang ST, Tseng CJ (2000). Reciprocal regulation of nitric oxide and glutamate in the nucleus tractus solitarii of rats. *Eur J Pharmacol* **407**: 83–89.

Lovick T, Key BJ (1996). Inhibitory effect of nitric oxide on neuronal activity in the periaqueductal grey matter of rat. *Exp Brain Res* **108**: 382–388.

Lundbeck A/S H (2003). Gaboxadol in phase III clinical trial. A news release from H. Lundbeck A/S 06/19/2003 01:43:44 PM GMT. http://www.pressi.com/int/release/68357.html.

Masood A, Banerjee B, Vijayan VK, Ray A (2003). Modulation of stress-induced neurobehavioral changes by nitric oxide in rats. *Eur J Pharmacol* **458**: 135–139.

Mantelas A, Stamatakis A, Kazanis I, Philippidis H, Stylianopoulou F (2003). Control of neuronal nitric oxide synthase and brain-derived neurotrophic factor levels by GABA-A receptors in the developing rat cortex. *Brain Res Dev Brain Res* **145**: 185–195.

Mathias S, Steiger A, Lancel M (2001). The $GABA_A$ agonist gaboxadol improves the quality of post-nap sleep. *Psychopharmacology* **157**: 299–304.

McDonald CE, Gagnon MJ, Ellenberger EA, Hodges BL, Ream JK, Tousman SA *et al* (1994). Inhibitors of nitric oxide synthesis antagonize nitrous oxide antinociception in mice and rats. *J Pharmacol Exp Ther* **269**: 601–608.

Monzon ME, Varas MM, De Barioglio SR (2001). Anxiogenesis induced by nitric oxide synthase inhibition and anxiolytic effect of melanin-concentrating hormone (MCH) in rat brain. *Peptides* **22**: 1043–1047.

Moore PK, Babbedge RC, Wallace P, Gaffen ZA, Hart SL (1993). 7-Nitroindazole, an inhibitor of nitric oxide synthase, exhibits antinociceptive activity in the mouse without increasing blood pressure. *Br J Pharmacol* **108**: 296–297.

Nakashima MN, Ajiki K, Nakashima K, Takahashi M (2003). Possible or nitric oxide in anxiety following transient cerebral ischemia in mice. *J Pharmacol Sci* **91**: 47–52.

Nastiti K, Benton D, Brain PF (1991). The effects of compounds acting at the benzodiazepine receptor complex on the ultrasonic calling of mouse pups. *Behav Pharmacol* **2**: 121–128.

Pokk P, Vali M (2002). The effects of the nitric oxide synthase inhibitors on the behaviour of small-platform-stressed mice in the plus-maze test. *Prog Neuro-Psychopharmacol Biol Psychiat* **26**: 241–247.

Quock RM, Emmanouil DE, Vaughn LK, Pruhs RJ (1992). Benzodiazepine receptor mediation of behavioral effects of nitrous oxide in mice. *Psychopharmacology* **107**: 310–314.

Quock RM, Nguyen E (1992). Possible involvement of nitric oxide in chlordiazepoxide-induced anxiolysis in mice. *Life Sci* **51**: PL255–PL260.

Sasaki M, Gonzalez-Zulueta M, Huang H, Herring WJ, Ahn S, Ginty DD *et al* (2000). Dynamic regulation of neuronal NO synthase transcription by calcium influx through a CREB family transcription factor-dependent mechanism. *Proc Natl Acad Sci USA* **97**: 8617–8622.

Sieghart W (1995). Structure and pharmacology of gamma-aminobutyric $acid_A$ receptor subtypes. *Pharmacol Rev* **47**: 181–234.

Sigel E (2002). Mapping of the benzodiazepine recognition site on GABAA receptors. *Curr Top Med Chem* **2**: 833–839.

Suzuki E, Nakaki T, Shintani F, Kanba S, Miyaoka H (2002). Antipsychotic, antidepressant, anxiolytic, and anticonvulsant drugs induce type II nitric oxide synthase mRNA in rat brain. *Neurosci Lett* **333**: 217–219.

Vale AL, Green S, Montgomery AM, Shafi S (1998). The nitric oxide synthesis inhibitor L-NAME produces anxiogenic-like effects in the rat elevated plus-maze test, but not in the social interaction test. *J Psychopharmacol* **12**: 268–272.

Volke V, Kõks S, Vasar E, Bourin M, Bradwejn J, Männistö PT (1995). Inhibition of nitric oxide synthase causes anxiolytic-like behaviour in an elevated plus maze. *NeuroReport* **6**: 1285–1288.

Volke V, Soosaar A, Kõks S, Bourin M, Männistö PT, Vasar E (1997). 7-Nitroindazole, a nitric oxide synthase inhibitor, has anxiolytic-like properties in exploratory models of anxiety. *Psychopharmacology* **131**: 399–405.

Volke V, Soosaar A, Kõks S, Vasar E, Männistö PT (1998). L-Arginine abolishes the anxiolytic-like effect of diazepam in the elevated plus-maze test in rats. *Eur J Pharmacol* **351**: 287–290.

Volke V, Wegener G, Bourin M, Vasar E (2003). Antidepressant- and anxiolytic-like effects of selective neuronal NOS inhibitor 1-(2-trifluoromethylphenyl)-imidazole in mice. *Behav Brain Res* **140**: 141–147.

Wall MJ (2003). Endogenous nitric oxide modulates GABAergic transmission to granule cells in adult rat cerebellum. *Eur J Neurosci* **18**: 869–878.

Wang QP, Guan JL, Nakai Y (1997). Electron microscopic study of GABAergic synaptic innervation of nitric oxide synthase immunoreactive neurons in the dorsal raphé nucleus in the rat. *Synapse* **25**: 24–29.

Whiting PJ, McKernan RM, Wafford KA (1995). Structure and pharmacology of vertebrate $GABA_A$ receptor subtypes. *Int Rev Neurobiol* **38**: 95–138.

Xiong H, Yamada K, Han D, Nabeshima T, Enikolopov G, Carnahan J *et al* (1999). Mutual regulation between the intercellular messengers nitric oxide and brain-derived neurotrophic factor in rodent neocortical neurons. *Eur J Neurosci* **11**: 1567–1576.