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Additional Information

A multi-omics study for uncovering molecular mechanisms associated with hyperammonemia-induced cerebellar function impairment in rats

Original article

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ABSTRACT

Patients with liver cirrhosis may develop covert or minimal hepatic encephalopathy (MHE). Hyperammonemia (HA) and peripheral inflammation play synergistic roles in inducing the cognitive and motor alterations in MHE. The cerebellum is one of the main cerebral regions affected in MHE. Rats with chronic HA show some motor and cognitive alterations reproducing neurological impairment in cirrhotic patients with MHE. Neuroinflammation and altered neurotransmission and signal transduction in the cerebellum from hyperammonemic (HA) rats is associated to motor and cognitive dysfunction but underlying mechanisms are not completely known. The aim of this work was to use a multi-omics approach to study molecular alterations in cerebellum from hyperammonemic rats to uncover new molecular mechanisms associated with hyperammonemia-induced cerebellar function impairment.

We analysed metabolomics, transcriptomics and proteomics data from the same cerebellums from control and HA rats and performed multi-omic integrative analysis of signaling pathways enrichment with PaintOmics tool. Histaminergic system, the corticotropin-releasing hormone, cyclic GMP-protein kinase G pathway and intercellular communication in the cerebellar immune system were some of the most relevant enriched pathways in HA rats. In summary, this is a good approach to find altered pathways, which helps to describe the molecular mechanisms involved in the alteration of brain function in rats with chronic HA and to propose possible therapeutic targets to improve MHE symptoms.

Keywords: hyperammonemia, multi-omics, cerebellum, signaling pathways, neurotransmission, immune system.

1. INTRODUCTION

Patients with liver cirrhosis may develop covert or minimal hepatic encephalopathy (MHE) with mild cognitive impairment, attention deficits and psychomotor slowing which impair quality of life, reduces life span and increases accidents, falls and hospitalizations. MHE affects several million people and is a serious health, social and economic problem (Felipo, 2013).

Hyperammonemia (HA) and peripheral inflammation play synergistic roles in inducing the cognitive and motor alterations in MHE (Shawcross et al., 2004; Montoliu et al., 2009; Felipo et al., 2012).

The cerebellum is one of the main cerebral regions affected in MHE. Cerebellar blood flow in cirrhotic patients correlate with bimanual and visuomotor coordination tests and with cGMP and nitric oxide metabolites (Felipo et al 2014). Neuroinflammation in the cerebellum must be involved in these alterations. Cerebellums from patients dead with different grades of liver disease, from steatosis to steatohepatitis, show neuroinflammation (Balzano et al, 2018 a, b).

Rats with chronic HA show some motor and cognitive alterations reproducing neurological impairment in cirrhotic patients with MHE. This animal model is widely used to study the mechanisms involved in such motor and cognitive alterations. Some of the impaired brain functions in these rats are associated with cerebellar alterations. Learning of a conditional discrimination task is impaired in HA rats, associated to a decrease of extracellular cGMP levels and decreased function of the glutamate (Glu)-nitric oxide (NO)-cGMP pathway (Hermenegildo et al, 1998; Erceg et al 2005). Motor incoordination is induced by increased extracellular GABA in the cerebellum in HA rats. The increased GABA is due to reversal of the GABA transporter GAT-3 in astrocytes, due to neuroinflammation (Hernández-Rabaza et al, 2016; Cabrera-Pastor et al, 2018a).

Chronic HA per se is enough to induce neuroinflammation with activation of microglia and increased inflammatory markers in the cerebellum, associated with impaired cognitive function. Reducing neuroinflammation with ibuprofen restores learning in a Y maze task in HA rats (Rodrigo et al, 2010).

However, the molecular mechanisms underlying HA-induced cerebellar neuroinflammation and alterations in neurotransmission and the cGMP pathway, as well as the relationship between the two, is not fully understood.

Recently, some omic approaches have been performed to study molecular mechanisms and biomarkers of different neurological diseases as Parkinson (Maver and Peterlin, 2011), glioblastoma, or aging and cognitive decline (Tasaki et al, 2018).

A multi-omic analysis allows discovering molecular alterations and affected pathways from a more global perspective, since each omic data type provides different and complementary information of the biological system under study. In this context, a multi-omic study can contributes to better understanding the mechanisms involved in neurological alterations, and this information can allow finding new therapeutic targets.

The aim of this work was to perform a multi-omic integration analysis in the cerebellum from HA rats to discover new altered pathways, which can explain some impaired brain functions associated to the cerebellum and understand the mechanisms involved.

2. MATERIAL AND METHODS

2.1. Animals

For this study, 10 male Wistar rats (Charles River), 125-150 g weight, were randomly distributed into two groups, control and HA rats. HA rats were made hyperammonemic by feeding them an ammonium-containing diet (30 % ammonium acetate, Panreac) for 4

weeks as previously described (Felipo et al., 1988). Animals remained during this period in a controlled environment with food and drink ad libitum and a 12:12 h light: dark cycle, with 55 ± 5% of humidity and 22 ºC of temperature. The experiments were approved by the Comite Ético de Experimentación Animal (CEEA) of our Center and by the Conselleria de Agricultura of Generalitat Valenciana, were performed in accordance with guidelines of the Directive of the European Commission (2010/63/EU) for care and management of experimental animals and comply with the ARRIVE guidelines for animal research.

The animals were sacrificed by decapitation. Cerebellums were dissected. One half was rapidly frozen in liquid nitrogen and stored at -80ºC. The other half cerebellum was put in RNA stabilizer (RNA Later, from Invitrogen) to transcriptomic analysis. The frozen cerebellums pulverized in liquid nitrogen to complete homogenization. From this sample, 35 mg sent to a proteomic facility of the University of Valencia and 35 mg used for the metabolomic analysis.

2.2. Proteomic analysis

The proteomic analysis was carried out by the proteomics facility of Central Service for Experimental Research Support at the University of Valencia that belongs to ProteoRed, PRB2-ISCIII. Proteins were extracted from the samples and quantified by colorimetric assay RC_DC Lowry (Biorad) following the manufacturer's instructions, and loaded in 1D SDS-PAGE gel. After that, samples were digested using grade Trypsin (Promega) in order to obtain peptides to sequence for the spectral library acquisition. Peptides were loaded onto an analytical column (Liquid Chromatography (LC) column, 3C18-CL, 75m x 12 cm, Nikkyo) and were analyzed in a mass spectrometer (MS) nanoESI qQTOF (5600 TripleTOF, from SCIEX). The tripleTOF was operated in information-dependent acquisition mode.

Once the spectral library was acquired, the software Protein Pilot v5.0 was used to identify proteins and associate the peaks to known proteins. Default parameters were used to generate peak list directly from 5600 TripleTof wiff file of the pooled sample. With these parameters Uniprot_mammals (UniProt Consortium, 2018) and RefSeq at NCBI (O'Leary et al, 2016) databases were interrogated. To avoid using the same spectral evidence for more than one protein, the identified proteins were grouped based on MS/MS spectra by the Protein-Pilot Progroup algorithm. Thus, the Uniprot_mammals database was used for library building.

For quantification of the proteins, a SWATH LC-MS/MS analysis was performed. For this analysis, peptides were loaded onto an analytical column (LC Column, 3 C18- CL, 75umx12cm, Nikkyo) and analyzed in a mass spectrometer nanoESI qQTOF (5600 TripleTOF, from SCIEX). The tripleTOF was now operating in swath mode. The used Swath windows were: 15 Da window widths from 450 to 1000 Da, 37 windows.

The wiff files obtained from Swath experiment were analyzed by Peak View 2.1; this software is used to identify and quantify proteins from data obtained in mass spectrometry experiments. In our experiment, 1081 proteins were quantified. The peaks obtained by Peak View were analyzed with Marker View 1.3, which is used to perform visualize data obtained by SWATH experiments.

2.3. Transcriptomic analysis

For the transcriptomic analysis, RNA extraction was performed in the genomic service of our center (CIPF)**.** Samples obtained were sent to the Centre for Genomic Regulation (CRG) located in Barcelona, to be sequenced by RNA-Seq technique and to obtain the quantification of mRNAs. The sequencing platform used was Illumina HiSeq 2500 High Output V4 and a Single-Read Sequencing (single-end) protocol in reads that had a length of 50 bp. The quality control of sequencing reads was performed with FastQC v0.11.5.

Next, reads were aligned to the reference genome from Ensembl (Zerbino et al, 2018) version 88 for Rattus norvegicus with STAR 2.5.3a software (Dobin et al, 2013). A total of 32624 genes were quantified.

2.4. Metabolomic analysis

Metabolomic analysis was performed with a kit of the BIOCRATES platform, the 180 phenotyping kit to analyze amino acids, biogenic amines and lipids. Tissue samples were homogenized in methanol by sonication (3*20s) and then centrifuged (5 min, 10000 g). Next, 10 µL were put in the plate of BIOCRATES for LC-MS analysis. The analysis was performed following the instructions for the BIOCRATES Kit in a QTRAP 4500 spectrometer from SCIEX. This kit has two main parts: an LC-MS protocol to quantify amino acids and biogenic amines, and the Field Injection Analysis (FIA) part, to analyze lipids (sphingolipids, glycerophospholipids, acylcarnitines and sugar).

Amino acid concentrations were calculated with de software Analyst from SCIEX. Data from FIA were analyzed with the MetIDQ software of BIOCRATES to obtain metabolite concentrations. As a result of this process, we obtained the concentrations of 34 amino acids, 14 sphingolipids, 9 acylcarnitines and 80 glycerophospholipids.

2.5 Measurement of extracellular histamine in the cerebellum.

For in vivo microdialysis, rats were anesthetized with isoflurane at 5% for induction and 1.5–3% for maintenance. A microdialysis guide was implanted in the cerebellum (AP-10.2, ML-1.6, and DV- 1.2), as in Cabrera-Pastor et al. (2016a). After 48 h a microdialysis probe was implanted in the freely moving rat. Probes were perfused (3 lL/ min) with artificial cerebrospinal fluid (in mM): NaCl, 145; KCl, 3.0; CaCl2, 2.26; buffered at pH 7.4 with 2 mM sodium phosphate. After a 2–3 h stabilization period, samples were collected every 30 min and stored at 80 C. The chromatographic separation was performed on a HPLC system with a Waters Atlantis HILIC Silica column (3.0 mm i.d.,

10 0mm 2.1 mm).The mobile phase was composed of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using the following gradient program: 90% A 0–1.5 min, 15% A at 1.7 min, 15% A 3 min; 90% A 3.1 min and 90% A 4.5 min. The flow rate was 0.4 mL/min; the column temperature was 30 C and the injection volume was 30 uL. A QTRAP 4500 from SCIEX equipped with an ESI ion source was operated in positive ion mode. The following conditions were used: Entrance potential 10, Curtain gas 20, Declustering potential 31 V, Collision energy 15 eV, GAS1 40 and GAS2 30, 600 C and 4500 V in MRM mode with the following transition for quantification of histamine: 112 *m/z* >95 m/z.

2.6 Primary cultures of cerebellar neurons.

Primary cultures of cerebellar granule neurons were prepared as previously described (Llansola et al, 2005 and 2009). Briefly, cerebella from 7-day-old Wistar rats were rapidly dissected and incubated with 3 mg/ml dispase (grade II) for 30 min in a 5% CO_2 incubator at 37ºC. The supernatant was removed, and tissue was incubated with basal Eagle medium (BME) containing 40 μg/ml DNase I for 20 minutes. The cellular suspension was filtered through a mesh with a pore size of 90 μm and rinsed three times with BME. Finally, the cells were resuspended in complete medium (BME containing 10% heat-inactivated fetal bovine serum (GIBCO), 2 mM glutamine, 100 μg/ml gentamicin, and 25 mM KCl). Cells were plated onto polylysine-coated plates, except for viability experiments in which cells were plated in coverslips coated with polylysine (312,000 cells/cm^2 in both cases, in 35-mm-diameter plates). After 20 min at 37ºC, medium containing unattached cells was removed, and fresh medium was added. The cells were grown at 37ºC in a 5% CO_2 atmosphere. To prevent proliferation of non-neuronal cells, 10 μM cytosine arabinoside was added 24 h after plating. Glucose (5.6 mM) was added to the culture medium twice a week.

2. 7 Determination of free intracellular calcium concentration

After 10-13 days of culture, basal and glutamate or NMDA-induced increase of intracellular calcium was determined using Fura2-AM as previously described (Llansola et al, 2009). Briefly, neurons were washed three times with pre-warmed Locke's solution without magnesium. Neurons were charged with the fluorescent probe (4μM Fura2-AM) for 45 minutes and then washed 4-5 times to eliminate excess of probe and incubated for 15 minutes to complete probe cutting by intracellular esterases. Plates were put on a chamber with controlled temperature (32 ºC) and with continuous superfusion of Locke's solution. Fluorescence was measured with a fluorescence microscopy connected to a Digital CCD camera (Hamamatsu), using as λ excitation 340 and 380 nm and λ emission 510 nm. The ratio F340/F380 was determined with the Aquacosmos software (Hamamatsu). To measure the increase of intracellular calcium induced by NMDA or glutamate we perfused 12 ml of 0.3 mM NMDA or the indicated concentration of glutamate in Locke's solution followed by Locke's solution perfusion. To calculate free calcium concentration in the neurons (nM) we measured the ratio R= F340/F380 and used the formula: [Ca](nM) = Kd*Fmax/Fmin*(R/Rmin)/(R/Rmax), were Fmax is the maximal fluorescence at 380 nm and Rmax is the maximal ratio F340/F380. Fmax and Rmax were measured in these cultures using a solution of Locke + 10 μM ionomicin + 20 mM calcium. Fmin is the fluorescence at 380 nm when calcium concentration is 0 and Rmin the ratio F340/F380 at calcium concentration =0. Fmin and R min were measured in these neurons using a solution of Locke without calcium + 10 μM ionomicin + 5 mM EGTA. The constant used in this formula is: Kd= 230, which is the value for Fura2 at the temperature and pH conditions used in these experiments. The other values were: Fmax/Fmin= 1,75; Rmin= 0,0385 and Rmax 0,101. The value of R is the mean of the ratio F340/F380 for at least 40 neurons.

2. 8 Determination of the content of proteins by western blot.

Homogenates of cerebellum were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting as previously described (Cabrera-Pastor et al, 2018a). Primary antibodies were against GluA4 (1:100, CHEMICON AB1508), Na+/K+-ATPase (1:1000, MILLIPORE 05-369), CD74 (1:500, MA5-32232 INVITROGEN) and Claudin 10 (1:1000, Biorbyt orb48053). Beta-actin was used as loading control. Secondary antibodies conjugated with alkaline phosphatase were from Sigma (St. Louis, MO, USA). After development using alkaline phosphatase images were captured with a Hewlett Packard scan Scanjet 5300C. The intensities of the bands were measured using the program AlphaImager 2200 (AlphaEaseFC 2200 for Windows, Cambridge CB4 0FW, UK)

2.9. Data preprocessing

For RNA-seq data, we used NOISeq R package (Tarazona et al, 2015) to check the existence of potential technical biases. According to these results, the count data were normalized with the Conditional Quantile Normalization method from CQN package (Hansen et al, 2012)**,** after removing genes with less than 1 count per million reads on average for both control and HA groups. A total of 13647 genes remained for subsequent analyses.

Regarding proteomics data, Total Area Sum normalization provided by the MarkerView software was applied, together with voom transformation (Law et al, 2014) to obtain normally distributed data.

For metabolomics data, we first filtered out metabolites with less than 8 non-missing values, following the “80 % rule” (Smilde et al, 2005). The missing values for the remaining 131 metabolites were imputed with the mice R package (Buuren and Groothuis-Oudshoorn, 2010), with method “cart”, which is based on classification and

regression trees to calculate the values to impute. Imputed data were normalized with VSN method (Huber et al, 2002).

2.10. Differential expression analysis

We applied Limma R package (Ritchie et al, 2015) to each omic independently in order to identify differentially expressed features between controls and HA rats. Omic features were declared as differentially expressed features when having a p-value lower than 0.05 and an absolute log fold change greater than 0.5.

2.11. Multi-omics integration: PaintOmics 3

PaintOmics 3 (Hernández-de-Diego et al, 2018) input was the matrix containing the fold changes and differentially expressed features obtained from Limma analysis for each one of the three omics. PaintOmics performs a functional enrichment analysis per omic with the Fisher's Exact Test, and returns a joint enrichment significance per pathway with the Fisher's combined probability test. When visualizing pathways, the fold change of each omic feature in the pathway is displayed on a scale of red for up regulated and blue for down-regulated features.

2.12. Statistic analysis.

Statistical analyses were performed using the software program GraphPad Prism. All data were checked for normality with Kolmogorov-Smirnov test and analyzed by a Student's t-test when only two groups were compared, and by a two-way ANOVA followed by Sidack's multiple comparison tests in dose-response curve data. Results are indicated in figure legends. Significance levels were set to p=0.05.

3. RESULTS AND DISCUSSION

The multi-omics functional enrichment analysis with PaintOmics 3 tool provided a holistic view of the paths altered. Out of the 313 KEGG pathways tested for enrichment in differentially expressed features, 23 were significant when combining all the three omics (Figure 1). Significant enriched pathways related to neurotransmission or immune system were the most relevant in our study.

3.1 Neurotransmission related pathways.

The *neuroactive ligand-receptor interaction* pathway (Figure 2) showed the most significant combined p-value (p=0.00074) within the neurotransmission related pathways (Figure 1).

Features significantly upregulated in genomics were: a cholinergic receptor (*Chrne*), two metabotropic receptors (*Grm6 and Gpr83*), one hormone receptor (*Crhr2*) and a hormone (*Lhb*). Significantly downregulated genes were an adrenergic receptor (*Adra1a*) and the neuropeptide Y receptor, *Npy1r,* whereas the ionotropic glutamate receptor subunit GluA4 (GRIA4), was downregulated in proteomics. Histamine was upregulated in metabolomics (Figure 2A).

These results indicate that, in addition to glutamatergic neurotransmission, which is known to be altered in HA rats cerebellum, other less studied neurotransmitter pathways like those of histamine, epinephrine or acetylcholine are altered in cerebellum from HA rats. In addition, signaling through peptides and hormones, i-e., neuroendocrine system, was also altered, as corticotropic-releasing hormone receptor, luteinizing hormone and neuropeptide Y receptor were differentially expressed.

Levels of histamine and the gene of its receptor (*Hrh1*) were upregulated (Figure 2A).

We wanted to check if the increase of histamine found in this metabolomic analysis is reflected in an increase in extracellular histamine in the cerebellum of HA rats. We measured, by LC-MS, the concentration of histamine in samples obtained by in vivo microdialysis in the cerebellum of control and HA rats. The concentration of extracellular histamine in cerebellum of HA rats was significantly higher (137±18 nM, n=12) than in control rats (60±13 nM, n=10, $p < 0.01$). Then, increased extracellular histamine levels in cerebellum of HA rats confirm the altered histaminergic neurotransmission in HA.

The histaminergic afferents in the cerebellum originate in the hypothalamus. Because of this particular circuit, the cerebellum could be considered a region of somato-visceral integration. It was reported that histaminergic transmission modulates motor balance and coordination in the cerebellum (Li et al, 2014; Zhang et al, 2016a). Histaminergic neurotransmission in the cerebellum also facilitated memory consolidation in tests of inhibitory avoidance (Silva-Marques et al, 2016).

Although there are few studies on the histaminergic system in HE, it was reported that rats with porta-cava anastomosis (PCS), a model of HE, have increased levels of histamine in the hypothalamus and in the rest of the brain regions (Fogel et al, 1991; Lozeva et al, 1998). The increased levels of histamine may contribute to the altered circadian rhythms, sleep-wake cycle and locomotor activity in HE (Lozeva et al, 2001 and 2003; Spahr et al, 2007). Recently it was reported a role of the histaminergic system in modulation of wakefulness in hyperammonemic disorders (Sergeeva et al, 2020). Histamine is also involved in modulation of the hypothalamus-pituitary-adrenal (HPA) axis (Tuomisto et al, 2001) and then related to the alterations found in the present work in corticotropic-releasing hormone (CRH) response, as the CRH receptor 2 (Crhr2) is significantly upregulated (Figure 2A). The CRH pathway modulates stress, anxiety and circadian rhythms. Activation of this receptor in cerebellar slices increases spontaneous

firing frequency of Purkinje neurons and inhibits P-currents in these cells (Tao et al, 2009) and CRH also increases Purkinje neuron excitability by modulating sodium, potassium, and Ih currents (Libster et al, 2015). CRH facilitates LTD in climbing fibers-Purkinje cells synapses by downregulation of excitatory transmission (Schmolesky et al, 2007). CRH also modulates GluRdelta2 expression in parallel fiber-Purkinje synapses (Gounko et al, 2005). Decreased levels of CRH in the inferior olive, the sole origin of cerebellar climbing fibers, were found in patients with spinocerebellar degeneration or olivopontocerebellar atrophy. Deficiency of CRH in the olivocerebellar system induces ataxia-like motor abnormalities. CRH releasing neurons in the inferior olive project directly to the cerebellar nuclei and CRH selectively excites glutamatergic projection neurons in the cerebellar interpositus nucleus via two CRH receptors, CRHR1 and CRHR2, and their downstream inward rectifier K+ channel and/or hyperpolarization-activated cyclic nucleotide-gated (HCN) channel. Furthermore, CRH promotes cerebellar motor coordination and rescues ataxic motor deficits (Wang et al, 2017). There are no reports on cerebellar CRH in HA, but increased content of CRH in the hypothalamus of rats with chronic HA, which leads to altered HPA axis and impairment of circadian rhythms was reported (Llansola et al, 2013).

We can suggest that increased histamine levels in cerebellum from HA rats contribute to motor coordination impairment and sleep and circadian rhythm alteration in HE.

In addition, a main cell type releasing histamine are mast cells, components of the immune system, considered the first inducers of microglia activation, inducing neuroinflammation and cognitive impairment (Dong et al, 2017 and 2019; Zhang et al, 2016b and 2016c), which could be also involved in HA-induced microglia activation in the cerebellum (Rodrigo et al, 2010).

Cerebellum is one of the brain areas with notable expression of receptors for the luteinizing hormone (LH, *Lhb* gene), significantly upregulated in cerebellum from HA rats. Some neural actions of LH were reported (Lei and Rao, 2001). Immunoendocrine interactions, including release of LH and CRH induced by proinflammatory cytokines through nitric oxide-mediated activation of Cyclooxigenase-2 and prostaglandin E2 production or activation of guanylate cyclase and cyclic GMP production, were described (Rettori et al, 2009). In addition, histamine in the brain can induce increase of LH in plasma (Niaz et al, 2018). Then, we can suggest that altered CRH and LH signaling in the cerebellum from HA rats may be due to neuroinflammation and altered cGMP pathway (see below).

Neuropeptide Y receptor 1 (NPY) was significantly downregulated in cerebellum from HA rats. NPY is involved in motor coordination alterations in the Machado-Joseph disease associated with neurodegeneration of specific brain regions, including the cerebellum and striatum. Overexpression of NPY alleviated the motor coordination impairments and attenuated the related neuropathological parameters, preserving cerebellar volume and granular layer thickness. Additionally, NPY mediated increase of brain-derived neurotrophic factor levels and decreased neuroinflammation markers (Duarte-Neves et al, 2015).

Even though the change was not significant, an important decrease in 5-hydroxytryptamine (5-HT, serotonin) levels was found, together with upregulation of the serotonin receptor Htr2c, in cerebellum from HA rats. A neuromodulatory role of serotonin in the cerebellum, affecting motor function, has been reported. All cerebellar subregions receive serotonin innervation mostly originated from the reticular formation and serotonergic projections from the cerebellum to midbrain suggest bidirectional interactions (Kawashima, 2018). Some subtypes of serotonin receptors are expressed in

the cerebellum, both in Purkinje and in granule neurons (Hoxha et al, 2017). It has been proposed that serotonin controls ‘responsibility’ of each cerebellar unit in cerebellar learning and control (Schweighofer et al, 2004). Serotonin modulates the firing rate of Purkinje cells acting upstream, on inhibitory interneurons, Lugaro cells and modulates spontaneous firing rate of granule neurons and neurons in deep cerebellar nuclei (DCN). Serotonin also increases tonic inhibition of granule neurons acting on Golgi cells (Fleming and Hull, 2019). Moreover, long-term depression (LTD) in parallel fiber-Purkinje cells synapses is facilitated by serotonin, whereas serotonin inhibits LTD between mossy fiber-DCN neurons synapses (Kawashima, 2018).

Serotonin decreased levels have been found in cerebellum from patients with cerebellar ataxia and serotonin based therapies to treat cerebellar ataxia, for example with an agonist of the receptor 5-HT1A, have been tested and improve cerebellar ataxia (Trouillas, 1993; Dieudonné, 2001; Takei et al, 2005; Hoxha et al, 2017; Kawashima, 2018).

There are no studies of serotonin neurotransmission in the cerebellum in HA or HE. The only references in the literature are an increased activity of the enzyme monoamino oxidase B (MAOB), which metabolizes serotonin, in the cerebellum from mice models of congenital HA (Rao et al, 1994). Our present findings suggest that serotonergic neurotransmission in the cerebellum should be analyzed in HA, regarding a possible implication on the motor function alterations in animal models and patients with HE. Decreased serotonin levels and increased MAO activity have also been observed outside cerebellum in rats with bile duct ligation (BDL), a model of HE with HA, also showing locomotor and motor coordination impairments (Dhanda and Sandhir, 2015). Lozeva et al (2004) reported a correlation between serotonin turnovers and hyperammonemia in PCS rats.

Other neurotransmitter systems affected by HA in the cerebellum were: 1) the adrenergic system, with a significant downregulation of the adrenergic receptor 1 alpha (*Adra1a*), which in vermis cerebellum was associated with positively motivated exploratory activities and a decrease of this function is associated with depressive behaviors (Stone et al, 2007). Depressive behavior is present in some hepatic encephalopathy patients and has been associated to ammonia-induced alteration of the serotonergic system (Yue et al, 2019). In addition, the beta subunit of the adrenergic receptor was also decreased. These results suggest that impaired adrenergic system can also contribute to depressive behaviors in EH 2) the nicotinic cholinergic receptor, epsilon subunit (*Chrne*) was significantly upregulated in the cerebellum from HA rats. Nicotinic receptors modulate GABA and glutamate neurotransmission, mainly in cerebellar mossy fibers (Jaarsma et al, 1997; De Filippi et al, 2005). Nicotine receptor agonists improve motor coordination in a model of olivocerebellar ataxia (Wecker et al, 2013).

The glycine receptor alpha 2 subunit (*Glra2*) and glycine were upregulated, although not significantly. Our previous results reported an effect of cerebellar extracellular cGMP on glycine receptors, which is involved in modulation of learning ability (Cabrera-Pastor et al, 2016a). In HA rats, glycinergic neurotransmission in the cerebellum is altered, as well as modulation of the Glu-NO-cGMP pathway by extracellular glycine. According with this, our multiomic results support the altered glycinergic neurotransmission, which can contribute to the alterations of cerebellar-dependent learning ability in HA rats.

Glutamate was slightly upregulated in HA rats, while the ionotropic glutamate receptor AMPA subunit 4, GluA4 (GRIA4), was significantly downregulated and the gene for metabotropic glutamate receptor, mGluR6 (*Grm6*), was upregulated (Figure 2A).

In addition, we performed western blots with homogenates of part of the sample used for proteomic analysis to validate relative content of the GluA4 subunit of AMPA receptor

(Gria4) in control and hyperammonemic rats. As shown Figure 2B, the cerebellar content of GluA4 was significantly decreased (69±8 %, $p<0.05$) in hyperammonemic rats compared with the content in control rats.

Alterations in membrane expression of other GluA subunits were previously reported in HA rats (Cabrera-Pastor et al, 2018b) and also altered glutamate metabotropic receptor signaling, which also modulates the Glu-NO-cGMP pathway (Llansola et al, 2005; Cabrera-Pastor et al, 2012). However, our multi-omics analysis revealed expression changes in other non-previously reported glutamate receptor subunits, such as GluA4 (AMPA receptor) and mGluR6 (a G-protein-coupled receptor) (Figure 2A), which may be also involved in impaired glutamatergic neurotransmission. This analysis also detected an increase of basal glutamate in the cerebellum of HA rats, as reported for extracellular glutamate (Cabrera-Pastor et al, 2019).

The *cGMP-PKG* pathway (Figure 3A) was significantly enriched (p= 0.003, for combined omics) (Figure 1). Glutamate, mainly through its NMDA-type receptors, activates the pathway glutamate-nitric oxide-cGMP and this pathway is impaired in cerebellum from HA rats (Hermenegildo et al, 1998; Cabrera-Pastor et al, 2016b).

In this pathway, we found several ATPases significantly downregulated, one of them in genomics and seven in proteomics. Also were significantly downregulated the inositol-three-phosphate receptor 1 (ITPR1) and a Na^+/Ca^{2+} exchanger (SLC8A2) in proteomics and in transcriptomics the gene *Adra1a* codifying the alpha 1a subunit of the adrenergic receptor (also in Figure 2A) (while the gene *Adrb2*, beta-2 subunit of the adrenergic receptor, was downregulated, but no significantly). A protein phosphatase 1 (PP1) subunit (PPP1CC, gamma catalytic subunit) was significantly upregulated at protein level (Figure 3A).

Directly involved in this pathway, cGMP-phosphodiesterase (PDE) 3B gene (*Pde3b*) was upregulated, whereas PDE5A (*Pde5a*) and cGMP-dependent protein kinase (PKG2) (*Prkg2*) genes were downregulated, but not significantly. The gene for atrial natriuretic peptide (ANP) receptor 2 (*Npr2*), with guanylate cyclase activity, was also downregulated, although not significantly (Figure 3A).

These results confirm the impairment of this pathway, as previously reported (Hermenegildo et al, 1998; Cabrera-Pastor et al 2016b) and adds new molecular alterations. Basal extracellular cGMP is decreased in cerebellum from HA rats (Cabrera-Pastor et al, 2016b) and altered expression of PDEs and the receptor of ANP can contribute to this alteration.

Connection between cGMP and neuroendocrine features (CRH and LH) is reported, as we commented above, as well as cGMP link to glutamatergic and glycinergic neurotransmission, indicating a link between two altered pathways, cGMP-PKG and neuroactive ligand-receptor interactions.

We can see in the cGMP-PKG pathway that PKG modulate almost all of the other significantly altered features, suggesting that phosphorylation by this kinase may, direct or indirectly, affects expression of ATPases, ITP receptor, Na^+/Ca^{2+} exchanger and PP1 and this can be a key alteration in cerebellums from HA rats.

In cGMP-PKG pathway (Figure 3A), three different class of ATPases were significantly downregulated in proteomics. Plasma membrane Ca-ATPases (PMCAs) (ATP2B2 and ATP2B3, isoforms 2 and 3) that transport calcium ions out of the cell and the *Atp2b4* gene (corresponding to Ca^{2+}-ATPase isoform 4, regulated by calcium-calmodulin). A sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA, ATP2A2) and different isoforms of the catalytic alpha subunit of Na^+/K^+-ATPase (ATP1A3, ATP1A2 AND ATP1A1).

We performed western blots with homogenates of part of the sample used for proteomic analysis to validate relative content of Na^+/K^+ ATPase alpha 1 subunit of in control and hyperammonemic rats. As shown Figure 3B, the cerebellar content of Na^+/K^+ ATPase alpha 1 subunit was significantly decreased (68±9 %, $p<0.05$) in hyperammonemic rats compared with the content in control rats.

Modulation of Na^+/K^+-ATPase by cGMP-PKG pathway in brain and association of impairment of this pathway to disruption of ion homeostasis has been widely reported in different brain areas and some pathologies, including ageing (Scavone et al, 2005; Munhoz et al, 2005; Carvalho et al, 2012 and Spong et al, 2016).

SERCA2 regulates calcium release from endoplasmic reticulum, modulating intracellular calcium concentration and homeostasis (Britzolaki et al, 2018). Modulation of SERCA activity by cGMP-PKG was also reported (Zhang et al, 2005). Plasma membrane Ca-ATPase (PMCA) dysregulation has been associated with altered calcium homeostasis and neurodegenerative disorders (Padanyi et al, 2016; Hajieva et al, 2018). PMCA isoform 2 is richly expressed in the brain and particularly in the cerebellum. It interacts with NMDA receptor subunits and PSD95 in postsynaptic sites and it is expressed in presynaptic membranes (Garside et al 2009). Related to calcium homeostasis regulation, were also significantly downregulated a Na^+/Ca^{2+}-exchanger (SLC8A2) and the Inositol-3-phosphate receptor 1 (InsP3R1) (ITPR1), which regulates calcium release from endoplasmic reticulum, supporting that there is altered calcium homeostasis in cerebellum from HA rats (Figure 3A). Considering the important role of calcium, both in presynaptic and postsynaptic signaling pathways, these molecules are candidates to contribute to altered neurotransmission.

As is shown in Cabrera-Pastor et al (2016a) intracellular calcium level is decisive to the biphasic modulation of the Glu-NO-cGMP pathway in the cerebellum and consequently

to the modulation of associated-learning ability. Hyperammonemia alters calcium homeostasis of Purkinje neurons in culture. This article shows that cerebellar slices from hyperammonemic rats have lower intracellular basal calcium concentration than control slices. In addition, extracellular cGMP increases intracellular calcium in slices from hyperammonemic or control rats and this modulates phosphorylation and activation of Calcium-calmodulin kinase II (CaMKII), leading to modulation of CaMKII-Nitric oxide synthase-cGMP pathway, in a biphasic way.

We also measured calcium concentration in cerebellar granule neurons in culture chronically (10-13 days of culture) exposed to 0.1 mM ammonia and in control neurons. We measured basal intracellular calcium concentration, as well as, the increase of intracellular calcium in response to activation of glutamate receptors with different glutamate concentrations, activating different types of glutamate receptors and with the specific NMDA receptor agonist, NMDA. NMDA receptors are the main ionotropic glutamate receptor subtype involved in calcium entry. As shown in Figure 3C-E, basal calcium concentration in significantly lower (150.2±28 in neurons exposed to ammonia vs 210± 40 nM in control neurons, $p<0.01$) whereas the increase in intracellular calcium induced by NMDA is significantly higher (940±200 % of basal in ammonia exposed neuros vs 421±52 % of basal in control exposed neurons, $p<0.05$) in cultured granule cerebellar neurons exposed to ammonia, compared with control neurons. Glutamate induce a lower increase of intracellular calcium at low concentrations (5 µM glutamate: 2.5±0.9 in control neurons vs 1±0.4 µM in ammonia exposed neurons, $n=6$, $p<0.05$; 20 µM glutamate: 2±0.9 in controls vs 0.8±0.5 µM in ammonia exposed neurons, $n=4$, $p<0.05$) but not at higher concentrations (Figure 3E). In these cerebellar granule neurons in culture it was shown altered glutamate-nitric oxide-cGMP pathway alteration by 0.1 mM chronic ammonia exposure (Hermenegildo et al, 1998).

These results support the alteration of basal calcium homeostasis in cerebellum of hyperammonemic rats and show an altered response to activation of some glutamate ionotropic receptors, indicating altered calcium signaling in cerebellum of hyperammonemic rats.

In addition, Ca^{2+}-ATPases also regulate energy metabolism (Boczek et al, 2014), as another downregulated ATPase, a Na^{+}/K^{+}-ATPase. Mutations in the Na^{+}/K^{+}-ATPase alpha-3 isoform were associated to motor alterations, as Parkinsonism or cerebellar ataxia (Holm and Lykke-Hartmann, 2016). These proteins modulate K ion homeostasis in brain and this has effects on learning (Hertz and Chen, 2016).

All these results indicate an evident dysregulation of ion homeostasis, which consequently should alter neurotransmission and alteration of ATP levels that could alter energetic metabolism in cerebellum of HA rats, associated to altered cGMP-PKG pathway.

3.2 Immune related pathways.

The *antigen processing and presentation pathway* showed a combined p-value=0.0041 (Figure 1). The most significant feature was the upregulation of gene for *Cd8a*, a surface marker of cytotoxic T-lymphocytes. The gene for the Cd4 surface marker of T-helper lymphocytes, also named L-selectin, was also upregulated, but not significantly. In addition, the genes codifying for RT1-A1 and A2 and for RT1-CE10, surface markers of the major histocompatibility complex I (MHCI) were significantly downregulated. The protein calreticulin (CALR), a calcium-binding protein located in the endoplasmic reticulum and associated to MHC I, was upregulated (whereas the gene was downregulated) (Figure 4A).

Previous reports clearly show that HA induce neuroinflammation, which alters neurotransmission. This is involved in some neurological alterations found in HA rats and

546 in cirrhotic patients with MHE (Rodrigo et al, 2010; Hernández-Rabaza et al, 2016;
547 Cabrera-Pastor et al, 2018a). However, the mechanisms involved in HA-induced
548 neuroinflammation remain unknown.
549 One of the mechanisms inducing neuroinflammation in brain is infiltration of immune
550 cells, due to altered function of the brain-blood barrier (BBB). Two main altered features
551 are the upregulation of cluster of differentiation (CD) CD4 and CD8, specific markers for
552 T-helper and cytotoxic lymphocytes, respectively, suggesting increased presence of
553 lymphocytes in the cerebellum from HA rats.
554 A significant downregulation of RT1-CE10, *Rt1-a1* and *Rt1-a2* genes, which are part of
555 the Major Histocompatibility Complex I (MHCI), also suggests altered immune response
556 in cerebellum of HA rats. MHCI acts inducing cytotoxicity mediated by T cells and
557 immune tolerance.
558 The gene *Cd74* was also upregulated, but not significantly. Western blot of the protein
559 CD74 showed a significant increase in cerebellum of hyperammonemic rats (124±7 % of
560 controls, $p<0.05$) (Figure 4B). CD74 is a chaperone for antigen presentation in the
561 MHCII, that induce $CD4^+$ T-helper and $CD8^+$ T-cytotoxic lymphocyte activation
562 (Mensali et al, 2019). The upregulation of Cd74 in hyperammonemic rat cerebellum
563 suggest that this protein is responsible to the upregulation of CD4 and CD8a (Figure 4A).
564 Then, these results indicate activation of T-lymphocytes induced by MHCII in cerebellum
565 of hyperammonemic rats. The increase of MHCII expression in activated microglia was
566 showed in cerebellum of hyperammonemic rats (Rodrigo et al, 2010).
567 Calreticulin is a low-affinity and high capacity calcium binding protein localized in lumen
568 of endoplasmic reticulum, which is widely expressed in different cerebellar neurons
569 (Purkinje, granule or Golgi cells) (Nori et al, 1993). Calreticulin is a possible biomarker
570 of ageing related disease (Cardoso et al, 2018) and then, upregulation of this protein could

be also a biomarker of disease in HA rats. In addition, calreticulin can be exposed by neurons, becoming then a signal for phagocytosis by microglial cells (Vilalta and Brown, 2018). Its upregulation in cerebellum from HA rats could be related to increased phagocytosis, which is related to some neurological diseases (Vilalta and Brown, 2018).

Mast cells can infiltrate in the brain activating astrocytes by direct contact and both exacerbate inflammatory response by release of inflammatory mediators activating microglia and leading to chronic neuroinflammation (Kempuraj et al, 2017; Skaper et al, 2018). These cells release serotonin and histamine, both metabolites altered in cerebellum of HA rats, in our metabolomic analysis. In addition, mast cells can be activated by CRH (which associated pathway was also affected in the present analysis). Portal hypertension is associated to liver injury. This alteration induces mast cell activation and associated neuroinflammation, leading to encephalopathy. Mast cells integrate splanchnic and systemic inflammation leading from liver steatosis to encephalopathy (Aller et al, 2007; Aller et al, 2019). These results suggest a possible role of mast cells in HA-induced neuroinflammation to be more deeply studied.

Another pathway related with immune system was the *cell adhesion molecules (CAMs*) (Figure 5A), with a combined p-value of 0.013 (Figure 1). In this pathway, we also found the up-regulation of *Cd8a* and *Cd4* and of *Cd274*, ligand for PD1, expressed in activated B and T lymphocytes or myeloid cells, supporting infiltration and activation of immune cells in the cerebellum of HA rats. As in the previous pathway, the genes codifying for RT1-A1 and A2 and for RT1-CE10, were significantly downregulated. In addition, the gene for the tight-junction (TJ) protein claudin 10 (*Cldn10*) was significantly downregulated as the gene for another member of claudin family, *Cldn11* (no significant

595 in this case) and another component of TJ, occludin (*Ocln* gene), was upregulated. The
596 myelin-associated protein (MAG) was significantly upregulated in HA (Figure 5A).
597 In cerebellar samples from same rats, we have validated by western blot a significant
598 decrease (73±7 % of controls, $p<0.05$) of the protein claudin 10 in hyperammonemic rats
599 (Figure 5B).
600 Several genes codifying intercellular adhesion molecules were differentially expressed in
601 cerebellum from HA rats: *Icam1* and *Glycam1* were upregulated, whereas *Icam2*, *Ncam1*
602 and *Vcam1* were downregulated, although not significantly. *Itgb7* was the only
603 upregulated integrin, whereas *Itgad, Itgb8, Itgb2, Itga6* were all downregulated.
604 Pertaining to the cadherin family, *Cdh3* (P-Cadherin) was upregulated whereas *Cdh5*
605 (VE-Cadherin) and *Cdh1* (E-cadherin) were downregulated, but not significantly. Other
606 CAMs were downregulated, as P-selectin (*Selplg*), Netrin G2 (*Ntng2*), involved in
607 synaptic cell adhesion, or Nectin 2, involved in T cell receptor signaling pathway. These
608 results suggest that intercellular communication should be impaired in cerebellar tissue
609 from HA rats.
610 CAMs have important roles in the inflammatory process, mainly in infiltration of immune
611 cells and in the function of BBB. There is recent evidence indicating that BBB is impaired
612 in cirrhotic patients (and correlated with HA) and in animal models of HE (Dhanda and
613 Sandhir, 2018; Vairappan et al, 2019). We show here that two neuronal tight junction (TJ)
614 component genes, *Cldn10* and *Cldn11* are downregulated, suggesting impaired BBB
615 integrity. Moreover, it was reported that cerebellar BBB seems especially vulnerable,
616 with faster and more pronounced increase in BBB permeability by reduced expression of
617 TJ proteins (Silwedel and Förster, 2006).
618 Dhanda and Sandhir (2018) reported downregulation of TJ proteins in different brain
619 regions of BDL rats. These authors reported downregulation of occludin and ZO-1,

whereas we found them upregulated and upregulation of VCAM-1, whereas we found downregulated VCAM-1, but this discrepancy can be due to the different animal model or also different brain region (Dhanda and Sandhir, 2018). In BDL rats it was also reported dislocalization of occludin from the TJ sites in the cerebellum but not in frontal cortex (Mavrakis et al, 2012). In another animal model of liver cirrhosis induced by CCl4 (carbon tetrachloride), was also reported reduction of different TJ in brain tissue (Vairappan et al, 2019).

Regarding CAMs, although we found some downregulated genes (*Vcam1, Ncam1 and Icam2*, *Alcam*), *Icam1* and *Glycam1* genes were significantly upregulated, supporting facilitation of leukocyte trafficking into the cerebellum (Bittner et al, 2013; Liddelow and Hoyer, 2016). In the present study, we also found alteration of some adhesion proteins of another family, also involved in BBB integrity: two cadherins were downregulated, whereas another was upregulated.

P-selectin was downregulated in HA. This protein mediates monocyte-endothelial interactions in the brain, which were found, associated to early time point of liver inflammation (D'Mello and Swain, 2014). Nectin2, expressed in neurons and astrocytes, is important to maintenance of astrocytic perivascular endfoot processes (Miyata et al, 2016).

The final effects of these alterations and the function of each molecule requires further studies, but our results support alteration of BBB function in the cerebellum of HA rats.

The myelin-associated protein (MAG) was significantly upregulated in HA. It is expressed in Schwann cells and oligodendrocytes. This protein inhibits axonal, neurite growth and in the cerebellum, this process is mediated by p75 neurotrophic receptor and induce apoptosis of cerebellar granule neurons (Domeniconi et al, 2005; Fernández-Suárez et al, 2019). It was found a decrease of cerebellar granule neurons in port-mortem

cerebellums of patients with steatohepatitis, associated to cerebellar neuroinflammation and infiltration of lymphocytes (Balzano et al, 2018a) and MAG upregulation can contribute to this neuronal death.

Our results supports that chronic HA alters immune system-related pathways in the cerebellum and these alterations may be involved in the mechanisms leading to cerebellar neuroinflammation.

4. CONCLUSIONS

In summary, our multi-omic analysis revealed new pathways altered in cerebellums from HA rats. The most relevant ones that should contribute to the circadian and sleep alterations and to motor and cognitive impairment induced by HA are: glutaminergic and histaminergic neurotransmission, CRH response, ion and specially calcium homeostasis, energy metabolism, immune response (T cells and MHCI complex), intercellular communication, BBB integrity and infiltration of immune cells in the cerebellum of HA rats.

Our results supports that neuroinflammation and altered neurotransmission are the main contributors to neurological impairment in HA.

Our multi-omic study suggests new mechanisms by which HA modules brain function, which should to be more deeply studied in the future.

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

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FIGURE LEGENDS

Fig. 1 PaintOmics 3 pathway enrichment significant results. P-values are displayed for each omic and pathway, and the joint p-value combining all the omics is in the last column. Red color indicates that the pathway is significantly enriched in DE features for that particular omic or for the combination of all of them ($p<0.05$)

Fig. 2 A. Neuroactive Ligand-Receptor Interaction Pathway generated with PaintOmics. Cell color indicates the fold-change between HA and control rats (red is upregulation in HA, while blue is downregulation). Boxes with a thicker black frame represent significantly altered features and shown in more detail at the bottom. B, Western blot of GluA1 subunit. A representative image shown. C: control and HA: hyperammonemia. Data are expressed as percentage of control samples and it is mean±SEM of 8 samples per group, in duplicated. Two-tailed Student's t-test was applied ($t=2.488$, $df=26$, $p<0.05$). Values significantly different from controls are indicated by asterisk $*p < 0.05$.

Fig. 3 A. cGMP-PKG Signaling Pathway generated with PaintOmics. The color of the cells indicates the fold-change between HA and control rats (red is for upregulation in HA, while blue is downregulation). Boxes with thicker black frame represent significantly altered features and shown in more detail at the bottom. B. Western blot of Na^+/K^+ ATPase alpha 1 subunit. A representative image shown. C: control and HA: hyperammonemia. Data are expressed as percentage of control samples and it is mean±SEM of 8 samples per group. Two-tailed Student' t-test was applied ($t=2.245$, $df=11$, $p<0.05$). Intracellular calcium concentration was determined as is described in Methods. In C, basal calcium concentration (nM) in control and ammonia exposed

neurons is represented. Data are mean±SEM of 16 culture plates. Paired Student's t-test was applied (t=3.533, df=15, p<0.01). The NMDA-induced increase of intracellular calcium shown in D, as percentage of basal values. Data are mean±SEM of 8 culture plates. Welch's t-test was applied to correct different variances (t=2.503, df=7.926, p<0.05). In E shown the dose-response curve of glutamate-induced increase of intracellular calcium in µM. Data are mean±SD of 4-6 culture plates. Two-way ANOVA indicated significant effect of ammonia exposure (F(1, 32)=6.363, p<0.05) and Sidack's multiple comparison tests shown significant differences at 5 and 20 µM glutamate. Values significantly different from controls are indicated by asterisk *p < 0.05, **p<0.01.

Fig. 4 Antigen processing and presentation pathway generated with PaintOmics. The color of the cells indicates the fold-change between HA and control rats (red is for upregulation in HA, while blue is downregulation). Boxes with thicker black frame represent significantly altered features and they are shown in more detail at the bottom. B, Western blot of CD74. A representative image shown. C: control and HA: hyperammonemia. Data are expressed as percentage of control samples and it is mean±SEM of 8 samples per group. Two-tailed Student's t-test was applied (t=2.819, df=13, p<0.05). Values significantly different from controls are indicated by asterisk *p < 0.05.

Fig. 5 Cell Adhesion Molecules Pathway generated with PaintOmics. The color of the cells indicates the fold-change between HA and control rats (red is for upregulation in HA, while blue is downregulation). Boxes with thicker black frame represent significantly altered features and shown in more detail at the bottom. B, Western blot of Claudin 10. A representative image shown. C: control and HA: hyperammonemia. Data

are expressed as percentage of control samples and it is mean±SEM of 8 samples per group, in duplicated. Two-tailed Student's t-test was applied ($t=2.208$, $df=17$, $p<0.05$). Values significantly different from controls are indicated by asterisk $*p < 0.05$.