### http://www.hh.um.es

Cellular and Molecular Biology

# Expression of certain proteins in the subfornical organ and cerebrospinal fluid of spontaneously hypertensive rats

I. González-Marrero<sup>1,2</sup>, E.M. Carmona-Calero<sup>1,2</sup>, P. Fernández-Rodríguez<sup>1</sup>,

H. Pérez-González<sup>2</sup>, C. Ormazabal-Ramos<sup>3</sup>, L. Castañeyra-Ruiz<sup>1</sup>, C.G. Pérez-García<sup>2</sup>,

I. Martínez-Peña-Valenzuela<sup>2</sup>, A. Castañeyra-Ruiz<sup>1</sup>, A. Castañeyra-Perdomo<sup>1,2</sup> and R. Ferres-Torres<sup>2</sup>

<sup>1</sup>Department of Biotechnology, Institute of Research and Sciences of Puerto del Rosario, Fuerteventura, Spain,

<sup>2</sup>Department of Anatomy, Faculty of Medicine, University of La Laguna, Tenerife, Spain and

<sup>3</sup>Department of Pediatric Medicine, Faculty of Medicine, University of La Laguna, La Laguna, Tenerife, Spain

Summary. The objective of this study was to analyze the proteins in the cerebrospinal fluid of spontaneously hypertensive rats and to study their possible role in the relationship between hydrocephalus, arterial hypertension and variations in the subfornical organ. Brains and cerebrospinal fluid from control Wistar-Kyoto rats and spontaneously hypertensive rats sacrificed with chloral hydrate were used. Cerebrospinal fluid and extract of subfornical organ were processed by protein electrophoresis. Antisera against protein bands of 141, 117 and 48 kDa and Concanavalin A were used for immunohistochemical and western blot study of the subfornical organ, adjacent circumventricular structures and cerebrospinal fluid. Ventricular dilation in the spontaneously hypertensive rats and the presence of quite a lot of protein bands in the cerebrospinal fluid of the hypertensive rats, which were either not observed or scarcely present in the cerebrospinal fluid of the Wistar-Kyoto rats, were confirmed. The subfornical organ, third ventricle ependyma and choroideus plexus showed immunoreactive material for antibodies against 141kDa, 117 and 48 kDa proteins band (anti-B1, anti-B2 and anti-B3). The larger amount of the immunoreactive material was found in the subfornical organ of the spontaneously hypertensive rat. Our results and the alterations observed by other authors in the subfornical organ in hydrocephalic and hypertensive rats support the possibility that this circumventricular organ, some proteins of the cerebrospinal fluid and ventricular dilation could be connected with the physiopathology of this type of hypertension.

**Key words:** Cerebrospinal fluid, Protein, Hypertensive rats, Subfornical organ

# Introduction

The cerebrospinal fluid (CSF) is a dynamic, multifunctional system (Starcevic et al., 1988; Bonadio, 1992) secreted from several different central nervous system (CNS) structures, and any change or alteration in the CSF composition characterizes many pathological processes of the central nervous system (Starcevic et al., 1988). The human CSF (Starcevic et al., 1988; Bonadio, 1992; Yuan and Desiderio, 2005) contains various proteins, two of the main components are albumin (70%) and gammaglobulin (10 to 15%), and has an overall albumin: globulin ratio of about 5:1. The CSF absolute protein concentration is age-dependent and the CSF mean protein concentrations range from 15-45 mg/dl (being greatest at birth: 45-120 mg/dl) (Starcevic et al., 1988; Yuan and Desiderio, 2005). It has been demonstrated that the CSF protein composition is altered in the hydrocephalus (Rodriguez et al., 2001) and that the spontaneously hypertensive rats experience a progressive increase in the brain ventricles size (Ritter and Dinh, 1986).

The subfornical organ (SFO) like other circumventricular organs, is characterized by the absence of the blood-brain barrier. The SFO has connections with the brain regions involved in the central regulation of drinking, blood pressure and cardiovascular function. Among these regions is the anteroventral region of the third ventricle (AV3V) (Akert and Steiner 1970; Lind et al., 1982; Collister and Hendel 2005). It is also described that the orexin-A (neuropeptide primarily produced in

*Offprint requests to:* Romualdo Ferres-Torres, Departamento de Anatomía, Facultad de Medicina, Universidad de la Laguna, 38071 La Cuesta, La Laguna, Tenerife, Spain. e-mail: roferres@ull.es

the lateral and perifornical hypothalamus) injected into the SFO induce hypotension and decrease heart rate by a reduction in sympathetic tone (Smith et al., 2007). A high sodium diet also activates neurons in the SFO and in the other hypothalamic nuclei (Budzikowski et al., 1997; Collister and Hendel 2005), and it has been suggested that the SFO is necessary for the complete hypertensive response to chronic Angiotensin II administration (Hendel and Collister, 2005). Moreover, alterations in the receptors have been described in the SFO of the SHR (Saavedra et al., 1986a, b). Furthermore underdevelopment of the SFO and variations of the angiotensin II receptor content of the subfornical organ have also been found in hydrocephalic animals (Açikgöz et al., 1999).

Since SHR rats present ventricular dilation (Ritter and Dinh, 1986; Ritter et al., 1988), the SFO is altered in the hydrocephalus (Açikgöz et al., 1999) and moreover, the angiotensin II receptors were found to be increased in the SFO in hypertensive and hydrocephalic rats when compared to the control animals (Saavedra et al., 1986a,b; Açikgöz et al., 1999), the aim of the present work is to analyze the proteins in the CSF of spontaneously hypertensive rats in order to study their possible role in the relationship between hydrocephalus, arterial hypertension and variations of the subfornical organ.

# Material and methods

30 normotensive male Wistar-Kyoto rats (WKY) and 30 spontaneously hypertensive male rats (SHR) from Charles River Laboratories Spain S.A. (Barcelona, Spain) were used. The 30 WKY rats were divided into two control groups formed of 15 animals each: the WKY-26 group which was composed of rats sacrificed at 26 weeks after birth and the WKY-38 group which was composed of rats sacrificed at 38 weeks after birth. 30 SHR rats were also divided into two groups: SHR-26 and SHR-38 groups sacrificed at the same ages as the controls. The rats were anesthetized with chloral hydrate (200 ml/100g of body weight at 160mg/ml) and before sacrifice 100 ml of CSF from the cistern magna of each animal was extracted. Extract of SFO were prepared from 7 rats of each group, which were processed by protein electrophoresis according to Laemmli (1970) (sodium docecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE, 5-15% gradient). 8 rats from each group were fixed by intracardiac perfusion with Bouin's fluid, dehydrated and embedded in paraffin under standard conditions. Brains were cut into four serial coronal sections. One of the serial coronal sections was stained by the Klüver-Barrera method.

The polyclonal antibodies raised against CSF protein band of 141, 117 and 48 kDa in mice, referred to as anti-B1, anti-B2 and anti-B3 as in a previous work (Martinez-Peña y Valenzuela et al., 2006), were used as the primary antibodies. The sections at the level of the SFO from the WKY and SHR rats were incubated simultaneously in the same coplin jar each containing: anti-B 1:500, Anti-B2 1:300 and anti-B3 1:100. Incubation was for 24 h at room temperature, followed by "DAKO" StreptABCcomplex/HRP Duet, Mouse/ Rabbit procedure. The peroxidase reaction product was visualized using nickel intensified diaminobenzidine reaction. Densitometry of the immunoreactive reaction (ir) was performed using a "Magiscan" Analysis Imagen System (Joyce Loebl).

Western blot of CSF (equal volume of WKY and SHR) protein and SFO extracts protein (equal weight) were used to show CSF protein bands of 141, 117 and 48 kDa (B1, B2 and B3). The blotted proteins were incubated in Tris-saline (TBS) non-fat milk 5% for 60 minutes and then incubated in the primary antibodies anti-B1, anti-B2 and anti-B3 at 1:500 for 2 h. Antimouse IgG labeled with peroxidase (PIERCE) was used as the secondary antibody at a dilution of 1:5000 for 1.30 h at room temperature. The membranes were incubated with Concanavalin A (Con A) 5.0 mg/ml (Con A peroxidase labeled Sigma) for 1h and washed in Trissaline-Tween (TBS-T) (Tween 20, 0.1% Sigma) to visualize the lectin binding. The peroxidase reaction products from western blot and lectin binding were visualized by quimioluminiscence (PIERCE).

To validate the controlled method specificity the primary antibody was omitted. The specificity of the antiserums was evaluated by means of absorption test incubating the antiserum overnight with the homologous antigen. The antigen was able to eradicate the immunostaining.

# Results

#### Ventricular dilatation

The brain ventricles in the SHR-26 showed a slight dilatation, but SHR-38 rats presented a greater increase in ventricle size mainly in lateral and third ventricle than those of the normal size of the WKY rats (Fig. 1). The Sylvian aqueduct is permeable and the fourth ventricle is also lightly dilated in the SHR groups.

#### Electrophoresis and western blot

#### CSF

In the electrophoresis study of the CSF, we found quite a lot of protein bands in the CSF of the SHR rats that were scarcely present or not expressed in the CSF of WKY rats and antibodies against three of them (bands of 141, 117 and 48 kDa) were obtained.

We observed that anti-B1, anti-B2 Anti-B3 marked their correspondent band of 141, 117 and 48, kDa and the reaction was more intense in the SHR than WKY.

Con A reactive material was found in the 141 and 117 kDa band of CSF and in the several other bands of different molecular weight, in general the reaction was higher in SHR than WKY, but 48 kDa band was not

# SFO-Extracts

Anti-B3 immunoreactive material was mainly observed in a band that corresponded with a molecular weight of 48 kDa in the SFO extracts, and the intensity of the reaction was higher in SHR subfornical organ than in the control rats. The anti-B1 and anti-B2 immunoreactive reaction was observed in the bands of their own molecular weight. The expression of anti-B2 and anti-B1 was also higher in SFO-extract of SHR group (Fig. 2).

Immunohistochemistry

Subfornical organ (Fig. 3, Graphic 1)

A weak immunochemical reaction for anti-B1 was found in the in the SFO of the WKY rats (Fig. 3A,C).



Fig. 1. Photographs with the ventricular dilation at the level of the SFO, optic chiasm and lateral and third ventricle, WKY (**A**) and SHR (**B**) Bar: 500  $\mu$ m. AC: anterior commissure; CP: choroids plexus; FC: fornix commissure; LV: lateral ventricle; V3: thirst ventricle; \*: subfornical organ.



**Fig. 2.** Western blot of cerebrospinal fluid (CSF) and subfornical organ (SFO) extract. Anti B1, B2 and B3 in SFO extract of WKY and SHR. B1: 141 kDa, B2: 117 kDa and, B3: 48 kDa bands. Concanavalin A (Con A) in CSF of WKY and SHR rats groups. B1: 141 kDa, B2: 117 kDa. WKY: Wistar-Kyoto rats; SHR: spontaneously hypertensive rats; SFO: subfornical organ; B1, B2, B3: Anti Bands B1, B2 and B3; Concanavalin A: Con A.



**Fig. 3.** Photographs of the SFO of the WKY and SHR rats immunostained with anti-B1. Coronal view of the SFO (**A**: WKY and **B**: SHR). Bar: 80 μm. Ependymal layers and parenchyma of the SFO. (**C**: WKY and **D**: SHR). Bar: 20 μm. WKY: Wistar-Kyoto rats; SHR: spontaneously hypertensive rats; SFO: subfornical organ; N: neurons, V3: thirst ventricle.

The greater amount of the anti-B1-ir was found in the SFO organ of the SHR (Fig. 3B,D); the anti-B1-ir was evenly distributed in the parenchyma, perivascular space in the ependymal and subependymal layer; besides, immunoreactive material was also found in some neurons of the SHR SFO (Fig. 3D). The immunoreactive densitometry study showed that the SFO of the SHR rats was significantly bigger than the WKY (Graphic 1).

Anti-B2 immunoreactive material was found in the ependymal layer, in groups of neurons and in a generalized form in the parenchyma of the SFO organ of WKY and SHR rats. The amount of anti-B2-ir was slightly, higher but not significantly, in the hypertensive group (Fig. 4, Graphic 1).

The presence of the anti-B3-ir was observed in several neurons and in the ependymal and subependymal layers in the SFO of both WKY and SHR, but the biggest anti-B3 staining and expression were observed in SHR groups (Fig. 5). The SFO of the SHR rats also showed anti-B3-ir significantly higher than the WKY by densitometry analysis (Graphic 1).

# Choroideus plexus (CP)

Immunoreactive material for the three antibodies was present in the ventricular ependyma and Choroideus





Fig. 5. Photographs of the SFO of the WKY and SHR rats immunostained with anti-B3. Coronal view of the SFO (A: WKY and B: SHR). Bar: 240  $\mu$ m. (C: WKY and D: SHR). Bar: 80  $\mu$ m Ependymal layers and parenchyma of the SFO. (E: WKY and F: SHR). Bar: 20  $\mu$ m. SFO: subfornical; CP: choroids plexus; Ep: ependyma; F: fórnix; N: neurons; V3: thirst ventricle.



**Graphic 1.** Densitometry of the anti-bands (B1, B2 and B3) immunoreactions (ir) in the subfornical organ (SFO) of Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR). \*: significant difference.

Plexus (CP) of the III ventricle, and no differences between SHR and WKY rats were observed (Fig.5 A, B).

# Discussion

The cerebrospinal fluid proteins are increased in the hydrocephalus and the elevated levels of brain specific proteins indicate brain damage due to hydrocephalus. The reported changes suggest disturbance of oxidative metabolism and neurotransmission in the hydrocephalic brain, damage in the periventricular cells, and on the other hand the blood brain barrier function (CSF/serum albumin ratio) increased with age (Ritter et al., 1988; Beems et al., 2003). We found hydrocephalus and an increase in the number of the protein bands in the CSF of the hypertensive rats (SHR), and more angiotensin II receptors are present in the SHR rats (Saavedra et al., 1986b).

A correlation between the duration of the angiotensin II action and the degree of hydrocephalus was observed after the prolonged action of angiotensin II application on macrostructures of the brain (Artychina et al., 1980). Moreover, Ritter and Dinh (Ritter and Dinh, 1986; Ritter et al., 1988) found a progressive increase of ventricular size from 4 to 56 weeks of age. In a previous study (Carmona-Calero et al., 2005), we did not find qualitative ventricular dilation in SHR rats of 15 weeks of age but, in agreement with Ritter and Dinh (1986) the results of the present work showed a slight ventricular dilation at 26 weeks and a clearly present hydrocephalus at 38 postnatal weeks.

The SFO is necessary for the full hypertensive response to chronic angiotensin II administration (Hendel and Collister, 2005). The angiotensin II receptors are increased in number and the angiotensin II content decreased in the SFO of SHR group (Saavedra et al 1986a,b; Carmona-Calero et al., 2005). The captopril treatment of the SHR rats produced a decrease in arterial hypertension and in the number of the angiotensin II receptors but an increase in angiotensin II content (Saavedra et al., 1986a,b; Carmona-Calero et al., 2005). However, captopril treatment and experimental hypertension did not alter the brain ventricle size (Ritter et al., 1988, 1988), but the angiotensin II application produced a hydrocephalus in rats (Artychina et al., 1980).

Arterial hypertension occurs in the human obstructive hydrocephalus (Verrees et al., 2003) and, although the neurological deterioration produced by the lateral and III ventricles dilation were resolved swiftly following placement of ventricular catheters and administration of diuretic agents, systemic blood pressure did not decrease with the release of cerebrospinal fluid and resolution of increased intracranial pressure. A decrease in systemic blood pressure lagged well behind improvement in neurological status; the patients remained morbidly hypertensive until systemic blood pressure was controlled with medications that produce a focal transudation of protein and fluid (Verrees et al., 2003).

The subcommissural organ (SCO) showed anti-B1 and anti-B2 immunoreactive material but not anti-B3, besides which the anti-B1 was lower and anti-B2 was higher in the hypertensive groups with respect to the control groups (Martinez-Peña y Valenzuela et al., 2006). In the present work, we found anti-B1, anti-B2 and anti-B3 immunoreactive material in the SFO and choroideus plexus (CP), moreover, when comparing the SHR and WKY groups, the expression of the three antibodies was higher in the SFO of the hypertensive groups but not in the CP, the greater amount of the anti-B1 and anti-B3 was found in the SFO of the SHR.

Glycoproteins play important roles in cell interaction tissue morphogenesis, immune reactions, and pathologies. CSF contains many glycoproteins, and each glycoprotein has different glycosylated isoforms (Yuan and Desiderio, 2005). We found several bands marked with lectin Con A and two of them were 141 and 117 kDa protein bands but not the 48 kDa, which agrees with the fact that lectin ConA is found SCO (Rodriguez et al., 1989; Ueda et al., 1997)) and SFO (Verrees et al., 2003). Anti-B1 and anti-B2 but not anti-B3 were expressed in the SCO. These findings could mean that the 141 and 117 kDa protein bands are common to the SCO, SFO and CP that the 48 kDa protein band is more specific to the SFO and CP. Thereafter, the 48 kDa band is probably more connected with this type of hypertension since it is increased in SHR groups. On the other hand, lectin ConA reaction was also observed in the other CSF protein band of higher and lower molecular weight (against which no antibodies were obtained). The lectin histochemical reaction was higher in the SHR than in WKY groups.

The hypertensive animals showed an increase in ventricular size and the angiotensin II receptor content in the SFO and other circumventricular organs increased after experimental hydrocephalus and hypertension (Artychina et al., 1980, Açikgöz et al., 1999). Therefore, the variations in the amount of the AT1 receptors and angiotensin II are closely connected with hypertension and ventricular dilation. We think that in the SHR rats, the circumventricular structures that participate in blood pressure cerebral autoregulation led to breakdown of the blood-brain barrier which it is week or lacking in the SFO, therefore proteins could pass throughout SFO to CSF. This findings support the possibility that the SFO alterations, the variations in the proteins of the CSF and the modification in the ventricular size could be connected with the etiologic and development aspects of this type of the hypertension.

Acknowledgements. This work was supported by the Canary Foundation for Research and Health Foundation (FUNCIS) project n° 74/03.

# References

- Açikgöz B., Akpinar G., Bingöl N. and Usseli I. (1999). Angiotensin II receptor content within the circumventricular organs increases after experimental hydrocephalus in rats. Acta Neurochir. 141, 1095-1099.
- Akert K. and Steiner F.A. (1970). The ganglion psalterii. A brief review of anatomical and physiological aspects of the subfornical organ in mammals. Curr. Res. Neurosc. 10, 1-14.
- Artychina N.I., Ljowschina I.P., Kuwajewa O.F. and Hecht K. (1980). Experimental hydrocephalus using prolonged angiotensin II application. Psychiatr. Neurol. Med. Psychol. 32, 695-703.
- Beems T., Simons K.S., Van Geel W.J., De Reus H.P., Vos P.E. and VerbeeK M.M. (2003). Serum- and CSF-concentrations of brain specific proteins in hydrocephalus. Acta Neurochir. 145, 37-43.
- Bonadio W.A. (1992). The cerebrospinal fluid: physiologic aspects and alterations associated with bacterial meningitis. Pediatr. Infect. Dis. J. 11, 423-431.
- Budzikowski A.S., Vahid-Ansari F., Robertson G.S, and Leenen F.H. (1997). Patterns of neuronal activation during development of sodium sensitive hypertension in SHR. Hypertension 30, 1572-1577.
- Carmona-Calero E., Pérez-González H., Martínez-Peña y Valenzuela I., González-Marrero I Pérez-García C.G., Marrero-Gordillo N., Ormazabal-Ramos C., Castañeyra-Perdomo A. and Ferres-Torres
  R. (2005). Effect of the arterial hypertension and captopril treatment on the angiotensin II content in the subfornical organ. A study in SHR rats. Histol. Histopathol. 20, 135-138.
- Collister J.P. and Hendel M.D. (2005). Chronic effects of angiotensin II and receptor antagonists in subfornical organ-lesioned rats. Clin. Exp. Pharmacol. Physiol. 32, 462-466.

Hendel M.D. and Collister J.P. (2005). Contribution of the subfornical

organ to angiotensin II-induced hypertension. Am. J. Physiol. Heart. Circ. Physiol. 288, H680-H685.

- Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.
- Lind R.W., Van Hoesen G.W. and Jhonson A.K. (1982). An HRP study of the connections of the subfornical organ in the rat. J. Comp. Neurol. 210, 265-277.
- Martínez-Peña y Valenzuela I., Carmona-Calero E.M., Pérez-Gonzalez H., Ormazabal-Ramos C., Fernández-Rodríguez P., González-Marrero I., Castañeyra-Perdomo A. and Ferres-Torrer R. (2006).
  Alterations of the cerebrospinal fluid proteins and subcommissural organ secretion in the arterial hypertension and ventricular dilatation. A study in SHR rats. Histol. Histopathol. 21, 179-185.
- Ritter S. and Dinh T.T.(1986). Progressive postnatal dilation of brain ventricles in spontaneously hypertensive rats. Brain Res. 370, 327-332.
- Ritter S., Dinh T.T., Stone S. and Ross N. (1988). Cerebroventricular dilation in spontaneously hypertensive rats (SHRs) is not attenuated by reduction of blood pressure. Brain Res. 450, 354-359.
- Rodriguez E.M., Rodriguez S., Schoebitz K., Yulis C.R., Hoffmann P., Manns V. and Oksche A. (1989). Light- and electron-microscopic investigation of the rat subcommissural organ grafted under the kidney capsule, with particular reference to immunocytochemistry and lectin histochemistry. Cell Tissue Res. 258, 499-514.
- Rodriguez E.M., Oksche A. and Montecinos H. (2001). Human subcommissural organ, with particular emphasis on its secretory activity during the fetal life. Micros. Res. Tech. 52, 573-590.
- Saavedra J.M., Correa F.M., Kurihara M. and Shigematsu K.(1986). Increased number of angiotensin II receptors in the subfornical organ of spontaneously hypertensive rats. J. Hypertens. Suppl. 4, S27-S30.
- Saavedra J.M., Correa F.M., Plunkett L.M., Israel A., Kurihara M. and Shigematsu K. (1986). Binding of angiotensin and atrial natriuretic peptide in brain of hypertensive rats. Nature 320, 758-760.
- Smith P.M., Samsom W.K. and Ferguson A.V. (2007). Cardiovascular actions of orexin-a in the rat subfornical organ. J. Neuroendocrinol. 19, 7-13.
- Starcevic V.P., Morrow B.A., Farner L.A., Keil L.C. and Severs W.B. (1988). Long-term recording of cerebrospinal fluid pressure in freely behaving rats. Brain. Res. 462, 112-117.
- Ueda T., Fujimori O. and Yamada K. (1997). Lectin-binding glycoconjugates in the subfornical organ of rat. Okajimas Folia Anat. Jpn. 73, 325-334.
- Verrees M., Fernandes Filho J.A., Suarez J.I. and Ratcheson R.A. (2003). Primary hypertension induced cerebellar encephalopathy causing obstructive hydrocephalus. Case report Neurosurg. 98, 1307-1311.
- Yuan X. and Desiderio D.M. (2005). Proteomics analysis of human cerebrospinal fluid. J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 815,179-189.

Accepted June 22, 2007