Role of brain nitric oxide in the cardiovascular control of bullfrogs

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ABSTRACT

The goal of the present study was to determine if nitric oxide (NO) acting on the brain of bullfrog (*Lithobates catesbeianus*) is involved in arterial pressure and heart rate (HR) control by influencing sympathetic activity. We investigated the effect of intracerebroventricular injections of L-NMMA (a nonselective NO synthase inhibitor) on mean arterial blood pressure (MAP), HR and cutaneous vascular conductance (CVC) of pelvic skin after intravenous injection of α or β adrenergic blockers, prazosin or sotalol, respectively. Arterial pressure was directly measured by a telemetry sensor inserted in the aortic arch of animals. L-NMMA increased MAP, but did not change HR. This hypertensive response was inhibited by the pre-treatment with prazosin, but accentuated by sotalol. The effect of L-NMMA on MAP was also inhibited by i.v. injections of the ganglionic blocker, hexamethonium. Thus, NO acting on the brain of bullfrog seems to present a hypotensive effect influencing the sympathetic activity dependent on α and β adrenergic receptors in the periphery.

1. Introduction

There is evidence that NO acts on the periphery as a vasodilator, not only in mammals (Rees et al., 1990), but also in amphibians and other vertebrates (Hoffmann and Romero, 2000; Donald and Broughton, 2005; Skovgaard et al., 2005). In amphibians, NO has been attributed to participate in physiological processes besides vascular resistance (Knight and Burnstock, 1996; Rea and Parsons, 2001; Broughton and Donald, 2002), such as microvascular permeability (Nguyen et al., 1995; Rumbaut and Huxley, 2002) and water uptake (Rea and Parsons, 2001; Rea et al., 2002). Much less is known, however, about the physiological role of encephalic NO in amphibians. We and others demonstrated previously that NO acting on brain of toads and frogs is involved in the reduction of preferred body temperature during hypoxia (Guerra et al., 2008) and the control of breathing (Hedrick et al., 1998; Gargaglioni and Branco, 2001), respectively. Moreover, nitrergic cells are reported to be ubiquitously distributed throughout the anuran brain (Bruning and Mayer, 1996; Huynh and Boyd, 2007), and there are NO-producing neurons in the hypothalamic regions and brainstem, including the *nucleus tractus solitarius* (NTS), of some anuran species (Bruning and Mayer, 1996; Munoz et al., 1996; Lazar and Losonczy, 1999) besides bullfrogs (Huynh and Boyd, 2007). To date, no study has investigated the possible role of brain NO in the cardiovascular control of amphibians. At least for mammals, a number of studies support the idea of a role for NO acting on specific encephalic nuclei, such as the paraventricular nuclei (Zhang et al., 1997; Zhang and Patel, 1998) and the NTS (Harada et al., 1993; Tseng et al., 1996), in decreasing sympathetic nerve activity, especially that which involves cardiovascular control (cf. Krukoff, 1999; Chen et al., 2001; Patel et al., 2001; Guo et al., 2009).

The sympathetic nervous system of anurans is organized such that its paravertebral chains extend from the second to the tenth spinal nerve, and a mixed sympatovagal trunk innervates the three-chambered heart (Nilsson, 2011). Adrenaline, released from the sympathetic nerves, is the major mediator of chronotropic and inotropic effects in the heart via activation of β-adrenergic receptors, namely β2 receptors (Herman and Sandoval, 1983; Herman and Mata, 1985). At least in bullfrogs, β2 receptors account for the majority of the receptors found in the atria and the ventricle (Herman et al., 1996). In terms of the peripheral vasculature, there is evidence for the vasoconstrictor effect of α-adrenergic receptors in anurans (Kimmel, 1992; Bianchi-da-Silva et al., 2000), which are activated by adrenaline, released from sympathetic nerves, and circulating noradrenaline (Azuma et al., 1965). In fact, adrenaline, noradrenaline and phenylephrine were all demonstrated to cause arterial pressure (AP) increases by activation of α receptors in amphibians (Erlij et al., 1965; Kirby and Burnstock, 1969; Lillo, 1979; Herman and Sandoval, 1983). Moreover, both α and β adrenergic receptors are present in the cutaneous vessels of frogs and contribute to vasoconstriction and vasodilation, respectively (Herman and Sandoval, 1983; Malvin and Riedel, 1990). It is also known that β receptors are important for water

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absorption in the ventral pelvic skin (Viborg and Rosenkilde, 2004). No study has addressed the possible influence of brain NO on peripheral adrenergic activity in amphibians.

Based on the considerations above, our aim was to investigate if brain NO is involved in the control of AP and HR via peripheral adrenergic and/or noradrenergic activity. By using a telemetry sensor to measure AP directly, we investigated the effect of brain NO inhibition, induced by intracerebroventricular injection of L-NMMA, on the peripheral α- and β-adrenergic influences on AP, HR and skin blood flow in bullfrogs.

2. Material and methods

2.1. Animals

We used bullfrogs, *Lithobates catesbeianus* (Shaw, 1802), of both sexes, weighing 180–350 g. Frogs were obtained from the bullfrog farm (CAUNESP) at the College of Agricultural and Veterinarian Sciences — UNESP, São Paulo state, Brazil. In our laboratory at the Department of Animal Morphology and Physiology (UNESP), all the animals were maintained at 25 °C in containers, with free access to tap water from an artesian well and a basking area, for at least two weeks before experimentation. The animals were fed *Tenebrio molitor* larvae and/or commercial carnivorous fish food two to three times per week. The study was conducted with the approval of the local Animal Care and Use Committee (Protocol 008519-10).

2.2. Surgical procedures

The animals were anesthetized by submergence in an aqueous 0.3% solution of 3-aminobenzoic acid ethyl ester (MS-222, Sigma, St. Louis, USA) buffered to pH 7.7 with sodium bicarbonate. After loss of the eye reflex, the animals were fixed to a David Kopf stereotaxic apparatus (Model 900 Small Animal Stereotaxic, Tujunga, CA, USA). The skin covering the skull was removed with the aid of a bone scraper and an opening was made in the skull above the telencephalon using a small drill (LB100, Beltec, Araraquara, Brazil). A guide cannula, prepared from a hypodermic needle segment, 14 mm in length and 0.55 mm in outer diameter, was attached to the tower of the stereotaxic apparatus and lowered into the lateral cerebral ventricle. These coordinates were adapted from the encephalic atlas of anurans (Donkelaar, 1998). The displacement of the meniscus in a water manometer confirmed correct positioning of the cannula within the lateral ventricle. The orifice around the cannula was filled with a paste consisting of a mixture of equal parts of paraffin and glycerin. The cannula was attached to the bone with stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion and infection. The experiments were performed seven days after brain surgery.

To measure pulsatile arterial pressure (PAP) and body temperature, the frogs were instrumented with a telemetry transmitter (TR43P, Telemetry Research, New Zealand). This telemetry system, originally developed for rat instrumentation, was adapted for use in anurans as follows: the animal was positioned in lateral decubitus, and an incision was made in skin and muscles at the left side of the abdomen to expose the left aortic arch (Fig. 1). The aortic arch was isolated using surgical threads to diminish blood flow, and a catheter, connected to the telemetry transmitter, was inserted into a tiny hole made in the artery using a needle (25 × 0.6 mm). The catheter was fixed to the artery wall with 2-octyl cyanoacrylate (Dermabond® Topical Skin Adhesive, Johnson & Johnson, Brazil) surgical glue to allow uninterrupted blood flow in the cannulated vessel. A surgical mesh joined the vessel with the catheter and was fixed to the surrounding tissue with surgical glue to reinforce the whole structure. The body of the telemetry transmitter, which houses the temperature sensor, was carefully placed inside the abdominal cavity and sutured to the obliquus internus muscle.

After the surgery, the frogs were treated with a prophylactic antibiotic (enrofloxacin, Flotril®; Schering-Plough, 5.0 mg kg^{-1}, s.c.) and an analgesic (Flunixina Meglumina, Banamine®; Schering-Plough, 1.0 mg kg^{-1}, s.c.), according to recommended dosages for amphibians (Gentz, 2007; Smith, 2007). Five days after guide cannula and telemetry transmitter implantations, the animals were again anesthetized (see protocol above) and a polyethylene cannula (PE50) was implanted in the femoral vein for the administration of adrenergic agonists and antagonists. For measurements of regional skin blood flow (SkBF), a laser Doppler probe (MSP100XP Standard Surface Probe, ADInstruments®, Sydney, Australia) was sutured to the pelvic surface skin. After the surgery, the animals were again treated with antibiotic and analgesic agents (see above).

2.3. Intracerebroventricular (i.c.v.) microinjections

Microinjections were performed via a thin dental needle (Mizzy, 30 Gauge), which was inserted until its tip was 0.4 mm below the guide cannula. A volume of 1 μL was injected over a period of 45 s with a 5-μL Hamilton syringe using a microinjection pump (model 310, Stoelting Co., IL, USA). The movement of an air bubble inside the PE-10 polyethylene tubing connecting the microsyringe to the dental needle confirmed drug flow. Thirty minutes were allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux and interference in PAP recordings.

At the end of each experiment, 1 μL of 2% Evans blue solution was microinjected into the lateral ventricle. The animals were euthanized by submergence in an aqueous 0.025% solution of benzocaine hydrochloride buffered to pH 7.7 with sodium bicarbonate (AVMA, 2007). Upon dissection, we observed that the dye had diffused into the periventricular tissue and spread along the ventricular system.

2.4. Determination of mean arterial blood pressure (MAP), heart rate (HR) and body temperature (T_b)

Pulsatile AP and T_b from each animal were continuously monitored during all experimental protocols by telemetry transmission. The sensor implanted within the animal's abdominal cavity detects, amplifies and transmits PAP and T_b data to a receiver (Telemetry Research Receiver, Auckland, New Zealand). This signal is transmitted to a system of data acquisition and analysis (PowerLab System, ADInstruments®/Chart Software, version 7.3, Sydney, Australia). The telemetry transmitters have been calibrated by the manufacturer using a Mensor 6100 series reference pressure sensor (pressure range from 760 to 1040 mm Hg; temperature range from 22 to 40 °C). Heart rate was calculated by counting the peaks of the PAP recording. Mean arterial pressure was automatically calculated as MAP = 2/3 DP + 1/3 SP (DP = diastolic pressure; SP = systolic pressure) from the pulsatile arterial pressure recording in real-time using the cyclic measurements tool from the Chart Software.

2.5. Measurements of skin blood flow (SkBF) and calculation of cutaneous vascular conductance (CVC)

Skin BF was continuously monitored in real-time using a laser Doppler probe (MSP100XP Standard Surface Probe, ADInstruments®, Sydney, Australia) sutured to the pelvic surface skin. Signals from the laser Doppler probe were monitored by a blood flow meter (ML191, ADInstruments®, Sydney, Australia) connected to a computer (PowerLab System, ADInstruments®/Chart™ Software, Sydney, Australia). This methodology was based on previous studies in toads (Viborg and Rosenkilde, 2004; Viborg and Hillyard, 2005; Viborg et al., 2006) and crocodiles (Seebacher and Franklin, 2007). The flow meter was calibrated in a colloidal solution of suspended latex spheres of standard size and concentration, according to recommendations in the manufacturer's manual (MLA191 calibration kit, ADInstruments).
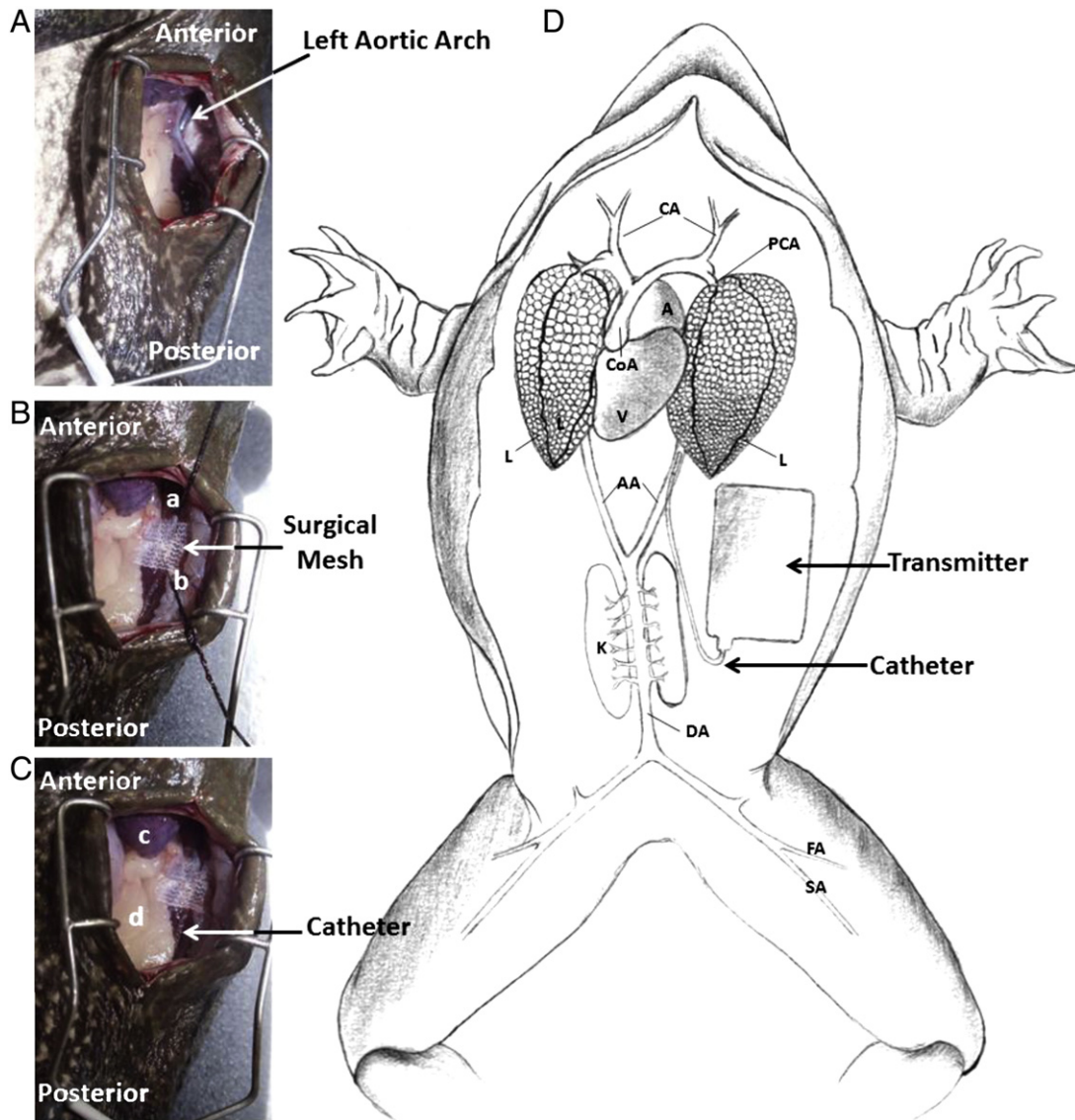
Fig. 1. Surgical procedure for telemetry transmitter implantation in the left aortic arch (LAA). See text for details. In A, B and C are sequential pictures of localization (A), isolation, catheter insertion (B), and catheter fixation to the left aortic arch and tissue surrounding the vessel (C). In B, a and b refer to the transitory interruption of blood flow through the aortic arch by threads, and the arrow indicates the location of a polypropylene surgical mesh (see text). In D, schematic drawing of a bullfrog, showing the positioning of the transmitter body within the abdominal cavity and the catheter inserted into the left aortic arch. A, atrium; AA, aortic arch; CA, carotid artery; DA, dorsal aorta; FA, femoral artery; PCA, pulmocutaneous artery; CoA, conus arteriosus; L, lung; K, kidney; SF, sciatic artery; V, ventricle. Source: Prepared by the author[1]

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[1] The illustration was done by the author based on the drawing of Mr. Lazaroff.

At each designated time-point, SkBF was assessed by averaging laser Doppler flow, measured in perfusion units (PU) in mV, over a steady 1 to 2-min interval, and CVC was then calculated as PU/MAP.

2.6. Experimental protocols

Experiments were performed on unanesthetized and unrestrained frogs previously prepared as described above.

Once all hemodynamic parameters had stabilized following venous cannulation (approximately 48 h later), a basal recording was taken for at least 30 min before each animal was submitted randomly to all protocols. After the basal recording, an intravenous (i.v.) bolus injection of prazosin (α1-adrenergic antagonist; 1.0 mg kg^{-1}; Sigma-Aldrich, USA), sotalol (β-adrenergic antagonist; 0.5 mg kg^{-1}; Sigma, USA) or Ringer's solution was combined with an i.c.v. microinjection of L-NMMA (a nonselective NOS inhibitor; 500 μg per animal;

Tocris, USA) or mCSF (vehicle) 5 min later. Pulsatile AP was recorded for approximately 20–30 min after i.c.v. injection. The combinations among i.v. and i.c.v. injections were performed in a randomized sequence. Phenylephrine or isoproterenol was used in order to test the α or β antagonists' blockade efficacy, respectively. All the experimental protocols were completed in about one week and each animal received a total of six i.c.v. microinjections (one per day). Another group of frogs was used to verify if i.v. injection of hexamethonium (25 mg kg^{-1}; Sigma) is able to inhibit the effect of i.c.v. L-NMMA (total of four i.c.v. microinjections per animal; one per day). Hexamethonium is a neuronal acetylcholine receptor antagonist that blocks preganglionic sites in both sympathetic and parasympathetic nervous systems. The dose of hexamethonium used in the present study, based on studies in reptiles (Eme et al., 2011), birds (Crossley and Altimiras, 2000) and mammals (Cheung, 1990), was effective as a ganglionic blocker since it was sufficient to inhibit parasympathetic

activity in bullfrogs, i.e., it blocked the tachycardic effect of atropine (3.0 mg kg^{-1}; Sigma; data not shown).

Doses of L-NMMA, phenylephrine, isoproterenol, sotalol and atropine were chosen according to previous studies (Herman and Sandoval, 1983; Herman and Mata, 1985; Skals et al., 2005; Guerra et al., 2008; Taylor et al., 2012) and pilot experiments. We used the maximum dose of L-NMMA that induced minimum behavioral alterations (see explanation in Discussion). Regarding prazosin, we used a dose (1 mg kg^{-1}) based on its maximum solubility (1 mg mL^{-1}; Bianco, 1978). A lower dose of prazosin (0.5 mg kg^{-1}), as well as the 1 mg kg^{-1} dose, induced similar inhibitions of the hypertensive effect of phenylephrine; however, 0.5 mg kg^{-1} of prazosin did not induce a significant reduction in the bradycardic effect of the α1 agonist (data not shown).

The animals were euthanized once the protocols were completed. The ionic composition of the mCSF in mEq/L [pH = 7.8 at 25 °C (Branco et al., 1992)] was 56.6 NaCl, 2.7 KCl, 0.9 CaCl$_2$, 0.45 MgSO$_4$, and 27.0 NaHCO$_3$.

2.7. Data analysis and statistics

Changes in mean arterial pressure (ΔMAP), heart rate (ΔHR) and cutaneous vascular conductance (Δ%CVC) were determined by comparing baseline values with maximal drug effects (Minson et al., 2002; Green et al., 2006; Seebacher and Franklin, 2007). A T-test was performed to verify that differences between the ΔMAP, ΔHR and Δ%CVC were significantly different from zero (P < 0.05). The assumptions of analysis of variance of normality of residuals (Cramer-von Mises, P < 0.05) and the presence of outliers were also tested. ANOVA was then used to assess the influence of the four drugs over ΔMAP, ΔHR and Δ%CVC and to assess the significant differences between agonist (phenylephrine and isoproterenol) effects before and after injection of antagonists (prazosin and sotalol). Differences among mean values were identified using a post hoc Tukey's test and considered significant for P < 0.05. Statistical analyses were performed using SAS GLM procedure (Littell et al., 2004).

3. Results

3.1. Resting hemodynamic values

Table 1 shows SP, DP, MAP and HR at the time of guide cannula and telemetry transmitter implantations in anesthetized animals, and 1 and 7 days after surgeries. CVC values are presented only at 7 days post-surgery because the flow transducer was fixed to the animal skin 5 days after brain surgery. Bullfrogs exhibited normal behavior and stability of cardiovascular variables 7 days after brain surgery and telemetry device implantation. Heart rate increased during anesthesia (PS) and decreased during the first day, becoming stable at the beginning of experiments, 7 days after surgeries (F$_{(2,22)}$ = 11.54; P < 0.001; repeated measures ANOVA). There was no significant change in MAP induced by surgeries.

Table 1
Mean arterial pressure (MAP) and heart rate (HR) of the bullfrog, *Lithobates catesbeianus*, at 24 °C, just after (post-surgery: PS), and 1 (D1), and 7 (D7) days after surgery.

	SP (mm Hg)	DP (mm Hg)	MAP (mm Hg)	HR (bpm)	CVC (mV/mm Hg)
Post-surgery	28.4 ± 1.3	21.5 ± 1.3	25.3 ± 1.2	48.9 ± 1.78	–
Day 1	28.7 ± 1.8	22.4 ± 1.5	25.7 ± 1.6	37.4 ± 2.3[a]	–
Day 7	29.2 ± 1.6	23.3 ± 1.4	26.4 ± 1.5	36.3 ± 2.2[a]	0.00259 ± 0.00091

N = 12 animals.
[a] Significant difference from PS value.

3.2. Effect of brain NO synthase blockade associated with the antagonism of systemic α- or β-adrenergic receptors in cardiovascular function of bullfrogs

Fig. 2 shows representative recordings of PAP, MAP and HR after i.c.v. injection of L-NMMA plus pretreatment with i.v. injection of Ringer's (Fig. 2A), prazosin (Fig. 2B) or sotalol (Fig. 2C). L-NMMA injection (Fig. 2A) caused no change in HR but increased MAP, a response that was inhibited by prazosin (Fig. 2B) but accentuated by sotalol (Fig. 2C).

Fig. 3 shows that i.c.v. injections of L-NMMA caused significant increase in MAP (F$_{(3,43)}$ = 40.17; P < 0.0001; Tukey's test) and decrease in CVC in the seat patch skin of bullfrogs (effect of treatment: F$_{(3,26)}$ = 11.15; P < 0.0001; Tukey's test), but did not change HR compared with i.c.v. injection of mCSF. Intravenous injection of prazosin alone caused no significant decrease in MAP or an increase in CVC, but significantly inhibited L-NMMA-induced hypertension (F$_{(3,43)}$ = 40.17; P < 0.0001; Tukey's test; Fig. 3). Neither prazosin nor L-NMMA affected HR. Prazosin inhibited both the hypertensive (F$_{(2,31)}$ = 42.47; P < 0.0001) and the bradycardic (F$_{(2,31)}$ = 34.38; P < 0.0001) effects of the α1-adrenergic agonist, phenylephrine (100 µg kg^{-1}) (Fig. 4). A lower dose of phenylephrine, 50 µg kg^{-1}, was tested but it increased AP with a small reduction in HR (data not shown). Phenylephrine significantly decreased %CVC in the ventral pelvic skin, which was not influenced by prazosin (Fig. 4).

Fig. 5 shows that the β-adrenergic antagonist, sotalol, used in tandem with mCSF (i.c.v.), did not change MAP, HR and %CVC, but significantly increased the pressor response of L-NMMA (F$_{(3,44)}$ = 43.89; P < 0.0001; Tukey's test) without affecting the effect of L-NMMA on %CVC. No treatment affected HR. The effectiveness of sotalol as a β antagonist was confirmed as it completely blocked the tachycardic effect of the β agonist, isoproterenol (F$_{(2,34)}$ = 89.42; P < 0.0001; Fig. 6). A higher dose of sotalol (3 mg kg^{-1}) was also capable of blocking the tachycardic effect of isoproterenol (data not shown); however, as 3 mg kg^{-1} of sotalol alone caused a slight reduction in HR (−19.44 ± 3.4%), the dose of 0.5 mg kg^{-1} was used in the present study. Intravenous injection of isoproterenol increased CVC in the seat patch skin, which was completely blocked by sotalol (F$_{(2,19)}$ = 7.81; P < 0.05; Fig. 6).

3.3. Effect of brain NO synthase blockade associated with the ganglionic blockade on cardiovascular function in bullfrogs

In Fig. 7, i.v. injection of the ganglionic blocker, hexamethonium, combined with i.c.v. microinjection of mCSF, did not change MAP and HR. However, pretreatment with hexamethonium inhibited the hypertensive effect evoked by L-NMMA (F$_{(3,30)}$ = 31.03; P < 0.0001). None of the treatments caused significant changes in HR.

4. Discussion

To our knowledge, this is the first study to demonstrate that brain NO seems to play a role in MAP control in bullfrogs by affecting sympathetic activity in a manner dependent on vascular α1- and β-adrenergic receptors.

4.1. Direct measurements of pulsatile arterial pressure by telemetry in bullfrogs: considerations about the method

As far as we know, this is the first time blood pressure was directly measured in anurans using telemetry. The anatomical differences between anuran amphibians and mammals make further procedural adjustments necessary for the cannulation of blood vessels and arrangement of the transmitter device inside the body cavity, which does not present a thoracic cavity limited by ribs and a diaphragm. Cannulation of the dorsal artery of amphibians (homologous to the mammalian abdominal aorta (Van Vliet and West, 1994), which is generally used for measuring AP by telemetry in these animals) has several ramifications to the kidneys
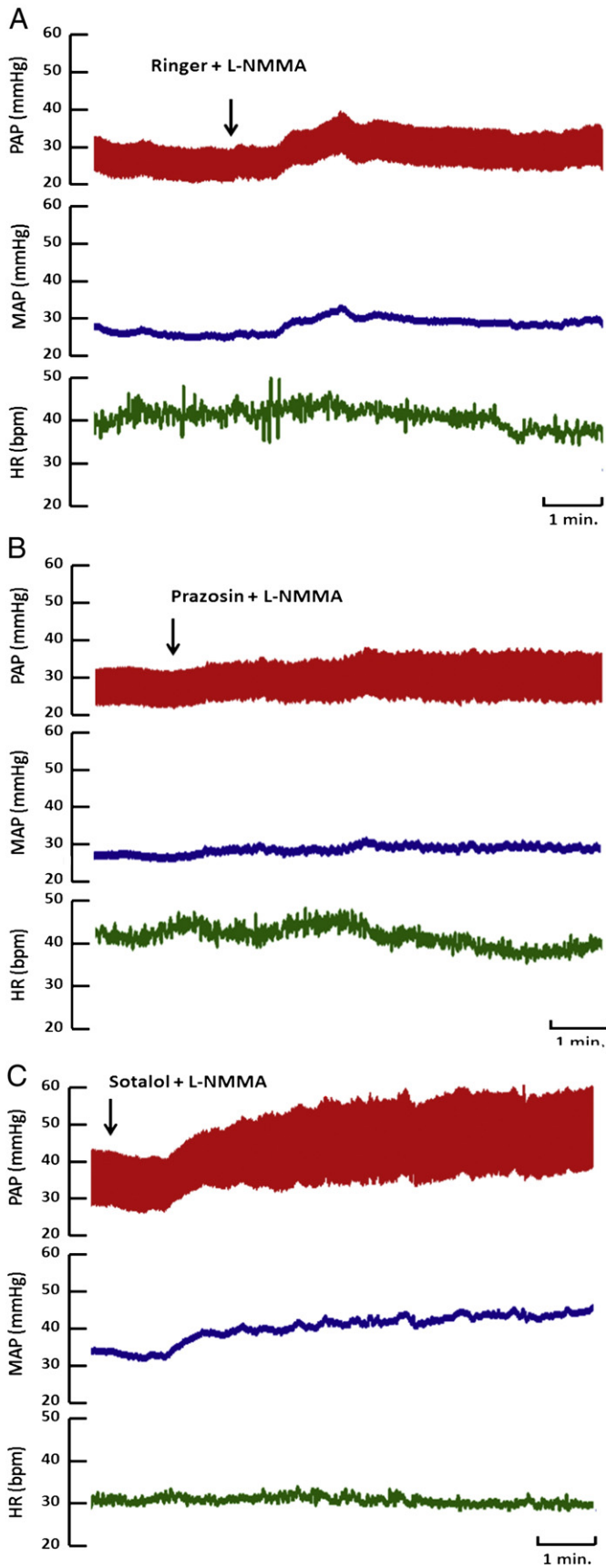
Fig. 2. Representative recordings of pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR) of a bullfrog. Effect of intracerebroventricular (i.c.v.) injection of L-NMMA (500 μg per animal) after intravenous (i.v.) injection of Ringer's solution (A), 1.0 mg kg^{-1} of prazosin (B) or 0.5 mg kg^{-1} of sotalol (C) on PAP, MAP and HR.
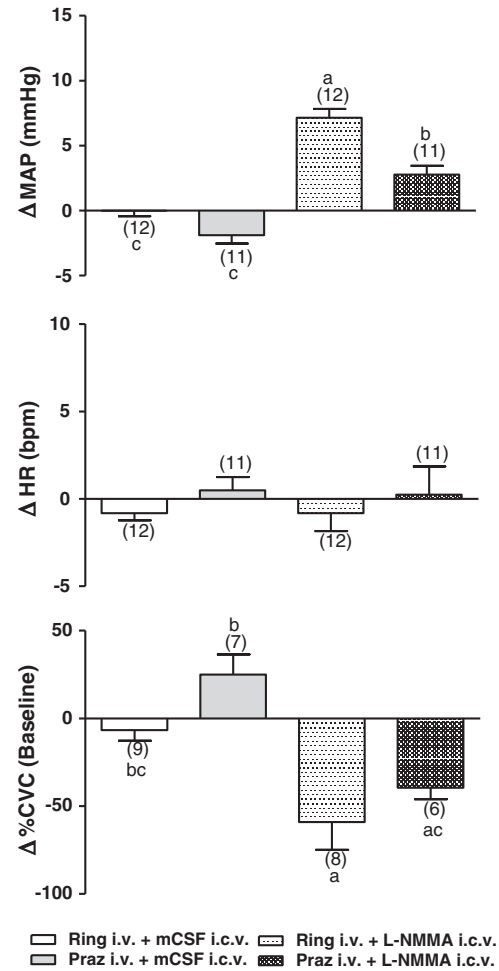
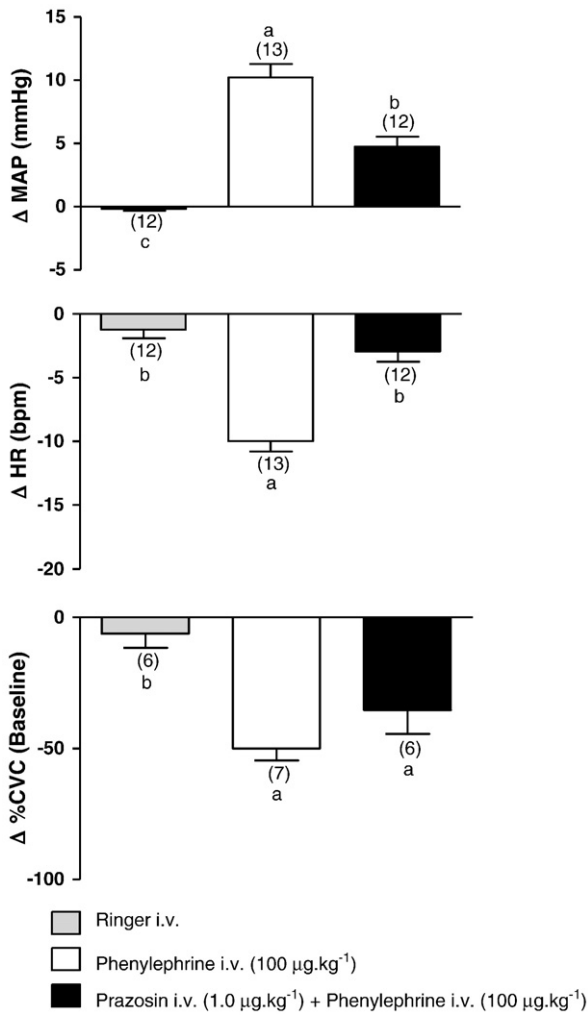Fig. 3. Changes in mean arterial blood pressure (ΔMAP), heart rate (ΔHR) and cutaneous vascular conductance (Δ%CVC) after intracerebroventricular (i.c.v.) injection of L-NMMA (500 μg per animal) or mock cerebrospinal fluid (mCSF; 1 μL), preceded by intravenous (i.v.) injection of prazosin (Praz; 1.0 mg kg^{-1}) or Ringer (Ring). CVC is expressed as % of baseline (%CVC baseline). Data are expressed as means ± s.e.m. Values indicated by different letters are significantly different from each other by Tukey's test (P < 0.05). Number of the animals is between parentheses.

(urogenital arteries) and insertion of the catheter into that vessel proved to be unfeasible, as we could not isolate it. In anurans, catheterization of the dorsal aorta would increase the chance of organ damage in this region, compromising important organs such as the urinary bladder, which is a physiological reservoir of water in these animals (Shoemaker and Nagy, 1977). Thus, the aortic arch was chosen for catheter insertion, as it was easily accessible when the surgical incision was performed laterally.

The implantation of a telemetry transmitter for PAP measurements proved to be a valuable method for this kind of long experimental protocol used in the present study, thereby reducing the number of animals used and avoiding the likely occlusion of the femoral arterial catheter after extended use. This is particularly important when using amphibians collected from their natural environment. Thus, monitoring blood pressure for long periods can be a very reliable method for assessing the influence of seasonality, feeding, infection or even pharmacological manipulations on cardiovascular function in the same animal, excluding possible influences from handling and anesthesia.

Comparing the present study with those that used a catheter in the femoral artery, we can notice that MAP and HR values of our bullfrogs at 24 °C (Table 1) are within the range previously reported at temperatures from 20 to 25 °C (Herman and Mata, 1985; Rocha and Branco, 1998; Bicego-Nahas and Branco, 1999; Taylor et al., 2012).

Fig. 4. Changes in mean arterial blood pressure (ΔMAP), heart rate (ΔHR) and cutaneous vascular conductance (Δ%CVC) after intravenous (i.v.) injection of phenylephrine preceded by Ringer (vehicle) or prazosin. CVC is expressed as % of baseline (%CVC baseline). Data are expressed as means ± s.e.m. Values indicated by different letters are significantly different from each other by Tukey's test (P < 0.05). Number of the animals is between parentheses.

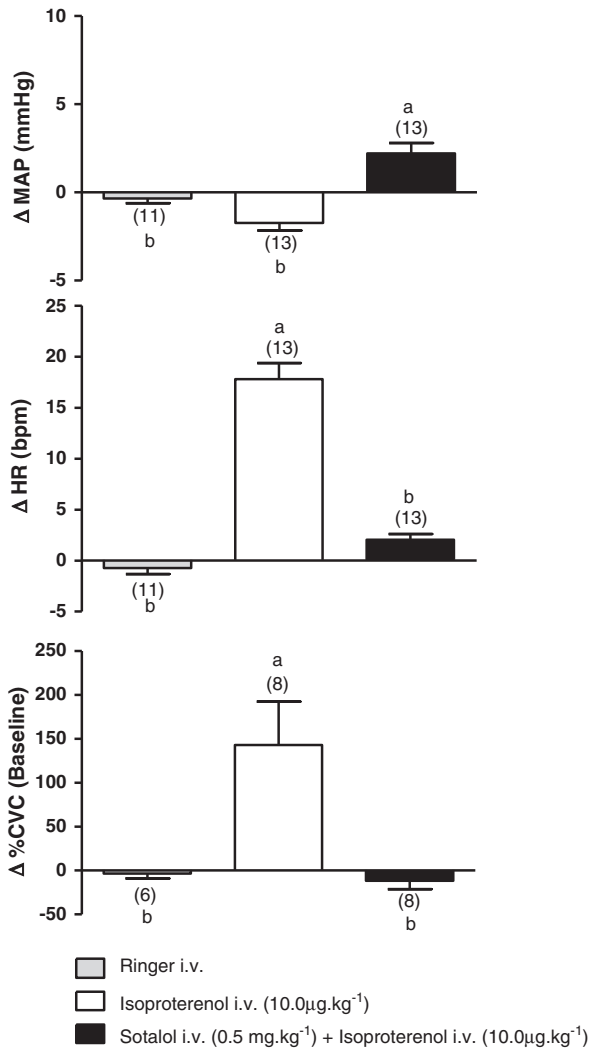### 4.2. Effect of the i.c.v. injection of L-NMMA on cardiovascular function in bullfrogs

There are many studies reporting increases in MAP, sympathetic activity and plasma levels of catecholamines in response to brain NOS inhibition in mammals (Matsumura et al., 1998; Shankar et al., 1998). While a clear hypertensive response is observed following the inhibition of NO production in the mammalian brain, HR responses remain unclear as some data indicate tachycardia, while others indicate bradycardia, associated with NO inhibition (cf. Krukoff, 1999). These different results seem to be related to the dose of the NOS inhibitor and/or the site of action in the central nervous system (CNS) (cf. Krukoff, 1999). NO can modulate preganglionic parasympathetic neurons in the nucleus ambiguus and in the dorsal vagal motor nucleus, acting on HR control (Hornby and Abrahams, 2000; Fletcher et al., 2006; cf. Zanzinger, 1999). The absence of a bradycardic reflex response to the pressor effect caused by L-NMMA in the present study (Fig. 3) may suggest an influence of NO on baroreflex sensitivity in bullfrogs, interfering with the parasympathetic cardiac limb. There is evidence in mammals of the involvement of NO in the increase (Qadri et al., 1999; Souza et al., 2001; Carvalho et al., 2006; Fletcher et al., 2006; Souza et al., 2009), as well as in the decrease (Liu et al., 1996; Murakami et al., 1998), of baroreflex sensitivity.
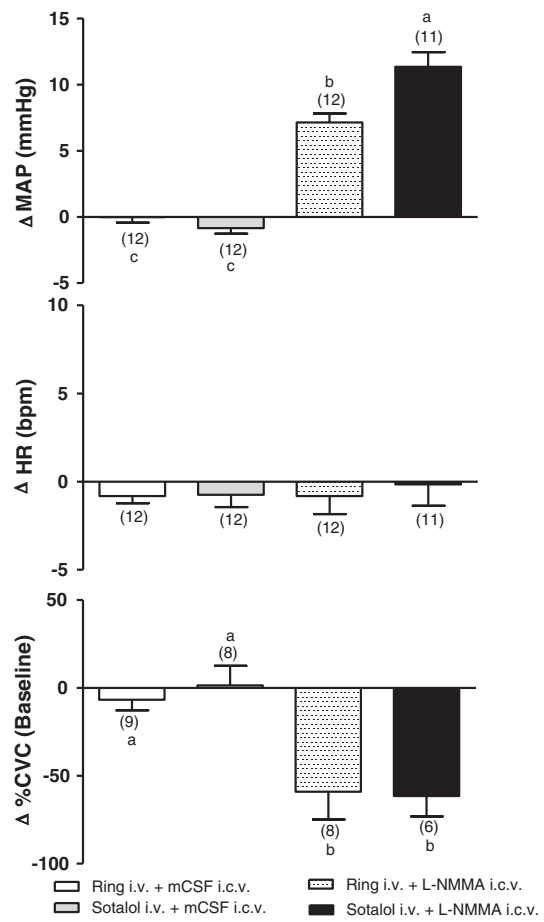
Fig. 5. Effect of intracerebroventricular (i.c.v.) injection of L-NMMA (500 μg per animal) or mock cerebrospinal fluid (mCSF), preceded by intravenous (i.v.) injection of sotalol (0.5 mg kg⁻¹) or Ringer (Ring) on mean arterial blood pressure (ΔMAP), heart rate (ΔHR) and cutaneous vascular conductance (Δ%CVC). CVC is expressed as % of baseline (Δ%CVC baseline). Data are expressed as means ± s.e.m. Values indicated by different letters are significantly different from each other by Tukey's test (P < 0.05). Data for "Ring i.v. + mCSF i.c.v." and "Ring i.v. + L-NMMA i.c.v." are the same plotted in Fig. 2. Number of the animals is between parentheses.

In the present study it is not possible to specify the exact site where NO acts to affect blood pressure, since i.c.v. injections of L-NMMA affected a large portion of the brain in frogs (all ventricles became blue after i.c.v. injection of 1 μL of Evans blue). At least in mammals, there are several important regions of the CNS related to cardiovascular regulation by NO, such as: i) the hypothalamus, especially the paraventricular (PVN) and supraoptic nuclei (Mizukawa et al., 1989; Yang et al., 1999); ii) the brainstem, where NO-producing neurons are found in the NTS and several subdivisions of the ventrolateral medulla, and iii) the spinal cord, which includes NO-producing sympathetic preganglionic neurons of intermediolateral cell column (Chen et al., 2001; cf. Krukoff, 1999). Regarding amphibians, there is evidence that the NTS is involved in blood pressure control in toads (Bianchi-da-Silva et al., 2000). Moreover, NO-producing neurons are found in hypothalamic and brainstem regions, including the NTS of some anuran species, such as *Rana perezi* (Munoz et al., 1996), *Xenopus laevis* (Bruning and Mayer, 1996; Munoz et al., 1996; Lazar and Losonczy, 1999), and *L. catesbeianus* (Huynh and Boyd, 2007). However, the role of NO at specific sites controlling MAP in amphibians is a matter of further investigation in our laboratory.

There is evidence to suggest that the reduction of brain perfusion induces a sympathetic hypertensive response in mammals (Paton et al., 2009), which could lead us to interpret that the MAP increase is caused by i.c.v. injection of L-NMMA (~1.5 mg kg⁻¹) in our bullfrogs

Fig. 7. Effect of intracerebroventricular (i.c.v.) injection of L-NMMA (500 μg per animal) or mock cerebrospinal fluid (mCSF), preceded by intravenous (i.v.) injection of hexametho-nium (Hex; 25.0 mg kg^{-1}) or Ringer's (Ring) on mean arterial blood pressure (ΔMAP) and heart rate (ΔHR). Data are expressed as means ± s.e.m. Values indicated by different letters are significantly different from each other by Tukey's test (P < 0.05). Number of the animals is between parentheses.

Fig. 6. Effect of intravenous (i.v.) injection of isoproterenol preceded by Ringer's (vehicle) or sotalol injection in mean arterial pressure (ΔMAP) and heart rate (ΔHR) and cutaneous vascular conductance (Δ%CVC). CVC is expressed as % of baseline. Data are expressed as means ± s.e.m. Values indicated by different letters are significantly different from each other by Tukey's test (P < 0.05). Number of the animals is between parentheses.

as an indirect consequence of an overall vasoconstriction in the brain. However, in contrast to mammals, the absence of an endothelial NO system is well documented in anurans, at least in large conductance blood vessels, such as systemic and pulmonary arteries, and mesenteric resistance arteries (Broughton and Donald, 2002; Donald and Broughton, 2005; Jennings and Donald, 2008, 2010; Trajanovska and Donald, 2011). Recently, Trajanovska and Donald (2011) showed that the eNOS gene is expressed in several non-vascular tissues, but not in the vascular endothelium of the frog, *Xenopus tropicalis*. The authors concluded that eNOS in adult amphibians has no role as an endothelial regulator of the vasculature. In fact, NO control of systemic vessel resistance in toads is provided by nitrergic nerves (Broughton and Donald, 2002; Donald and Broughton, 2005; Jennings and Donald, 2008, 2010). Furthermore, it was documented that peripheral injection of 50 mg kg^{-1} of L-NAME in bullfrogs (Rea et al., 2002) increases skin permeability without any change in vascular resistance or blood pressure.

Microinjection of L-NMMA into the lateral ventricle of bullfrogs occasionally induced an exploratory behavior in these animals. Behavioral responses may indirectly cause baseline cardiovascular alterations. In fact, activation of the grooming reflex was also demonstrated to occur in rats after intra-PVN injection of L-NAME (Cruz and Machado, 2009),
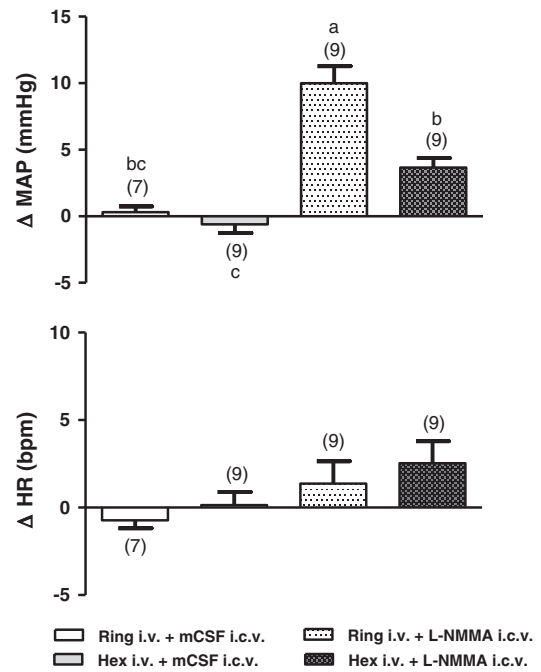
which in this case appeared not to be related to the pressor effect of L-NAME, since Zhang et al. (1997) and Zhang and Patel (1998) showed that an increase in MAP and HR occurs even in anesthetized animals. In our frogs, the MAP increase caused by L-NMMA injection was always observed before behavioral changes, excluding an indirect influence on cardiovascular modulation by brain NO.

4.3. Role of peripheral α1- and β-adrenergic receptors on i.c.v. L-NMMA-induced cardiovascular effects in bullfrogs

According to our results, L-NMMA in the CNS of bullfrogs increases MAP via α1-adrenoreceptor activation by adrenaline and/or circulating noradrenaline. This result suggests the existence of an inhibitory tonus of brain NO on sympathetic activity on the cardiovascular system that is dependent on α1-adrenoreceptors, corroborating previous studies in mammals (cf. Krukoff, 1999). The α1-antagonist, prazosin, inhibited the hypertensive effects of both L-NMMA (Figs. 2B and 3) and phenyl-ephrine (Fig. 4) in frogs, which confirms the vasoconstrictor action of α1 receptors in these animals.

In contrast to the results with the α-adrenoceptors, the blockade of the β-adrenergic receptors with sotalol accentuated the hypertensive effect of L-NMMA in the CNS of bullfrogs. Therefore, in these animals, L-NMMA seems to activate the sympathetic nervous system (SNS) to release adrenaline/noradrenaline that acts on both α- (Fig. 3) and β- (Fig. 5) adrenoceptors to cause vasoconstriction and vasodilation, respectively.

Neither prazosin nor sotalol significantly influenced the reduction in CVC of ventral pelvic skin microvasculature induced by L-NMMA (Figs. 3 and 5), indicating the involvement of α- and β-adrenoceptors of other vascular beds in these responses. In contrast, sotalol completely blocked the increased CVC induced by isoproterenol (Fig. 6). This result indicates that β-adrenoreceptors in the microvasculature of ventral pelvic skin may be important for water uptake, not only in more terrestrial anurans, such as the toads, *Bufo woodhouseii*, *Bufo punctatus* and *Bufo bufo*

(Viborg and Rosenkilde, 2004; Viborg and Hillyard, 2005), but also in bullfrogs (present study), a more aquatic species.

The participation of the SNS in the L-NMMA effect was confirmed by the ganglionic blockade with hexamethonium (Fig. 7). However, one cannot rule out possible humoral influences, such as the renin–angiotensin system or the non-mammalian vasopressin analogue, arginine vasotocin (AVT; Acharjee et al., 2004). At least in mammals, there is evidence that the pressor effect of i.c.v. L-NAME is mediated partly by the SNS, and partly by the renin–angiotensin system (Moriguchi et al., 1998). Moreover, brain NO can influence MAP by inhibiting vasopressin production, thereby maintaining the baseline MAP (Liu et al., 1998). In anurans, the renin–angiotensin system is known to play an important role in responses to hypotension, which may be related to hemorrhage or dehydration conditions (West et al., 1998). Regarding AVT, there is high vasotocinergic fiber density (Van Vossel-Daeninck et al., 1981; Mathieson, 1996) and a dense accumulation of mRNA for AVT receptors in the CNS, more specifically in hypothalamic areas (Kohno et al., 2003; Acharjee et al., 2004; Hasunuma et al., 2007). The association of brain NO with renin–angiotensin system and/or AVT remains to be elucidated in bullfrogs.

In summary, our data indicate that, NO acting on the CNS of bullfrogs seems to be important for controlling blood pressure via inhibition, at least in part, of the sympathetic activity dependent on α- and β-adrenergic receptors in systemic vessels.

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