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ORIGINAL ARTICLE

Mechanism of the cardiovascular responses caused by L-proline microinjected into the supraoptic nucleus of the hypothalamus in unanesthetized rats

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Abstract In the present study, we report on the cardiovascular effects caused by the microinjection of L-proline (L-Pro) into the supraoptic nucleus (SON) in unanesthetized rats: the possible involvement of ionotropic glutamate receptors in the SON, as well as the peripheral mechanisms involved in the mediation of its cardiovascular effects. We compared the L-Pro effects with those caused by the injection of L-glutamate (L-Glu) into the SON. Microinjection of increasing doses of L-Pro into the SON caused dose-related cardiovascular responses in unanesthetized rats that were similar to those observed after the injection of L-Glu. Pretreatment of the SON with either a selective non-NMDA (NBQX) or a selective NMDA (LY235959) glutamate receptor antagonist blocked the cardiovascular response to L-Pro. The dose–effect curve for the pretreatment with increasing doses of LY235959 was shifted to the left in relation to the curve for NBQX, showing that LY235959 is more potent than NBQX in inhibiting the cardiovascular response to L-Pro. On the other hand, the cardiovascular response to L-Glu was only significantly reduced by pretreatment with NBQX (2 nmol/100 nL), but not affected by LY235959 (2 nmol/100 nL). The pressor response to L-Pro was not affected by intravenous pretreatment with the ganglion blocker pentolinium, but it was blocked by intravenous pretreatment with the V_1-vasopressin receptor antagonist dTyr$(CH_2)_5$(Me)AVP. In conclusion, these results suggest that L-Pro has a selective receptor that is sensitive to ionotropic glutamate receptor antagonists. Its activation in the SON results in vasopressin release into the systemic circulation, causing pressor and bradycardiac responses.

Keywords L-Proline · Supraoptic nucleus · NMDA and non-NMDA receptors · LY and NBQX antagonist · Vasopressin · Cardiovascular response

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Introduction

L-Proline (L-Pro) is a nonessential endogenous amino acid that can act as a neurotransmitter or neuromodulator in the central nervous system (CNS) of mammals (Snyder et al. 1973; Mulder and Snyder 1974; Felix and Kunzle 1976; Yoneda and Roberts 1982; Hauptmann et al. 1983; Fremeau et al. 1992; Renick et al. 1999).

L-Ornithine is the main source of L-Pro in the rat brain. The enzyme ornithine-aminotransferase is responsible for converting L-ornithine into L-glutamic-semi aldehyde that is spontaneously converted to L-Δ-pyrroline-5-carboxylic acid (P5C). The enzyme pyrroline-5-carboxylate reductase (P5CR) reverts P5C into L-Pro. L-Pro may regulate this pathway in the CNS via a negative feedback mechanism in which it selectively inhibits P5CR (Yoneda and Roberts 1982).

Studies using synaptosomes suggested the existence of a sodium-dependent uptake of L-Pro in nerve terminals, from which it can be released following depolarization caused by exposure to potassium ions in a calcium-dependent manner (Bennett et al. 1972; Snyder et al. 1973; Mulder and Snyder 1974). The uptake and accumulation of L-Pro were reported to occur in synaptosomes from the midbrain, striatum, hippocampus, hypothalamus, cortex, pons, medulla and cerebellum (Hauptmann et al. 1983). Furthermore, the transport of L-Pro has also been reported to occur in putative

glutamatergic neurons of the rat brain, suggesting a possible synaptic role for L-Pro in CNS excitatory pathways (Fremeau et al. 1992). Immunohistochemical studies revealed several groups of strongly labeled L-Pro-containing neurons in the arcuate nucleus, the supraoptic nucleus of the hypothalamus (SON), the area postrema and the brainstem (Takemoto and Semba 2006), which are structures known to modulate the cardiovascular system in rats.

Central effects of L-Pro on the cardiovascular system were first described by Takemoto (1990), who has reported pressor response after the microinjection of L-Pro into the cisterna magna of conscious rats. Such effect was reported to involve the activation of central ionotropic excitatory amino acid receptors as well as a vasopressin release into the systemic circulation (Takemoto 1995). In anesthetized animals, the microinjection of L-Pro into medullary areas induced a depressor response that was associated with a reduced vascular resistance in the hindquarters (Takemoto 2001, 2004), which was mediated by ionotropic glutamate receptors (Takemoto 2005). Recently, we have reported cardiovascular effects after the microinjection of L-Pro into supra medullary structures (Lopes-Azevedo et al. 2012). In that study, microinjection of L-Pro into the third ventricle (3 V) of unanesthetized rats evoked a blood pressure increase that was mediated by an acute release of vasopressin into the circulation. It was also proposed that such effect was mediated by structures located in or near the PVN, because similar effects were observed after the microinjection of L-Pro into the PVN, a structure in close proximity to the wall of the 3 V (Lopes-Azevedo et al. 2012). Despite evidence indicating a possible involvement of L-Pro in central cardiovascular control (Takemoto 1990, 2001, 2005), little is known about the role of this amino acid in the SON, a supra medullary structure that also modulates the cardiovascular system. The SON presents several groups of strongly labeled L-Pro-containing neurons (Takemoto and Semba 2006), and the microinjection of L-glutamate (L-Glu) into the SON was reported to cause cardiovascular responses via activation of ionotropic glutamate receptors and vasopressin release into the circulation (Busnardo et al. 2007).

The hypothesis of this study is that the microinjection of L-Pro into the SON causes cardiovascular responses in unanesthetized rats by the activation of excitatory amino acid ionotropic receptors, thus resulting in an acute release of vasopressin into the systemic circulation.

Materials and methods

Animal preparation

One hundred and twenty-eight male Wistar rats weighing between 250 and 270 g were used. Animals were kept in the Animal Care Unit of the Department of Pharmacology of the School of Medicine of Ribeirao Preto, University of São Paulo. Rats were housed in plastic cages in a 23–25 °C temperature-controlled room, with free access to water and commercial food. Housing conditions and experimental procedures were approved by the University of São Paulo Animal Ethical Committee, which complies with the Guiding Principles for Research Involving Animals and Human Beings of the American Physiological Society.

Rats were anesthetized with tribromoethanol (Aldrich Chemical Co. Inc., Milwaukee, USA), 250 mg/kg (i.p.). After local anesthesia with 2 % lidocaine, the skull was surgically exposed and a stainless steel guide cannula (0.6 mm o.d., 23G) was implanted 1 mm above the injection site, using a stereotaxic apparatus (Stoelting, Wood Dale, IL, USA). Stereotaxic coordinates for cerebral cannula implantation in the SON were selected from the brain atlas of Paxinos and Watson (2007): AP = +6.9 mm, $L = 1.8$ mm from the medial suture and $V = -7.8$ mm deep from the skull. Cannulae were fixed to the skull with dental cement and one metal screw. A tight-fitting mandrel was kept inside the guide cannula to avoid its occlusion. After the surgery, animals received 100,000 U of a polyantibiotic comprised streptomycins and penicillins (Pentabiotico®, Fontoura-Wyeth, SP, Brazil, 80,000 UI) intramuscularly (i.m.) to prevent infection and 2.5 mg/kg of the non-steroidal anti-inflammatory flunixin meglumine (Banamine®, Schering Plough, Brazil), subcutaneous (s.c.) for postoperative analgesia.

Three to five days later, animals were anesthetized with tribromoethanol and a polyethylene catheter was implanted into the femoral artery for blood pressure recording. The arterial catheter consisted of a piece of PE-10 tubing (4–5 cm) heat-bonded to a longer piece of PE-50 tubing (Clay Adams, Parsippany, NJ, USA) (12–14 cm). The catheter was filled with heparin (15 U/mL) in sterile saline (150 mM NaCl). The PE-10 segment was introduced into the femoral artery until its tip reached the aorta. The catheter was secured in the position with threading, and the PE-50 segment was passed under the skin to be extruded on the dorsum of the animal. Animals were allowed to recover for 24 h. When the i.v. route was used for drug injection, another catheter was simultaneously inserted into the femoral vein. The anti-inflammatory flunixin meglumine (2.5 mg/kg, s.c.) was used for post-operative analgesia.

Measurement of cardiovascular responses

After the surgery, animals were kept in individual cages in the Animal Care Unit, which were transported to the experimental room. Animals were allowed 1 h to adapt to the conditions of the experimental room, such as sound and illumination, before starting blood pressure and heart rate

recordings. The experimental room was acoustically isolated and had constant background noise produced by an air exhauster. Pulsatile arterial pressure (PAP) of freely moving animals was recorded using an amplifier (model 7754A, Hewlett Packard, Palo Alto, CA, USA) coupled to a computerized acquisition system (MP100, Biopac, Systems Inc.,Goleta Santa Barbara, CA, USA). The mean arterial pressure (MAP) and heart rate (HR) were derived from PAP data using the Acknowledge III software (Biopac, USA). The MAP was calculated according to the equation: diastolic pressure + (systolic − diastolic)/3. The HR (beats/min, bpm) was calculated from PAP peak intervals integrated every 6 s. After connecting the intra-arterial cannula to the transducer, the pulsatile arterial pressure was acquired for an initial period of at least 15 min, as baseline recording, before the microinjections were performed. MAP and HR were considered stable when recorded values were around, respectively, 100 mmHg and 365 bpm. The injection needle was then slowly introduced into the guide cannula, without touching or restraining the animals. Control MAP and HR values were calculated as the average of the 5-min period recorded just before each injection.

Drug injections

Drugs were dissolved in artificial cerebrospinal fluid (ACF). Injections into the SON were performed as bolus in a final volume of 100 nL. Drug was injected over a 5-s period using a 1 μL syringe (KH7001, Hamilton, Reno, NV, USA) that was connected to a 33G (Small Parts, Miami Lakes, FL, USA) microinjection needle, through a piece of PE-10. After a 30-s period, the needle was removed. The microinjection needle was 1 mm longer than the guide cannula. Each rat received either 2 or 3 injections during the experiment, with a minimum 20 min interval between them.

Drugs

The following drugs were used: L-proline and L-glutamic acid monosodium salt (Sigma, St. Louis, MO, USA), dTyr$(CH_2)_5$(Me)AVP (Península, Belmont, CA, USA), [3S-(3a,4aa,6b,8aa)]-Decahydro-6-(phosphonomethyl)-3-isoquinolinecarboxylic acid (LY 235959) and 2,3-Dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) (TOCRIS, Westwoods Business Park, Ellisville, MO, USA), pentolinium tartrate (ACROS Organics, Morris Plains, NJ, USA), tribromoethanol (Aldrich Chemical Co. Inc., Milwaukee, USA) and urethane (Sigma, St. Louis, MO, USA), Vehicle—artificial cerebrospinal fluid (ACF) had the following composition: 100 mM NaCl; 2 mM Na_3PO_4; 2.5 mM KCl; 1 mM $MgCl_2$; 27 mM $NaHCO_3$; 2.5 mM $CaCl_2$ and pH 7.4.

Experimental protocol

Animals were divided into the following experimental groups: (1) ACF group, receiving vehicle microinjection into the SON; (2) L-Pro group, which received 3, 30, 56, 100, 300 or 900 nmol/100 nL of L-Pro microinjected into the SON. Each rat received either 2 or 3 microinjections during the experiment, with a minimum 20-min interval between them. The injections were performed randomly, and no tachyphylaxis was observed; therefore, L-proline injections could be repeated at short-time intervals; (3) L-Glu group, receiving 10 nmol/100 nL of L-Glu microinjected into the SON; (4) ACF, NBQX or LY/L-Pro group, which received microinjections of L-Pro (56 nmol/100 nL) into SON at least 20 min before and again 15 min after the local pretreatment with ACF (100 nL), NBQX (0.5, 1.0, and 2.0 nmol/100 nL) or LY235959 (0.0005, 0.005, 0.05, 0.5 and 2.0 nmol/100 nL); 5) ACF, NBQX or LY/L-Glu group, which received microinjections of L-Glu (10 nmol/100 nL) into the SON at least 20 min before and again 15 min after the local pretreatment with ACF (100 nL), NBQX (2 nmol/100 nL) or LY235959 (2 nmol/100 nL); (6) Pentolinium/L-Pro group, receiving L-Pro microinjections into the SON at least 20 min before and again 15 min after the pretreatment with the ganglion blocker pentolinium (5 mg/kg, i.v.); and (7) dTyr/L-Pro group, receiving L-Pro microinjections into the SON at least 20 min before and again 15 min after the pretreatment with the V1-vasopressin receptor antagonist dTyr(CH2)5(Me)AVP (50 μg/kg, i.v.). The doses of the drugs used were selected based on the previous reports in the literature (Scopinho et al. 2006; Busnardo et al. 2007).

Histological procedure

At the end of the experiments, rats were anesthetized with urethane (1.25 g/kg i.p.) and 100 nL of filtered 1 % Evan's blue dye was injected into the SON as a marker of the injection site. The chest was surgically opened; the descending aorta occluded; the right atrium severed and the brain perfused with 10 % formalin through the left ventricle. Brains were post-fixed for 48 h at 4 °C, and later 40-μm sections were cut using a cryostat (CM1900, Leica, Wetzlar, Germany). Brain sections were stained with 0.5 % cresyl violet. Injection sites were verified in serial sections using the rat brain atlas of Paxinos and Watson (2007) as reference.

Statistical analysis

Nonlinear regression analysis was used to compare mean arterial pressure (MAP) and heart rate (HR) results of the different doses of L-Pro administered into the SON, and the

dose–response data were fitted to a sigmoid equation. The maximal MAP and HR responses were compared using the paired Student's t test (before treatment vs. after treatment). Two-way ANOVA (treatment vs. time) was used to compare the effects of the microinjection of L-Pro or L-Glu on the ΔMAP and ΔHR, before and after treatments, applying Bonferroni's correction for multiple comparisons when using the t test. Although PAP was recorded throughout the experimental procedure, curves for statistical analysis or illustrative figures were generated with points obtained with different data sampling. For statistical purposes, curves were generated with sampling at 0.2, 0.25 and 0.3 min to generate 4, 5 and 6 points, respectively. Illustrative curves were generated with sampling at 1/min for more accurate representation. Values of $P < 0.05$ were taken as shown with statistically significant differences between the groups. Percentage inhibition of L-Pro responses by SON pretreatment with either LY 235959 or NBQX antagonists was analyzed utilizing nonlinear regression analysis. Data are represented as mean ± standard error of the mean (SEM). We used the Prism software (GraphPad, USA) to perform the statistical analysis. $P < 0.05$ was assumed to be statistically significant.

Results

Cardiovascular responses to the microinjection of L-Pro or L-Glu into the SON of unanesthetized rats (dose–response curve)

The microinjection of ACF 100 nL into the SON did not affect the MAP (before: MAP = 95.5 ± 3.2 mmHg, after: MAP = 96.0 ± 3.0 mmHg, $t = 0.54$, $P > 0.05$, $n = 6$) and HR baseline (before: HR = 353.1 ± 10.9 bpm, after: HR = 355.0 ± 11.5 bpm, $n = 6$, $t = 2.3$, $P > 0.05$).

ACF vs. L-Pro

Injections of 3, 30, 56, 100, 300, 900 nmol/100 nL of L-Pro into the SON caused dose-related pressor ($R^2 = 0.88$) and bradycardiac ($R^2 = 0.79$) responses (Fig. 1). A dose–response curve was generated using a group of 16 rats that received up to three injections each, applied randomly at minimum 20-min intervals. No tachyphylaxis was observed after microinjections of L-Pro into the SON. The dose of 56 nmol/100 nL of L-Pro was used in all experimental protocols.

The administration of L-Pro in areas adjacent to the SON did not affect the MAP (before: MAP = 95.7 ± 2.0, after: MAP = 95.7 ± 2.2; $t = 0.06$, $n = 19$) and HR baseline (before: HR = 363.2 ± 7.1, after: HR = 361.3 ± 8.2; $t = 0.78$, $n = 19$) of unanesthetized rats. A diagrammatic representation showing the distribution of microinjection sites throughout the SON is presented in Fig. 2a. Filled circles indicate injection sites in the SON. Open circles indicate injection sites immediately outside the SON. Photomicrography of a coronal section of a rat brain depicting the site of microinjection in the SON is shown in Fig. 2b.

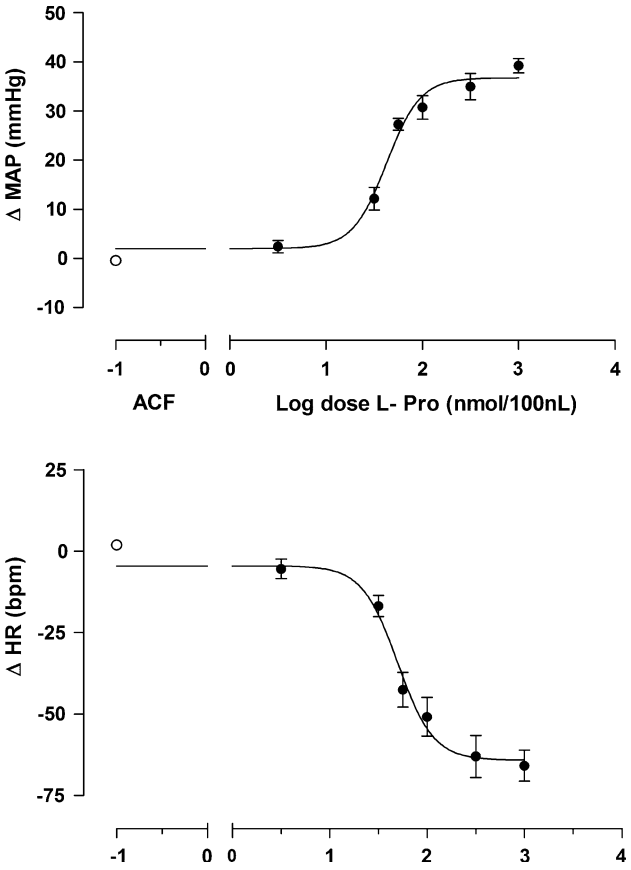

Fig. 1 Dose–effect curves for L-proline (L-Pro, *filled circles*) and artificial cerebrospinal fluid (ACF ($n = 6$), *open circles*) microinjected into the SON of unanesthetized rats on the mean arterial pressure (ΔMAP) and heart rate (ΔHR). Doses of L-Pro were, respectively, 3 ($n = 6$), 30 ($n = 6$), 56 ($n = 6$), 100 ($n = 6$), 300 ($n = 6$) and 900 ($n = 6$) nmol/100 nL. Circles represent means and bars the SEM. Dose–effect curves were generated by nonlinear regression analysis and were statistically significant (ΔMAP, $R^2 = 0.88$ and ΔHR, $R^2 = 0.79$)

The microinjection of L-Pro (56 nmol/100 nL) into the SON of normotensive rats (MAP = 95.9 ± 5.1 mmHg; HR = 360.5 ± 11.8 bpm, $n = 6$) caused a significant pressor response (ΔMAP = 27.3 ± 1.2 mmHg, $t = 22.9$, $P < 0.0001$, $n = 6$) and HR decrease (ΔHR = −42.6 ± 5.3 bpm, $t = 8.4$, $P < 0.001$, $n = 6$). On the other hand, the microinjection of ACF (100 nL) into the SON caused no significant changes in blood pressure (ΔMAP = −0.4 ± 0.8 mmHg, $n = 6$) or heart rate (ΔHR = 1.9 ± 0.8 bpm, $n = 6$). There were effects of L-Pro on the MAP and HR (MAP: $F_{1,60} = 183.3$; HR: $F_{1,60} = 34.94$):

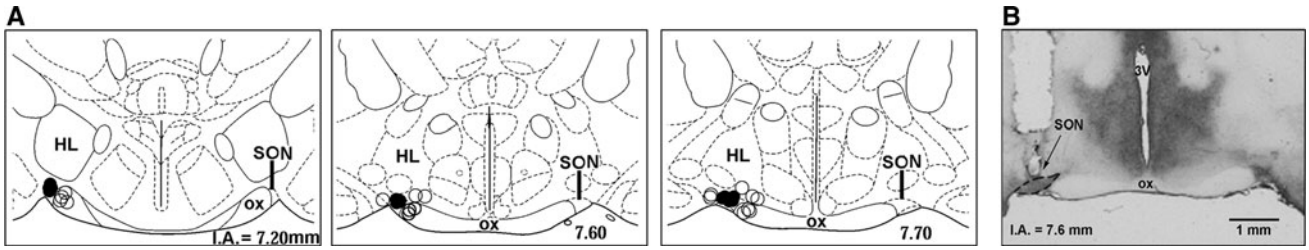

Fig. 2 **a** A diagrammatic representation based on the rat brain atlas of Paxinos and Watson (2007) showing the dispersion of injection sites among experiments. *Filled circles* indicate injection sites in the SON. *Open circles* indicate injection sites immediately outside the SON. **b** A photomicrography of a coronal section of a rat brain depicting a site of microinjection in the SON. The center of the microinjection is indicated by the *arrow*. *IA* interaural, *SON* supraoptic nucleus, *OX* optic chiasm, *3 V* third ventricle

a significant effect over time (MAP: $F_{5,60} = 21.91$, HR: $F_{5,60} = 9.50$) and an interaction between treatment and time (MAP: $F_{5,60} = 20.56$; HR: $F_{5,60} = 6.89$) (Fig. 3a). The latencies to the onset and duration of response were 17.1 ± 1.6 s and 12.6 ± 0.7 min, respectively.

ACF vs. L-Glu

The microinjection of L-Glu (10.0 nmol/100 nL) into the SON of normotensive rats (MAP = 96.0 ± 3.6 mmHg; HR = 369.8 ± 6.2 bpm, $n = 6$) caused a significant pressor response (ΔMAP = 26.0 ± 1.6 mmHg, $t = 14.1$, $P < 0.0001$, $n = 6$) and HR decrease (ΔHR = −50.3 ± 5.0 bpm, $t = 8.6$, $P < 0.001$, $n = 6$). On the other hand, the microinjection of ACF (100 nL) into the SON caused no significant changes in blood pressure (ΔMAP = −0.4 ± 0.9 mmHg, $n = 6$) or heart rate (ΔHR = −0.2 ± 1.0 bpm, $n = 6$). There were effects of L-Glu on the MAP and HR (MAP: $F_{1,60} = 258.0$; HR: $F_{1,60} = 124.0$): a significant effect over time (MAP: $F_{5,60} = 37.21$; HR: $F_{5,60} = 16.84$) and an interaction between treatment and time (MAP: $F_{5,60} = 36.45$; HR: $F_{5,60} = 14.93$) (Fig. 3b).

Effect of local pretreatment with ACF or NBQX on cardiovascular response to L-Pro or L-Glu microinjection into the SON of unanesthetized rats

ACF/L-Pro

Pretreatment of the SON with 100 nL of ACF ($n = 5$) did not affect MAP (before: 89.0 ± 0.7 mmHg; after: 91.0 ± 3.0 mmHg, $t = 0.65$, $P > 0.05$) or HR baseline (before: 351.8 ± 3.3 bpm; after: 350.4 ± 3.1 bpm, $t = 0.86$, $P > 0.05$). Pretreatment with ACF did not affect the

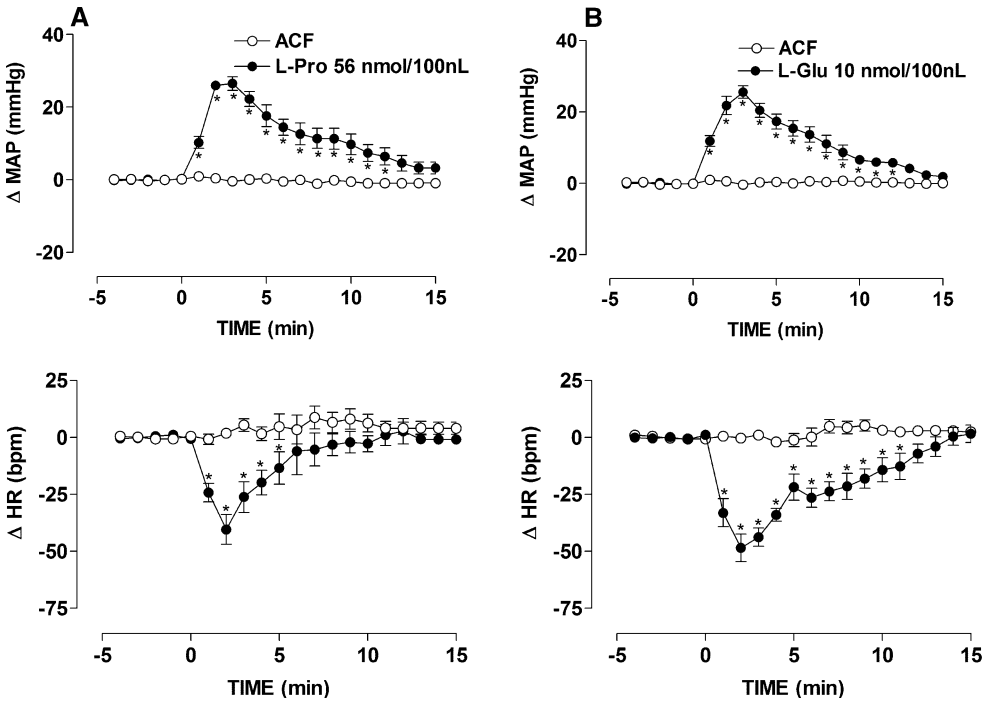

Fig. 3 **a** Time course of the effect of microinjection of 56 nmol/100 nL of L-Pro (*filled circles*) or 100 nL of ACF (*open circles*) into the SON on ΔMAP and ΔHR. L-Pro injections were made at time 0. *Circles* represent the mean and *bars* the SEM, $n = 6$, $^*P < 0.001$. **b** Time course of the effect of 10 nmol/100 nL L-Glu (*filled circles*) or 100 nL ACF (*open circles*) microinjected into the SON on ΔMAP and ΔHR. L-Glu injections were made at time 0. The *circles* represent the mean and *bars* the SEM, $n = 6$, $^*P < 0.001$

pressor and bradycardiac responses to the microinjection of 56 nmol/100 nL L-Pro into the SON (ΔMAP before ACF = 31.8 ± 2.8 mmHg and after = 30.1 ± 2.0 mmHg, $t = 1.8$, $P > 0.05$; ΔHR before ACF: −51.5 ± 5.5; after: −50.4 ± 6.0 bpm, $t = 0.2$, $P > 0.05$, $n = 5$).

NBQX/L-Pro

Microinjection of different doses of the selective non-NMDA glutamate receptor antagonist -NBQX into the SON did not affect basal MAP (MAP before NBQX: 96.5 ± 2.5 mmHg; after: 97.4 ± 2.6 mmHg; $P > 0.05$, $n = 14$) or HR (HR before NBQX: 364.8 ± 3.8 bpm; after: 368.2 ± 4.6 bpm, $P > 0.05$; $n = 14$). Local pretreatment with NBQX [0.5 ($n = 4$), 1.0 ($n = 4$) or 2.0 ($n = 6$) nmol/100 nL] caused a dose-related blockade of pressor ($R^2 = 0.98$) and bradycardiac ($R^2 = 0.97$) responses to L-Pro microinjection into the SON (Fig. 4). The higher dose of NBQX inhibited the pressor (−91.2 ± 3.5 %) and bradycardiac (−88.1 ± 1.1 %) responses to microinjected L-Pro.

Microinjection of 2 nmol NBQX into the SON did not affect baseline MAP (before: MAP = 104.6 ± 3.2 mmHg; after: MAP = 106.4 ± 2.8 mmHg, $t = 0.76$, $P > 0.05$, $n = 6$) or HR (before: HR = 374.6 ± 7.2 bpm; after: HR = 374.3 ± 5.8 bpm, $t = 0.57$, $P > 0.05$, $n = 6$). Pretreatment with NBQX blocked the pressor (before: ΔMAP = 27.8 ± 4.6 mmHg; after: ΔMAP = 3.9 ± 2.0 mmHg, $t = 4.9$, $P < 0.01$, $n = 6$) and bradycardiac (before: ΔHR = −41.1 ± 3.5 bpm; after: ΔHR = −2.3 ± 4.2 bpm, $t = 5.2$, $P < 0.01$, $n = 6$) responses to L-Pro microinjection into the SON. However, pretreatment with ACF did not affect the pressor (before: ΔPAM = 31.1 ± 3.0 mmHg; after: ΔPAM = 33.0 ± 4.3 mmHg, $n = 3$, $t = 0.98$, $P > 0.05$) and bradycardiac (before: ΔFC = −44.5 ± 5.7 bpm; after: ΔFC = −51.1 ± 8.7 bpm; $t = 2.2$; $P > 0.05$; $n = 3$, paired Student's t test) responses to L-Pro (56 nmol/100 nL) microinjection into SON of the normotensive rats. There were effects of L-Pro on MAP and HR (MAP: $F_{1,60} = 76.61$; HR: $F_{1,60} = 55.72$): a significant effect over time (MAP: $F_{5,60} = 21.97$; HR: $F_{5,60} = 18.39$, and an interaction between treatment and time (MAP: $F_{5,60} = 12.34$; HR: $F_{5,60} = 13.14$) (Fig. 5a).

ACF or NBQX/L-Glu

The microinjection of 2 nmol NBQX into the SON did not affect the baseline MAP (before: MAP = 94.3 ± 4.8 mmHg; after: MAP = 95.6 ± 5.5 mmHg, $t = 0.93$, $P > 0.05$, $n = 4$) or HR (before: HR = 374.6 ± 7 bpm; after: HR = 380.0 ± 5.6 bpm, $t = 3$, $P > 0.05$, $n = 4$). Pretreatment with NBQX significantly reduced the pressor (before: ΔMAP = 26.2 ± 2.4 mmHg; after: ΔMAP = 9.2 ± 0.7 mmHg, $t = 7.5$, $P < 0.01$; $n = 4$) and bradycardiac (before: ΔHR = 47.5 ± 5.0 bpm; after: ΔHR = −13.3 ± 1.9 bpm, $t = 5.9$, $P < 0.01$, $n = 4$) responses to L-Glu microinjection in the SON. However, pretreatment with ACF did not affect the blood pressure (ΔMAP before: 26.0 ± 1.9 mmHg; ΔMAP after: 24.6 ± 2.1 mmHg, $t = 3.5$, $P > 0.05$, $n = 3$) or the heart rate (ΔHR before: −41.9 ± 6.1 bpm; ΔHR after: −42.8 ± 6.4 bpm, $t = 0.2$, $P > 0.05$, $n = 3$) response to L-Glu into the SON. There were effects of L-Glu on the MAP and HR (MAP: $F_{1,24} = 57.48$; HR: $F_{1,24} = 40.53$): a significant effect over time (MAP: $F_{3,24} = 74.18$; HR: $F_{3,24} = 32.95$) and an interaction between treatment and time (MAP: $F_{3,24} = 17.19$; HR: $F_{3,24} = 12.24$) (Fig. 5b).

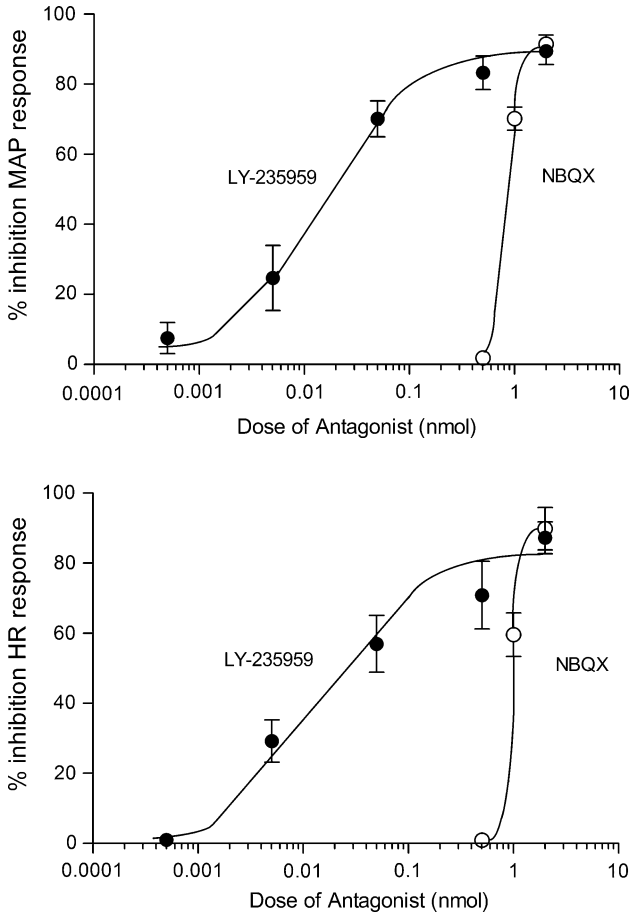

Fig. 4 Inhibition of pressor and bradycardiac responses (expressed as % inhibition) by microinjection of L-Pro (56 nmol/100 nl) into the SON after local pretreatment with different doses of the non-NMDA glutamate receptor antagonist NBQX [0.5 ($n = 4$), 1 ($n = 4$) and 2 ($n = 6$) nmol]. Inhibition of pressor and bradycardiac responses (expressed as % inhibition) by the microinjection of L-Pro (56 nmol/100 nL) into the SON after local pretreatment with different doses of the NMDA glutamate receptor antagonist LY (0.0005 ($n = 4$), 0.005 ($n = 4$), 0.05 ($n = 5$), 0.5 ($n = 4$) and 2 ($n = 6$) nmol/100 nL). *Curves* were generated by nonlinear regression analysis. *Symbols* represent means and *vertical lines* indicate the SEM

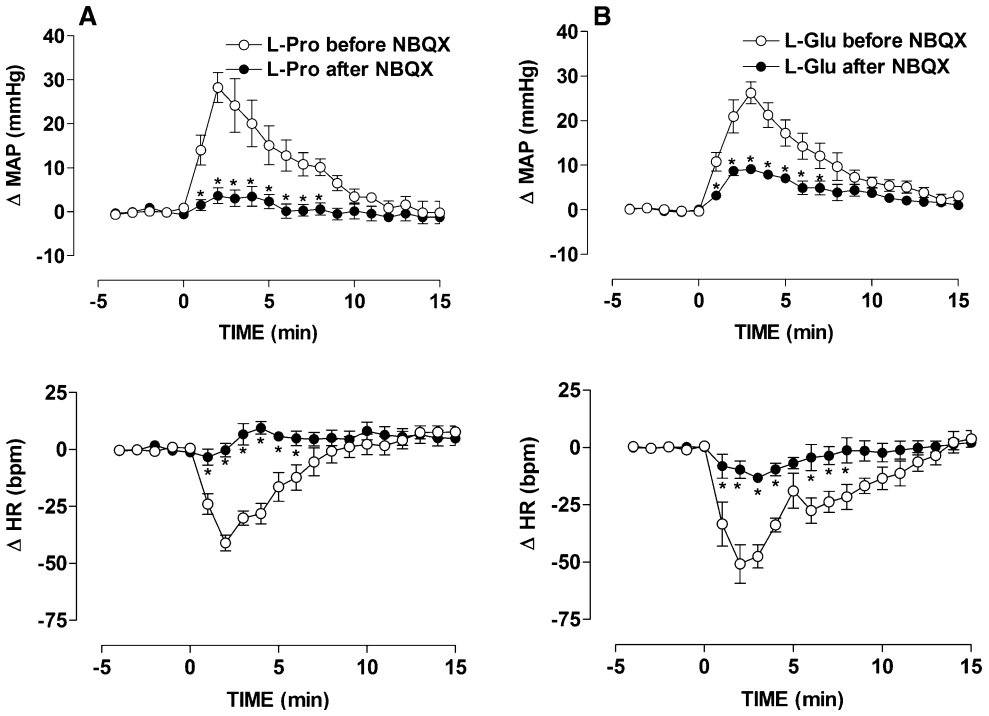

Fig. 5 **a** Time course of the effect of the microinjection of 56 nmol/100 nL of L-Pro into the SON on the mean arterial pressure (ΔMAP) and heart rate (ΔHR) before (*open circle*) and after (*filled circle*) local pretreatment with the selective non-NMDA ionotropic glutamate receptor antagonist NBQX (2 nmol/100 nL, $n = 6$). **b** Time course of the effect of microinjection of 10 nmol/100 nL of L-Glu into the SON on the mean arterial pressure (ΔMAP) and heart rate (ΔHR) before (*open circle*) and after (*filled circle*) local pretreatment with the selective non-NMDA ionotropic glutamate receptor antagonist, NBQX (2 nmol/100 nL, $n = 4$). Microinjections of L-Pro or L-Glu were made at the time 0. *Points* represent the mean and *bars* the SEM; $^*P < 0.05$, two-way ANOVA, applying the Bonferroni's correction for multiple comparisons when using the *t* test

Effect of local pretreatment with ACF or LY 235959 on cardiovascular response to L-Pro or L-Glu microinjection into the SON of unanesthetized rats

ACF/L-Pro

Pretreatment of the SON with 100 nL of ACF ($n = 6$) did not affect basal MAP (before: 92.2 ± 3.3 mmHg; after: 93.9 ± 4.2 mmHg, $t = 0.5$, $P > 0.05$) or HR (before: 351.5 ± 5.3 bpm; after: 351.3 ± 5.1 bpm, $t = 0.2$, $P > 0.05$). Pretreatment with ACF did not affect the pressor and bradycardiac responses to the microinjection of 56 nmol/100 nL L-Pro into the SON (ΔMAP before ACF: 32.7 ± 1.4 mmHg; after: 29.0 ± 1.8 mmHg, $t = 1.6$, $P > 0.05$; ΔHR before ACF: −56.7 ± 9.8; after: −55.8 ± 7.3 bpm, $t = 0.3$, $P > 0.05$, $n = 6$).

LY235959/L-Pro

The microinjection of different doses of the selective NMDA glutamate receptor antagonist LY235959 into the SON did not affect basal MAP (MAP before LY235959: 101.9 ± 2.4 mmHg; after: 101.9 ± 2.0 mmHg; $P > 0.05$, $n = 23$) or HR (HR before LY: 371 ± 2.8 bpm; after: 375.4 ± 2.2 bpm; $P > 0.05$ $n = 23$). The local pretreatment with LY [0.0005 ($n = 4$), 0.005 ($n = 4$), 0.05 ($n = 5$), 0.5 ($n = 4$) or 2.0 ($n = 6$) nmol/100 nL] caused a dose-related blockade of pressor ($R^2 = 0.90$) and bradycardiac ($R^2 = 0.85$) responses to L-Pro microinjection into the SON (Fig. 4). The higher dose of LY inhibited the pressor (−89.5 ± 3.8 %) and bradycardiac (−87.3 ± 4.5 %) responses to microinjected L-Pro.

The microinjection of 2 nmol LY 235959 into the SON did not affect baseline MAP (before: MAP = 102.9 ± 3.4 mmHg; after: MAP = 104.4 ± 2.5 mmHg, $t = 1.1$, $P > 0.05$, $n = 6$) or HR (before: HR = 372.4 ± 3.3 bpm; after: HR = 375.3 ± 3.3 bpm, $t = 2.4$, $P > 0.05$, $n = 6$). Pretreatment with LY 235959 blocked the pressor (before: ΔMAP = 29.8 ± 2.4 mmHg; after: ΔMAP = 4.3 ± 2.0 mmHg, $t = 12.2$, $P < 0.01$, $n = 6$) and bradycardiac (before: ΔHR = −44.6 ± 4.5 bpm; after: ΔHR = −8.7 ± 4.5 bpm, $t = 6.4$, $P < 0.01$, $n = 6$) responses to L-Pro microinjection into the SON. However, pretreatment with ACF did not affect the pressor (before: ΔPAM = 27.3 ± 4.4 mmHg; after: ΔPAM = 26.3 ± 2.6 mmHg, $n = 3$, $t = 0.54$, $P > 0.05$) and bradycardiac (before: ΔFC = −43.3 ± 2.7 bpm; after: ΔFC = −44.1 ± 6.7 bpm; $t = 0.06$; $P > 0.05$; $n = 3$, paired Student's *t* test) responses to

L-Pro (56 nmol/100 nL) microinjection into SON. There were effects of L-Pro on the MAP and HR (MAP: $F_{1,60} = 151.3$; HR: $F_{1,60} = 60.12$): a significant effect over time (MAP: $F_{5.60} = 29.27$; HR: $F_{5,60} = 12.36$) and an interaction between treatment and time (MAP: $F_{5,60} = 19.35$; HR: $F_{5,60} = 9.12$) (Fig. 6a).

ACF or LY235959/L-Glu

While the microinjection of 2 nmol LY 235959 into the SON did not affect the baseline MAP (before: MAP = 95.6 ± 3.6 mmHg; after: MAP = 100.7 ± 3.9 mmHg, $t = 2.9$, $P > 0.05$, $n = 4$) or HR (before: HR = 363 ± 3 bpm; after: HR = 365 ± 4 bpm, $t = 0.9$, $P > 0.05$, $n = 4$) neither did it affect the pressor (before: ΔMAP = 27.9 ± 2.8 mmHg; after: ΔMAP = 24.8 ± 2.3 mmHg, $t = 0.8$, $P > 0.05$; $n = 4$) nor bradycardiac (before: ΔHR = −49.04 ± 4.3 bpm; after: ΔHR = −47.2 ± 3.0 bpm, $t = 0.3$, $P > 0.05$, $n = 4$) response to L-Glu (10 nmol/100 nL) microinjection in the SON. Similarly, the pretreatment with ACF did not affect the blood pressure (ΔMAP before: 26.7 ± 2.6 mmHg; ΔMAP after: 24.9 ± 2.4 mmHg, $t = 2.6$, $P > 0.05$, $n = 3$) or the heart rate (ΔHR before: −43.9 ± 6.9 bpm; ΔHR after: −44.5 ± 6.0 bpm, $t = 0.14$, $P > 0.05$, $n = 3$) response to L-Glu microinjected into the SON. There were no interactions between treatment and time for either MAP or HR (MAP: $F_{3,24} = 0.48$; HR: $F_{3,24} = 0.65$) or effects of L-Glu (MAP: $F_{1,24} = 2.83$; HR: $F_{1,24} = 1.89$), but there was significant effect over time (MAP: $F_{3,24} = 75.63$; HR: $F_{3,24} = 41.51$) (Fig. 6b).

Effect of the systemic pretreatment with the ganglion blocker pentolinium on the cardiovascular response to the microinjection of L-Pro into the SON of unanesthetized rats

Intravenous pretreatment with pentolinium (5 mg/kg) significantly reduced the MAP (before pentolinium: MAP = 97.2 ± 3.6 mmHg; after pentolinium: MAP = 64.1 ± 3.0 mmHg, $t = 11.3$, $P < 0.001$, $n = 5$) but did not affect the HR baseline (before: HR = 366.6 ± 6.3 bpm, after pentolinium: HR = 365.6 ± 5.6 bpm; $t = 0.14$, $P > 0.05$, $n = 5$).

Pretreatment with pentolinium did not block the pressor response to the microinjection of L-Pro into the SON, but significantly increased the response (L-Pro before pentolinium: ΔMAP = 29.8 ± 2.5 mmHg and L-Pro after

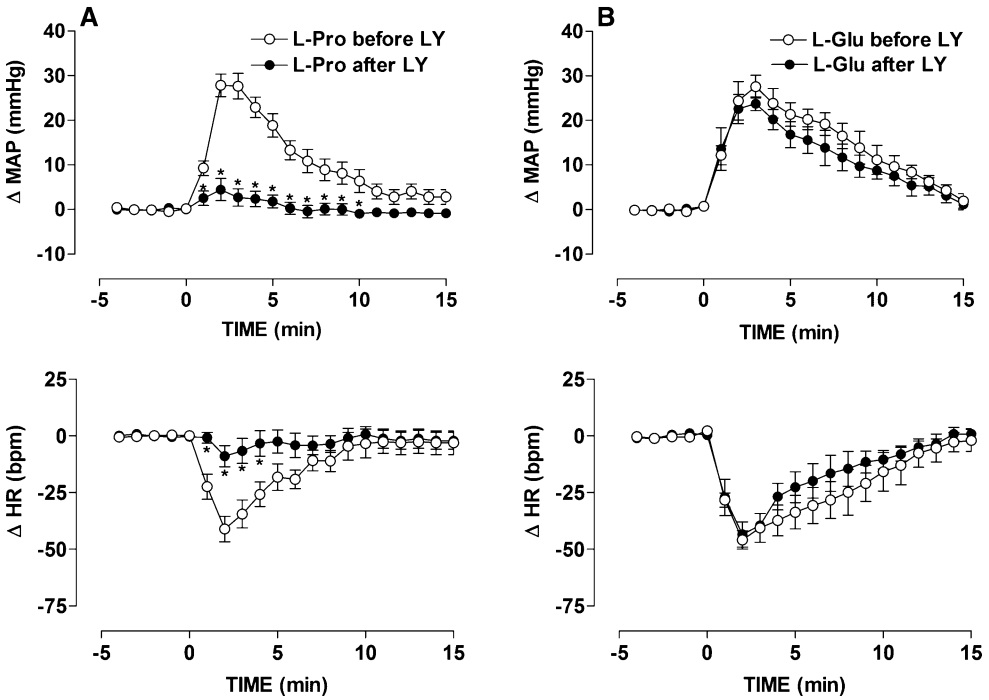

Fig. 6 **a** Time course of the effect of the microinjection of 56 nmol/100 nL of L-Pro into the SON on the mean arterial pressure (ΔMAP) and heart rate (ΔHR) before (*open circle*) and after (*filled circle*) local pretreatment with the selective NMDA ionotropic glutamate receptor antagonist, LY235959 (LY 2 nmol/100 nL, $n = 6$). **b** Time course of the effect of the microinjection of 10 nmol/100 nL of L-Glu into the SON on the mean arterial pressure (ΔMAP) and heart rate (ΔHR) before (*open circle*) and after (*filled circle*) local pretreatment with the selective NMDA ionotropic glutamate receptor antagonist LY235959 (LY 2 nmol/100 nL, $n = 4$). Microinjections of L-Pro or L-Glu were made at time 0. Points represent the mean and bars the SEM; $^*P < 0.05$, two-way ANOVA, applying Bonferroni's correction for multiple comparisons when using the *t* test

pentolinium: $\Delta MAP = 45.4 \pm 7.3$ mmHg, $t = 2.9$, $P < 0.05$, $n = 5$), and blocked the bradycardiac response (L-Pro before pentolinium: $\Delta HR = -46.3 \pm 1.5$ bpm and L-Pro after pentolinium: $\Delta HR = -6.2 \pm 3.8$ bpm; $t = 9.3$, $P < 0.01$, $n = 5$). There was a significant effect of pentolinium on the cardiovascular effect of L-Pro (MAP: $F_{1,40} = 8.45$; HR: $F_{1,40} = 27.25$): a significant effect over time (MAP: $F_{4,40} = 30.7$; HR: $F_{4,40} = 9.8$) and an interaction between treatment and time (MAP: $F_{4,40} = 2.68$; HR: $F_{4,40} = 5.27$) (Fig. 7a).

Effect of the systemic pretreatment with the V(1)-vasopressin receptor antagonist dTyr$(CH_2)_5$(Me)AVP on the cardiovascular responses to the microinjection of L-Pro into the SON of unanesthetized rats

The intravenous pretreatment with dTyr$(CH_2)_5$(Me)AVP (50 μg/kg) did not affect the basal MAP (MAP before dTyr: MAP = 96.3 ± 3.5 mmHg and after dTyr: MAP = 98.4 ± 4.2 mmHg, $t = 0.98$, $P > 0.05$, $n = 5$) or HR baseline (before dTyr: HR = 367.1 ± 6.5 bpm and after dTyr: HR = 370.3 ± 6.3 bpm, $t = 0.5$, $P > 0.05$, $n = 5$).

Pretreatment with the vasopressin antagonist significantly blocked the pressor (L-Pro before dTyr: $\Delta MAP = 30.9 \pm 1.8$ mmHg and L-Pro after dTyr: $\Delta MAP = 2.1 \pm 1.7$ mmHg, $t = 9.2$, $P < 0.05$, $n = 5$) and bradycardiac responses (L-Pro before dTyr: $\Delta HR = -63.1 \pm 4.5$ bpm and L-Pro after dTyr: $\Delta HR = -8.8 \pm 8.3$ bpm, $t = 5.0$, $P < 0.05$, $n = 5$). There was a significant effect of dTyr$(CH_2)_5$(Me)AVP on the cardiovascular effect of L-Pro (MAP: $F_{1,40} = 81.87$; HR: $F_{1,40} = 70.82$): a significant effect over time (MAP: $F_{4,40} = 28.48$; HR: $F_{4,40} = 21.25$) and an interaction between treatment and time (MAP: $F_{4,40} = 22.31$; HR: $F_{4,40} = 9.48$) (Fig. 7b). Recordings showing the cardiovascular effect of the microinjection of L-Pro into the SON before and after dTyr, in one unanesthetized rat, are shown in Fig. 8.

Discussion

In the present study, the microinjection of the amino acid L-Pro into the SON of unanesthetized rats caused cardiovascular responses that were characterized by a dose-related blood pressure increase and heart rate decrease, with an ED50 of approximately 56 nmol/100 nL. The dose–response curves indicated the absence of tachyphylaxis to L-Pro as there was a positive relationship between doses and responses. The doses used in our study were within the dose range recommended by Gaede and Pilowsky (2012).

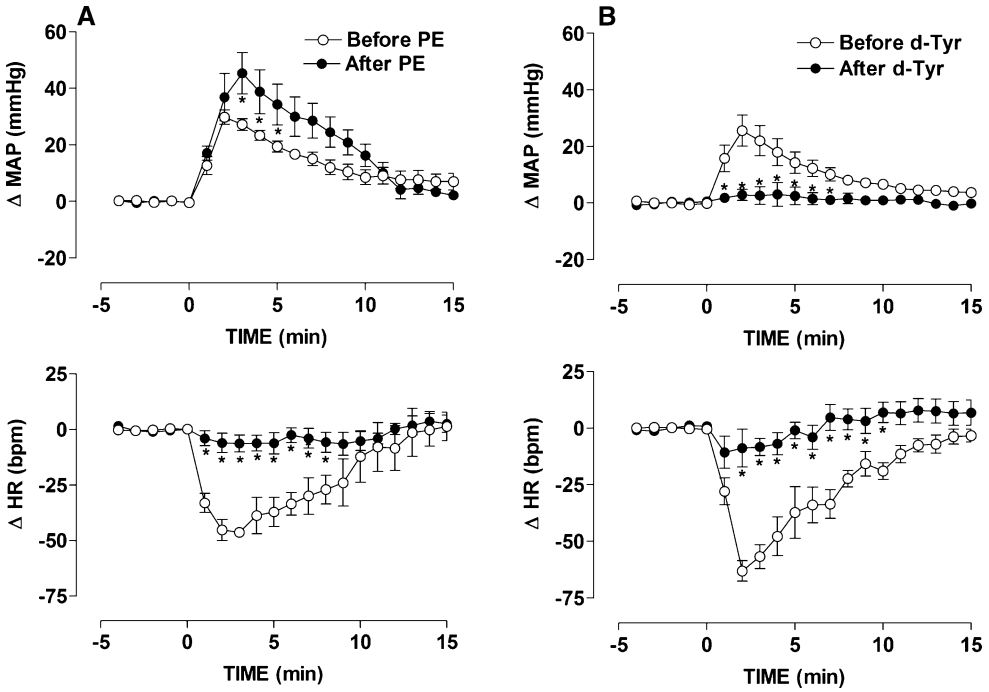

Fig. 7 **a** Time course of the effect of the microinjection of 56 nmol/100 nL of L-Pro into the SON on the mean arterial pressure (ΔMAP) and heart rate (ΔHR) before (*open circle*) and after (*filled circle*) i.v. pretreatment with the ganglion blocker pentolinium (5 mg/kg, $n = 5$). **b** Time course of the effect of the microinjection of 56 nmol/100 nL of L-Pro into the SON on the mean arterial pressure (ΔMAP) and heart rate (ΔHR) before (open circle) and after (filled circle) i.v. pretreatment with the V_1-vasopressin receptor antagonist dTyr(CH2)5(Me)AVP (d-Tyr, 50 μg/kg, $n = 5$). Microinjections of L-Pro were made at time 0. *Points* represent the mean and *bars* the SEM; $^*P < 0.05$, two-way ANOVA, applying Bonferroni's correction for multiple comparisons when using the *t* test

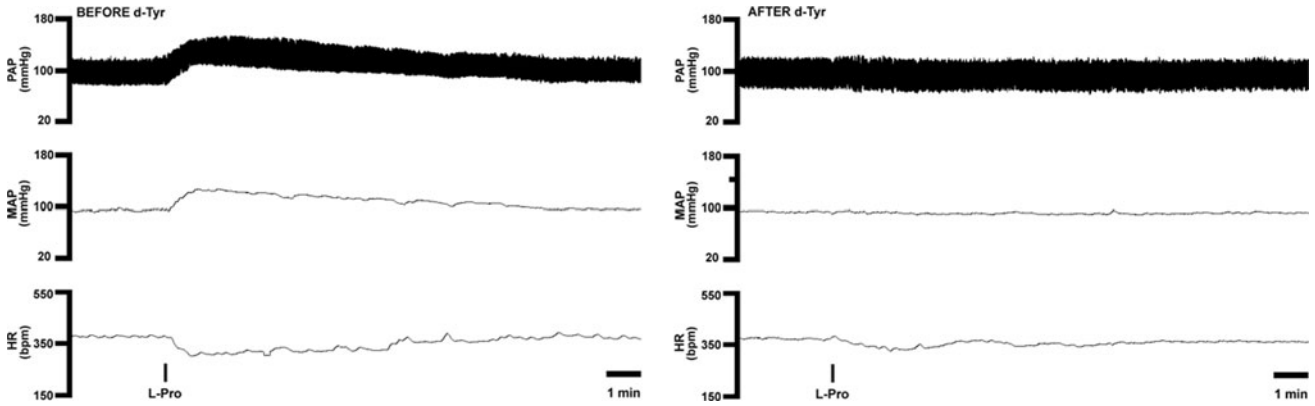

Fig. 8 Pulsatile arterial pressure (*PAP*), Mean arterial pressure (*MAP*) and heart rate (*HR*) recordings of one unanesthetized rat illustrating the pressor and bradycardiac responses to the microinjection of 56 nmol/100 nL of L-proline (L-Pro) into the SON before and after i.v. pretreatment with the V1-vasopressin receptor antagonist dTyr(CH2)5 (Me)AVP (d-Tyr, 50 μg/kg)

The volume injected was tailored to the specific nuclei being studied, i.e. the SON, and finally our study also demonstrate that the L-Pro effect depends principally on the dose. The microinjection of ACF into the SON caused no significant cardiovascular responses, thus excluding the possibility that responses observed after the L-Pro microinjection into the SON were due to a mechanical stimulation. In addition, no significant cardiovascular responses were observed when L-Pro was microinjected into areas surrounding the SON, thus reinforcing the idea of an action of L-Pro in the SON. The microinjection of L-Glu (10 nmol/100 nL), a known amino acid neurotransmitter in the CNS, into the SON of unanesthetized rats caused cardiovascular responses that were similar to those caused by L-Pro.

Cardiovascular effects caused by the microinjection of L-Pro into medullary structures have been previously reported (Takemoto 1995, 2001, 2005). The first indication that L-Pro causes cardiovascular effects when microinjected into supramedullary areas was the observation that L-Pro microinjection into the third ventricle caused pressor and bradycardiac responses in unanesthetized rats that could involve the stimulation of the magnocellular neurosecretory cells of the PVN (Lopes-Azevedo et al. 2012). Moreover, it is well established in the literature that there is an involvement of L-Glu neurotransmission in cardiovascular control in medullary as well as supra medullary areas, including the SON (Busnardo et al. 2007; Busnardo et al. 2009; Crestani et al. 2010; Machado and Bonagamba 1992; Mauad and Machado 1998; Resstel and Correa 2008). Immunohistochemical and pharmacological studies have shown that the hypothalamus, among the areas involved in cardiovascular control, contains high levels of ionotropic glutamate receptors (Meeker et al. 1994; Singewald and Philippu 1996). Specifically, the magnocellular neurons of the hypothalamus are densely innervated by glutamatergic synapses (Boudaba et al. 2003). L-Pro, at high concentrations (>100 μM), had been proposed to activate three classes of receptors: both the non-NMDA and NMDA-type ionotropic glutamate receptors and the strychnine-sensitive glycine receptor (Nistri and Morelli 1978; Ault et al. 1987; Henzi et al. 1992; Pace et al. 1992). In addition, competitive NMDA receptor antagonists, such as the 2-amino-7-phosphonoheptanoic acid (AP-7), (+)-3-3(2-carboxypiperazin-4yl)-propyl-1-phosphonic acid (CPP) and cis-4-(phosphonomethyl) piperidine-2-carboxylic acid (CGS 19755), as well as the NMDA receptor channel blocker MK-801, were reported to inhibit [H^3]Pro binding to mouse brain synaptic membranes (Ortiz et al. 1997), suggesting a common site for L-Pro and L-Glu. However, there is also evidence suggesting that the site of L-Pro could not be the same as L-Glu, because the specific binding of L-Pro in hippocampal synaptic membranes was not displaced by the excitatory amino acids L-Glu and L-aspartato (Greene et al. 1986).

Ionotropic glutamate receptors are coupled to ion channels, which allow the flow of Na^+, K^+, and sometimes Ca^{2+} through the pre and postsynaptic membranes. These receptors are classified according to the selectivity of the agonist and the homology of the amino acid in subtype *N*-methyl-D-aspartate (NMDA) and non-NMDA receptors, which are subdivided into receptors sensitive to α-amino-3-hydroxy-5-methyl-4-isoxazol propionate (AMPA) and kainate-sensitive receptors (KA) (Hollmann and Heinemann 1994). The AMPA and kainate receptors mediate fast depolarization in the majority of synapses in the brain and spinal cord. The binding of glutamate to these receptors promotes the influx of sodium ions and efflux of potassium ions, leading to depolarization of the postsynaptic neuron. This localized action potential, generated by activation of non-NMDA receptors, reaches a threshold of excitation that can activate NMDA receptors. This is happening

because NMDA receptors are blocked by magnesium ions in the resting membrane potential and they need a predepolarization for activation. In addition, NMDA channels are only activated in the presence of glutamate and a co-agonist, glycine (von Euler and Liu 1993; Ozawa et al. 1998). Therefore, the cardiovascular effects of microinjection of L-Pro in the SON could be caused by an activation of non-NMDA and/or NMDA glutamate receptors.

To investigate whether glutamate receptors were involved in the pressor and bradycardiac responses to the microinjection of L-Pro into the SON, we pretreated animals with different doses of selective non-NMDA or NMDA glutamate receptor antagonists and compared the results to those obtained after the microinjection of L-Glu into the SON. The microinjection of increasing doses of the selective non-NMDA glutamate receptor antagonist NBQX (0.5, 1.0, 2.0 nmol/100 nL) into the SON had no effect on blood pressure and heart rate baseline in unanesthetized rats. However, local pretreatment with NBQX inhibited the pressor and bradycardiac responses to the microinjection of L-Pro into SON of unanesthetized rats in a dose-related manner. Similarly, the microinjection of increasing doses of the selective NMDA glutamate receptor antagonist LY235959 (0.0005, 0.005, 0.05, 0.5, 2 nmol/100 nL) into the SON did not affect the blood pressure and heart rate baseline and the pretreatment with LY235959 also blocked the pressor and bradycardiac responses to the microinjection of L-Pro into SON of unanesthetized rats in a dose-related manner. The ID50 for NBQX was approximately 1 nmol/100 nL, while for LY235959 it was approximately 0.05 nmol/100 nL, thus indicating that LY235959 was at least 20 times more potent than NBQX in inhibiting the pressor and bradycardiac responses to the microinjection of L-Pro into the SON. The pressor and bradycardiac responses caused by the microinjection of L-Glu into the SON of unanesthetized rats were blocked by local pretreatment with the selective non-NMDA glutamate receptor antagonist NBQX (2 nmol/100 nL), but were not affected by local pretreatment with the selective NMDA glutamate receptor LY235959 (2 nmol/100 nL), the latter at a dose that completely blocked the cardiovascular effects of the injection of L-Pro into the SON. These results evidence a discrepancy between the responses to L-Pro and L-Glu, with respect to their block by local pretreatment with selective glutamate receptor antagonists.

The present results indicate that the pressor and bradycardiac responses caused by L-Glu microinjection into the SON of unanesthetized rats were mediated by an activation of non-NMDA glutamate receptors, without a significant involvement NMDA glutamate receptors in the response to L-Glu, as previously reported (Busnardo et al. 2007). Considering that LY235959 is a more potent and selective NMDA antagonist than AP-5 or AP-7 (Benveniste and Mayer 1991), the lack of inhibition of the L-Glu response would not be explained by an ineffective blockade of the NMDA receptor by LY235959.

L-Pro and L-Glu caused similar cardiovascular responses when injected into the SON of unanesthetized rats, which could indicate that L-Pro could be acting on glutamate receptors as an agonist. If so, it would be expected that its effects should be blocked only by pretreatment with the non-NMDA antagonist NBQX, and not affected by pretreatment with LY235959, like L-Glu. Because the cardiovascular responses were blocked by both antagonists, one could propose that these responses were mediated by both subtypes of glutamate receptors. On the other hand, the discrepancy between the effects of the antagonists on the responses to L-Pro and L-Glu evidences the possibility that L-Pro is acting on a L-Pro selective receptor, which is blocked by ionotropic glutamate receptor antagonists. If this is the case, although these antagonists are capable of differentiating NMDA from non-NMDA glutamate receptor subtypes, with a selectivity window over 1,000-fold, they would not be selective to L-Glu receptors and would also be capable of recognizing ionotropic L-Pro receptors, in this case with an inverse order of potency.

This set of results agrees with studies from the literature showing that the cardiovascular responses of L-Pro in medullary structures are similar to those of L-Glu, but with different mechanisms of action. The injection of L-Pro and L-Glu into the cisterna magna of conscious rats was reported to cause similar increases in blood pressure and mesenteric vascular resistance, but L-Pro responses were completely blocked by pretreatment with the non-selective ionotropic glutamate receptor antagonist kynurenic acid while L-Glu responses were only attenuated (Takemoto 1999). Also, the microinjection of L-Pro or L-Glu into the nucleus tractus solitarius (NTS) of anesthetized rats caused similar depressor and bradycardiac responses that were also differentiated by pretreatment with kynurenic acid: the responses to L-Pro were blocked, whereas the responses to L-Glu were potentiated (Takemoto 2001). Moreover, the microinjection of L-Pro into the caudal ventrolateral medulla (CVLM) of anesthetized rats caused a significant reduction in the blood pressure, which was blocked by local pretreatment with the non-NMDA receptor-selective antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and significantly attenuated by pretreatment with the NMDA receptor-selective antagonist MK801 (Takemoto 2005). Considering this, the present results showing the cardiovascular responses to the injection of L-Pro into the SON blocked by both NBQX and LY235959 favor the idea of a selective receptor for L-Pro in the SON and argue for the idea that L-Pro would be acting on L-Glu receptors, as a glutamatergic agonist. If this is the case, the results also indicate that although NBQX and LY235959 are capable of differentiating between subtypes of L-Glu

ionotropic receptors, they are not specific antagonists for L-Glu receptors, and would also recognize L-Pro receptors, and that on the L-Pro receptor LY235959 is 20 times more potent than NBQX.

Because pressor responses were observed after the microinjection of L-Pro into the SON, and the sympathetic nervous system is the main neural control of the cardiovascular system, it is important to test if the latter mediates the cardiovascular response to the injection of L-Pro into the SON. To check this hypothesis, we pretreated animals with the ganglionic blocker pentolinium before the microinjection of L-Pro into the SON. Intravenous pretreatment with pentolinium significantly lowered the blood pressure baseline, thus confirming an effective sympathetic blockade. Nonetheless, the pressor response to L-Pro was increased after the ganglion blockade, thus excluding a major involvement of the sympathetic nervous system in the mediation of the response to L-Pro. The increase in the pressor response to L-Pro that was observed after the treatment with pentolinium could be consequent upon the reduction in the baseline blood pressure caused by the autonomic blockade or because pentolinium blocked the compensatory bradycardic reflex. The treatment with pentolinium blocked the bradycardiac response to L-Pro, without affecting its pressor effect, thus agreeing with the idea that the HR effects observed after the injection of L-Pro, and L-Glu into the SON are due to baroreflex as a vagal response. Similarly, the pressor responses to microinjection of noradrenaline, either intracortical or i.c.v., as well as the pressor response to the intraseptal injection of acetylcholine were shown to be increased in unanesthetized rats pretreated with pentolinium (Correa et al. 1985; Peres-Polon and Correa 1994; Fernandes et al. 2003; Busnardo et al. 2007).

Vasopressin is synthesized mainly by magnocellular neurosecretory cells within the paraventricular nucleus of hypothalamus (PVN) and in the SON, being transported axonally and stored in the neurohypophysis until it is released into the systemic circulation (Swaab et al. 1975) to act on V_1-vasopressin receptors in the vascular smooth muscle (Phillips et al. 1990). Previously, Takemoto (1995) had reported that the cardiovascular response to the intracisternal microinjection of L-Pro in conscious rats was mediated by vasopressin release into the bloodstream. To verify whether vasopressin also mediated the pressor responses to L-Pro microinjection into the SON, we pretreated animals with the potent V_1-vasopressin receptor antagonist dTyr$(CH_2)_5$(Me)AVP. Pretreatment with dTyr$(CH_2)_5$(Me)AVP blocked the cardiovascular responses to the microinjection of L-Pro into the SON, thus suggesting that an acute vasopressin release mediated the pressor response to L-Pro. This antagonist has also been used to evidence the involvement of vasopressin in the cardiovascular responses to the injection of L-Glu into the diagonal band of Broca (Tavares and de Aguiar Correa 2003), SON (Busnardo et al. 2007) and PVN (Busnardo et al. 2009). However, the physiological role of the pressor response observed after microinjection of L-Pro into the SON is not yet clear. It may be part of cardiovascular adjustments to stress situations, since circulating vasopressin has been reported to be increased during the exposure to aversive situations (Jorgensen et al. 2002), and especially after a physical stress such as hypovolemia, since the main stimuli for vasopressin release are decreased blood pressure or blood volume and increased plasma osmolality (Cunningham et al. 2004).

In conclusion, our results indicate that the microinjection of L-Pro into the SON causes dose-related pressor and bradycardiac responses similar to those caused by the microinjection of L-Glu into the SON. The cardiovascular response to L-Glu was blocked by pretreatment of the SON with NBQX (a selective non-NMDA receptor antagonist), but not affected by pretreatment with LY235959 (a selective NMDA receptor antagonist). The cardiovascular response to L-Pro was blocked by pretreatment with either NBQX or LY235959, the latter being 20 times more potent than NBQX in blocking the effects of L-Pro. These results argue for the idea that L-Pro would act as an agonist on the L-Glu receptor, and favor the idea that L-Pro would act on specific prolinergic receptors. The results also suggest that ionotropic excitatory amino acid receptor antagonists, such as NBQX and LY235959, could not be specific for L-Glu receptors, where they can clearly discriminate between non-NMDA and NMDA glutamate receptor subtypes, but also could block L-Pro receptors. They also indicate that the pressor response to the microinjection of L-Pro into the SON is mediated by a release of vasopressin into the systemic circulation.

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Conflict of interest The authors declare that they have no conflict of interest.

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