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Research Report

Hypothalamic disconnection caudal to paraventricular nucleus affects cardiovascular and drinking responses to central angiotensin II and carbachol

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ABSTRACT

The paraventricular nucleus of the hypothalamus (PVN) is an important area of the brain involved in the control of cardiovascular system and fluid-electrolyte balance. In the present study we evaluated the effects of hypothalamic disconnection (HD) caudal to PVN in the pressor and dipsogenic responses induced by intracerebroventricular (icv) injections of angiotensin II (ANG II) or carbachol (cholinergic agonist). Male Holtzman rats (280–320 g) with a stainless steel cannula implanted into the lateral ventricle and submitted to sham or HD surgery were used. HD (2 or 15 days) reduced the pressor responses to ANG II (50 ng/1 μl) icv (8±3 and 11±3 mm Hg, respectively, vs. sham: 23±3 and 21±2 mm Hg) or carbachol (4 nmol/1 μl) icv (8±2 and 21±3 mm Hg, respectively, vs. sham: 33±3 and 33±3 mm Hg), without changing baseline arterial pressure. Acutely (2–4 days), HD also reduced water intake to icv ANG II (3.3±2.2 vs. sham: 14.2±3.0 ml/60 min) or carbachol (4.4±1.8 vs. sham: 11.4±1.6 ml/60 min); however, chronically (15–17 days), HD produced no change on ANG II- and carbachol-induced water intake, in spite of the increased daily water intake and urinary volume. The results suggest that medial projections caudal to PVN are important for pressor and dipsogenic responses to central angiotensinergic and cholinergic activation.

1. Introduction

One important area of the brain involved in the control of the cardiovascular system and of the fluid-electrolyte balance that receives signals from more rostral areas, including inputs from osmoreceptor and angiotensin II (ANG II) sensitive areas in the lamina terminalis, is the paraventricular nucleus of the hypothalamus (PVN) (Antunes et al., 2006; Bains and Ferguson, 1995; Camacho and Phillips, 1981; Coote, 1995; Gutman et al., 1988; Li et al., 2008; Shi et al., 2008; Swanson and Kuypers, 1980; Swanson and Sawchenko, 1980; Toney et al., 2003). Osmotic stimuli or central ANG II activate projections from the magnocellular PVN to the neurohypophysis to release vasopressin and also activate descending projections from the parvocellular PVN to the medulla oblongata, mainly to the rostral ventrolateral medulla (RVLM) and to the spinal cord, increasing sympathetic nerve activity and arterial pressure (Antunes et al., 2006; Hoffman et al., 1977; Johnson et al., 1978;

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Shafton et al., 1998; Stocker et al., 2006; Swanson and Kuypers, 1980; Swanson and Sawchenko, 1983).

The importance of medial projections from more rostral areas, including lamina terminalis, to the PVN for the control of fluid-electrolyte balance and cardiovascular regulation was investigated by different studies using hypothalamic disconnection (HD) rostral to PVN performed with a microknife (Bealer, 1982; Hartle and Brody, 1984). The HD rostral to PVN increases daily and water deprivation-induced water intake and urinary volume and reduces the pressor responses to intracerebroventricular (icv) injection of ANG II (Bealer, 1982; Hartle and Brody, 1984). Other studies, using electrophysiology approaches demonstrated that subfornical organ (SFO), which lays in the lamina terminalis, has an excitatory input to the PVN neurons, probably using ANG II as a neurotransmitter (Bains and Ferguson, 1995; Ferguson, 2009). In addition, results showing that the SFO disconnection reduces hypertonic saline-induced drinking and c-fos expression in the PVN are also evidence of the importance of medial hypothalamic projections to the PVN, particularly those from the lamina terminalis, for the control of fluid-electrolyte balance (Starbuck et al., 2002). Therefore, these medial descending projections from rostral areas located in the lamina terminalis like the SFO, organum vasculosum of the lamina terminalis (OVLT) and median preoptic nucleus (MnPO) or even from the septal area or diagonal band to the hypothalamus and more specifically to the PVN convey important signals related to the control of fluid-electrolyte balance and of cardiovascular regulation.

In spite of the importance of the PVN for the control of fluid-electrolyte balance and of cardiovascular regulation, particularly, sympathetic activation, clearer information about projections from the PVN to the caudal areas is still lacking. A previous study, in anaesthetized rats, showed that HD caudal to PVN induces a strong hypotension (−30 mm Hg, starting approximately 30 min after the disconnection), suggesting that medial projections descending from the PVN are essential for the maintenance of arterial pressure in anesthetized rats (Colombari et al., 2002). The suggestion is that these descending projections from the PVN convey signals important for sympathetic activation that arise from the action of ANG II or another neurotransmitter in rostral areas strongly involved with sympathetic and cardiovascular regulation like the anteroventral third ventricle (AV3V) region, which includes the OVLT, or the SFO. However, no study has investigated yet if neural projections caudal to the PVN are important for the pressor response to ANG II acting centrally or to the activation of any other central pressor mechanism.

Therefore, in the present study, we investigated the effects of HD caudal to the PVN in the pressor and dipsogenic responses induced by icv injections of ANG II or carbachol (cholinergic agonist) in rats. The dipsogenic response to subcutaneous (sc) injection of the ß-adrenergic agonist isoproterenol, the baseline arterial pressure in conscious rats, daily water intake, urinary excretion and body weight were also evaluated in HD rats. Central injections of ANG II or carbachol are effective tools that activate central angiotensinergic and cholinergic mechanisms involved in sympathetic activation, hypertension, vasopressin secretion and thirst (Hoffman et al., 1977; Johnson and Thunhorst, 1997; McKinley et al., 1999; McKinley and Johnson, 2004). Isoproterenol injected sc induces thirst by increasing circulating levels of ANG II (Hosutt et al., 1978; Leenen and McDonald, 1974).

2. Results

2.1. Histological analysis

Fig. 1A shows the typical HD caudal to PVN in a rat representative of the animals tested. HD was located at the level of the dorsomedial and ventromedial hypothalamus. The lateral cut extends until the border of the fornix, sparing almost all medial forebrain bundle. Although not shown in Fig. 1, the cut extends ventrally down to the surface of the brain. Fig. 1B shows the typical site of LV injection.

2.2. Basal levels of MAP and HR in HD rats

Acute (1 day) or chronic (15 days) HD did not affect baseline MAP (118±3 and 122±2 mm Hg, respectively, vs. sham: 117±3 and 119±3 mm Hg, respectively). Chronic HD also did not affect baseline HR (366±10 bpm vs. sham: 387±10 bpm). However, acute HD increased basal HR (423±17 bpm vs. sham: 346±8 bpm, $p<0.05$, Student's t test).

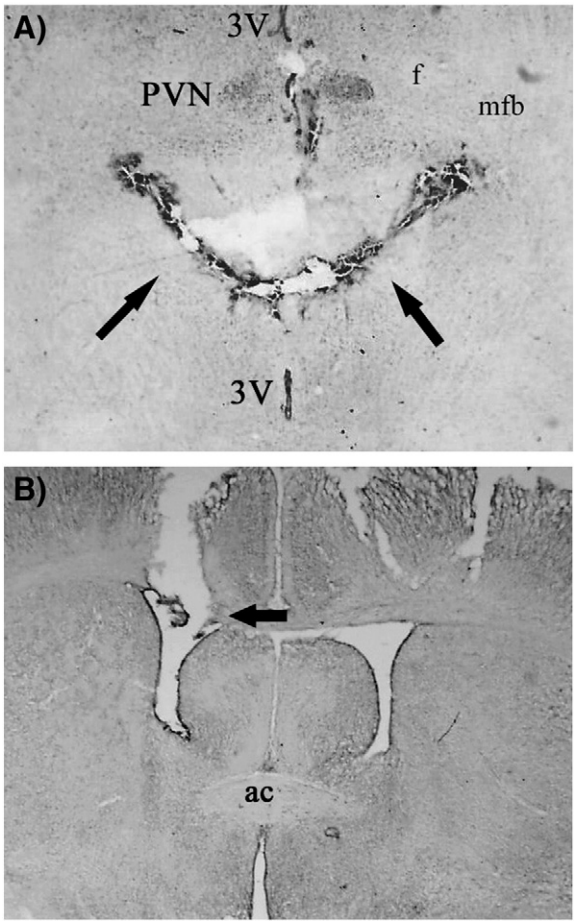

Fig. 1 – Photomicrographs showing (black arrows) (A) the typical position of the hypothalamic disconnection caudal to paraventricular nucleus in a horizontal section of the forebrain of a representative rat and, (B) the site of injection into the lateral ventricle in a coronal section of the forebrain. ac = anterior comissure; f = fornix; mfb = medial forebrain bundle; 3V = third ventricle.

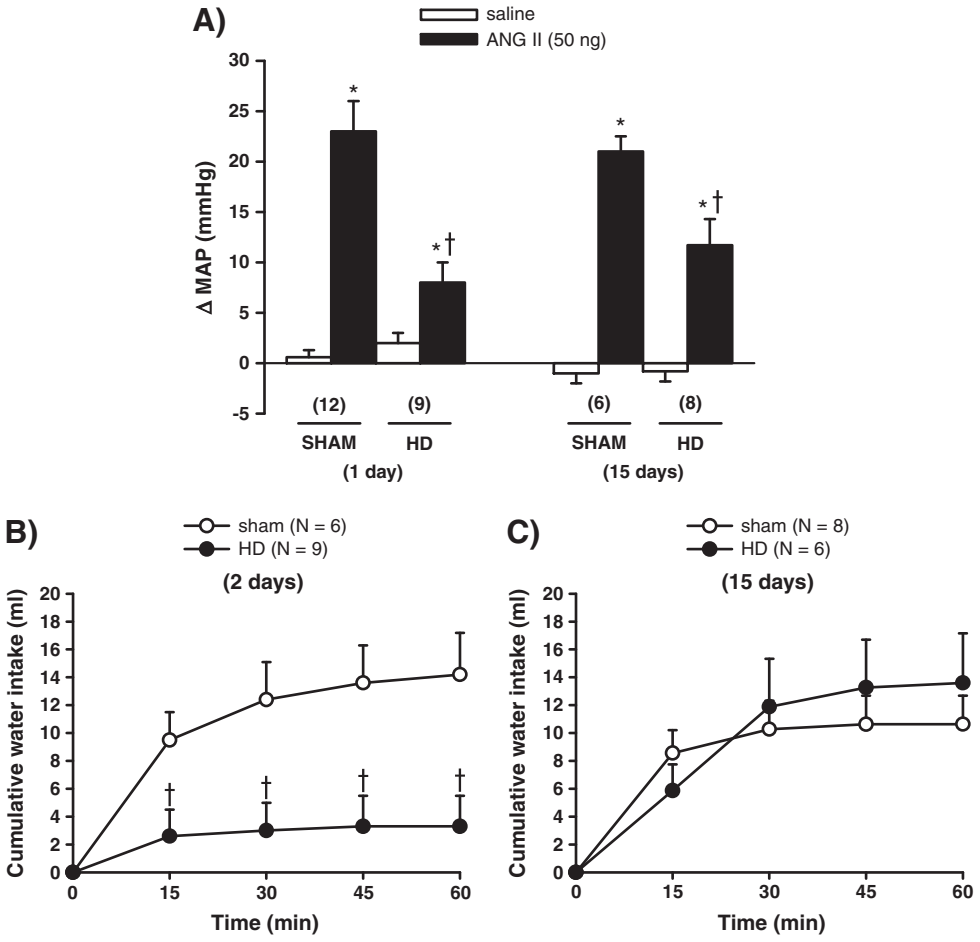

Fig. 2 – (A) Changes in MAP and (B and C) cumulative water intake induced by icv injections of saline or ANG II (50 ng/1 μl) in acute (1–2 days) or chronic (15 days) sham or HD rats. The results are represented as means ± SEM. The number of animals is indicated in parenthesis.*, different from saline; †, different from sham + ANG II (two-way ANOVA, followed by Student-Newman–Keuls test, $p<0.05$).

2.3. *Cardiovascular responses and water intake induced by icv ANG II in HD rats*

Acute (1 day) or chronic (15 days) HD similarly reduced the pressor response induced by ANG II (50 ng/1 μl) icv (8 ± 2 and 11 ± 3 mm Hg, respectively, vs. sham: 23 ± 3 and 21 ± 2 mm Hg, respectively) (Fig. 2A), [$F(3,62)=6.77$; $p<0.05$], without changes in HR (Table 1).

Table 1 – Effects of acute (1 day) and chronic (15 days) sham or HD caudal to PVN on changes in HR induced by icv injections of angiotensin II (50 ng/1 μl) or carbachol (4 nmol/1 μl).

Groups	ΔHR(bpm)	
	ANG II	Carbachol
Sham (1 day)	−2 ± 11 (N = 12)	−28 ± 12 (N = 12)
HD (1 day)	24 ± 9 (N = 9)	−3 ± 8 (N = 7)
Sham (15 days)	25 ± 19 (N = 6)	−27 ± 12 (N = 6)
HD (15 days)	13 ± 15 (N = 8)	−7 ± 10 (N = 8)

Results are shown as the mean ± S.E.M. N = number of rats.

Water intake induced by icv ANG II (50 ng/1 μl) was also strongly reduced by acute (2 days) HD (3.3 ± 2.2 ml/60 min vs. sham: 14.2 ± 3.0 ml/60 min), [$F(1,52)=30.18$, $p<0.001$], (Fig. 2B). Conversely, ANG II-induced water intake was not modified by chronic (15 days) HD (13.6 ± 3.6 ml/60 min vs. sham: 10.6 ± 2.0 ml/60 min), [$F(1,48)=0.40$, $p>0.05$], (Fig. 2C).

2.4. *Cardiovascular responses and water intake induced by icv carbachol in HD rats*

Acute (1 day) or chronic (15 days) HD also reduced the pressor response to carbachol (4 nmol/1 μl) icv (8 ± 2 and 21 ± 3 mm Hg, respectively, vs. sham: 33 ± 3 and 33 ± 3 mm Hg, respectively), [$F(3,58)=9.32$; $p<0.05$], (Fig. 3A), without changes in HR [$F(3,58)=2.052$; $p>0.05$], (Table 1). The reduction of the pressor response to carbachol produced by chronic HD was less intense than that produced by acute HD (Fig. 3A).

Water intake induced by icv carbachol (4 nmol/1 μl) was also reduced by acute (4 days) HD (4.4 ± 1.8 ml/60 min vs. sham: 11.4 ± 1.6 ml/60 min) [$F(1,40)=43.5$; $p<0.001$], (Fig. 3B). Similar to icv ANG II, water intake induced by icv carbachol was not modified by chronic (17 days) HD (10.4 ± 1.2 ml/60 min vs. sham: 9.8 ± 1.8 ml/60 min), [$F(1,48)=0.13$; $p>0.05$], (Fig. 3C).

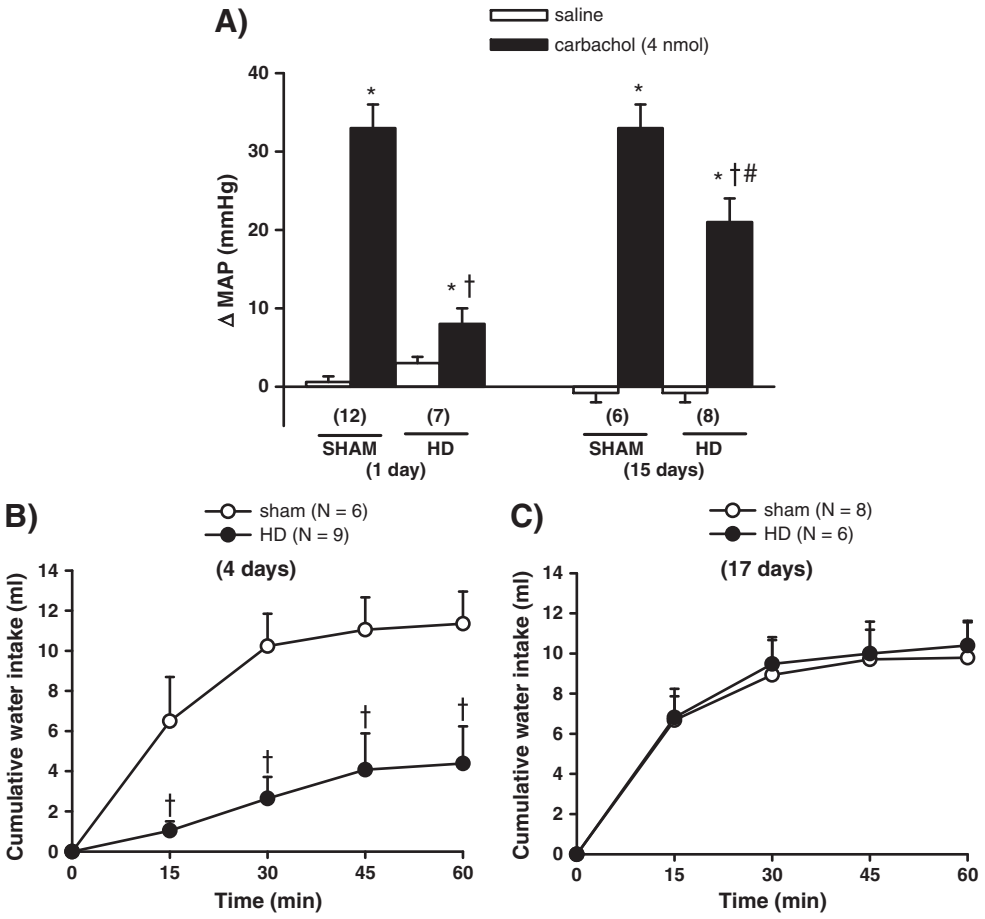

Fig. 3 – (A) Changes in MAP and (B and C) cumulative water intake induced by icv injections of saline or carbachol (4 nmol/1 μl) in acute (1–4 days) or chronic (15–17 days) sham or HD rats. The results are represented as means ± SEM. The number of animals is indicated in parenthesis.*, different from saline; †, different from sham + carbachol; #, different from 1 day HD + carbachol (two-way ANOVA, followed by Student-Newman–Keuls test, $p < 0.05$).

2.5. *Water intake induced by sc isoproterenol in HD rats*

Water intake induced by sc isoproterenol (30 μg/kg of body weight) was also reduced by acute (4 days) HD (4.6 ± 0.9 ml/120 min vs. sham: 6.7 ± 0.7 ml/120 min) [$F(1,64) = 9.95$; $p < 0.05$], not by chronic (15 days) HD (6.5 ± 1.1 ml/120 min vs. sham: 5.8 ± 1.1 ml/120 min), [$F(1,64) = 0.74$; $p > 0.05$], (Fig. 4).

2.6. *Daily water intake, body weight and urinary excretion by HD rats*

Daily water intake sustained increased in HD rats from day 6 until the end of the recording (14th day after HD) [$F(1,272) = 75.76$; $p < 0.001$] (Fig. 5). Daily water intake also increased in the first 2 days after HD; however, it was not different from control levels on days 3 to 5 after HD (Fig. 5).

In water replete rats, 24 h urinary volume strongly increased in both acute [$F(3,23) = 10.07$; $p < 0.001$] or chronic [$F(3,23) = 14.87$; $p < 0.001$] HD rats (4 and 15 days, respectively) compared to sham (Table 2). However, in 24 h water deprived rats, urinary volume was only slightly increased in HD rats (Table 2).

After HD, body weight was reduced until day 9 after the disconnection and it was fully recovered 2 weeks after HD [$F(1,66) = 14.24$; $p < 0.05$], (Table 3).

2.7. *Daily water intake and pressor and dipsogenic responses to icv ANG II or carbachol in unilateral HD rats*

Compared to sham, unilateral HD did not affect daily water intake during the 14 days of recording [($F(1,224) = 0.58$; $p > 0.05$].

Acute or chronic unilateral HD did not change the pressor responses induced by either icv ANG II (50 ng/1 μl) or carbachol (4 nmol/1 μl) (Table 4) and acute unilateral HD did not modify ANG II-induced water intake (14.4 ± 5.9 ml/60 min vs. sham 14.2 ± 3.0 ml/60 min; $p > 0.05$; $N = 5$).

3. Discussion

The results show that acute and chronic HD caudal to PVN reduced the pressor responses to both central angiotensinergic

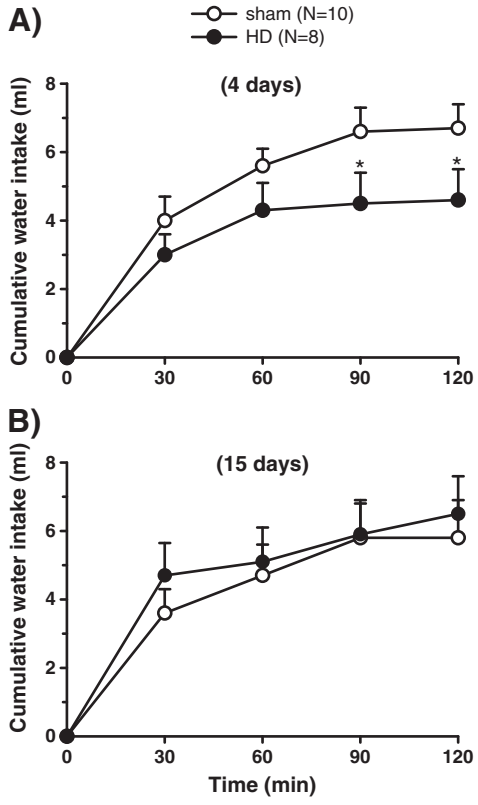

Fig. 4 – Cumulative water intake induced by sc isoproterenol (30 μg/kg of body weight) in (A) acute (4 days) or (B) chronic (15 days) sham or HD rats. The results are represented as means ± SEM. The number of animals is indicated in parenthesis.*, different from sham (two-way ANOVA, followed by Student-Newman–Keuls test, $p<0.05$).

and cholinergic activation whereas only acute HD reduced water intake induced by these stimuli. Contrary to the reduction of induced water intake by acute HD, daily water intake and urinary volume increased in both acute and chronic HD rats. The results suggest that descending projections that cross the

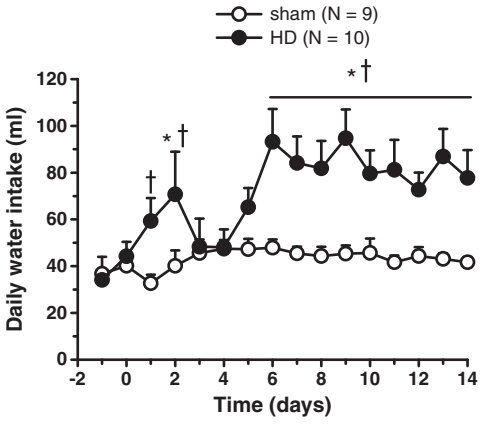

Fig. 5 – Daily water intake by sham or HD rats. Day 0: day of surgery. *, different from before surgery; †, different from sham ($p<0.001$).

Table 2 – Twenty four hour urinary volume by sham or HD rats that had water ad libitum or were water deprived.

	N	Water ad libitum (ml/24 h)	Water deprivation (ml/24 h)
Sham (4 days)	7	11.4±1.4	7.2±0.9
HD (4 days)	6	27.2±3.9 *	12.1±3.4 †
Sham (15 days)	7	12.9±1.3	9.0±0.2
HD (15 days)	6	45.8±8.7 *	18.7±2.6 †

Results are shown as means ± S.E.M. N = number of rats.
* Different from sham in the same condition.
† Different from ad libitum water intake (one-way ANOVA, followed by Student-Newman–Keuls; $p<0.05$).

medial hypothalamus are strongly involved in the cardiovascular responses to the forebrain angiotensinergic and cholinergic activation and on the control of fluid-electrolyte balance.

A previous study (Colombari et al., 2002) demonstrated that HD caudal to PVN reduced the MAP in anesthetized rats, suggesting that projections caudal to PVN are important for the maintenance of the baseline MAP in those animals. On the other hand, the present data shows that HD caudal to PVN produces no change in the baseline MAP in conscious rats. Perhaps, in anesthetized HD rats, the lack of signals from the rostral forebrain, including those from the PVN, to the hindbrain areas are not compensated by other cardiovascular regulatory mechanisms and then a reduction in sympathetic activity may cause hypotension immediately after HD. Similar to the present study, it was previously demonstrated that the acute (1 day) electrolytic lesion of the PVN also did not change the baseline MAP in conscious rats (Olivan et al., 2001). Although acute (1 day) HD caudal to PVN did not change the MAP, it produced a tachycardia, similar to that previously reported for HD caudal or rostral to PVN (Bealer, 1986; Colombari et al., 2002). There are two possible explanations for this tachycardia. One is that any tendency of hypotension detected by baroreceptors may result in increased sympathetic activity and a decrease in parasympathetic activity, resulting in vasoconstriction and tachycardia to maintain the MAP at normal levels. Another is the existence of descending signals from the rostral forebrain including AV3V region and PVN that tonically affect brainstem mechanisms involved in the control of HR (Colombari and Cravo, 1999; Menani et al., 1988).

Injections of ANG II or carbachol icv induce an array of responses, such as c-fos expression in the lamina terminalis, PVN and supraoptic nucleus, and increases in MAP, sympathetic nerve activity, vasopressin secretion and water intake (Hatzinikolaou et al., 1981; Hoffman et al., 1977; Kato et al., 2004; Miyajima and Bunag, 1984; Rowland et al., 1994; Xu et al., 2001). The HD performed in the present study removes the projections caudal to PVN, which probably include most of the projections to the brainstem areas, such as the RVLM and the intermediolateral cell column, that are involved in the control of sympathetic nerve activity to the cardiovascular system. Glutamatergic projections from PVN to RVLM are suggested to be involved in sympathetic activation produced by increased plasma osmolarity and ANG II levels during water deprivation (Andrews and Brenner, 1981; Burnier et al., 1983; Stocker et al., 2006). Thus, it is possible that HD caudal to PVN reduces the pressor responses to central ANG II and

Table 3 – Body weight (g) before and after sham or HD surgery.

Group	N	0 days	3 days	6 days	9 days	12 days	15 days
Sham	7	306±5	304±6	315±11	332±7	348±8	361±8
HD	6	305±5	285±7	290±11 *	301±7 *	334±6	347±10

Results are shown as means±S.E.M. N=number of rats. 0 to 15 days after surgery.
* Different from sham (two-way ANOVA, followed by Student-Newman–Keuls; $p<0.05$).

carbachol by removing these pathways from the PVN to the RVLM. Moreover, HD performed in the present study reached the ventral surface of the forebrain, likely damaging projections from the PVN to the posterior pituitary, resulting in a reduction of vasopressin secretion. Therefore, HD caudal to PVN may reduce the pressor responses to central ANG II and carbachol affecting sympathetic activation and vasopressin release. However, it is not possible to completely exclude a reduction of the pressor responses caused by hypovolemia and/or fluid-electrolyte imbalance, similar to the reduction in the pressor response to icv ANG II or carbachol produced by the treatment with the diuretic and natriuretic drug furosemide (Colombari et al., 1992).

The pressor responses induced by ANG II and carbachol injected centrally were reduced, but not completely abolished, by acute or chronic HD caudal to PVN. This may suggest that the pressor responses induced by icv administration of these substances also depend on neural pathways spared by HD caudal to PVN. It is well known that the pressor response induced by icv ANG II is abolished by HD rostral to PVN, that removes most of the medial forebrain bundle (MFB) whereas the pressor response to icv ANG II is only reduced by HD rostral to PVN, that spares the MFB (Kawabe et al., 1995). In the present study, HD spared most of the MFB and then, the remaining pressor response may depend on this pathway.

Both increased water intake and urine volume were reported in rats with coronal knife cuts between the OVLT and the PVN, which transect the supraoptic-neurohypophysial tract (Kawabe et al., 1993). These authors attributed the polyuria and the resulting polydipsia to a reduction in vasopressin release after this transection. The present data show that rats submitted to HD caudal to PVN also displayed a consistent polydipsia mainly after the 6th day of HD. This polydipsia might be due to reduced vasopressin level leading to an increased daily urinary excretion as shown by the present results. However, besides changes in vasopressin secretion, HD might also disrupt descending projections that control sympathetic discharges to the kidney which might change renal water and electrolyte excretion. Daily water intake increased in the first 2 days after HD, returned to control levels from day 3 to 5 after HD and became consistently increased chronically (6–14 days after HD). The delay to consistently increase daily water intake is coincident with the period in which HD reduces ANG II- and carbachol-induced water intake. It seems that although signals to increase daily water intake were present since the first day after HD, the effects of HD reducing dipsogenic responses may affect the total daily water intakes. That is, HD causes excessive diuresis that results in dehydration and increases in ANG II levels. However, simultaneously, HD impairs dipsogenic responses as suggested by the reduction of icv ANG II- and carbachol-induced water intakes. HD affects dipsogenic responses only acutely and, therefore, chronically all the dehydration resulting from the excessive diuresis might be corrected by increasing daily water intake. Therefore, it is likely that AVP secretion is impaired in HD rats and this may account for the increased urinary excretion and increased daily water intake. Nonetheless, both acute and chronic HD rats were able to reduce urinary volume when they had no water available to ingest, which may suggest that HD rats are still able to secrete vasopressin in response to dehydration. Although it was not investigated in the present study, it is also possible that an intense dehydration and reduction of extracellular volume results in a reduced renal perfusion pressure which may affect renal function.

Table 4 – Effects of acute (1 day) and chronic (15 days) of unilateral HD on mean arterial pressure (MAP) responses induced by icv injections of angiotensin II (50 ng/1 μl) or carbachol (4 nmol/1 μl).

Groups	ΔMAP (mm Hg)	
	ANG II	Carbachol
Sham (1 day)	23±3 (N=6)	33±3 (N=6)
Unilateral HD (1 day)	24±3 (N=3)	25±3 (N=3)
Sham (15 days)	21±2 (N=6)	33±4 (N=6)
Unilateral HD (15 days)	24±1 (N=5)	30±2 (N=5)

Results are shown as the mean±S.E.M. N=number of rats.

A previous study demonstrated that chemical lesion of the PVN reduces water intake induced by icv ANG II (Gutman et al., 1988). The present study shows that water intake induced by icv ANG II or carbachol was reduced by acute HD, suggesting that medial pathways caudal to PVN are important for ANG II- and carbachol-induced thirst. In addition, acute HD also reduced sc isoproterenol-induced water intake, a response dependent on increased circulating levels of ANG II acting in the lamina terminalis, particularly in the SFO and OVLT (Ferguson, 2009; McKinley and Johnson, 2004; Simpson, 1981). As previously demonstrated using c-fos expression as a marker, icv ANG II activates MnPO and juxtaventricular parts of SFO and OVLT whereas intravenous (iv) ANG II activates mainly SFO and OVLT (McKinley et al., 1995). Nevertheless, regardless of the route of administration, drinking responses to icv ANG II or circulating ANG II (by treating rats with sc isoproterenol) were reduced by HD caudal to PVN. Thus, caudal HD seems to decrease water intake by disrupting common central pathways activated either by centrally-administered stimuli or peripherally-generated ANG II.

However, chronic HD did not affect water intake induced by icv ANG II or carbachol or by sc isoproterenol. The recovery of ANG II- and cholinergic-induced water intake chronically is

probably a consequence of neural plasticity involving the activation of alternative pathways to replace those damaged by HD. It is important to remember that daily water intake was normal or even increased after HD, which suggests that, in spite of some impairment, rats still have active mechanisms to induce water intake. Lesions of the lateral hypothalamus also affects induced and daily water intake, which suggests that mechanisms related to the control of water intake involve both medial and lateral pathways of the hypothalamus. Therefore, the deactivation or lesion of one of these pathways might be compensated by the other (Camargo et al., 1991; Goncalves et al., 1992; Haibara et al., 1994; Kucharczyk and Mogenson, 1975).

Although disruption of the descending projections seems to be the main reason for the effects of HD, hindbrain adrenergic inputs to the forebrain are also suggested to facilitate ANG II-induced drinking and pressor responses (for review (Johnson and Edwards, 1990). Disrupting the pathways caudal to PVN by HD might also remove these ascending adrenergic inputs to the forebrain affecting at least part of the dipsogenic and pressor responses to ANG II. However, it is also necessary to consider that ascending pathways from the hindbrain, particularly those from the lateral parabrachial nucleus and the nucleus of the solitary tract, may also inhibit water intake (Blanch et al., 2007; Menani and Johnson, 1995; Ohman and Johnson, 1986). Therefore, more studies are necessary to determine if the effects produced by HD are related to disruption of descending or ascending pathways or to impaired vasopressin secretion.

In contrast to the effects of complete bilateral HD, the unilateral knife-cut produced no change in daily water intake in the pressor responses to central ANG II or carbachol or in the dipsogenic response to central ANG II, suggesting that intact unilateral projections caudal to PVN are sufficient for the cardiovascular and drinking responses produced by the activation of forebrain angiotensinergic or cholinergic mechanisms. In addition, the unilateral knife cut results also demonstrate that the changes in cardiovascular and fluid-electrolyte responses after HD are not due to any traumatic effect of the knife-cut, especially acutely when a potential brain inflammatory response might be present.

In conclusion, the results show that HD caudal to PVN permanently reduces the pressor responses induced by central ANG II or carbachol whereas drinking responses were reduced only acutely. These results suggest that medial projections caudal to PVN are important for pressor and dipsogenic responses to the central angiotensinergic and cholinergic activation, as well as for the dipsogenic response induced by peripherally generated ANG II. Although HD did not affect induced water intake chronically, it increased daily water intake possibly as a consequence of the diuresis resulting from reduced vasopressin release.

4. Experimental procedures

4.1. *Animals*

Male Holtzman rats weighing 280–320 g were used. The animals were housed individually in stainless steel cages in a room on a 12:12 light/dark cycle (lights on from 7:00 AM to 7:00 PM), controlled room temperature at 23±2 °C and humidity at 55±10%. Guabi rat chow (Paulinia, SP, Brazil) and tap water were available ad libitum, except in the protocol for testing urinary volume. The Ethical Committee for Animal Care and Use from Dentistry School of Araraquara, UNESP approved the experimental protocols used in the present study. The experiments followed the U.S. National Institutes of Health Guide for the Care and Use of Laboratory (NIH publication no. 80–23, 1996).

4.2. *Brain surgery*

Animals were anesthetized with intraperitoneal ketamine (Francotar, Virbac, Jurubatuba, SP, Brazil, 80 mg/kg body weight) combined with xylazine (Xilazin, Syntec, Cotia, SP, Brazil, 7 mg/kg body weight) and adapted to a stereotaxic apparatus (model 900, David Kopf Instruments, Tujunga, CA, USA). The skull was leveled between bregma and lambda. The HD caudal to the PVN was achieved by means of a stereotaxically placed special double-edged microknife of bayonet shape (1 mm radius, 2 mm height), lowered 1.5 mm caudal to the bregma, along the midline down to the inner surface of the sphenoid and the cut was achieved by rotating it 90° left and 90° right. Sham HD surgery (sham-cut) consisted of lowering the knife at the same coordinates, but no rotation was performed. Immediately after the HD or sham-cut, using the second arm of the stereotaxic, a stainless steel cannula (10×0.7 o.d.) was implanted into the lateral ventricle (LV) according to the following coordinates: 0.5 mm caudal to bregma, 1.5 mm lateral to bregma and 3.4 mm below the dura-mater. The LV cannula was fixed to the cranium using dental acrylic resin and jeweler screws.

4.3. *Drugs*

Angiotensin II (50 ng/1 μl) and carbachol (4 nmol/1 μl) from Sigma Chemical Co. were injected into the LV with a 10 μl Hamilton syringe connected by PE-10 polyethylene tubing to a needle, introduced into the brain through the guide cannula. The needles for injection into the LV were 2 mm longer than the guide cannula. Angiotensin II, carbachol or saline (0.15 M NaCl) were injected in a volume of 1.0 μl into the LV. Isoproterenol (30 μg/kg of body weight) was injected subcutaneously (sc).

4.4. *Arterial pressure and heart rate recordings*

One day before blood pressure recording, sham and HD rats were anesthetized with ketamine (80 mg/kg of body weight) and xylazine (7 mg/kg of body weight). Catheters were inserted into the femoral vein for drug administration and into the aorta through the femoral artery to record arterial pressure and heart rate (HR). To record mean arterial pressure (MAP) and HR the arterial catheter was connected to a Statham Gould (P23 Db) pressure transducer coupled to a pre-amplifier (model ETH-200 Bridge Bio Amplifier) that was connected to a Power Lab computer data acquisition system (model Power Lab 16SP, ADInstruments).

In the groups of rats tested acutely (1 day), arterial and venous cannulas were implanted immediately after the brain surgery. In the group of rats tested chronically (15 days), the arterial and venous cannulas were implanted 1 day before the test.

On the day of the test, freely moving conscious rats had MAP and HR recorded for 20 min and then the following sequential injections into the LV were performed: saline (0.15 M NaCl, 1 μl), ANG II (50 ng/1 μl) and carbachol (4 nmol/1 μl). The interval between saline and ANG II injections was 30 min and between ANG II and carbachol it was 4–5 h. MAP and HR were recorded for approximately 20 min after central injections.

In the group of sham and HD rats tested chronically, daily water intake was measured from 1 day before to the 14th day after brain surgery, using 100 ml burettes with 1.0 ml division fitted with a metal spout.

4.5. *Water intake tests*

Water intake was recorded using burettes with 0.1 ml division fitted with a metal spout. In one group of rats, water intake was measured each 15 min for 60 min immediately after injections of ANG II (50 ng/1 μl) or carbachol (4 nmol/1μl) into the LV. In the group of acute sham or HD, ANG II and carbachol were injected 2 and 4 days after the brain surgery, respectively. In the group of chronic sham or HD, ANG II and carbachol were injected 15 and 17 days after the brain surgery, respectively.

In another group of rats, water intake was measured each 30 min for 120 min after sc isoproterenol (30 μg/kg of body weight). The same animals were used for acute (4 days after brain surgery) or chronic (15 days after brain surgery) tests.

Animals had no access to food during water intake tests.

4.6. *Urinary excretion*

Other groups of rats were housed in metabolic cages to have urine collected for 24 h on days 4 and 15 after sham or HD surgery. Sham and HD animals were randomly divided to those that had water available ad libitum and those that were water deprived during the 24 h period of urine collection.

In the groups of sham and HD rats that had water available ad libitum, body weight was measured just before the brain surgery and every 3 days for the next 15 days.

4.7. *Histology*

At the end of the experiments, animals were deeply anesthetized with sodium thiopental (60 mg/kg of body weight, ip) and received an injection of 0.2 μl of 1% Evans Blue solution into the LV. Saline, followed by 10% formalin, were perfused through the heart. Brains were then removed, frozen, cut, stained with Giemsa stain and analyzed by light microscopy to confirm the position of the HD and the injections into the LV. Coronal sections were used to confirm the site of injections into the LV and horizontal sections to analyze HD.

4.8. *Statistical analysis*

The results are presented as means ± SEM. Cardiovascular responses and water intake to icv or sc treatments and daily water intake were compared by two-way ANOVA followed by Student-Newman–Keuls test. Baseline MAP and HR were analyzed by Student's *t* test. Urinary excretion was evaluated by one-way ANOVA followed by Student-Newman–Keuls test. Differences were considered significant at $p<0.05$.

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