Autonomic Neuroscience: Basic and Clinical 164 (2011) 34–42

Contents lists available at ScienceDirect

Autonomic Neuroscience: Basic and Clinical

journal homepage: www.elsevier.com/locate/autneu

Central mechanisms involved in pilocarpine-induced pressor response

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ARTICLE INFO

Article history:
Received 7 February 2011
Received in revised form 25 May 2011
Accepted 26 May 2011

Keywords:
Cholinergic mechanisms
Arterial pressure
Hypertension
Sympathetic activity
Vasopressin

ABSTRACT

Pilocarpine (cholinergic muscarinic agonist) injected peripherally may act centrally to produce pressor responses; in the present study, using c-fos immunoreactive expression, we investigated the forebrain and brainstem areas activated by pressor doses of intravenous (i.v.) pilocarpine. In addition, the importance of vasopressin secretion and/or sympathetic activation and the effects of lesions in the anteroventral third ventricle (AV3V) region in awake rats were also investigated. In male Holtzman rats, pilocarpine (0.04 to 4 μmol/kg b.w.) i.v. induced transitory hypotension followed by long lasting hypertension. Sympathetic blockade with prazosin (1 mg/kg b.w.) i.v. or AV3V lesions (1 day) almost abolished the pressor response to i.v. pilocarpine (2 μmol/kg b.w.), whereas the vasopressin antagonist (10 μg/kg b.w.) i.v. reduced the response to pilocarpine. Pilocarpine (2 and 4 μmol/kg b.w.) i.v. increased the number of c-fos immunoreactive cells in the subfornical organ, paraventricular and supraoptic nuclei of the hypothalamus, organ vasculosum of the lamina terminalis, median preoptic nucleus, nucleus of the solitary tract and caudal and rostral ventrolateral medulla. These data suggest that i.v. pilocarpine activates specific forebrain and brainstem mechanisms increasing sympathetic activity and vasopressin secretion to induce pressor response.

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1. Introduction

Peripheral treatment with muscarinic cholinergic agonists usually produces vasodilation and reduction in arterial pressure (Feigl, 1975; Inoue et al., 1984), whereas central muscarinic cholinergic mechanisms predominantly increase arterial pressure due to increases in sympathetic activity and vasopressin secretion (Hoffman et al., 1977; Punnen et al., 1986; Giuliano et al., 1989; Imai et al., 1989; Arneric et al., 1990; Buccafusco, 1996; Kubo et al., 2000; Padley et al., 2007).

Cholinergic muscarinic agonists like pilocarpine are usually used as therapeutic agent to stimulate salivary secretion in patients with xerostomia (Ferguson, 1993). Besides salivation, in anesthetized rats, small doses of pilocarpine injected intraperitoneally (i.p.) induce a long-lasting pressor response (Trendelenburg, 1961; Moreira et al., 2003; Takakura et al., 2005). Injection of pilocarpine directly in the central nervous system (CNS) induces an intense vasoconstriction in the mesenteric vascular bed and an increase in arterial pressure (Moreira et al., 2003). Lesions of the tissue surrounding the anteroventral portion of the third ventricle (AV3V region) abolish peripheral pilocarpine-induced pressor responses in anesthetized rats, suggesting that pilocarpine acts on the central cholinergic receptors to produce at least part of its cardiovascular responses (Takakura et al., 2005; Borella et al., 2008).

The AV3V region that includes the organ vasculosum of the lamina terminalis (OVLT), the preoptic periventricular area, the median preoptic nucleus (MnPO), and the anterior hypothalamic periventricular area is strongly involved in fluid-electrolyte balance and cardiovascular regulation (Brody et al., 1978; Brody and Johnson, 1980; Menani et al., 1990). The AV3V region plays a critical role in the increase in blood pressure and vasopressin release elicited by injection of angiotensin II (Johnson et al., 1978). Electrolytic lesions of this region abolish the development of different forms of experimental hypertension like renin-dependent-Goldblatt-hypertension, hypertension in Dahl salt-sensitive rats and hypertension by the treatment with deoxycorticosterone and salt (Buggy et al., 1977; Brody et al., 1978; Brody and Johnson, 1980; Goto et al., 1982; Haywood et al., 1983). The pressor, dipsogenic, natriuretic and kaliuretic responses to central cholinergic activation are also strongly affected by AV3V lesions (Menani et al., 1990; Colombari et al., 1992a, b; Gonçalves et al., 1992).

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doi:10.1016/j.autneu.2011.05.008

The pressor response induced by pilocarpine acting in the central nervous system is due to activation of different nuclei that control efferent mechanisms like vasopressin secretion and/or sympathetic system. A previous study using c-fos as a marker of neuronal activity suggested the involvement of different forebrain areas like the subfornical organ (SFO), MnPO, OVLT and, perhaps, paraventricular (PVN) and supra-optic (SON) nuclei of the hypothalamus on peripheral pilocarpine-induced water intake (Inenaga et al., 2008). Activation of these areas might also be the mechanism involved in the pressor responses to peripheral pilocarpine. However, the dose of pilocarpine used in the previous study (12 μmol/kg of body weight) is higher than the ones (2 and 4 μmol/kg of body weight) that has been used to produce pressor response and salivation (Moreira et al., 2003; Takakura et al., 2005; Borella et al., 2008). Thus, the central areas and efferent mechanisms activated by small doses of pilocarpine injected peripherally are not clear yet. Therefore, in the present study, we sought to investigate: 1) the effects of different doses of intravenous (i.v.) pilocarpine on mean arterial pressure (MAP) and heart rate (HR) in conscious rats and compared these effects with those of another muscarinic cholinergic agonist (carbachol) also injected i.v. at similar doses to reinforce how specific and particular are the cholinergic mechanisms activated by peripheral pilocarpine; 2) the importance of sympathetic activation and vasopressin secretion for i.v. pilocarpine-induced pressor responses; and 3) the neural activity revealed by c-fos expression in central areas related to cardiovascular regulation, like the SFO, PVN, SON, OVLT, MnPO, nucleus of the solitary tract (NTS) and caudal (CVLM) and rostral (RVLM) ventrolateral medulla in rats treated with low doses of i.v. pilocarpine. Although a previous study (Takakura et al., 2005) showed that acute AV3V lesions abolish the pressor responses to low dose of pilocarpine i.p. in anesthetized rats, to exclude any possible difference between anesthetized and awake rats, in the present study, we tested the effects of AV3V lesions on cardiovascular responses to i.v. pilocarpine in awake rats.

2. Methods

2.1. Animals

Experiments were performed in adult male Holtzman rats weighing 300 to 320 g. The animals were housed individually in stainless steel cages in a room with controlled temperature (23 ± 2 °C) and humidity (55 ± 10%). Lights were on from 7:00 am to 7:00 pm. Standard Guabi rat chow (Paulinia, SP, Brazil) and tap water were available *ad libitum*. All experimental protocols were approved by Animal Experimentation Ethics Committee of the Federal University of São Paulo (UNIFESP).

2.2. Arterial pressure and heart rate recordings

To record mean arterial pressure (MAP) and heart rate (HR), one day before the experiments, rats were anesthetized with ketamine (80 mg/kg of body weight) combined with xylazine (7 mg/kg of body weight) and a polyethylene tubing (PE-10 connected to a PE-50) was inserted into the abdominal aorta through the femoral artery. At the same time, a second polyethylene tubing was inserted in the femoral vein for drugs administration. Arterial and venous catheters were tunneled subcutaneously and exposed on the back of the rat to allow access in unrestrained freely moving rats. To record pulsatile arterial pressure, MAP and HR, the arterial catheter was connected to a Stathan Gould (P23 Db) pressure transducer coupled to a pre-amplifier (model ETH-200 Bridge Bio Amplifier) that was connected to a Powerlab computer data acquisition system (Powerlab 16SP, ADInstruments). Recordings were performed 1 day after the surgery and began 10–15 min after the connection of the arterial line to the pressure transducer. MAP and HR values recorded immediately before and those recorded at the maximum peak of change after i.v. injections of saline, pilocarpine or carbachol were used as reference to calculate the changes in MAP and HR.

2.3. Drugs

Pilocarpine (0.04, 0.4, 2 and 4 μmol/kg of body weight), carbachol (2 and 4 μmol/kg of body weight), prazosin (1 mg/kg of body weight), [β-Mercapto-β-, β-cyclopentamethylenepropionyl, O-me-Tyr^2, Arg^8] vasopressin — Manning Compound (10 μg/kg of body weight) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All drugs were dissolved in sterile saline.

2.4. Immunohistochemical procedure for c-fos detection

All histology was performed using brain tissue from rats deeply anesthetized with sodium thiopental (70 mg/kg of body weight) and perfused transcardially with 100 ml of buffered saline followed by 500 ml of freshly prepared 4% paraformaldehyde in 100 mM phosphate buffer, pH 7.4. Brains were removed and stored in the perfusion fixative for 24–48 h at 4 °C before being cut into 40-μm-thick coronal slices. Tissue was kept in cryoprotectant solution (20% glycerol plus 30% ethylene glycol in 50 mM phosphate buffer, pH 7.4) at −20 °C until processed.

Immunohistochemistry was processed with anti-fos serum raised in rabbit (Ab-5, lot D09803; Oncogene Science) at a dilution of 1:10,000. The primary antiserum was localized using a variation of the avidin–biotin complex system (ABC). In brief, sections were firstly incubated for 24 h in the primary antibody, before the incubation for 90 min at room temperature in a solution of biotinylated goat anti-rabbit IgG (Vector Laboratories). Then the sections were placed in the mixed avidin–biotin–horseradish peroxidase complex solution (ABC Elite kit; Vector Laboratories) for the same period of time. Between each step of the above immunohistochemistry processing sections were rinsed 3 × 10 min with potassium–PBS. The peroxidase complex was visualized by a 10 min exposure to a chromogen solution containing 0.02% 3,3′ diaminobenzidine tetrahydrochloride with 0.3% nickel–ammonium sulfate (DAB–Ni) in 0.05 M Tris buffer, pH 7.6, followed by incubation for 10 min in chromogen solution with hydrogen peroxide (1:3000) to produce a blue-black product. The reaction was stopped by extensive washing in potassium–PBS, pH 7.4. At the end, sections were mounted on gelatin-coated slides and then dehydrated and coverslipped with DPX.

Counts of the number of fos-immunoreactive neurons as a function of experimental status were generated for each area (OVLT, MnPO, SFO, SON, PVN, NTS, CVLM and RVLM) by using the 10× objective of a Zeiss Axioskop 2 microscope (Oberkochen, Germany) equipped with a camera lucida. To be considered as positive for fos-like immunoreactivity, the nucleus of the neurons had to be of appropriate size (ranging approximately from 8 to 15 μm) and shape (oval or round), show the characteristic blue-black staining of oxidized DAB–Ni, and be distinct from the background at magnification of 10×. For each animal, fos-positive cells were plotted and counted at three distinct rostrocaudal levels of each area (OVLT, MnPO, SFO, SON, PVN, NTS, CVLM and RVLM).

The number of c-fos-positive nucleus was identified and counted in a one-in-six series of transverse sections (1 section every 240 μm). Counts were made on both sides of the brain and throughout the portion of the forebrain and brainstem. Summing up the cells identified at all levels of the region of interest in the forebrain and in the brainstem and multiplying this number by 6 produced an uncorrected total number of c-fos-positive neurons counted in each rat. After applying a 0.81 Abercrombie correction factor previously determined on identically prepared histological material (Takakura et al., 2008), a more accurate estimate of the actual number of c-fos-positive neurons in the three rat groups was obtained.

2.5. Electrolytic AV3V lesions

Rats were anesthetized with ketamine (80 mg/kg of body weight) combined with xylazine (7 mg/kg of body weight) and placed in a stereotaxic frame (model 900, David Kopf Instruments). Bregma and lambda were positioned at the same horizontal level. A tungsten wire electrode (0.4 mm in diameter), bared at the tip (0.5 mm), was inserted into the brain using the following coordinates: 0.0 mm from bregma, in the midline and 7.0 mm below the dura mater. Electrolytic lesions were performed using a cathodal current (2 mA during 10 s). A clip attached to the tail was used as the indifferent electrode. Sham-lesioned rats had the electrode placed along the same coordinates, except that no current was passed.

To avoid acute adipsia that follows AV3V lesions (Buggy et al., 1977), besides water and food pellets, AV3V-lesioned rats had access to 10% sucrose solution during 5 days following the lesions. From a total of 22 rats submitted to AV3V lesions, 13 rats had lesions placed correctly.

2.6. Histology to confirm AV3V lesions

At the end of the experiments, the animals were deeply anesthetized with sodium thiopental (70 mg/kg of body weight, i.p.). Saline followed by 10% buffered formalin was perfused through the heart. The brains were removed, frozen, cut coronally into 50 μm sections, stained with Giemsa stain and analyzed by light microscopy to confirm the AV3V lesions.

2.7. Statistical analysis

Statistical analysis was done with Sigma Stat version 3.0 (Jandel Corporation, Point Richmond, CA). Data are reported as means ± standard error of means (SEM). One-way parametric analysis of variance followed by the Newman Keuls multiple comparisons test was used. The hypotensive and hypertensive responses to i.v. pilocarpine were analyzed separately. Significance was set at $p<0.05$.

3. Experimental protocols

3.1. MAP and HR in conscious rats that received i.v. pilocarpine or carbachol

Baseline MAP and HR were recorded for 10 min and then rats received i.v. injections of saline (control). Ten minutes later, rats received i.v. injections of pilocarpine (0.04, 0.4, 2 or 4 μmol/kg of body weight) or carbachol (2 or 4 μmol/kg of body weight) and the recordings continued for an additional 40 min. Each dose of pilocarpine or carbachol was tested in one group of animals.

3.2. MAP and HR in conscious rats that received i.v. pilocarpine combined with i.v. prazosin or vasopressin antagonist

Baseline MAP and HR were recorded for 10 min and then rats received i.v. injections of saline, prazosin (1 mg/kg of body weight) or vasopressin V1 receptor antagonist (10 μg/kg of body weight). Fifteen min later rats received i.v. injections of pilocarpine (2 μmol/kg of body weight) and the recordings continued for an additional 40 min. Different groups of rats were used to test each combination of treatments. The dose of pilocarpine used to test the effects of the pre-treatment with prazosin or vasopressin antagonist was the smallest dose that injected i.v. produced strong effects on MAP.

3.3. Expression of c-fos in forebrain and brainstem areas in conscious rats treated with i.v. pilocarpine

The expression of c-fos in different central areas (SFO, MnPO, OVLT, PVN, SON, NTS, CVLM and RVLM) was investigated in three groups of conscious rats. A control group (n = 4) received i.v. injections of saline, another group (n = 4) received i.v. injections of pilocarpine (2 μmol/kg of body weight) and a third group (n = 5) received i.v. injections of pilocarpine (4 μmol/kg of body weight). Sixty min after the i.v. injections, rats were anesthetized, perfused and had the brains removed and submitted to immunohistochemical procedures for c-fos detection. Each rat was assigned a random letter code and the cells were counted by a 'blinded' observer.

3.4. MAP and HR in conscious AV3V-lesioned rats that received i.v. pilocarpine

MAP and HR were tested in conscious rats with acute (1 day) or chronic (15 days) sham or electrolytic AV3V lesions. Baseline MAP and HR were recorded for 10 min and then the rats received i.v. injections of saline (control). Ten minutes later rats received i.v. injections of pilocarpine (2 μmol/kg of body weight) and the recordings continued for an additional 40 min. The dose of pilocarpine used to test the effects of AV3V lesions was the smallest dose that injected i.v. produced strong effects on MAP in awake rats.

4. Results

4.1. Cardiovascular effects of pilocarpine or carbachol i.v. in conscious rats

Pilocarpine (0.04, 0.4, 2 and 4 μmol/kg of body weight) i.v. induced an immediate and transitory (less than 10 s of duration) hypotension

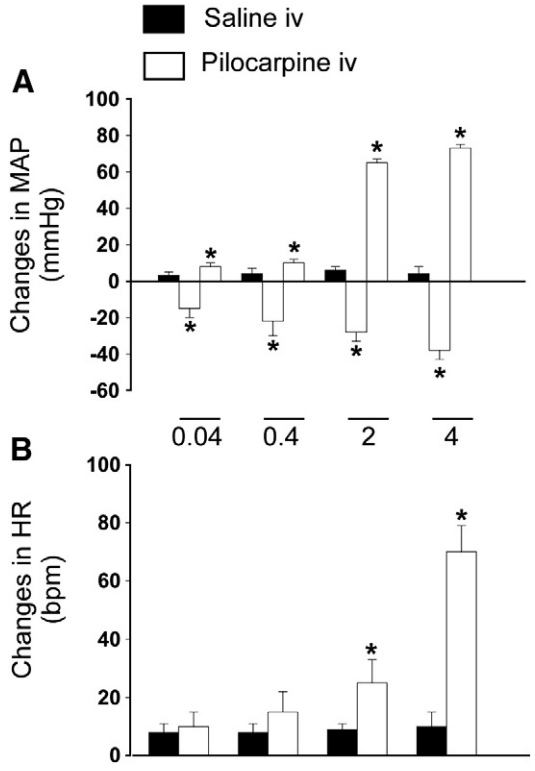

Fig. 1. Changes in (A) mean arterial pressure (MAP) and (B) heart rate (HR) produced by different doses of pilocarpine (0.04, 0.4, 2 and 4 μmol/kg of body weight) injected intravenously in conscious rats. The results are represented as mean ± SEM. n = 8 rats/group. *Different from saline (Newman–Keuls test, $p<0.05$).

Table 1
Changes in MAP and HR produced by i.v. injections of saline or carbachol in conscious rats.

Treatment	Changes in MAP (mm Hg)	Changes in HR (bpm)
Saline	+4±3	+10±6
Carbachol (2 μmol/kg)	−25±4*	−208±14*
Carbachol (4 μmol/kg)	−33±6*	−214±19*

Values are mean±SEM. Carbachol (2 and 4 μmol/kg of body weight). *Significantly different from saline. n=6 rats/group.

(−17±5, −22±8, −28±5 and −38±5 mm Hg, respectively, vs. saline: 2±3 mm Hg) [F(7, 66)=46.54; p<0.01] followed by long lasting (around 30 min duration) pressor responses (8±2, 10±2, 65±2 and 73±2 mm Hg, respectively, vs. saline: 2±3 mm Hg) [F(7, 66)=54.12; p<0.01] (Fig. 1A). Pilocarpine (2 and 4 μmol/kg of body weight, i.v.) also increased HR (25±8 and 70±9 bpm, respectively, vs. saline: 8±5 bpm) [F(7, 66)=23.75; p<0.01] (Fig. 1B).

Carbachol (2 and 4 μmol/kg of body weight) induced only a fast (less than 10 s of duration) hypotension (−25±4 and −33±6 mm Hg, respectively, vs. saline: 4±3 mm Hg) [F(2, 18)=22.37; p<0.01] (Table 1). Carbachol also decreased HR in conscious rats (−208±14 and −214±19 bpm, respectively, vs. saline: 10±6) [F(2, 12)=18.49; p<0.01] (Table 1).

4.2. Cardiovascular effects to i.v. pilocarpine combined with i.v. prazosin or vasopressin antagonist in conscious rats

The pre-treatment with i.v. vasopressin V1 antagonist (Manning Compound, 10 μg/kg of body weight) reduced the pressor response to pilocarpine (2 μmol/kg of body weight) i.v. (37±8 mm Hg, vs. saline: 62±5 mm Hg), whereas the α_1 adrenoceptor antagonist prazosin (1 mg/kg of body weight) almost abolished the pressor response to pilocarpine i.v. (12±3 mm Hg) [F(3, 26)=45.72; p<0.01] (Fig. 2A). Prazosin or vasopressin V1 antagonist did not affect i.v. pilocarpine-induced hypotension and tachycardia (Fig. 2A and B).

Prazosin (1 mg/kg of body weight) i.v. abolished the response to i.v. injection of the α_1-adrenoceptor agonist phenylephrine (5 μg/kg of body weight, i.v.) and the vasopressin antagonist (10 μg/kg of body weight, i.v.) abolished the pressor response to vasopressin (12 ng/0.1 ml, i.v.) (data not shown).

4.3. Expression of c-fos in the forebrain and brainstem areas of conscious rats treated with i.v. pilocarpine

Pilocarpine (2 and 4 μmol/kg of body weight) i.v. increased the number of c-fos imunorreactive cells in the OVLT [F(2, 14)=21.35; p<0.05], MnPO [F(2, 14)=31.54; p<0.05], SFO [F(2, 14)=26.17; p<0.05], SON [F(2, 14)=56.11; p<0.01], PVN [F(2, 14)=64.78; p<0.01], dorsomedial NTS [F(2, 11)=31.24; p<0.01], commissural NTS [F(2, 11)=36.61; p<0.01], CVLM [F(2, 11)=26.61; p<0.01] and RVLM [F(2, 11)=9.27; p<0.05] (Figs. 3 and 4).

4.4. Cardiovascular effects to i.v. pilocarpine in conscious AV3V-lesioned rats

As shown by previous studies (Vieira et al., 2004, 2006; Takakura et al., 2005), AV3V lesions (1 and 15 days) had no effect on resting MAP (113±4 and 115±4 mm Hg, respectively, vs. sham lesions: 110±6 and 113±6 mm Hg, respectively), however, AV3V lesions (1 day) increased resting HR (455±18 bpm, vs. sham lesion: 315±9 bpm).

One day AV3V-lesion almost abolished the pressor response to pilocarpine (2 μmol/kg of body weight) i.v. (7±5 mm Hg, vs. sham: 63±6 mm Hg), [F(5, 32)=27.55; p<0.01] without changing the tachycardic response (Fig. 5). The initial hypotension to i.v. pilocarpine was not modified by 1 day AV3V lesion, except that it had a longer duration (around 40 s vs. sham: 4 s).

The pressor and tachycardic response to i.v. pilocarpine was not modified by 15-day AV3V lesion (Fig. 5).

The AV3V lesion was located between the anterior commissure and the floor of the third ventricle with bilateral damage of the periventricular tissues from the organum vasculoso of the lamina terminalis through the preoptic and anterior hypothalamus, never extending caudally to the arcuate nucleus or medial hypothalamus (Brody et al., 1978; Menani et al., 1990; Vieira et al., 2004, 2006; Takakura et al., 2005). Fig. 6 shows a typical AV3V lesion in one rat representative of the lesioned group.

5. Discussion

Similar to anesthetized rats, pilocarpine injected i.v. produced a dose-dependent and transitory hypotension followed by hypertension in conscious rats (Trendelenburg, 1961; Moreira et al., 2003;

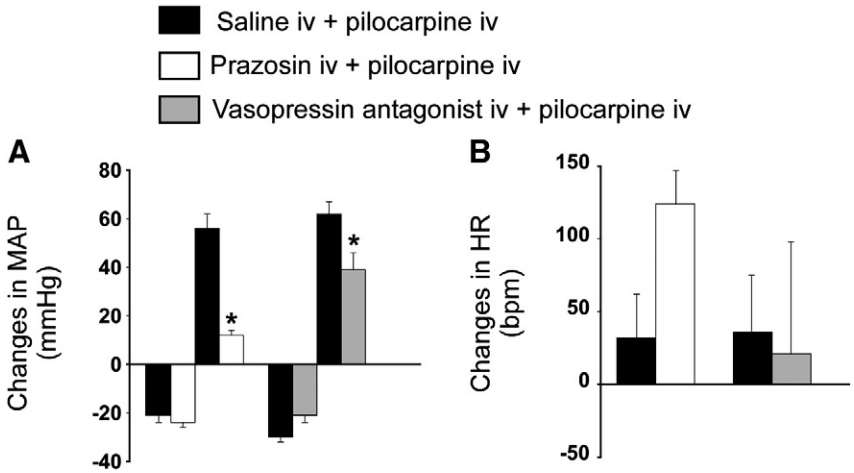

Fig. 2. Changes in (A) mean arterial pressure (MAP) and (B) heart rate (HR) produced by pilocarpine (2 μmol/kg of body weight) before and after intravenous administration of prazosin (1 mg/kg of body weight) or vasopressin V1 antagonist (Manning Compound — 10 mg/kg of body weight) in conscious rats. The results are represented as means±SEM. n=8 rats/group. *Different from saline+pilocarpine (Student–Newman–Keuls test, p<0.05).

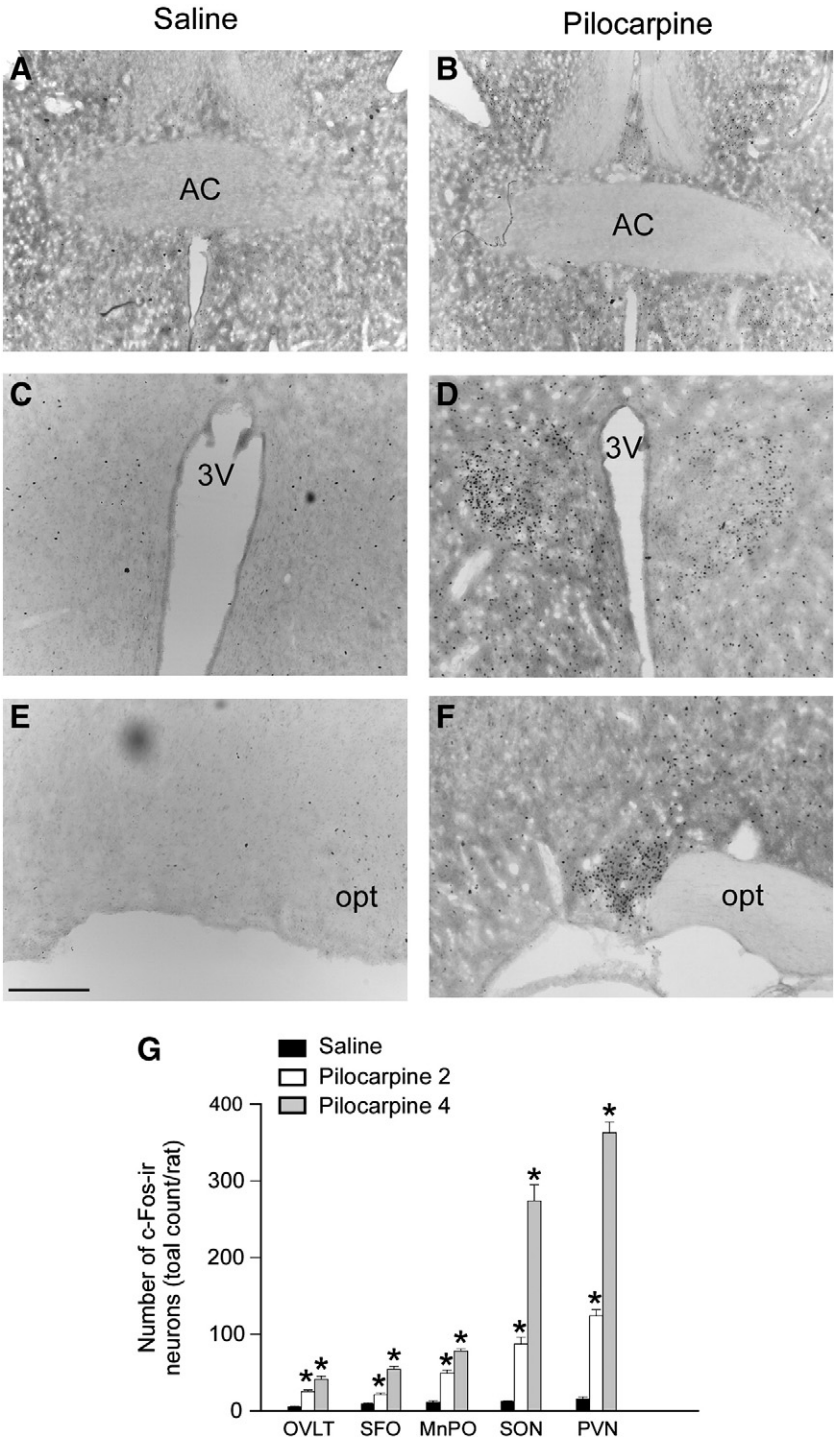

Fig. 3. (A, B, C, D, E and F) photomicrographs showing c-fos expression in (A and B) MnPO, (C and D) PVN and (E and F) SON in rats representative of the rats treated with intravenous injection of (A, C and E) saline or (B, D and F) pilocarpine (4 μmol/kg of body weight). (G) Number of c-fos immunoreactive cells in forebrain areas in rats treated with intravenous injection of saline or pilocarpine (2 and 4 μmol/kg of body weight). Results in panel G are expressed as means ± SEM. n = 4–5 rats/group. *Different from saline (Newman–Keuls test, $p<0.05$). Scale bar: 0.5 mm in E, applies to all panels. Abbreviations: AC, anterior commissure; MnPO, median preoptic nucleus; Opt, optic tract; OVLT, organ vasculosum of the lamina terminalis; PVN, paraventricular nucleus; SFO, subfornical organ; SON, supra optic nucleus; 3 V, third ventricle.

Takakura et al., 2005). Low doses of pilocarpine (2 and 4 μmol/kg of body weight) i.v. produced an intense and long-lasting pressor response and tachycardia. The pressor response to pilocarpine i.v. was almost abolished by the blockade of peripheral α_1-adrenoceptors with prazosin or acute (1 day) AV3V lesions, whereas the antagonism of vasopressin V1 receptors only reduced this response. These low doses of pilocarpine injected i.v. also activated forebrain areas (MnPO, OVLT, SFO, SON and PVN) and the NTS, similarly to the results of a previous study that investigated the effects of a higher dose of pilocarpine (Inenaga et al., 2008). In addition, the present results expand the conclusion of the previous study showing that pilocarpine i.v. also activated the RVLM and CVLM. Therefore, peripheral pilocarpine acting centrally activates forebrain and brainstem areas to induce pressor responses that result from increased vasopressin

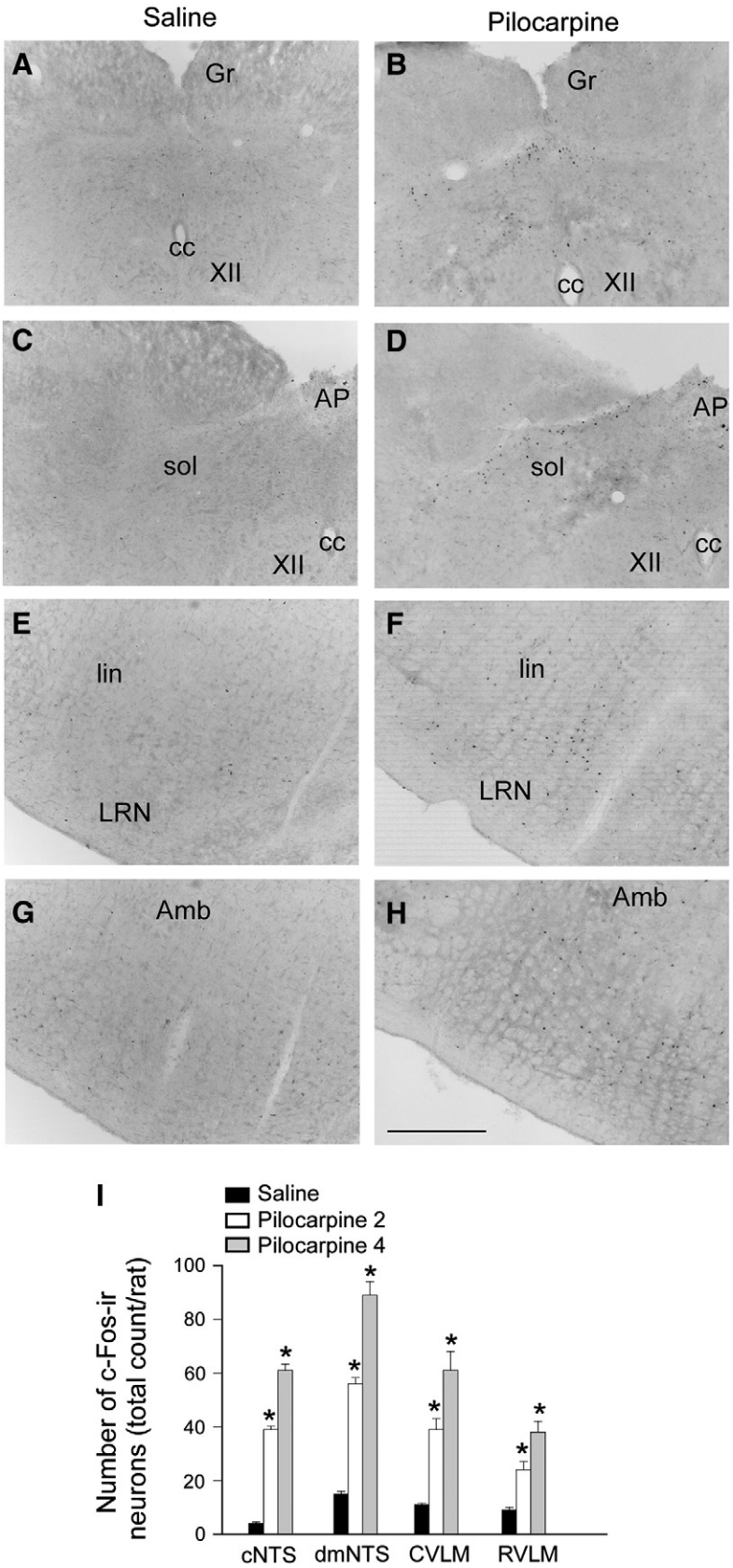

Fig. 4. (A, B, C, D, E, F, G and H) photomicrographs showing c-fos expression in (A and B) commissural NTS, (C and D) dorsomedial NTS (E and F) CVLM and (G and H) RVLM in rats representative of the rats treated with intravenous injection of (A, C, E and G) saline or (B, D, F and H) pilocarpine (4 μmol/kg of body weight). (I) Number of c-fos immunoreactive cells in brainstem areas in rats treated with intravenous injection of saline or pilocarpine (2 and 4 μmol/kg of body weight). Results in panel I are expressed as means ± SEM. n = 4–5 rats/group. *Different from saline (Newman–Keuls test, $p<0.05$). Scale bar: 0.5 mm in H, applies to all panels. Abbreviations: Amb, nucleus ambiguous; AP, area postrema; cc, central canal; CVLM, caudal ventrolateral medulla; Gr, gracilis nucleus; lin, linearis nucleus; LRN, lateral reticular nucleus; cNTS, commissural nucleus of the solitary tract; dmNTS, dorsomedial nucleus of the solitary tract; RVLM, rostral ventrolateral medulla; sol, solitary tract; XII, hypoglossal nucleus.

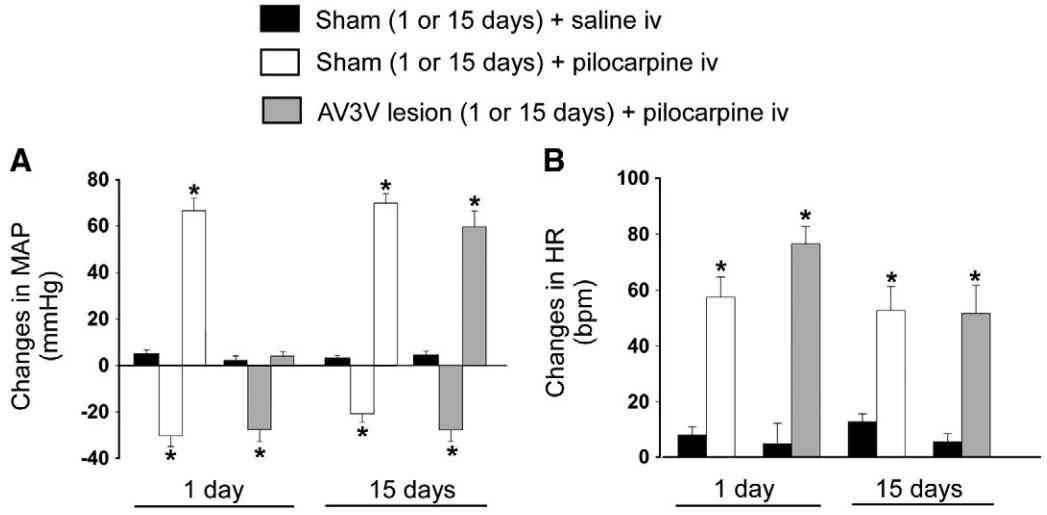

Fig. 5. Changes in (A) mean arterial pressure (MAP) and (B) heart rate (HR) produced by the treatment with pilocarpine (2 μmol/kg of body weight) or saline injected i.v. in 1 or 15 day sham or AV3V-lesioned rats. The results are represented as means ± SEM. n = 7 rats/group. *Different from sham + saline (Student–Newman–Keuls test, $p<0.05$).

secretion and mainly sympathetic activation through an AV3V region-dependent mechanism. The pressor response produced by pilocarpine leads to baroreflex activation and thus the activation of NTS and CVLM as revealed by c-fos immunoreactivity.

The initial hypotension to i.v. pilocarpine is probably a result of the vasodilation produced by the peripheral action of pilocarpine before it access to the CNS areas that activate pressor mechanisms (sympathetic activation and vasopressin secretion) which overcome peripheral vasodilation inducing pressor responses. Differently from pilocarpine, other cholinergic muscarinic agonists like carbachol injected peripherally produce only peripheral effects (hypotension and intense bradycardia) (Inoue et al., 1984). Therefore, due to some particular characteristics, pilocarpine easily crosses the blood-brain barrier (Freedman et al., 1989), reaching central areas to produce pressor responses.

As suggested by the present results, pilocarpine-induced pressor response is dependent on sympathetic activation and vasopressin secretion similar to previous reports for the activation of central muscarinic receptors (Hoffman et al., 1977; Imai et al., 1989). A previous study suggested that sympathetic activation might be the result of a ganglionic action of i.v. pilocarpine (Trendelenburg, 1961), whereas other studies have suggested a central action of low doses of pilocarpine to produce pressor response (Takakura et al., 2005; Borella et al., 2008). The present results show that low doses of pilocarpine increase the number of c-fos immunoreactive cells in central areas involved in the control of sympathetic activity and vasopressin secretion like the MnPO, OVLT, SFO, SON, PVN, and RVLM. Pilocarpine also increased the number of c-fos immunoreactive neurons in the NTS and CVLM that together with the RVLM are the main areas of the brainstem circuitry related to cardiovascular control (Ciriello et al., 1994; Dampney, 1994; Guyenet, 2006). Pilocarpine strongly increases arterial pressure which activates baroreceptors, thereby enhancing the activity of NTS and CVLM neurons, before the release of inhibitory signals in the RVLM trying to reduce sympathetic activation. In spite of the activation of these inhibitory mechanisms, arterial pressure increases as a consequence of the strong activation of pressor mechanisms due to direct or indirect activation of RVLM, AV3V region, PVN and SON by pilocarpine.

The increase of c-fos immunoreactive cells in the AV3V region (OVLT and MnPO) in rats treated with i.v. pilocarpine suggests that this region is involved in the responses produced by peripheral pilocarpine. The effects of AV3V lesions abolishing the pressor responses to pilocarpine confirm that mechanisms present in the AV3V region are essential for pilocarpine-induced pressor response. Pilocarpine i.v. also increases c-fos expression in the SFO, an area strongly connected with the AV3V region (Brody et al., 1978; Brody and Johnson, 1980). Injection of pilocarpine directly into the SFO activates neurons in this area (Inenaga et al., 2008) and cholinergic activation of the SFO produces strong pressor response dependent on the AV3V region (Colombari et al., 1992a). Therefore, pilocarpine may activate the AV3V region directly or indirectly (through the action in the SFO). Connections between the AV3V region and the PVN or SON may also be involved in the control of vasopressin secretion induced by pilocarpine, whereas sympathetic activation may involve mainly descending connections from the AV3V region to brainstem areas through the PVN that in turn may also connect directly to the intermediolateral column (IML) (Knuepfer et al., 1984; Yang and Coote, 1998; Westerhaus and Loewy, 1999; Geerling et al., 2010). Differently from the AV3V region, a previous study showed that the MSA is not involved in the pressor response to i.v. pilocarpine (Paulin et al., 2009) and, interestingly in the present study, no increase in c-fos expression in the MSA was detected (data not shown).

Acute AV3V lesions abolished the pressor responses to i.v. pilocarpine, whereas the hypotension was not affected, which suggests that these lesions impair the central pressor mechanisms activated by pilocarpine leaving intact the peripheral mechanisms responsible for the hypotension. In the absence of the central pressor mechanisms, long lasting hypotensive responses were expected due to activation of peripheral mechanisms alone. However, although longer (37 s in AV3V-lesioned rats, vs. 4 s in sham) the hypotension to i.v. pilocarpine in AV3V-lesioned rats was still transitory, followed by the return of arterial pressure close to baseline levels. These results suggest that pressor mechanisms are still partially acting in acute AV3V-lesioned rats and the action of these mechanisms is enough to counterbalance the peripheral vasodilation, reducing the duration of hypotension. In chronic AV3V-lesioned rats, the pressor responses to i.v. pilocarpine are completely recovered, which suggests that the central pressor mechanisms are activated by i.v. pilocarpine in these rats. The neural plasticity is probably the mechanism involved in the recovery of the central effects of pilocarpine in chronic AV3V-lesioned rats.

Although pilocarpine-induced salivation is suggested to depend on activation of central mechanisms (Moreira et al., 2001, 2002; Takakura et al., 2003, 2009; Borella et al., 2008), similar to a previous study (Inenaga et al., 2008) no increase in the number of c-fos immunoreactive cells in the salivary nuclei of the brainstem was

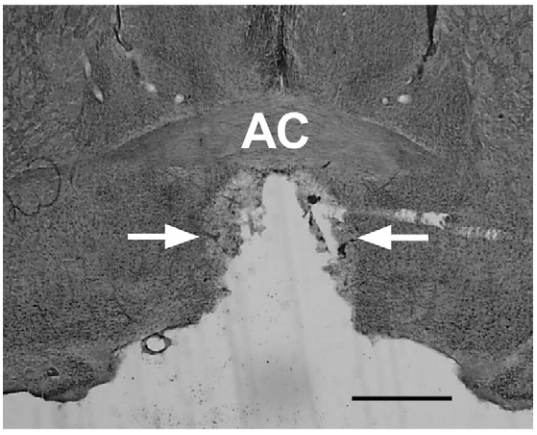

Fig. 6. Photomicrograph showing the typical AV3V lesion (arrows). AC, anterior commissure. Scale: 1 mm.

found in the present study (data not shown). In addition, we found no evidence in the literature suggesting that peripheral pilocarpine-induced salivation involves the activation of the salivary nuclei in the brainstem. Therefore, it seems that pilocarpine increases salivary gland secretion acting in other brain regions that might be even the areas investigated in the present study related to cardiovascular control.

In summary, i.v. pilocarpine, differently from carbachol, produces a transitory hypotension probably due to its peripheral action followed by sustained hypertension dependent on its central action in the forebrain (MnPO, OVLT, SFO, SON and PVN) and brainstem areas (RVLM) increasing vasopressin secretion and mainly sympathetic activation through an AV3V-dependent mechanism. Therefore, the efficiency of pilocarpine as a therapeutic agent to correct salivary gland dysfunction compared to other cholinergic muscarinic agonists is probably the result of a combination of salivary gland stimulation together with efficient maintenance of arterial pressure due to a balance between two opposite mechanisms: peripheral hypotensive and central hypertensive mechanisms activated by pilocarpine. However, on the other side, it is necessary to consider a possible hypertensive effect of this treatment.

Acknowledgments

We thank Silas Pereira Barbosa, Reginaldo da Conceição Queiróz and Silvia Fóglia for the technical support, Silvana A. D. Malavolta for secretarial assistance, and Ana V. de Oliveira for animal care. This study was supported by public funding from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (grants: 10/09776-3 to ACT; 06/60174-9 to TSM; 07/06430-6 to JVM;), Conselho Nacional de Pesquisa (CNPq) (grant: 300472/2005-6 to JVM and 501971/2007-6 to EC) and CAPES.

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