NMDA AND NON-NMDA GLUTAMATE RECEPTORS IN THE PARAVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS MODULATE DIFFERENT STAGES OF HEMORRHAGE-EVOKED CARDIOVASCULAR RESPONSES IN RATS

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Abstract—Here we report the involvement of *N*-Methyl-D-Aspartate (NMDA) and non-NMDA glutamate receptors from the paraventricular nucleus of the hypothalamus (PVN) in the mediation of cardiovascular changes observed during hemorrhage and post-bleeding periods. In addition, the present study provides further evidence of the involvement of circulating vasopressin and cardiac sympathetic activity in cardiovascular responses to hemorrhage. Systemic treatment with the V_1-vasopressin receptor antagonist dTyr $(CH_2)_5(Me)AVP$ (50 µg/kg, i.v.) increased the latency to the onset of hypotension during hemorrhage and slowed post-bleeding recovery of blood pressure. Systemic treatment with the β_1-adrenergic receptor antagonist atenolol (1 mg/kg, i.v.) also increased the latency to the onset of hypotension during hemorrhage. Moreover, atenolol reversed the hemorrhage-induced tachycardia into bradycardia. Bilateral microinjection of the selective NMDA glutamate receptor antagonist LY235959 (2 nmol/100 nL) into the PVN blocked the hypotensive response to hemorrhage and reduced the tachycardia during the post-hemorrhage period. Systemic treatment with dTyr$(CH_2)_5(Me)AVP$ inhibited the effect of LY235959 on hemorrhage-induced hypotension, without affecting the post-bleeding tachycardia. PVN treatment with the selective non-NMDA receptor antagonist NBQX (2 nmol/100 nL) reduced the recovery of blood pressure to normal levels in the post-bleeding phase and reduced hemorrhage-induced tachycardia. Combined blockade of both NMDA and non-NMDA glutamate receptors in the PVN completely abolished the hypotensive response in the hemorrhage period and reduced the tachycardiac response in the post-hemorrhage period. These results indicate that local PVN glutamate neurotransmission is involved in the neural pathway mediating cardiovascular responses to hemorrhage, via an integrated control involving autonomic nervous system activity and vasopressin release into the circulation. © 2016 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: cardiovascular system, glutamate neurotransmission, hemorrhagic shock, paraventricular nucleus of hypothalamus, sympathetic activity, vasopressin.

INTRODUCTION

Hemorrhagic shock is a serious complication that may occur as a result of trauma, surgery, gastrointestinal disease, and anticoagulant therapy (Levi et al., 2002; Siqueira and Schmidt, 2003; Gutierrez et al., 2004). Tissue perfusion is reduced during hemorrhagic shock, due to a loss of circulating blood volume (Garrioch, 2004), which can lead to death. Indeed, hemorrhage is implicated in millions of deaths worldwide (Levi et al., 2002; Siqueira and Schmidt, 2003; Gutierrez et al., 2004), and is the leading cause of trauma-associated deaths (Bellamy, 1984; Abjean, 1986; Moore et al., 1996; Cuschieri et al., 2012; Malinoski et al., 2012). Early emergency care and treatment for severe trauma are extremely important, because about 40% of trauma-induced deaths occur 5–30 min after trauma (Cherkas, 2011; Liu et al., 2013).

Blood loss triggers a complex set of neural and hormonal responses intended to preserve blood flow to vital organs and to reduce tissue energy consumption (Garrioch, 2004). These responses are triggered by arterial baroreceptors and atrial volume receptors, which transmit information about changes in blood volume and pressure to the nucleus tractus solitarius (NTS) (Loewy, 1990; Jaworski and Blair, 2004). These sensory signals are transmitted to supramedullary structures through ascending projections that originate in the NTS and ventrolateral regions of the medulla (Loewy, 1990). However, the central organization of neural networks responsible for the nervous and hormonal regulation of blood pressure during hemorrhage remains unclear.

The paraventricular nucleus of the hypothalamus (PVN) is comprised of magnocellular neurosecretory

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Abbreviations: ACF, artificial cerebrospinal fluid; ACTH, adrenocorticotropin; AVP, vasopressin; CMM, caudal midline medulla; HR, heart rate; MAP, mean arterial pressure; NMDA, *N*-Methyl-D-Aspartate; NTS, nucleus tractus solitarius; PAP, pulsatile arterial pressure; PVN, paraventricular nucleus of the hypothalamus; RVLM, rostral ventrolateral medulla; vlPAG, ventrolateral column of the periaqueductal gray.

neurons as well as preautonomic and neuroendocrine parvocellular neurons (Swanson and Kuypers, 1980). The preautonomic parvocellular region of the PVN contains neurons projecting directly to the intermediolateral cell column of the thoracolumbar spinal cord and the rostral ventrolateral medulla (RVLM), which constitute important regions involved in the regulation and generation of sympathetic activity (Kuypers and Maisky, 1975; Shafton et al., 1998; Pyner et al., 2001). Magnocellular neurons synthesize the neurohypophysial hormones oxytocin and vasopressin, which are axonally transported down to the neurohypophysis and subsequently secreted into the bloodstream (Bisset and Chowdrey, 1988). Labor and milk ejection involve an oxytocin release, whereas vasopressin is released in response to decreased blood pressure or blood volume and increased plasma osmolality (Cunningham and Sawchenko, 1991; Renaud and Bourque, 1991; Cunningham et al., 2002, 2004).

It has been reported that hemorrhage causes a massive activation of both magnocellular neurosecretory and parvocellular neurons in the PVN (Roberts et al., 1993; Li and Dampney, 1994; Petrov et al., 1995; Badoer, 1996; Badoer and Merolli, 1998; Krukoff, 1999). Accordingly, previous studies reported an involvement of the PVN in the elevation of circulating corticosterone and adrenocorticotropin (ACTH) induced by hemorrhagic stimuli (Darlington et al., 1988; Blair et al., 1998). However, the possible role of this hypothalamic nucleus in the control of cardiovascular function during hemorrhage is poorly understood (Darlington et al., 1988; Blair et al., 1998).

We have previously reported that glutamate neurotransmission is an important local signaling mechanism in the PVN, being involved in the regulation of cardiovascular function (Busnardo et al., 2009, 2013). We observed that PVN stimulation with L-glutamate (L-glu) caused pressor and tachycardiac responses that were mediated by activation of N-Methyl-D-Aspartate (NMDA) glutamate receptors and subsequent sympathetic stimulation. When NMDA receptors were blocked, the microinjection of L-glu into the PVN caused pressor and bradycardiac responses that were mediated by activation of local non-NMDA glutamate receptors with a vasopressin release into the circulation (Busnardo et al., 2009). Therefore, the control exerted by PVN glutamate neurotransmission on the cardiovascular system is mediated by both neural (sympathetic) and humoral (vasopressin) factors. Nevertheless, to the best of our knowledge, the involvement of PVN glutamate neurotransmission in the control of cardiovascular responses evoked by hemorrhagic stimuli has never been evaluated. Thus, our hypothesis is that glutamate neurotransmission in the PVN modulates the cardiovascular system during hemorrhage by integrating sympathetic and vasopressin mechanisms.

EXPERIMENTAL PROCEDURES

Subjects

Experimental procedures were carried out following protocols approved by the Ethical Review Committee of the School of Medicine of Ribeirão Preto (Protocol number 075/2015), which comply with requirements established by the National Institutes of Health (NIH). Male Wistar rats weighing approximately 250 g were used in the present experiment. Animals were housed in plastic cages in a temperature-controlled room (25 °C) in the Animal Care Unit of the Department of Pharmacology, School of Medicine of Ribeirão Preto. Animals were kept under a 12:12-h light–dark cycle (lights on between 06:00 and 18:00 h). Animals had free access to water and standard laboratory food, except during the experimental period.

Surgical preparation

To implant guide cannulas bilaterally in the PVN, five days before the trial, the animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and their heads fixed to a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). The skull was surgically exposed and trepanned with a dental drill at 1.9 mm from the medial line and 7.2 mm anterior to the interaural line (Paxinos and Watson, 1986). Bilateral stainless steel guide cannulas (24G, 13 mm-long) were lowered 8 mm from the skull, at a 12° angle to both sides. Guide cannulas were positioned 1 mm above the intended stimulation sites, and fixed to the skull with a metal screw and dental cement. After surgery, the animals received a poly-antibiotic formulation with streptomycins and penicillins to prevent infection (80.000 UI, i.m.) and the nonsteroidal anti-inflammatory flunixine meglumine for post operation analgesia (2.5 mg/kg, s.c.).

Twenty-four hours before the experiment, animals were anesthetized with tribromoethanol (250 mg/kg, i.p.) and polyethylene catheters were implanted into the right femoral artery for cardiovascular recording and into the left femoral artery for blood withdrawal (hemorrhage) and drug injection when it was necessary. The catheters were exposed on the dorsum of the animals and attached to the skin, allowing cardiovascular recording and blood withdrawal of unanesthetized rats in their own cage. Flunixine meglumine (2.5 mg/kg s.c.) was used for post operation analgesia.

Cardiovascular recording

The catheter was connected to a pressure transducer and pulsatile arterial pressure (PAP) was recorded using a HP-7754A pre-amplifier (Hewlett Packard, Palo Alto, CA, USA) and an acquisition board (MP100A, Biopac Systems Inc., Goleta, Santa Barbara, CA, USA) connected to a personal computer. Mean arterial pressure (MAP) and heart rate (HR) values were derived from PAP recordings using the Acknowledge III software (Biopac, USA). MAP was calculated according to the equation: diastolic pressure + (systolic − diastolic)/3. HR (bpm) was calculated from PAP peak intervals integrated every 6 s.

Hemorrhage

All animals underwent a fixed volume hemorrhage of 24 mL/kg (estimated as 30% of total blood volume) over

a period of 20 min (1.2 mL/min/kg). This rate evokes clear periods of compensation, decompensation, and recompensation (Troy et al., 2003). Blood was withdrawn via the left femoral artery using a withdrawal pump (K.D. Scientific, Holliston, MA, USA). MAP and HR were recorded for a further 40-min period after the completion of hemorrhage.

Drugs and solutions

LY235959 ([3S-(3a,4aa,6b,8aa)]-Decahydro-6-(phospho nomethyl)-3-is oquinolinecarboxylic acid) (TOCRIS, Westwoods Business, Park Ellisville, MO, USA) and NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]qui noxaline-7-sulfonamide) (TOCRIS) were dissolved in artificial cerebrospinal fluid (ACF), which had the following composition: NaCl 100 mM, Na_3PO_4 2 mM, KCl 2.5 mM, $MgCl_2$ 1.0 mM, $NaHCO_3$ 27 mM, $CaCl_2$ 2.5 mM, pH 7.4. DTyr$(CH_2)_5$(Me)AVP (Peninsula, Belmont, CA, USA), atenolol (SIGMA, St. Louis, MO, USA), tribomoethanol (SIGMA) and urethane (SIGMA) were dissolved in saline (NaCl 0.9%). Flunixine meglumine (Banamine®, Schering Plough, RJ, Brazil) and the poly-antibiotic preparation of streptomycins and penicillins (Pentabiotico®, Fort Dodge, SP, Brazil) were used as provided.

Drug microinjection into the PVN

The needles (33 gauge; Small Parts, Miami Lakes, FL, USA) used for microinjection of drugs into the PVN were 1 mm longer than the guide cannulas. The injection needles were connected to a 1-μL syringe (7001KH; Hamilton, Reno, NV, USA) through PE-10 tubing and bolus injections of 100 nL were made into the PVN (Busnardo et al., 2007, 2009; Crestani et al., 2009). Microinjections were performed within a 5-s period. After microinjection, the needle was left within the guide cannula for 1 min before being removed. Drugs were prepared before the experiments and stored at −20 °C. On the day of the experiment, the drugs were thawed and kept at 0 °C during experiments.

Experimental procedure: hemorrhage

Rats were transported to the experiment room in their own cages and were allowed at least 60 min to adapt to the experimental room conditions, such as sound and illumination, before starting the experiments. Cardiovascular recording began at least 30 min before hemorrhage onset and was continuously performed during the 20-min period of hemorrhage up to 40 min after the completion of hemorrhage. All pharmacological treatments (see below) were performed 10 min before hemorrhage onset. Experiments were performed during the morning period in order to minimize possible circadian rhythm interferences.

Effect of systemic treatment with dTyr$(CH_2)_5$(Me)AVP or atenolol on the cardiovascular responses to hemorrhage in unanesthetized rats. This protocol aimed to investigate the role of circulating vasopressin and cardiac sympathetic activity in cardiovascular responses to hemorrhage in unanesthetized rats. For this, an independent set of animals was treated intravenously with either saline (control, 1 mL/kg), the selective V_1-vasopressinergic receptor antagonist dTyr$(CH_2)_5$(Me) AVP (50 μg/mL/kg) (Busnardo et al., 2009), or the selective β_1-adrenoceptor antagonist atenolol (1 mg/mL/kg) (Dos Reis et al., 2014).

Effect of PVN treatment with LY235959, NBQX, or the combination of LY235959 + NBQX on the cardiovascular responses to hemorrhage in unanesthetized rats. This protocol aimed to investigate the involvement of glutamate neurotransmission in the PVN in cardiovascular changes induced by hemorrhage. For this, animals were divided into four experimental groups: (1) ACF, in which the vehicle ACF (100 nL) was microinjected bilaterally into the PVN; (2) NBQX, in which the selective non-NMDA glutamate receptor antagonist NBQX (2 nmol/100 nL) was microinjected bilaterally into the PVN; (3) LY235959, in which the selective NMDA glutamate receptor antagonist (2 nmol/100 nL) was microinjected bilaterally into the PVN; and (4) LY235959 + NBQX, in which a combined microinjection of LY235959 (2 nmol/100 nL) and NBQX (2 nmol/100 nL) was made bilaterally into the PVN (Busnardo et al., 2009, 2013).

Effect of PVN treatment with LY235959 in animals treated systemically with dTyr$(CH_2)_5$(Me)AVP on cardiovascular responses to hemorrhage in unanesthetized rats. This protocol aimed to investigate the involvement of circulating vasopressin in the modulation of cardiovascular responses to hemorrhage involving NMDA glutamate receptors in the PVN. For this, animals were subjected to combined treatment with dTyr$(CH_2)_5$(Me)AVP (50 μg/mL/kg) into the left femoral artery and LY235959 (2 nmol/100 nL) bilaterally into the PVN.

Histological determination of the microinjection sites

At the end of experiments, animals were anesthetized with urethane (1.25 g/kg, i.p.) and 100 nL of 1% Evan's blue dye was injected into the brain as a marker of the injection site. They were then submitted to intracardiac perfusion with 0.9% NaCl followed by 10% formalin. Brains were removed and postfixed for 48 h at 4 °C. Then, serial 40-μm-thick sections were cut with a cryostat (CM1900, Leica, Wetzlar, Germany). Sections were treated with propylene glycol and stained with 4% cresyl violet for light microscopy analysis. The actual placement of the microinjection needles was determined by analyzing serial sections and identified according to the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1986). Data from animals for which microinjection sites were located in regions surrounding the PVN were excluded from the study.

Statistics

Data were expressed as mean ± SEM. Basal values of MAP and HR before and after pharmacological treatments were compared using the Student's paired t-test. The time-course curves of MAP and HR changes were analyzed using a two-way ANOVA, with treatment as the main independent factor and time as the repeated measurement. When interaction between factors was observed, groups were compared using a one-way ANOVA followed by Bonferroni's post-test. $P < 0.05$ was assumed to be statistically significant.

RESULTS

Effect of systemic treatment with dTyr(CH2)5(Me)AVP or atenolol on the cardiovascular responses to hemorrhage in unanesthetized rats

DTyr(CH$_2$)$_5$(Me)AVP. Systemic treatment with the selective V$_1$-vasopressinergic receptor antagonist dTyr(CH$_2$)$_5$(Me)AVP (50 µg/kg, i.v., $n = 5$) did not affect baseline values of either MAP (102 ± 0.7 vs. 102 ± 0.9 mmHg, $t = 1.8$, $P = 0.1$) or HR (348 ± 4 vs. 356 ± 3 bpm, $t = 1.7$, $P = 0.1$). Analysis of the time-course curves of MAP and HR changes evoked by hemorrhage indicated a significant effect of treatment with dTyr(CH2)5(Me)AVP (ΔMAP: $F_{(1,136)} = 46$, $P < 0.0001$; ΔHR:

$F_{(1,136)} = 15$, $P = 0.0002$) and an effect over time (ΔMAP: $F_{(16,136)} = 16$, $P < 0.0001$; ΔHR: $F_{(16,136)} = 17$, $P < 0.0001$), as well as an interaction between treatment and time only for MAP response (ΔMAP: $F_{(16,136)} = 14$, $P < 0.0001$; ΔHR: $F_{(16,136)} = 0.8$, $P = 0.7$) (Fig. 1). *Post hoc* analysis revealed that dTyr(CH$_2$)$_5$(Me)AVP increased the latency to the onset of hypotension during hemorrhage and slowed the post-bleeding recovery of MAP ($P < 0.05$). Moreover, treatment with the V$_1$-vasopressinergic receptor antagonist facilitated the tachycardiac response during hemorrhage ($P < 0.05$) (Fig. 1).

Atenolol. Systemic treatment with the selective β$_1$-adrenoceptor antagonist atenolol (1 mg/kg, i.v., $n = 5$) did not affect baseline values of either MAP (95 ± 0.8 vs. 97 ± 1 mmHg, $t = 1.4$, $P = 0.2$) or HR (352 ± 3 vs. 353 ± 3 bpm, $t = 0.8$, $P = 0.4$). Analysis of the time-course curves of cardiovascular responses to hemorrhage indicated a significant effect of atenolol (ΔMAP: $F_{(1,136)} = 15$, $P = 0.0002$; ΔHR: $F_{(1,136)} = 213$, $P < 0.0001$) and an effect over time (ΔMAP: $F_{(16,136)} = 10$, $P < 0.0001$; ΔHR: $F_{(16,136)} = 4.3$, $P < 0.0001$), as well as an interaction between treatment and time (ΔMAP: $F_{(16,136)} = 5$, $P < 0.0001$; ΔHR: $F_{(1,136)} = 8$, $P < 0.0001$) (Fig. 1). *Post-hoc* analysis revealed that atenolol increased the latency to the onset of hypotension during hemorrhage ($P < 0.05$)
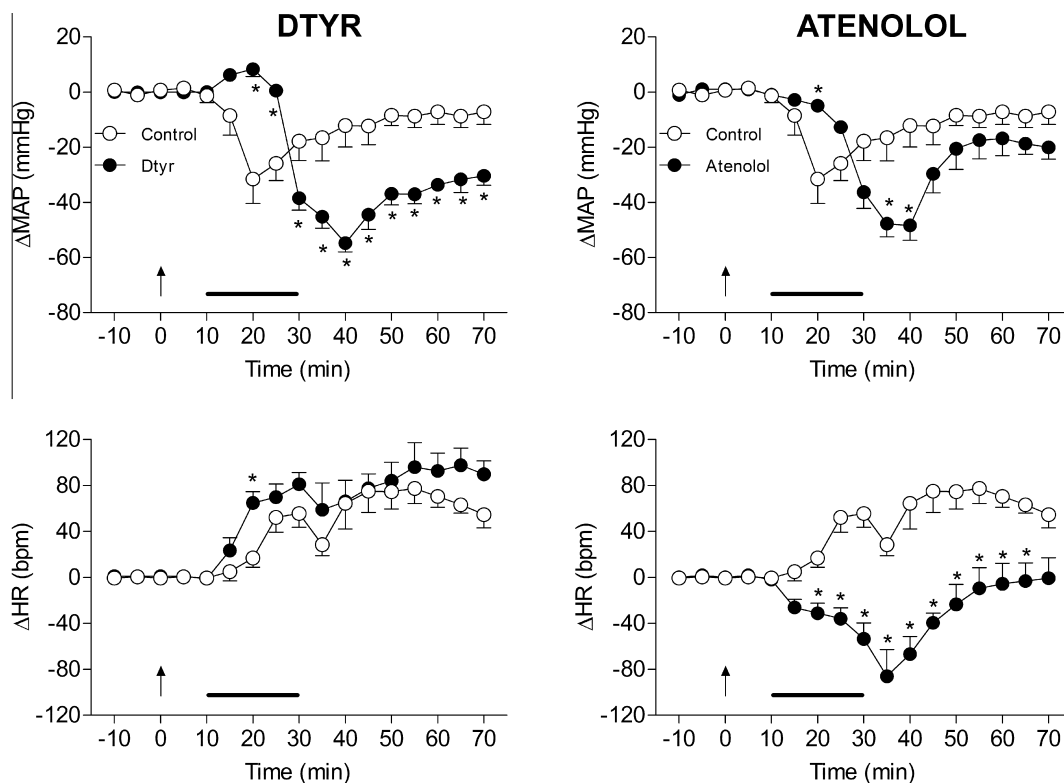

Fig. 1. Time-course curves of the effect of systemic treatment with saline (vehicle, $n = 5$), the selective V$_1$-vasopressinergic receptor antagonist dTyr(CH$_2$)$_5$(Me)AVP (50 µg/kg, $n = 5$) (left graphs), or the selective β$_1$-adrenoceptor antagonist atenolol (1 mg/kg, $n = 5$) (right graphs) in MAP and HR changes induced by hemorrhage. Drug injections were done at time 0, indicated by the arrow. Bleeding started at time 10 and finished at time 30; the black line represents the period of hemorrhage. Points represent the mean and bars the SEM. *, significantly different from control (vehicle) at the same timepoint (Bonferroni *post hoc* test, $P < 0.05$).

and reversed the hemorrhage-induced tachycardia into bradycardia ($P < 0.05$) (Fig. 1).

Effect of PVN treatment with LY235959, NBQX or the combination of LY235959 + NBQX on the cardiovascular responses to hemorrhage in unanesthetized rats

LY235959. Bilateral microinjection of the selective NMDA glutamate receptor antagonist LY23595 into the PVN (2 nmol/100 nL, $n = 5$) did not affect baseline values of either MAP (95 ± 0.3 vs. 95 ± 1 mmHg, $t = 0.3$, $P = 0.7$) or HR (372 ± 3.2 vs. 365 ± 1.4 bpm, $t = 2$, $P = 0.06$). Analysis of the time-course curves of cardiovascular changes evoked by hemorrhage indicated a significant effect of PVN treatment with LY235959 (ΔMAP: $F_{(1,136)} = 16$, $P < 0.0001$; ΔHR: $F_{(1,136)} = 73$, $P < 0.0001$) and an effect over time (ΔMAP: $F_{(16,136)} = 3$, $P = 0.0008$; ΔHR: $F_{(16,136)} = 10$, $P < 0.0001$), as well as an interaction between treatment and time (ΔMAP: $F_{(16,136)} = 3$, $P = 0.01$; ΔHR: $F_{(16,136)} = 6$, $P < 0.0001$) (Fig. 2). *Post hoc* analysis revealed that LY235959 abolished the hemorrhage-induced hypotension ($P < 0.05$) and reduced the tachycardiac response during the post-hemorrhage period ($P < 0.05$) (Fig. 2).

NBQX. Bilateral microinjection of the selective non-NMDA glutamate receptor antagonist NBQX (2 nmol/100 nL, $n = 5$) into the PVN did not affect baseline values of MAP (92 ± 0.7 vs. 93 ± 0.5 mmHg, $t = 1.3$, $P = 0.2$) and HR (377 ± 2.8 vs. 379 ± 1.3 bpm, $t = 0.5$, $P = 0.6$). Analysis of the time-course curves for hemorrhage-evoked cardiovascular changes indicated a significant effect of PVN treatment with NBQX (ΔMAP: $F_{(1,136)} = 21$, $P < 0.0001$; ΔHR: $F_{(1,136)} = 14$, $P = 0.0003$) and an effect over time (ΔMAP: $F_{(16,136)} = 17$, $P < 0.0001$; ΔHR: $F_{(16,136)} = 29$, $P < 0.0001$), as well as an interaction between treatment and time (ΔMAP: $F_{(16,136)} = 2.4$, $P = 0.003$; ΔHR: $F_{(16,136)} = 2.4$, $P = 0.004$) (Fig. 2). *Post-hoc* analysis revealed that NBQX reduced the MAP recovery in the post-bleeding phase ($P < 0.05$) and decreased the tachycardia during both hemorrhagic ($P < 0.05$) and post-hemorrhagic periods ($P < 0.05$) (Fig. 2).

LY235959 + NBQX. Combined treatment of the PVN with LY235959 (2 nmol/100 nL) + NBQX (2 nmol/100 nL) ($n = 5$) did not affect baseline values of either MAP (104 ± 0.4 vs. 105 ± 0.7 mmHg, $t = 0.6$, $P = 0.6$) or HR (337 ± 5 vs. 349 ± 3 bpm, $t = 1.6$, $P = 0.1$). Analysis of the time-course curves for cardiovascular responses to hemorrhage indicated a significant effect of treatment (ΔMAP: $F_{(1,136)} = 57$, $P < 0.0001$; ΔHR: $F_{(1,136)} = 6$, $P = 0.01$) and an effect over time (ΔMAP: $F_{(16,136)} = 4.2$, $P < 0.0001$; ΔHR: $F_{(16,136)} = 10.2$, $P < 0.0001$), as well as an interaction between treatment and time (ΔMAP: $F_{(16,136)} = 5.6$, $P < 0.0001$; ΔHR: $F_{(16,136)} = 3.7$, $P < 0.0001$) (Fig. 2). *Post-hoc* analysis revealed that combined treatment of the PVN with LY235959 + NBQX completely abolished the
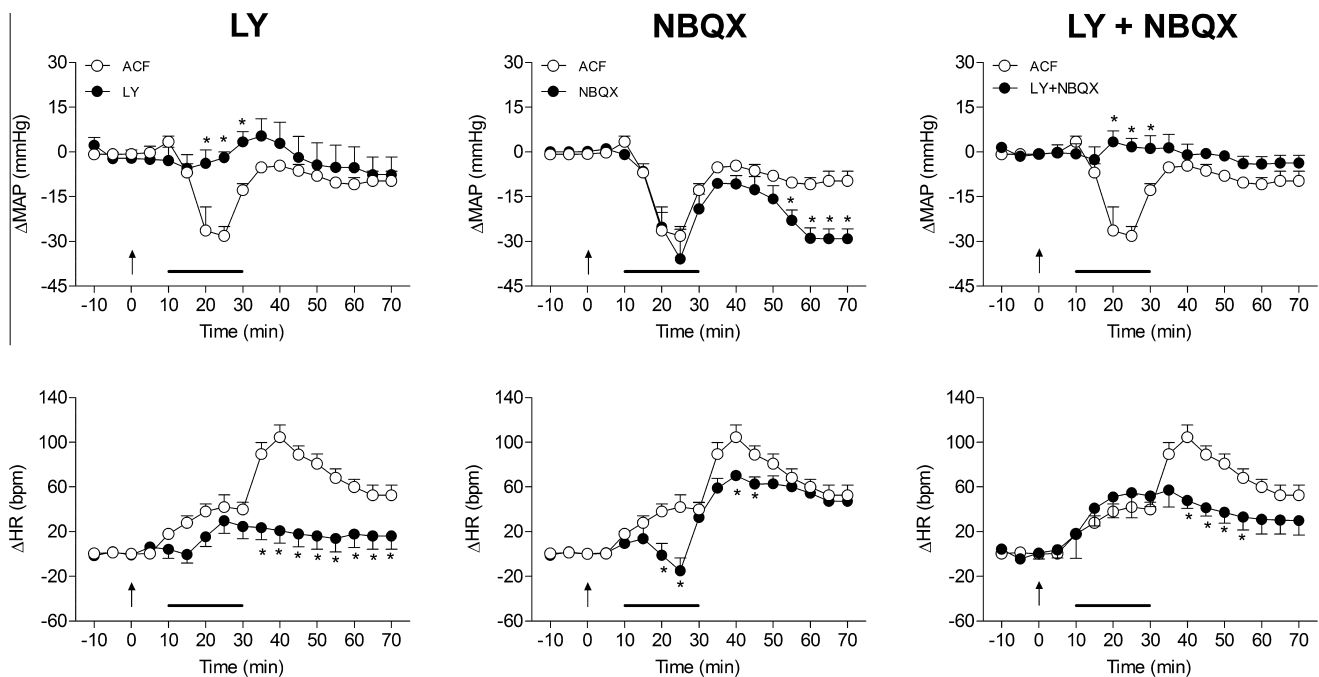

Fig. 2. Time-course curves of the effect of PVN treatment with vehicle (ACF, 100 nL, $n = 5$), the selective NMDA glutamate receptor antagonist LY235959 (2 nmol/100 nL, $n = 5$) (left graphs), the selective non-NMDA glutamate receptor antagonist NBQX (2 nmol/100 nL, $n = 5$) (middle graphs), or combined treatment with LY235959 + NBQX (2 nmol/100 nL/drug, $n = 5$) (right graphs) on MAP and HR changes induced by hemorrhage. Drug injections were done at time 0, indicated by the arrow. Bleeding started at time 10 and finished at time 30; the black line represents the period of hemorrhage. Points represent the mean and bars the SEM. *, significantly different from control (ACF) at the same timepoint (Bonferroni *post hoc* test, $P < 0.05$).

hemorrhage-induced hypotension ($P < 0.05$) and reduced the tachycardiac response in the post-hemorrhage period ($P < 0.05$) (Fig. 2).

Effect of PVN treatment with LY235959 in animals treated systemically with dTyr(CH2)5(Me)AVP on the cardiovascular responses to hemorrhage in unanesthetized rats

Bilateral microinjection of LY235959 into the PVN (2 nmol/100 nL) associated with systemic treatment with dTyr(CH2)5(Me)AVP (50 µg/kg) ($n = 5$) did not affect baseline values of MAP (97 ± 3 vs. 98 ± 2 mmHg, $t = 0.3$, $P = 0.8$) and HR (353 ± 5 vs. 345 ± 7 bpm, $t = 0.8$, $P = 0.4$). Analysis of the time-course curves for HR responses to hemorrhage indicated a significant effect of treatment ($F_{(1,136)} = 3.4$, $P = 0.06$) and an effect over time ($F_{(16,136)} = 16.7$, $P < 0.0001$), as well as an interaction between treatment and time ($F_{(16,136)} = 3.7$, $P = 0.003$) (Fig. 3). However, analysis of hemorrhage-evoked MAP changes did not indicate an effect of treatment ($F_{(1,136)} = 2.5$, $P = 0.1$) or an interaction ($F_{(1,136)} = 0.3$, $P = 1.0$) (Fig. 3). *Post hoc* analysis revealed that combined treatment with dTyr (CH$_2$)$_5$(Me)AVP systemically and LY235959 in the PVN reduced the tachycardiac response during the post-

hemorrhagic period ($P < 0.05$) without affecting the blood pressure response (Fig. 3).

Determination of microinjection sites in the PVN

A diagrammatic representation showing bilateral microinjection sites in the PVN of all animals used in the present study is presented in Fig. 4. Also, Fig. 4 presents a photomicrograph of a coronal brain section depicting bilateral microinjection sites in the PVN of one representative animal.

DISCUSSION

Results obtained in humans and animals have indicated that hemorrhage-induced cardiovascular responses occur in three progressive phases (Evans et al., 2001; Troy et al., 2003). In phase I, called compensatory, there is an increase in vasomotor and cardiac sympathetic
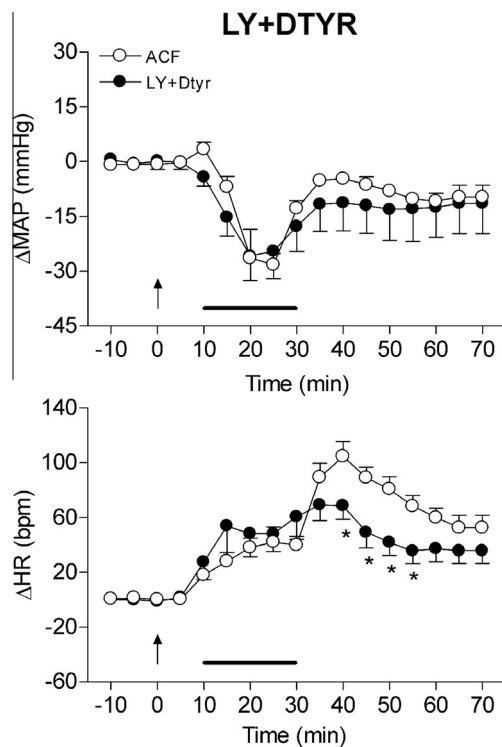

Fig. 3. Time-course curves of the effect of vehicle (ACF, 100 nL, $n = 5$) microinjected into the PVN (open circles) or the selective NMDA receptor antagonist LY235959 (2 nmol/100 nL) microinjected into the PVN associated with systemic treatment with the V$_1$-vasopressinergic receptor antagonist dTyr(CH$_2$)$_5$(Me)AVP (50 µg/kg) ($n = 5$) (solid circles) on MAP and HR changes induced by hemorrhage. Drug injections were done at time 0, indicated by the arrow. Bleeding started at time 10 and finished at time 30; the black line represents the period of hemorrhage. Points represent the mean and bars the SEM. *, significantly different from control (ACF) at the same timepoint (Bonferroni *post hoc* test, $P < 0.05$).
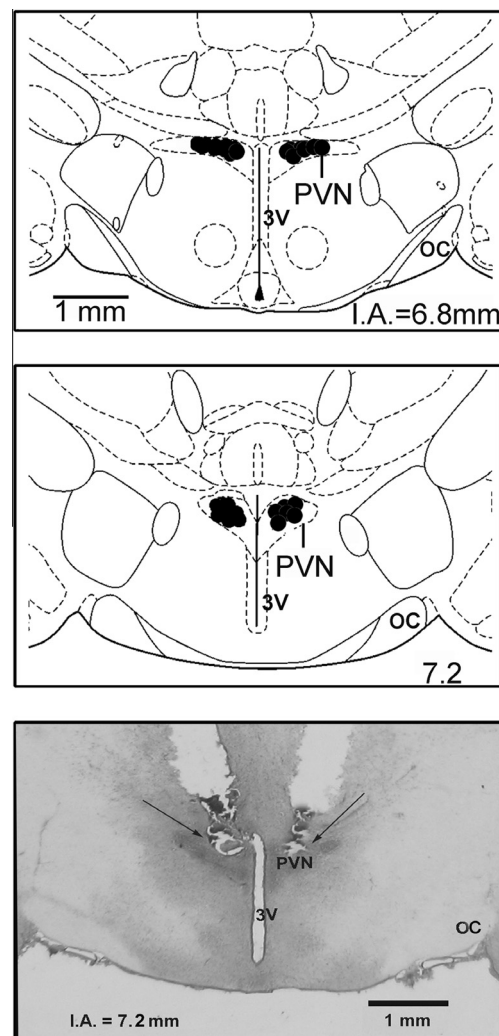

Fig. 4. Diagrammatic representation showing bilateral microinjection sites in the PVN of all animals used in the present study and a representative figure of a coronal section of a rat brain depicting the site of bilateral microinjection in the PVN. The center of the microinjection is indicated by arrows. IA – distance from interaural line, PVN – paraventricular nucleus of the hypothalamus; OC – chiasm optic tract; 3V – third ventricle.

drives and a decrease in cardiac parasympathetic activity, which leads to the maintenance of blood pressure within normal levels and to a HR increase (Victor et al., 1989; Schadt and Ludbrook, 1991; Evans et al., 1994; Thrasher and Shifflett, 2001; Troy et al., 2003). When bleeding reaches about a 30% decrease in total blood volume, phase II, called decompensatory, is established. This phase is characterized by rapid hypotension and variable responses in HR, which are mediated by a paradoxical cardiac parasympathetic stimulation and sympathetic nerve activity inhibition (Ludbrook et al., 1988; Victor et al., 1989; Schadt and Ludbrook, 1991; Scislo and O'Leary, 2006). Although during phase II an increase in renin-angiotensin-aldosterone activity (Michailov et al., 1987; Starc and Stalcup, 1987; Botelho et al., 1994) and circulating vasopressin occurs (Share, 1988; Shoji et al., 1993; Kakiya et al., 2000), these responses are not enough to sustain the blood pressure at normal levels resulting in an abrupt blood pressure fall. If the bleeding stops, phase III occurs, called recompensatory, when MAP and HR return to near basal levels (Schadt and Ludbrook, 1991; Troy et al., 2003) due to, at least in part, increased circulating levels of vasopressin and cardiac sympathetic activation (Kakiya et al., 2000; Troy et al., 2003).

Role of circulating vasopressin and peripheral β-adrenoceptors in the cardiovascular responses to hemorrhage

The present findings show a postponement of the hypotension during hemorrhage and a blunting of MAP post-bleeding recovery in animals treated with the V_1-vasopressinergic receptor antagonist dTyr(CH$_2$)$_5$(Me) AVP. These results are in line with previous results suggesting an important role for vasopressin in the recovery following hemorrhage (Zerbe et al., 1982; Chapman et al., 1986; Johnson et al., 1988; Fujisawa et al., 1994; Imai et al., 1996), which seems to be mediated by both central and peripheral actions of the peptide (Johnson et al., 1988). However, previous studies have indicated an involvement of vasopressin in the paradoxical sympathoinhibitory response during hemorrhagic shock (Peuler et al., 1990; Fujisawa et al., 1994; Budzikowski et al., 1996; Imai et al., 1996), thus supporting our results of postponement of the hypotension in animals treated with dTyr(CH$_2$)$_5$(Me)AVP. Indeed, vasopressin facilitates baroreflex-mediated inhibition of sympathetic activity (Share, 1988), which may contribute to the sympathoinhibitory action during hemorrhage. However, a role for central V_1-vasopressinergic receptors in eliciting hypotension during hemorrhage has also been suggested (Budzikowski et al., 1996). In fact, vasopressin acting on the brainstem inhibits sympathetic activity (Suzuki et al., 1989). In addition, a mechanism of central action for circulating vasopressin would be the activation of V_1-receptors in circumventricular organs lacking blood–brain barrier, such as the area postrema, which possesses vasopressinergic neurons and could activate a downstream pathway that in turn inhibits sympathetic outputs (Suzuki et al., 1989; Scislo et al., 2005; Scislo and O'Leary, 2006; Yang and Hwang, 2007).

Antagonism of peripheral $β_1$-adrenoceptors reversed the tachycardiac response into a bradycardia. The present findings corroborate previous studies indicating an involvement of cardiac sympathetic activity in hemorrhage-induced tachycardia (Hintze and Vatner, 1982), which is possibly mediated by an increase in catecholamine release from the adrenal medulla, since inhibition of sympathetic nerve activity has been reported during hemorrhagic shock (Ludbrook et al., 1988; Victor et al., 1989; Schadt and Ludbrook, 1991; Scislo and O'Leary, 2006; Frithiof et al., 2011). The bradycardia observed in atenolol-treated animals is in line with results indicating a coactivation of cardiac sympathetic and parasympathetic activity during hemorrhagic shock (Gonzalez Gonzalez et al., 1995; Porter et al., 2009).

Blockade of $β_1$-adrenoceptors also postponed the hypotension during hemorrhage and accentuated this response during the post-bleeding period. The enhanced hypotension is possibly related to a conversion of the tachycardiac response into bradycardia, thus implicating the HR response as an important compensatory response counteracting the arterial pressure decrease during critical periods of hemorrhagic shock. The reduction of hypotension during hemorrhage in atenolol-treated animals was unexpected. A possible explanation could be the blockade of vascular β-adrenoceptors, whose activation causes vascular smooth muscle relaxation. Nevertheless, further experiments are necessary to clarify this effect.

Involvement of PVN glutamate neurotransmission in cardiovascular responses to hemorrhage

Increase in immunoreactive Fos protein in vasopressinergic and sympathetic preautonomic neurons was observed in the PVN following bleeding (Badoer et al., 1993; Krukoff, 1993; Roberts et al., 1993; Petrov et al., 1995; Badoer and Merolli, 1998). Indeed, previous studies have reported that either lesion or knife cut deafferentation of the PVN reduced the neuroendocrine responses to hemorrhage; however, without affecting arterial pressure and HR responses (Darlington et al., 1988; Blair et al., 1998). Therefore, to the best of our knowledge, the present findings provide the first direct evidence of PVN involvement in hemorrhage-evoked cardiovascular responses. The absence of evidence in previous studies may be due to the use of lesions instead of a specific neurochemical blockade (i.e., glutamatergic), an approach used in the present experiment. Indeed, previous studies reported that although lesion or nonselective synaptic blockade of the PVN did not affect cardiovascular parameters (Darlington et al., 1988; Callahan et al., 1992; Busnardo et al., 2010; Crestani et al., 2010), specific blockade of local signaling mechanisms (e.g., nitrergic, GABAergic, and glutamatergic) induced arterial pressure and HR changes (Busnardo et al., 2010; Martins-Pinge et al., 2012). These results are supported by evidence for the existence of both excitatory and inhibitory inputs to the PVN, so that the control of cardiovascular function by this nucleus is only revealed after blockade of specific local neurochemical mechanisms.

PVN glutamate neurotransmission has been implicated in the control of autonomic, cardiovascular, and neuroendocrine activities (Curras-Collazo and Dao, 1999; Kawasaki et al., 2005; Busnardo et al., 2009, 2012, 2013; Crestani et al., 2010). To determine the role played by ionotropic glutamate receptors of the PVN in hemorrhage-induced cardiovascular responses, we pre-treated the PVN with selective NMDA and/or non-NMDA glutamate receptor antagonists. PVN treatment with the NMDA receptor antagonist LY235959 reduced the tachy-cardiac response in the post-hemorrhagic period. Together with the findings that pretreatment with a β_1-adrenoceptor antagonist abolished the hemorrhage-induced tachycardia, the LY235959 results provide evidence that PVN NMDA receptors contribute to cardiac sympathetic activation during the post-bleeding period. Indeed, previous results indicated a high expression of NMDA receptors throughout the PVN, including the preautonomic parvocellular neurons (Herman et al., 2000). Moreover, a wide range of studies has shown that activation of NMDA glutamate receptors in the PVN increases sympathetic nerve activity (Badoer, 1996; Chen et al., 2003; Li et al., 2008; Busnardo et al., 2009).

Conversely, blockade of PVN NMDA receptors inhibited the hypotension induced by hemorrhage, indicating that activation of these receptors in the PVN contributes to hemorrhagic shock. Two mechanisms could trigger this effect: firstly, the activation of NMDA glutamate receptors located in GABAergic neurons can increase local GABA release, which in turn activates $GABA_A$ receptors causing sympathoinhibition-mediated hypotension (Martin et al., 1991; Decavel and van den Pol, 1992; Boudaba et al., 1996; Zhang and Patel, 1998; Mathew and Hablitz, 2011); secondly, PVN NMDA glutamate receptors can modulate vasopressin release into the bloodstream during hemorrhagic shock (Busnardo et al., 2009, 2012). To test this hypothesis, we investigated the effect of intra-PVN treatment with LY235959 in animals intravenously pretreated with the V_1-vasopressinergic receptor antagonist dTyr(CH$_2$)$_5$(Me) AVP. Systemic treatment with dTyr(CH$_2$)$_5$(Me)AVP inhib-ited the LY235959-induced blockade of the hemorrhage-evoked hypotension, suggesting that the involvement of PVN NMDA receptors in hemorrhagic shock is dependent on a modulation of vasopressin release to the circulation. Previous results have indicated an inhibitory role of PVN NMDA receptors on vasopressin release into the blood-stream (Badoer, 1996; Busnardo et al., 2009, 2012), thus suggesting that inhibition of hemorrhagic shock following blockade of PVN NMDA receptors may be mediated by an increase of vasopressin release into the circulation. Excessive circulating vasopressin levels may allow the potent vasoconstrictor action of vasopressin to overcome the sympathoinhibitory effect, thus protecting against hemorrhagic shock. However, expression of NMDA receptors has been reported in PVN magnocellular neu-rosecretory neurons (Herman et al., 2000). Therefore, we cannot exclude the possibility that PVN NMDA recep-tors are implicated in vasopressin release during hemor-rhage, so that inhibition of hemorrhagic shock in animals

treated with LY235959 would be mediated by a reduction of vasopressin-mediated sympathoinhibition.

The ventrolateral column of the periaqueductal gray (vlPAG) in the midbrain has been demonstrated to play a key role in the decompensatory responses during hemorrhage (Cavun and Millington, 2001; Troy et al., 2003; Dean, 2004). Although there is evidence of activa-tion of downstream vasodepressor signals in the caudal midline medulla (CMM) (Vagg et al., 2008), vlPAG neu-rons also control vasopressin release into the circulation (Pelosi et al., 2008). Therefore, the vlPAG may be an important source of PVN glutamatergic inputs involved in decompensatory responses through the control of vasopressin release.

Treatment of the PVN with the selective non-NMDA glutamate receptor antagonist NBQX slowed the post-bleeding recovery of MAP. Our results obtained in animals treated systemically with a V_1-vasopressinergic receptor antagonist indicated an important role of vasopressin in recovery from hypotension associated with hemorrhage, thus suggesting that control of arterial pressure during the recompensatory phase exerted by PVN non-NMDA receptors may be mediated by activation of magnocellular neurosecretory neurons. Indeed, previous studies indicated the expression of non-NMDA receptors in vasopressin-containing magnocellular neurons (Herman et al., 2000). Accord-ingly, we have previously reported that control of vaso-pressin release from PVN magnocellular cells by local glutamatergic neurotransmission is mediated mainly by the activation of non-NMDA receptors (Busnardo et al., 2009, 2012). Furthermore, several studies have shown an involvement of PVN non-NMDA glutamate receptors in vasopressin-mediated cardiovascular responses (Scopinho et al., 2008; Busnardo et al., 2009; Crestani et al., 2009; Pelosi et al., 2015).

PVN treatment with NBQX also reversed the tachycardiac response during the hemorrhagic shock into bradycardia, and reduced the HR increase during the post-hemorrhagic period. Pharmacological and electrophysiological studies have demonstrated that activation of PVN non-NMDA glutamate receptors increases sympathetic nerve activity (van den Pol et al., 1990; Wuarin and Dudek, 1991; Herman et al., 2000; Chen et al., 2003; Li et al., 2008), which support the involvement of this receptor in the sympathetic-mediated tachycardiac response induced by hemorrhage. Coad-ministration of the NMDA receptor antagonist abolished the bradycardia during hemorrhagic shock observed in animals treated with the non-NMDA receptor antagonist, implicating local NMDA receptors in negative chronotropic response during hemorrhagic shock (Gonzalez Gonzalez et al., 1995; Porter et al., 2009). Furthermore, this finding reinforces the idea of an involvement of PVN NMDA receptors in the paradoxical sympathoinhibition during hemorrhagic shock. As discussed above, this action seems to be mediated by a modulation of vasopressin release to the circulation, which in turn inhibits sympa-thetic activity via central and peripheral actions (Share, 1988; Suzuki et al., 1989; Badoer, 1996; Budzikowski
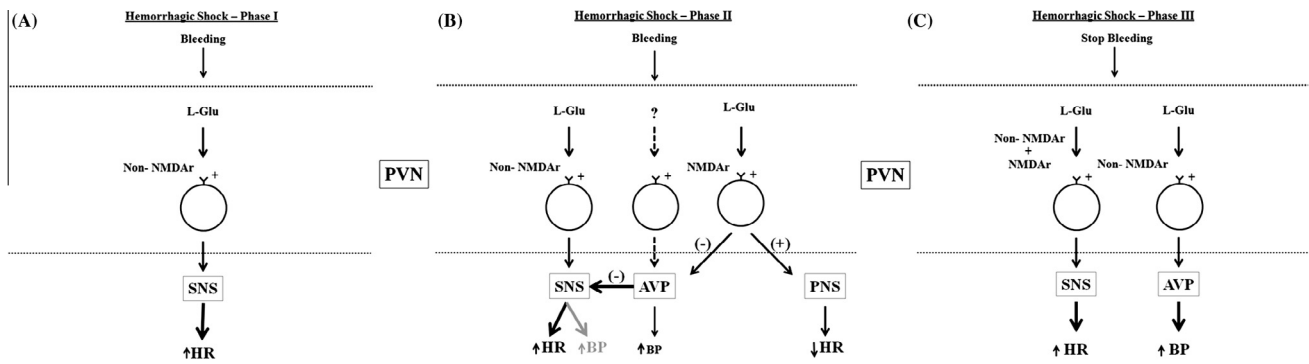
Fig. 5. Schematic representation illustrating the role of NMDA and non-NMDA glutamate receptors within the PVN controlling the three phases of hemorrhage-induced cardiovascular responses through modulation of vasopressin (AVP) release into the circulation and sympathetic (SNS) and parasympathetic (PNS) nervous system responses.

et al., 1996). However, previous results have also indicated that PVN glutamate neurotransmission facilitates cardiac parasympathetic activity (Crestani et al., 2010), suggesting that NMDA receptor-mediated negative chronotropic effect during hemorrhagic shock may be mediated by cardiac sympathetic inhibition and/or parasympathetic inhibition. However, the tachycardia during the post-bleeding period seems to be mediated by an activation of both NMDA and non-NMDA glutamate receptors in the PVN.

A schematic representation sketching the role of ionotropic glutamate receptors within the PVN in the three phases of hemorrhage-induced cardiovascular responses is presented in Fig. 5. Taken together, results reported in the present study indicate an involvement of PVN non-NMDA receptors in the sympathetic-mediated tachycardia during phase I and II, whereas both NMDA and non-NMDA receptors mediate this response during phase III. Furthermore, activation of NMDA receptors during phase II triggers decompensatory responses through a modulation of vasopressin release, whereas non-NMDA receptors are involved in vasopressin release during phase III.

CONCLUSION

In summary, the present findings confirm an involvement of circulating vasopressin in hemorrhagic shock, as well as in MAP recovery during the post-bleeding period. Cardiac sympathetic activity mediates hemorrhage-induced tachycardiac responses, which seem to be buffered by cardiac parasympathetic activation. Importantly, the present study provides the first evidence of a role of PVN glutamatergic neurotransmission in hemorrhage-induced cardiovascular changes. Our results indicate an involvement of NMDA receptors of the PVN in hemorrhagic shock, which is mediated by controlling vasopressin release to the circulation and a negative chronotropic effect. However, this receptor also seems to contribute to post-bleeding recovery, being involved in the tachycardiac response. Non-NMDA receptors in the PVN are involved in the tachycardiac response during hemorrhage and the post-

bleeding period. Moreover, this receptor is involved in the post-bleeding recovery of arterial pressure.

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