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Research Report

Effects of endothelial NOS antagonism within the periaqueductal gray on cardiovascular responses and neurotransmission during mechanical, heat, and cold nociception

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ABSTRACT

Nitric oxide (NO) is synthesized from L-arginine using NO synthase (NOS) enzyme that exists as 3 isoforms: endothelial (eNOS), neuronal (nNOS), and inducible (iNOS). We examined the role of eNOS within the dorsolateral periaqueductal gray mater (dlPAG) on cardiovascular responses along with glutamate and GABA concentrations during mechanical-, heat-, and cold-induced nociception in anesthetized rats. Mechanical stimulus was applied by a 10-second hindpaw pinch that increased mean arterial pressure (MAP) and heart rate (HR). Bilateral microdialysis of a selective eNOS antagonist, L-*N*(5)-(1-iminoethyl)ornithine (L-NIO; 10 μM), into the dlPAG had no effect on MAP or HR during a mechanical stimulation. Heat stimulus was generated by immersing a hindpaw metatarsus in a water-bath at 52 °C for 10 s which increased glutamate, GABA, MAP and HR. Administration of L-NIO into the dlPAG augmented cardiovascular responses and glutamate increase, but attenuated GABA changes during the heat stimulus. In contrast, application of a cold stimulus by immersing the hindpaw at 10 °C for 10 s resulted in decreases in MAP, HR, and glutamate. However, there was an increase in GABA concentration. Following microdialysis of L-NIO into the dlPAG, the responses to the cold stimulus was reversed i.e., the cold stimulus induced pressor and tachycardic responses, augmented glutamate, and attenuated GABA levels. These results demonstrate that eNOS within the dlPAG plays a differential role on the cardiovascular system during heat- and cold-mediated nociception via modulating glutamatergic/GABAergic neurotransmission. However, the mechanical stimulation had no effect on cardiovascular responses following eNOS antagonism within the dlPAG.

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1. Introduction

Nociception or pain evokes variable autonomic and behavioral responses (Zamir and Maixner, 1986; Keay and Bandler, 1993; Lovick, 1993; Bandler and Keay, 1996; Mason, 1999; Le Bars et al., 2001; Mason, 2005; Green et al., 2006). For example, cutaneous and superficial somatic pain elicit increases in mean arterial pressure (MAP), heart rate (HR), myocardial contractility, and sympathetic nerve activity; the classic "fight or flight" responses (Randich and Maixner, 1984; Zamir and Maixner, 1986; Lovick, 1993; Le Bars et al., 2001). The pain information arising from peripheral nociceptors is transmitted via myelinated (Aδ) and unmyelinated (C) fibers to different brain regions, including several cardiovascular regulatory centers, where the impulses are integrated in order to evoke the reflex autonomic responses (Randich and Maixner, 1984; Zamir and Maixner, 1986; Lovick, 1993; Le Bars et al., 2001). However, the mechanisms that coordinate the cardiovascular and autonomic reactions during pain are not fully understood.

The rostral ventrolateral medulla (RVLM) participates in the integration of pain impulses with changes in cardiovascular, autonomic and behavioral function, including localized increases in the concentration of the excitatory amino acid, glutamate, and γ-aminobutyric acid (GABA), the inhibitory neurotransmitter (Gebhart and Randich, 1990; Sun and Spyer, 1991; Lovick, 1993; Blessings, 1997; Ally, 1998; Le Bars et al., 2001). Our laboratory has shown that antagonism of α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors within the RVLM attenuates the increases in MAP, HR, and glutamate levels within the RVLM during mechanical, but not in response to heat-induced thermal nociception (Gray et al., 2001). Furthermore, blockade of *N*-methyl-D-aspartate (NMDA) receptors within the RVLM attenuates cardiovascular responses by decreasing localized serotonin and dopamine concentrations during mechanical stimulation, but not in response to hot thermal stimulation (Karlsson et al., 2006). Administration of L-arginine, a precursor of nitric oxide (NO), into the RVLM inhibits cardiovascular responses to heat-induced pain by attenuating glutamatergic neurotransmission (Ishide et al., 2003). The midbrain periaqueductal gray (PAG) matter also significantly contributes to cardiovascular regulation and pain modulation (Reichling et al., 1988; Lovick, 1993, 1996; Bandler and Keay, 1996; Le Bars et al., 2001). Particularly, the lateral (lPAG) and dorsolateral (dlPAG) portions of the PAG mediate increases in MAP and HR during cutaneous and somatic nociception (Reichling et al., 1988; Lovick, 1996; Le Bars et al., 2001). We have shown that NO mechanisms within the dlPAG attenuate cardiovascular responses and concentrations of glutamate during both mechanical and heat-induced thermal nociceptive stimuli (Ishide et al., 2005).

Nitric oxide, acting via the second messenger cyclic GMP (cGMP), plays a pivotal role in coordinating the cardiovascular system (Snyder, 1992; Zanzinger and Seller, 1997; Chikada et al., 2000; Chan et al., 2001a,b). Nitric oxide is synthesized from L-arginine via the enzyme NO synthase (NOS) that exists as three isoforms: calcium-dependent neuronal NOS (nNOS) or Type I (Bredt et al., 1990); calcium-independent inducible NOS (iNOS) or Type II (Murphy et al., 1993); and endothelial NOS (eNOS) or Type III (Forstermann et al., 1995). We have shown that blockade of the nNOS protein within the dlPAG augments cardiovascular activity to heat-induced thermal, but not in response to mechanical nociception; these effects are most probably mediated by increased glutamate and attenuated GABA levels (Karlsson et al., 2007). In the present study, we focused on the eNOS isoform to determine its effect on cardiovascular responses, glutamate, and GABA neurotransmission during mechanical- and heat-induced thermal nociception. In addition, we are introducing for the first time the cardiovascular events and neurotransmission changes that follow in response to a brief 10-second cold-induced thermal stimulus and investigated the role of eNOS within the dlPAG on the cold stimulus-induced responses. Microdialysis techniques were used to administer a specific eNOS antagonist, L-N(5)-(1-iminoethyl)ornithine (L-NIO) into the dlPAG and dialysates were collected to analyze the extracellular fluid concentrations of glutamate and GABA. Overall, the purpose of the present study was to delineate the interactions between eNOS-glutamatergic and eNOS-GABAergic neurotransmission on cardiovascular function in response to mechanical, heat and cold nociception.

2. Results

2.1. *Effects of L-NIO on MAP, HR, and concentrations of GABA within the dlPAG during mechanical stimulation*

After surgical setup and insertion of microdialysis probes into the dlPAG, baseline cardiovascular parameters were stable during the 120-minute stabilization period. In addition, the rats were deeply anesthetized and there were no fluctuations of MAP or HR. Also, the rats had no corneal reflex and a pinch on the earlobe or tail did not change MAP or HR. Thereafter, a mechanical stimulation for 10 s was applied to a hindpaw and cardiovascular responses were recorded that served as controls. Then, L-NIO was bilaterally administered into the dlPAG at sequential doses of 0.1 μM, 1 μM, and 10 μM for 30 min at a rate of 5 μL/min ($n=5$). These concentrations of L-NIO did not alter baseline MAP or HR. In addition, a subsequent mechanical stimulation had no effect on the increases in MAP or HR (Fig. 1). Because a higher log dose of 20 μM L-NIO increased baseline MAP and HR, it was not used in the dose–response or any other experiment.

Thereafter, we used 8 rats in a set of experiments to determine the effects of 10 μM L-NIO on cardiovascular responses, glutamate and GABA concentrations within the dlPAG during mechanical stimulations. Mechanical stimuli increased MAP and HR by 26±4 mmHg and 36±6 bpm, respectively, that returned to pre-stimulation levels almost immediately ($n=8$; Fig. 2). Thirty minutes of bilateral administration of 10 μM L-NIO into the dlPAG did not alter baseline MAP or HR, and had no effects on the cardiovascular variables in response to a second mechanical stimulation (Fig. 2). Finally, at 120 min after discontinuation of the drug, a third mechanical stimulation also produced no effects on the cardiovascular responses (Table 1). In 5 rats, glutamate and GABA concentrations within the dlPAG as determined by the high-performance liquid chromatography with electrochemical detection (HPLC-

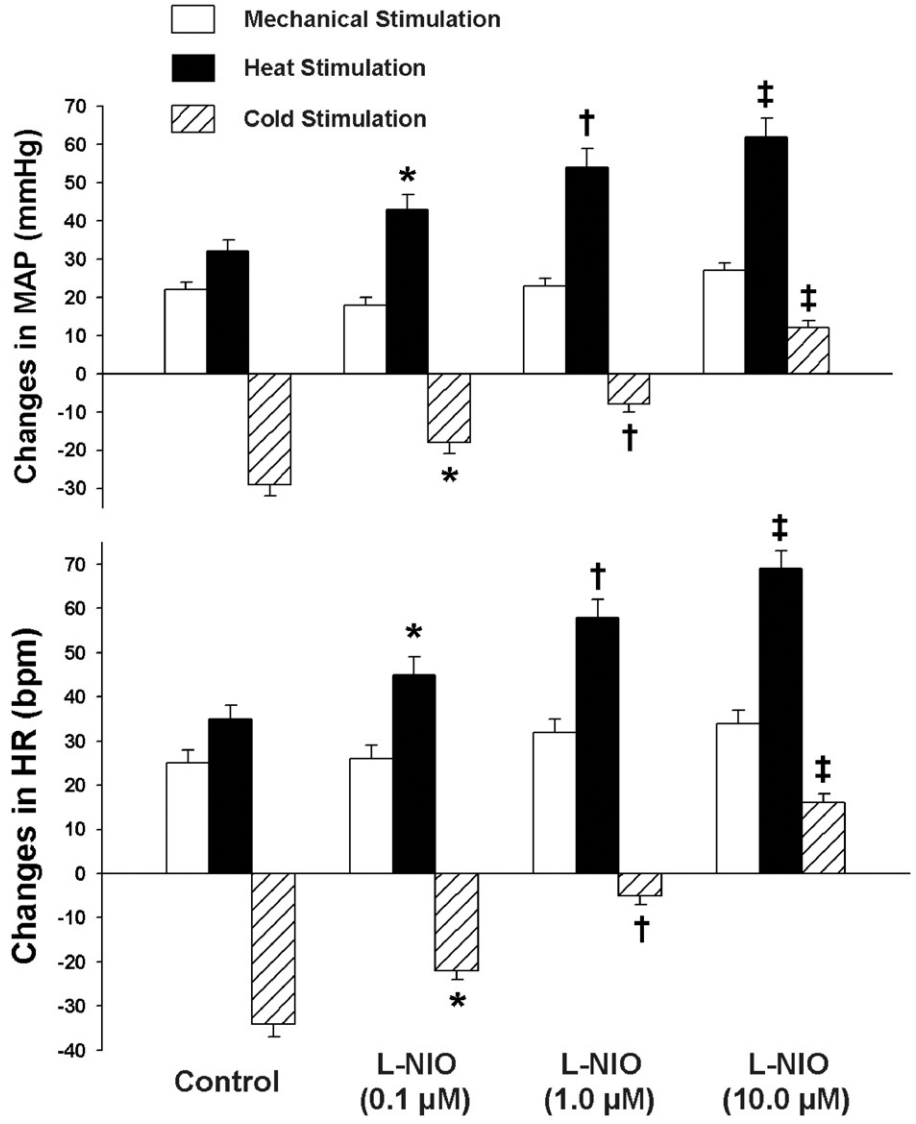

Fig. 1 – Dose–response data of changes in mean arterial pressure (MAP: upper panel) and heart rate (HR: lower panel) during a 10-second bilateral hindpaw mechanical stimulus (open bars), during a 10-second heat-induced thermal stimulus (closed bars), and a 10-second cold-induced thermal nociception before the perfusion of L-N(5)-(1-iminoethyl)ornithine (L-NIO; striped bars) and 30 min after sequential microdialysis of 0.1 μM, 1 μM, and 10 μM concentrations of the drug into the dorsolateral periaqueductal gray matter of anesthetized rats. Values represent means ± SEM ($n = 5$). *$P < 0.05$ versus control; †$P < 0.05$ versus control and prior dose; ‡$P < 0.05$ versus control and prior doses.

ECD) system were not significantly affected by eNOS blockade because of the large baseline differences. However, in 3 rats, mechanical stimulation increased glutamate concentration within the dlPAG. Following eNOS blockade within the dlPAG, a second mechanical stimulation raised glutamate by a similar level (18.8 ± 3.9 ng/mL versus 16.2 ± 2.7 ng/mL; $P < 0.05$). Baseline glutamate concentrations before and after administration of L-NIO were 4.75 ± 1.3 ng/mL and 5.12 ± 2.4 ng/mL, respectively. Likewise, for GABA, mechanical stimuli in 4 rats increased GABA concentrations by 2.2 ± 0.3 ng/mL and after eNOS blockade within the dlPAG, mechanical stimuli evoked similar increases in GABA levels (2.7 ± 1.1 ng/mL; $P < 0.05$ compared to controls). Baseline GABA before and after the drug were similar (1.25 ± 0.8 ng/mL and 1.51 ± 0.7 ng/mL, respectively). The glutamate and GABA concentrations were similar to those observed in previous studies (Gray et al., 2001; Ishide et al., 2005; Karlsson et al., 2007). These results suggested that eNOS played no role in modulating glutamate or GABA concentrations within the dlPAG during mechanical nociception.

2.2. *Effects of L-NIO on MAP, HR, and concentrations of glutamate and GABA within the dlPAG during heat-induced thermal stimulation*

A dose-response experiment with 0.1, 1, and 10 μM L-NIO, administered sequentially for 30 min at 5 μL/min into the dlPAG did not alter baseline MAP or HR values ($n = 5$). However, at 30 min after each concentration of the eNOS antagonist,

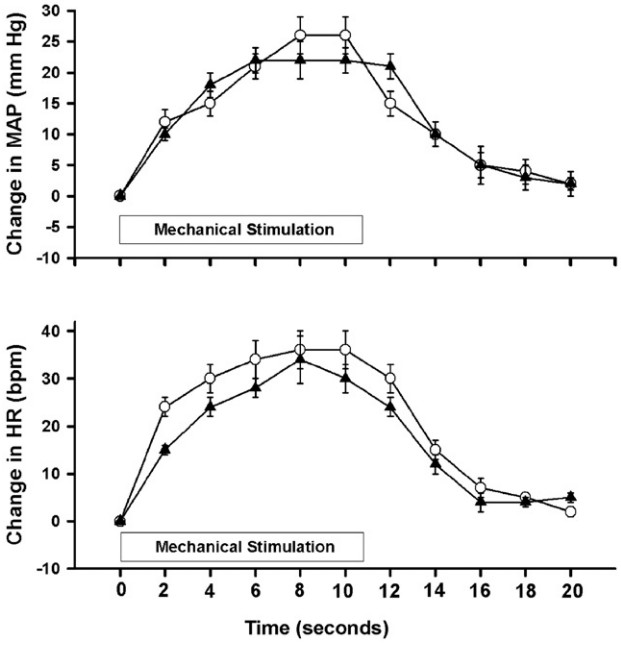

Fig. 2 – Average time–course changes in mean arterial pressure (MAP: upper panel) and heart rate (HR: lower panel) during a 10-second hindpaw mechanical stimulus 120 min after bilateral insertion of microdialysis probes into the dorsolateral periaqueductal gray matter (Control: open circles) and 30 min after microdialysis of L-*N*(5)-(1-iminoethyl) ornithine (L-NIO: 10 μM: closed triangles) into the same area of anesthetized rats. Values represent means ± SEM (*n* = 8).

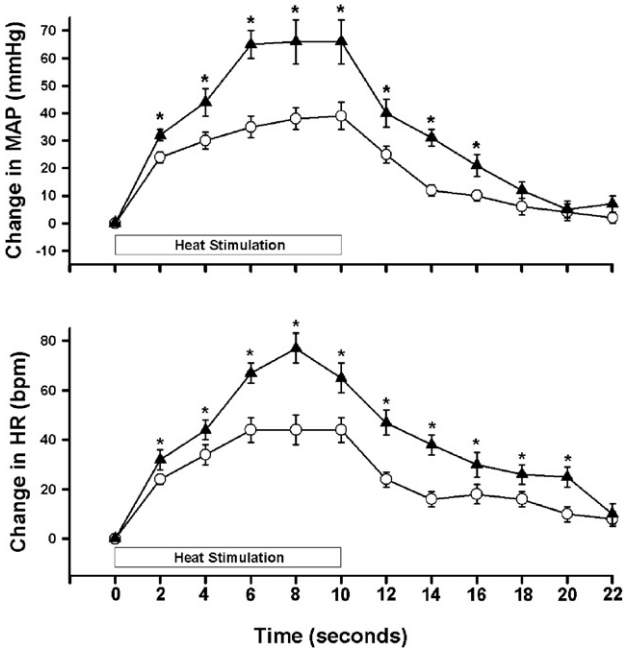

Fig. 3 – Average time–course changes in mean arterial pressure (MAP: upper panel) and heart rate (HR: lower panel) during a 10-second hindpaw immersion in a 52 °C hot-water bath at 120 min after bilateral insertion of microdialysis probes into the dorsolateral periaqueductal gray matter (Control: open circles) and 30 min after microdialysis of L-*N*(5)-(1-iminoethyl)ornithine (L-NIO: 10 μM: closed triangles) into the same region of anesthetized rats. Values represent means ± SEM (*n* = 8). *P < 0.05 versus control.

heat stimuli induced by immersing a hindpaw into a 52 °C hot water bath augmented the increases in MAP and HR in a dose-dependent manner (Fig. 1). Later, the 10 μM dose was used in a set of 8 experiments to determine its effects on cardiovascular responses and neurotransmitter concentrations with respect to 10-second heat-induced nociceptive stimulations. Before administration of L-NIO, a heat stimulation significantly increased MAP and HR by 39 ± 5 mmHg and 44 ± 6 bpm, respectively (Control; $P < 0.05$; Fig. 3). Then L-NIO (10 μM) was microdialyzed bilaterally into the dlPAG for 30 min. A subsequent heat-induced thermal stimulation potentiated the increases in both MAP and HR (ΔMAP = 66 ± 8 mmHg and ΔHR = 77 ± 8 bpm; Fig. 3). However, at 120 min after disconti-

Table 1 – Hemodynamic data during 10 s mechanical (*n* = 8), heat-induced thermal (*n* = 8), and cold-induced thermal (*n* = 8) stimulation before, 30 min after microdialysis of L-*N*(5)-(1-iminoethyl)ornithine (L-NIO: 10 μM) into the dorsolateral periaqueductal gray matter, and 120 min after discontinuation of L-NIO (recovery)

	Before L-NIO		After L-NIO		Recovery	
	Baseline	Peak	Baseline	Peak	Baseline	Peak
Mechanical stimulation						
MAP (mmHg)	111 ± 4	137 ± 5*	115 ± 5	137 ± 5*	107 ± 6	129 ± 5*
HR (bpm)	334 ± 9	370 ± 9*	345 ± 9	379 ± 11*	350 ± 11	380 ± 9*
Heat stimulation						
MAP (mmHg)	104 ± 5	143 ± 6*	99 ± 5	165 ± 9*+	111 ± 5	147 ± 6*
HR (bpm)	313 ± 11	357 ± 9*	301 ± 8	378 ± 9*+	324 ± 9	364 ± 9*
Cold stimulation						
MAP (mmHg)	112 ± 5	82 ± 4*	117 ± 6	132 ± 5*+	119 ± 6	84 ± 3*
HR (bpm)	350 ± 9	306 ± 8*	366 ± 6	382 ± 6*+	349 ± 9	299 ± 11*

All values represent means ± SEM. Abbreviations: MAP: mean arterial pressure; HR: heart rate; bpm: beats per minute.
* Significantly different vs. corresponding baseline values ($P < 0.05$).
\+ Significantly different vs. corresponding peak values before L-NIO and during recovery ($P < 0.05$).

nuation of L-NIO, the heat stimulation increased MAP and HR similar to control levels. This result demonstrated recovery and suggests that the augmentations were the effects of eNOS blockade within the dlPAG (Table 1).

Heat stimuli before L-NIO administration significantly increased glutamate levels ($P<0.05$; $n=5$; Fig. 4) within the dlPAG. A second heat-induced thermal stimulation after 30 min of L-NIO perfusion into the dlPAG resulted in potentiated glutamate levels that were significantly higher than the levels observed in controls ($P<0.05$; Fig. 4). Glutamate concentrations before and after L-NIO were 34.5±5.7 ng/mL and 37.2±6.4 ng/mL, respectively, suggesting that the drug did not alter the baseline glutamate level. Concomitantly, the first heat stimuli significantly increased dlPAG concentrations of GABA ($P<0.05$; $n=5$; Fig. 4). However, after 30 min of L-NIO perfusion into the dlPAG, eNOS blockade significantly attenuated the GABA levels in response to another heat stimulus ($P<0.05$; Fig. 4). Baseline GABA levels before and after the drug were 3.3±0.6 ng/mL and 4.1±0.6 ng/mL, respectively. Although the cardiovascular responses to a third heat stimulation after 120 min following discontinuation of the drug were similar to the control values, it is unclear why both glutamate and GABA concentrations did not show recovery.

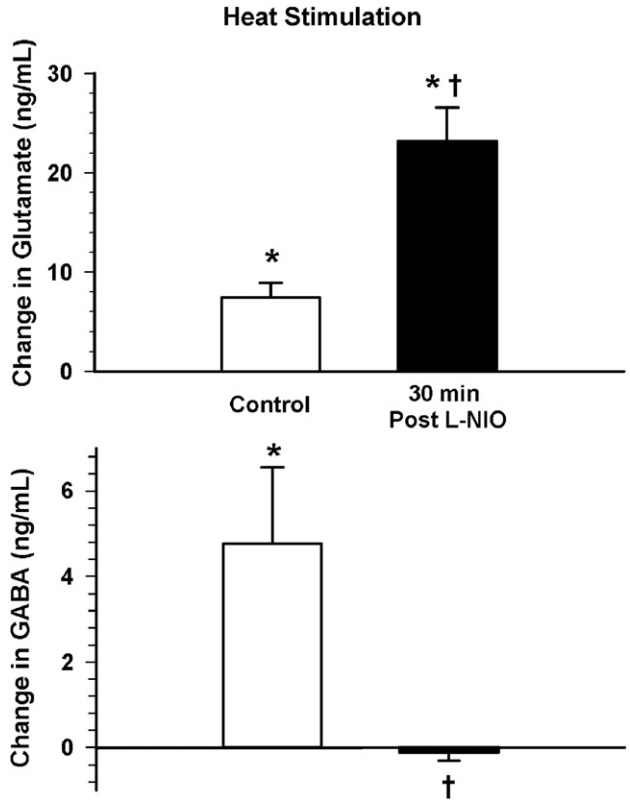

Fig. 4 – Average peak changes in extracellular glutamate concentrations ($n=5$: upper panel) and GABA ($n=5$: lower panel) concentrations in response to a 10-second hindpaw immersion in a 52 °C hot-water bath during control (open bars), 30 min after microdialysis of L-N(5)-(1-iminoethyl) ornithine (L-NIO: 10 μM: closed bars) into the dorsolateral periaqueductal gray matter. Values represent means±SEM. *$P<0.05$ versus baseline. †$P<0.05$ versus control level.

2.3. *Effects of L-NIO on MAP, HR, and concentrations of glutamate and GABA within the dlPAG during cold-induced thermal stimulation*

Interestingly, a cold stimuli evoked by immersing a hindpaw into a 10 °C cold water bath caused significant decreases in MAP and HR. Doses of 0.1, 1, and 10 μM L-NIO, administered sequentially for 30 min into the dlPAG did not alter baseline MAP or HR values ($n=5$). After 30 min of microdialyzing each concentration of L-NIO, cold-induced stimuli attenuated the decreases in MAP and HR in a dose-dependent manner (Fig. 1).

Thereafter, we used 8 rats to determine the effects of 10 μM L-NIO on cardiovascular responses, glutamate, and GABA concentrations within the dlPAG during a brief 10-second cold-induced thermal stimulation. Cold stimuli caused significant decreases in MAP and HR by 30±5 mmHg and 39±6 bpm, respectively, which immediately returned to pre-stimulation levels ($P<0.05$; Fig. 5). Thereafter, the administration of 10 μM L-NIO into the dlPAG did not alter baseline MAP or HR. However, a cold stimulus after 30 min produced significant pressor and tachycardic responses which were opposite to those seen in controls (Fig. 5). Finally, a third cold-induced thermal stimulation performed at 120 min after discontinuation of the drug elicited depressor and bradycardic responses similar to those seen in controls (Table 1).

Also, the cold stimuli decreased glutamate concentrations significantly in controls ($P<0.05$; $n=6$; Fig. 6). After bilateral administration of L-NIO for 30 min, a subsequent cold stimulation resulted in significantly elevated glutamate concentrations which were opposite to those concentrations seen before the drug ($P>0.05$; Fig. 6). The glutamate concentration before and after L-NIO were 16.7±3.4 ng/mL and 21.2±5.6 ng/mL, respectively, which suggests that the drug did not alter baseline glutamate levels. On the other hand, before L-NIO administration, cold stimulus significantly increased GABA ($n=8$; $P<0.05$; Fig. 6), however, after 30 min of L-NIO, the cold stimuli significantly reduced extracellular GABA levels (Fig. 6). The drug did not alter baseline GABA level as the concentration before and after L-NIO were 2.7±0.4 ng/mL and 2.2±0.6 ng/mL, respectively. However, glutamate and GABA concentrations during a third cold stimulation after 120 min of discontinuation of the drug did not recover to control levels.

2.4. *Control experiments and effects of L-NIO on MAP, HR, and extracellular fluid glutamate/GABA concentrations during mechanical, heat or cold stimulation*

Using 15 rats, time-control experiments for mechanical ($n=5$), heat ($n=5$), and cold ($n=5$) stimuli were performed with protocols that were similar to those mentioned in the above experiments with the exception that L-NIO was not administered into the dlPAG. In all experiments, there were no significant differences in changes in MAP or HR during any of the three types of stimulation suggesting that the modulation of cardiovascular responses during all stimuli was due to the effects of L-NIO within the dlPAG.

Further, using 15 rats in 3 separate sets of experiments, microdialysis probes were inserted bilaterally into areas 1.5 mm lateral to the dlPAG. The 3 types of stimulation protocols were repeated and cardiovascular parameters were

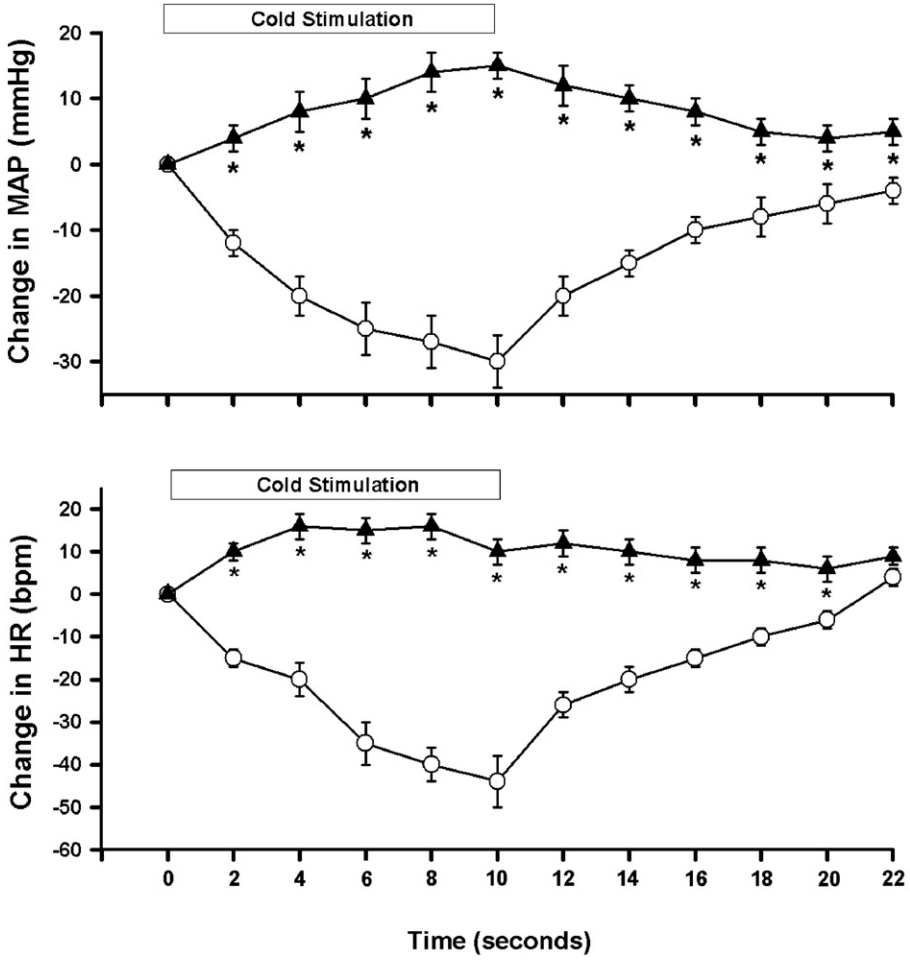

Fig. 5 – Average time–course changes in mean arterial pressure (MAP: upper panel) and heart rate (HR: lower panel) during a 10-second hindpaw immersion in a 10 °C cold water bath at 120 min after bilateral insertion of microdialysis probes into the dorsolateral periaqueductal gray matter (Control: open circles) and 30 min after microdialysis of L-N(5)-(1-iminoethyl)ornithine (L-NIO: 10 μM: closed triangles) into the same area of anesthetized rats. Values represent means ± SEM ($n=8$). *$P<0.05$ versus control.

measured. In all cases, L-NIO had no effects on cardiovascular responses during mechanical, heat, or cold stimulations.

2.5. *Histology*

Histological verification of the brain sections revealed that the membrane of each microdialysis probe was within the dlPAG region and the membranes were separated by 1.7 mm. The sections were photographed and compared with the rat brain atlas (Paxinos and Watson, 1998) and representative photomicrographs were shown in 2 publications from our laboratory (Ishide et al., 2005; Karlsson et al., 2007).

3. Discussion

The interactions between the "nociceptive system" and the "cardiovascular system" involve various neuronal mechanisms, alterations in neurotransmission, and activation of different regions of the central nervous system. The present study demonstrates increases in cardiovascular responses during mechanical stimulation that are unaffected by eNOS antagonism within the dlPAG. In addition, the data show that blockade of eNOS within the dlPAG significantly potentiates cardiovascular responses and extracellular glutamate levels, but attenuates GABA levels during heat-induced thermal nociception. In contrast, cold-induced thermal stimulation results in significant depressor and bradycardic responses associated with a decrease in glutamate and an increase in GABA levels within the dlPAG. The inhibition in cardiovascular function during the cold-stimuli is reversed to pressor and tachycardic effects following eNOS antagonism within the dlPAG, possibly mediated via increased glutamate and reduced GABA concentrations. Collectively, these results demonstrate that eNOS within the dlPAG plays a differential role in modulating cardiovascular function in response to heat- and cold-induced thermal nociception, possibly via altered glutamate and GABA neurotransmission. However, eNOS within the dlPAG appear to play no role on cardiovascular responses during mechanical stimulation.

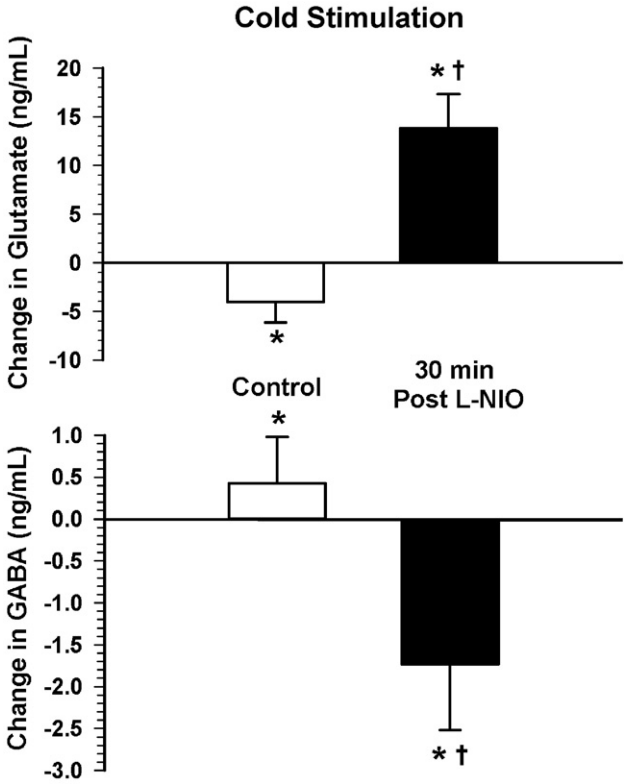

Fig. 6 – Average peak changes in extracellular glutamate concentrations ($n=6$: upper panel) and GABA ($n=8$: lower panel) concentrations in response to a 10-second hindpaw immersion in a 10 °C cold water bath during control (open bars), 30 min after microdialysis of L-N(5)-(1-iminoethyl) ornithine (L-NIO: 10 μM: closed bars) into the dorsolateral periaqueductal gray matter. Values represent means ± SEM. *$P<0.05$ versus baseline. †$P<0.05$ versus control level.

Acute cutaneous or superficial somatic pain evoked by chemical, electrical, thermal and mechanical modes of stimulation activates polymodal nociceptors. Next, the information is transmitted via the Aδ and C fibers to various spinal and supraspinal sites and elicits the classical defensive flight or withdrawal response, increased sympathetic nerve activity, tachycardia, and increases in blood pressure (Lovick, 1986; Sun and Spyer, 1991; Depaulis and Bandler, 1991; Blessings, 1997; Mason, 1999; Le Bars et al., 2001; Morgan and Clayton, 2005). In our study, heat-induced thermal stimuli cause pressor and tachycardic effects along with increases in glutamate and GABA concentrations within the dlPAG and are similar to those observed in a previous publication (Silva et al., 2000). In contrast, cold-mediated nociception elicits inhibitory cardiovascular responses associated with decreased glutamate and elevated GABA concentrations within the dlPAG. These hypotensive and bradycardic responses to cold appear to simulate defensive freezing or a recuperative response (Hammer and Kapp, 1986; Lovick, 1993; Depaulis et al., 1994). Conversely, eNOS blockade within the dlPAG has no effect on cardiovascular responses and glutamate or GABA neurotransmission during mechanical stimuli.

Several supraspinal sites integrate cutaneous, deep, somatic, and visceral nociception by evoking profound cardiovascular and behavioral changes such as vocalization and defensive flight (Randich and Maixner, 1984; Zamir and Maixner, 1986; Reichling et al., 1988; Le Bars et al., 1991; Depaulis and Bandler, 1991; Mason, 1999; Morgan et al., 2006). In addition to the varying recruitment of Aδ and C fibers during nociception, coordination of cardiovascular activity during pain depends on the modality of stimulation. For example, visceral or deep somatic pain elicits depressor responses and bradycardia (Lovick, 1993; Keay and Bandler, 2002; Lumb, 2002). In contrast, superficial cutaneous stimulation causes the opposite reactions: increases in MAP, HR, and sympathetic nerve activity (Taylor et al., 1997; Yoon and Yaksh, 1999). The regulation of cardiovascular activity during different types of pain may involve specific brain region where the signals are integrated. Particularly, the PAG plays a pivotal role in coordinating cardiovascular function and pain modulation (Reichling et al., 1988; Mason, 1999, 2005). In humans, the PAG serves as one of the most the important link between pain and the cardiovascular system (Green et al., 2006). However, cardiovascular responses during pain are mostly related to functionally distinct longitudinal columns of the PAG organization. For example, the lPAG and the dlPAG are involved in pressor responses, fight and flight responses, and behavioral activation when glutamate is administered into these areas: effects that are observed during cutaneous and superficial somatic pain (Lovick, 1993; Bandler and Keay, 1996). Superficial nociception also modulates the release of glutamate and GABA within the dlPAG neurons (Maione et al., 1999). The data of this present study correspond with the above concepts. We propose that the pressor and tachycardic responses during heat-induced thermal stimuli are integrated by the dlPAG neurons via modulating glutamate and GABA concentrations. The right and left dlPAG are reciprocally innervated and there are significant crossovers of nociceptive neural pathways arising from the periphery (Tortorici et al., 2007). Therefore, the collections of dialysates from bilateral dlPAG regions were justified even though the stimulation was unilateral.

In contrast, the ventrolateral PAG (vlPAG) is associated with depressor responses, defensive freezing, quiescence and inhibition: reactions that are similar to those caused by visceral pain (Depaulis et al., 1994; Keay et al., 1994; Morgan and Carrive, 2001). The vlPAG mediates hypotension during stimulation of visceral nociceptors and involves the Δ subtype of opioid receptors (Cavun et al., 2004). While somatic and visceral nociception have been studied extensively, a thorough search of the literature does not reveal a single experiment where an animal has been subjected to a brief 10-second period of cold-induced nociception. In the present study, this 10 °C cold stimulus evokes profound inhibitory cardiovascular responses: effects that are similar to those observed during visceral pain. The time frame of 10 s corresponds with the time periods for the mechanical and heat stimuli protocols. Moreover, a 20-second cold stimulus also evokes similar cardiovascular and neurochemical changes (data not shown). The hypotensive and bradycardic responses during cold-induced thermal stimuli suggest that the integration of the pain signals might have occurred, at least in part, within the vlPAG region. Because similar depressor and bradycardic responses to cold stimuli ($n=4$) are observed at temperatures ranging between 4 °C and 20 °C,

it appears that the cardiovascular responses to cold nociception are not temperature-dependent.

Nitric oxide (NO), a free radical, has been shown to evoke significant cardiovascular responses when injected into the dlPAG and accompanies defense reactions (Wang et al., 2001). Nicotinamide adenine dinucleotide phosphate-dependent diaphorase (NADPH-diaphorase), a marker for NOS, is abundant within the entire PAG area (Onstott et al., 1993; Lovick and Paul, 1999). NOS activity increases within the vlPAG during visceral pain (Rodella et al., 1998) and in rats expressing fear responses (Chiavegatto et al., 1998). Nitric oxide modulates glutamate release in both rat cerebrocortical nerve terminals (Sistiaga et al., 1997); such NO-glutamate interactions also regulate cardiovascular function (Lo et al., 1997). In addition, NOS co-localizes with GABA within the entire PAG region (Hall and Behbehani, 1998; Lovick and Paul, 1999), suggesting that NO-GABAergic interactions can affect both the nociceptive and the cardiovascular systems. We have shown that administration of the NO precursor, L-arginine, into the dlPAG attenuates cardiovascular responses by decreasing glutamate levels in response to either heat-induced thermal or mechanical stimuli, and subsequent perfusion of an NOS inhibitor reverses those attenuations (Ishide et al., 2005). In addition, blockade of nNOS activity within the dlPAG augments cardiovascular responses, increases glutamate levels, and decreases GABA concentrations in response to heat-induced thermal stimulation (Karlsson et al., 2007). However, mechanical stimuli following nNOS antagonism within the dlPAG have no effect on cardiovascular responses, glutamate or GABA concentrations (Karlsson et al., 2007). The effects of NO within the PAG are dependent on the functional divisions of the PAG (Wang et al., 2001). Moreover, each type of nociceptive input traveling via the C and Aδ fibers are processed separately in the different regions of the PAG (Lumb, 2002). Thus, it may be possible that heat-induced thermal pain activates the Aδ fibers primarily, whereas the pinch stimulus involves the C fibers (Karlsson et al., 2007). However, the data of the present study suggest that the role of eNOS protein is similar to the role of the nNOS isoform in modulating cardiovascular responses during mechanical and heat-induced thermal nociception.

The eNOS antagonism within the dlPAG completely reverses the cardiovascular responses from depressor responses to pressor effects during cold stimuli. Glutamate levels increase within the dlPAG while GABA concentrations decrease during the cold stimuli. The temporal relationship involving a pressor response and an augmentation in HR appears to be associated with concomitant increase in glutamate concentration within the dlPAG and a decrease in GABA response. Opposite changes in glutamate and GABA are associated with depressor response and bradycardia. As cited previously, there may be interneurons between the dlPAG and vlPAG areas that use glutamate and GABA as their neurotransmitters (Vanegas and Tortorici, 2007). It is possible that the rise in glutamate and fall in GABA concentrations within the dlPAG area following eNOS antagonism evoke excitatory post-synaptic potentials that, in turn, result in pressor and tachycardic responses during cold stimuli. Also, a rise in glutamate concentration within the dlPAG by the drug may lead to its diffusion into the adjacent vlPAG region and produce the increased cardiovascular effects in response to cold stimuli. Moreover, disinhibition of selective neurons by the eNOS antagonist within the dlPAG may shift the cardiovascular responses from inhibitory to excitatory during the cold stimulus. However, this shift may be a result of an artifact of anesthesia as anesthetics can greatly influence the cardiovascular system. Furthermore, a specific anesthetic can also influence cardiovascular function differentially not only in response to cold, but also during mechanical or heat nociception. Overall, the results of the present study demonstrate differential regulation of pain integration within the dlPAG which depends on the type of stimulus involved, percent recruitment of both afferent and efferent pathways, activation of different spinal and supraspinal regions, and protein–neurotransmitter interactions. Pain sensitivity varies across the estrus cycle in rats (Shane et al., 2007, Craft and Leitl, 2008). In the present study we did not test the estrus stage of the rats and it is unknown whether the estrus cycle influenced the responses.

The concentration of 10 μM L-NIO used in the study is presumed to be a selective eNOS antagonist (Rees et al., 1990, Benamar et al., 2003, Banwait and Rattan, 2003, Ishikawa and Quock, 2003). Intraperitoneal (i.p.) injections of L-NIO more than 30 mg/kg dose result in loss of eNOS selectivity and may inhibit nNOS activity (Ishikawa and Quock, 2003). Further, 10–20 mg/kg L-NIO given i.p. does not involve nNOS-mediated hyperthermia (Benamar et al., 2003). Considering that approximately 10% of 10 μM L-NIO diffuses out of the probe membranes, the amount of L-NIO being administered into the dlPAG is extremely miniscule in order to block the nNOS protein. Thus, the concentration of L-NIO can be assumed to be selective for eNOS.

In conclusion, the results of this study demonstrate that the blockade of eNOS activity within the dlPAG augments cardiovascular responses during heat-induced thermal stimulation, probably mediated via increases in the concentrations of glutamate and decreases in GABA levels. The eNOS antagonist, L-NIO, at a dose of 10 μm, has no effect on cardiovascular function during mechanical nociception. Finally, the present study shows depressor effects and bradycardia in response to a 10-second cold-induced thermal stimulation that changes into pressor and tachycardic effects following eNOS antagonism within the dlPAG. Overall, the data provide additional information regarding the link between the nociceptive and cardiovascular systems and the interaction between eNOS protein and glutamatergic/GABAergic neurotransmission within the dlPAG. However, further studies are crucial in order to identify the molecular, physiological, and therapeutic significance of the eNOS protein in relationship with heat-induced painful stimuli, as in burn injuries, or cold stimulus as in diving into cold water, or mechanically-evoked nociception caused by a blunt force injury.

4. Experimental procedures

4.1. *Surgery*

All surgical procedures and protocols were approved by the Institutional Animal Care and Use Committee of the Massachusetts College of Pharmacy and Health Sciences, and were performed according to regulations of the Animal Welfare Act

and the NIH Guide for the Care and Use of Laboratory Animals. Female Sprague Dawley rats (208–262 g: Charles River Labs., Wilmington, MA, USA) were used in the study. The surgical procedures were previously explained in details (Gray et al., 2001; Ishide et al., 2003; Ishide et al., 2005; Karlsson et al., 2006; Karlsson et al., 2007). Briefly, the rats were anesthetized with Na pentobarbital (35 mg/kg) and chloral hydrate (80 mg/kg) administered intraperitoneally (i.p.). The body temperature of each animal was maintained between 36–38 °C using a heating pad and infrared heat lamp. Supplemental doses of chloral hydrate-Na pentobarbital were administered i.p. whenever the rats exhibited a corneal reflex, responded to a pinch to the ear pinna, and/or when their blood pressure fluctuated. The left common carotid artery was catheterized with a tygone cannula coupled to a Model P231D Statham pressure transducer connected to a Model 79D physiological chart recorder (Grass Instruments, Braintree, MA, USA) in order to measure AP, MAP, and HR.

The rat's head was fixed in a stereotaxic frame with ear bars (Stoetling, Wood Dale, IL, USA). The lower body was positioned in a spinal unit with the pelvis and both knee joints firmly tied to steel posts. Two Burr holes were drilled in the skull bone and the dura was cut. Using stereotaxic guides, two CMA-11 microdialysis probes (CMA, North Chelmsford, MA, USA), each with 1 mm membrane tip and 0.22 mm outer diameter, were inserted bilaterally into the dlPAG at coordinates −6.3 from Bregma, 0.75 mm lateral to the sagittal suture, and 5.0 mm vertical from the surface of the brain (Paxinos and Watson, 1998). The probes were continuously perfused with artificial cerebrospinal fluid (aCSF; pH = 7.4; osmolality = ~305 mOs/kg) at 5 μL/min. The eNOS inhibitor, L-NIO, (Sigma Chemicals, St. Louis, MO, USA), was prepared in this aCSF solution. This L-NIO was presumed to be a potent and selective eNOS antagonist according to many publications (Rees et al., 1990, Benamar et al., 2003, Banwait and Rattan, 2003, Ishikawa and Quock, 2003). Thereafter, functional verification of probe placements into bilateral dlPAG was established after an administration of 1 mM L-glutamate (Sigma Chemicals) evoked immediate pressor and tachycardic responses. In addition, the locations of the probes were confirmed histologically at the end of each experiment after the animals were sacrificed.

4.2. Protocols

4.2.1. *Effects of L-NIO on MAP, HR, extracellular fluid glutamate and GABA concentrations during mechanical stimulation*

Following surgical setup, the animal was stabilized for 2 h. A dose–response relationship in MAP and HR was obtained after incremental log doses (0.1 μM, 1 μM, and 10 μM; $n = 5$) of L-NIO were microdialyzed for 30 min each into the dlPAG, followed by a mechanical nociceptive stimulus. The mechanical stimulation was similar to that as described previously (Sun and Spyer, 1991; Jones and Blair, 1995; Gray et al., 2001; Ishide at al., 2003, 2005; Karlsson et al., 2006, 2007). Briefly, it was an intense 10-second hindpaw pinch using a Kelly 14 cm straight artery forceps (Model 13018-14, Fine Science Tools, Foster City, CA, USA). The pressure was standardized in each manipulation and applied to three notches evenly over the metatarsus of each hindpaw, at an equal distance from the tip of the digits. A lower parameter of the mechanical stimulus, i.e., two notches was not used, because it did not evoke significant cardiovascular responses. Because repetitions of mechanical stimuli might alter paw sensitivity, the manipulations were performed using alternate hindpaw (Randall and Selitto, 1957). During these stimuli, MAP, and HR were continuously monitored and recorded. A higher log 20 μM dose of L-NIO increased baseline blood pressure and thus it was not used in any protocol.

After the dose–response experiments, we determined the effects of 10 μM L-NIO on cardiovascular responses and glutamate/GABA levels during mechanical stimulation using 8 rats. A 2-hour period was required for glutamate and GABA to stabilize as shown in previous studies and baseline samples were collected for 1 min in separate Eppendorf tubes (Gray et al., 2001; Ishide et al., 2005; Karlsson et al., 2007). Following the 2-hour stabilization, a 10-second mechanical stimulus was applied to determine the control cardiovascular responses. Simultaneously, the dialysate samples from both microdialysis probes were collected in a single Eppendorf tube in order to quantify glutamate and GABA concentrations using HPLC-ECD. The dialysate sample represented a 10-second collection during the stimulation period only. The precise timing for the aCSF to travel from the probe membrane to the Eppendorf tube was established before each experiment. Then 10 μM L-NIO was microdialyzed into bilateral dlPAG. After 30 min, a mechanical stimulation was performed on the other hindpaw with concomitant collections of dialysate samples and recordings of MAP and HR. Then, L-NIO perfusion was stopped and aCSF was administered bilaterally into the dlPAG for 120 min. A mechanical stimulation was repeated while recording MAP and HR, and collecting dialysate samples in order to determine whether recovery had occurred. All dialysates were coded and immediately stored at −80 °C. The samples were subsequently assayed for glutamate and GABA concentrations using HPLC-ECD. The samples were blindly quantified as the analyst who performed the assays was unaware of the protocol.

4.2.2. *Effects of L-NIO on MAP, HR, extracellular fluid glutamate and GABA concentrations during heat-induced thermal stimulation*

Heat-induced thermal stimuli were performed by immersing the metatarsus of a hindpaw in a hot water bath for 10 s at 52 °C (Sun and Spyer, 1991; Gray et al., 2001; Ishide et al., 2003). The protocol for these experiments was similar to that as described above. First, five rats were used to determine a dose–response relationship using incremental log doses (0.1 μM, 1 μM, and 10 μM) of L-NIO, microdialyzed for 30 min each into the dlPAG, followed by a stimulus. Thereafter, 10 μM L-NIO was administered into the dlPAG to determine its effect ($n = 8$) on cardiovascular responses, glutamate and GABA levels during the 10-second heat stimulations. After a stimulus, L-NIO was discontinued and aCSF was perfused into bilateral dlPAG for 120 min, followed by heat stimulation in order to determine recovery.

4.2.3. *Effects of L-NIO on MAP, HR, extracellular fluid glutamate and GABA concentrations during cold-induced thermal stimulation*

The protocol for this set of experiments was also similar to that described above except that the cold-induced thermal stimulation was performed by immersing the metatarsus of a

hindpaw in a 10 °C cold water bath for 10 s. First, 5 rats were used to determine a dose–response relationship (0.1 μM, 1 μM, and 10 μM). Then, 10 μM L-NIO was administered into the dlPAG in order to find the effects on cardiovascular responses, glutamate and GABA levels during a 10-second cold-induced nociceptive stimulus ($n=8$). Afterwards, L-NIO was discontinued and aCSF was perfused into bilateral dlPAG for 120 min, followed by a cold stimulation in order to determine if all responses recovered to baseline values.

4.2.4. *Analysis of glutamate and GABA concentrations using HPLC-ECD*

Glutamate and GABA concentrations were determined by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) as mentioned in our previous publications (Gray et al., 2001; Ishide et al., 2003, 2005; Karlsson et al., 2006, 2007). The apparatus consisted of an ESA 542 autosampler (ESA, MA), ESA 582 dual piston pump, ESA Coulochem II electrochemical detector with model 5020 guard cell and a 5011 analytical cell. The column was an ESA HR-80 RP C18 with a mobile phase of 0.1 M disodium hydrogen phosphate (Sigma, MO) in polished water. The ESA Coulochem II electrochemical detector analyzed both glutamate and GABA. Data was then integrated and analyzed by an integrator (Spectra Physics SP 4270). Equal volumes of microdialysate and working OPA/βME solution were manually mixed at room temperature by pipette for 30 s and injected precisely 2 min after initial mixing. Area under the curve was compared with standards of glutamate or GABA. Standards were prepared daily from stock standards stored at −80 °C. Glutamate and GABA concentrations were uncorrected for in vitro probe recovery, and standard calibration curves had $r2>0.998$.

4.2.5. *Histology*

At the end of each experiment, the rats were perfused transcardially with 10% phosphate-buffered formalin and after decapitation the brains were removed. The brains were fixed in a 10% phosphate-buffered formalin solution. Each brain was sliced into 44 μm transverse sections by a cryostat, and each slice was examined under a microscope to verify the probe tract locations. Each section was compared with the rat brain atlas (Paxinos and Watson, 1998) and with our previous publications (Gray et al., 2001; Ishide et al., 2005; Karlsson et al., 2007).

4.2.6. *Statistical analysis*

All data were mean ± standard error of the mean (SEM). Power and distribution analyses were performed before proceeding with any statistics. The baseline cardiovascular values were the average of a 4-minute pre-manipulation period. For the neurotransmitters, an average of their concentrations in 2–3 samples prior to any manipulation was taken as the baseline level. Peak changes in MAP and HR were the maximum values during the stimulation period. Values for changes in MAP, HR, extracellular fluid glutamate, and GABA concentrations during each stimulus before and after administration of L-NIO and after recovery, were analyzed by a one-way analysis of variance with repeated measures (RM-ANOVA). The dose–response data were also analyzed with one-way RM-ANOVA. *Post-hoc* Tukey's test was used when a significant F value was obtained. A $P<0.05$ value was considered to be statistically significant.

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