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Neurobiology of environmental enrichment in pigs: changes in monoaminergic neurotransmitters in several brain areas and in the hippocampal proteome

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Keywords: animal welfare, environmental enrichment, hippocampus, isobaric tags for relative and absolute Quantification (iTRAQ), neurotransmission, pig

Significance

Animal welfare has become an important aspect for the sustainability of animal production. The modification of the environment by enriching it with rooting materials and wider space allowance is known to have a positive effect on pigs' welfare. Searching for the underlying neurobiological mechanisms, we found that housing in an enriched environment increased the abundance of proteins related to protein synthesis, microtubule assembly, vesicle-mediated transport and energy metabolism in the hippocampus of pigs. Likewise, changes in the neurotransmitter profile in several brain areas were compatible with a better response to stress. This study expands the knowledge about the biological basis of animal welfare-promoting actions.

Abstract

Environmental enrichment in porcine farms improves animal welfare and leads to better public acceptance. To better understand the neurological mechanisms of the response to environmental enrichment, monoaminergic neurotransmitters were quantified in several brain areas from pigs after eight weeks of housing in barren or enriched conditions. Furthermore, iTRAQ labelling combined with LC-MS/MS was used to identify differentially abundant proteins in the hippocampus. Blood biochemical parameters related with stress and welfare were measured. Pigs under enriched conditions showed a decrease in plasma cortisol and lactate. The decrease in noradrenaline in the prefrontal cortex and amygdala, a general decrease in the dopaminergic system and an increase of serotonin in the striatum indicate a lower response to stress in enriched conditions. In the proteomic analysis, 2304 proteins were identified, of which 56 were differential between housing groups (46 upregulated and 10 downregulated). Bioinformatics analysis revealed that they were mainly related to ribosome, translation, microtubules and metabolic mitochondrial processes, indicating that pigs under enriched environments have higher abundance of proteins related to protein synthesis and neuronal activity. Together with previous behavioural studies, our results suggest that environmental enrichment provides a less stressful environment and that pigs cope better with stress conditions like the slaughterhouse.

Introduction

Environmental enrichment (EE) in porcine farms improves animal welfare and leads to a better public acceptance [1–9]. Information in pigs is scarce but it is widely accepted, from many studies performed mainly on rats, that physical enrichment, including increased space allowance and bedding enhanced with natural material such as straw, has been related to positive behavioural and physiological effects on animals [10,11] and to enhanced learning/memory, cognitive abilities, stress-coping abilities, reduced anxiety and depressive-like behaviour [12–16].

In rodents, it has been shown that these improvements in welfare are parallel to brain structural and molecular changes in response to external stimuli [10,17,18]. Some reports have shown that animals under EE undergo changes in molecular or cellular level of the prefrontal cortex [12,13,16] and hippocampus [19,20].

Chemical neurotransmission is an essential part of the brain function, including the response to stress, fear and reward [21–23]. The main components of these pathways are catecholamines (noradrenaline (NA); adrenaline (A); dopamine (DA) and their metabolites, homovanillic acid (HVA) and 3,4-dihydroxyphenyl acetic acid (DOPAC)), and indoleamines (serotonin (5-HT) and its metabolite 5-hydroxyindoleacetic acid (5-HIAA)), [21,24]. These neurotransmitter (NT) systems modulate the stress response through the integration of the activity among central nervous system areas, such as prefrontal cortex (PFC), hippocampus (HC), amygdala (Amy), hypothalamus (HPT) or striatum (Str), and the final activation of the hypothalamic-pituitary-adrenal (HPA) axis that results in the release of catecholamines and cortisol to plasma.

Not only stress but also positive conditions such as EE provoke changes in neurotransmitters and neurotrophic factors that correlate to behavioural changes, learning and memory in different animal species [10–12,14,16,17,25,26]. In laboratory animals, modifications in the monoamine NT profile linked to EE have been described. For example, EE alters the metabolism of DA and 5-HT in the PFC [27–30] and the serotonergic pathway in the HC [31].

EE also causes changes at cellular level, including hippocampal neurogenesis, an effect that has attracted much attention. The hippocampus has a unique anatomical structure, and it is essential for memory consolidation and storage, playing an important role in the neurogenesis and emotional mechanisms. Most of the research has been performed in rats and mice [17,19,20,32]. Morphological and structural changes would most probably be accompanied by changes in the protein composition and/or abundance in critical brain regions. In laboratory animals, changes in the brain proteome have been identified after EE [33–37], in models of depression [38,39], stress [40], behavioural disorders [41] or memory formation [42].

Our research group has recently analysed the changes in brain NTs provoked by the management of pigs at the slaughterhouse [43] and during road transport in pigs housed indoors or partially outdoors [44]. On the

other hand, we have described changes in the hippocampal proteome in conditions of intrauterine growth restriction in pigs [45]. The same animals involved in the present study were subjected to behavioural studies, that indicated that indeed EE pigs had better welfare behavioural scores (Qualitative behaviour assessment (QBA) (Welfare Quality®, 2009 [46]) and lower number of skin lesions on the carcass than pigs raised in BE conditions [47].

In the present study, we have analysed the changes in monoamine NT profile in several brain areas of pigs raised under barren or enriched conditions, as well as several plasma parameters related to stress and metabolism. Secondly, a quantitative proteomic analysis of the hippocampus has been undertaken as an approach to identify changes caused by long housing in EE conditions in this brain area.

Materials and methods

Experimental design and sample collection

The experimental design has been previously described [47]. A total of 44 female pigs aged 8 weeks coming from the same commercial farm were housed in four pens of 11 animals each, in the experimental facilities of IRTA (Monells, Spain). The pigs were crosses of Large White × Landrace RYR(1)- free (NN) sows with Pietrain heterozygous (Nn) boars. During the first 7 weeks, pigs were allocated under the same housing conditions, which consisted in a full slatted floor with a space allowance of 1.2 m2/pig. The following 8 weeks, the space allowance of two pens was reduced to 0.7 m2/pig (barren environment-BE) whereas on the other two pens the space allowance was maintained, the floor change to concrete and 700 g of straw/pig were provided every 2-3 days (enriched environment-EE). Animals were housed under natural light conditions at a constant environmental temperature of 22 ± 3 ºC. Each pen was provided with one steel drinker bowl (15 x 16 cm) connected to a nipple and a concrete feeder (58 x 34 cm) with four feeding places. Pigs had water and food ad libitum and were inspected daily.

Blood samples were obtained one week before beginning both treatments (14 weeks old), and at the end of the treatment (week 22). Afterwards, pigs were transported to the experimental slaughterhouse of IRTA (1.2 km distance) in pen groups. Afterwards, a 1 h lairage was carried out maintaining the housing pen groups

and pigs were stunned by exposure to 90% CO2 at atmospheric air for 3 min and exsanguinated after-wards. At the slaughterhouse, the skull was opened 5 min maximum after slaughter. The brain was removed and the Amy, HT, Str, HC and PFC were dissected, collected as quickly as possible (90 s maximum) in liquid N_2 and kept frozen at -80 °C. All bilateral areas (HC, Amy, Str) were collected together. The analysis of biochemical parameters and NTs were performed in samples from all the individuals included in the study.
The study was approved by the Institutional Animal Care and Use Committee (IACUC) of IRTA.

Serum biochemistry

Serum from all 44 animals was obtained by centrifugation at 2000 g for 10 min at room temperature. Supernatants were aliquoted and frozen at -80ºC until assay. Cholesterol (CHOP-PAP-method, OSR#6196), Creatine kinase (CK, IFCC method, OSR# 6179) and lactate (OSR#6193) were determined using the Olympus System Reagents (OSR, Olympus Diagnostica GmbH, Dublin, Ireland). Glutathione peroxidase (GPx) and Superoxide dismutase (SOD) were determined by using respectively Ransel and Ransod Kits (Randox Laboratories Ltd, Crumlin, UK). Haptoglobin (Hp) was determined spectrophotometrically (Phase Haptoglobin, Tridelta Ltd, County Kildare, Ireland). All techniques were adapted to the Olympus AU400 analyser. Cortisol concentrations were determined by ELISA (DRG Cortisol ELISA, DRG Diagnostics, Marburg, Germany).

Brain extracts preparation

Brain samples (Amy, HT, Str, PFC and HC) were weighted and homogenized in ice-cold 0.15 M NaCl, 0.05 M Tris-HCl pH 8.0 and 1.0 % Triton X-100 buffer with protease inhibitors (protease inhibitors cocktail, Sigma-Aldrich, St. Louis, MO) (0.3 g tissue/mL) and 100 pg/µL dihydroxybenzylamine (DHBA) as internal standard. The mixtures were homogenized by sonication (Branson Digital Sonifier, model 250, Branson Ultrasonics Corp., Danbury, CT) and the brain extracts were kept frozen in aliquots at -80 °C. Different aliquots of the brain extracts prepared as described were used for NT quantification (after acid precipitation of proteins) and for proteomic analysis [44].

Monoamine neurotransmitter quantification

Brain extracts from all 44 individuals included in the analysis were homogenized (1:2 v/v) in ice-cold 0.25 M perchloric acid containing 0.1 M NaS_2O_5 and 0.25 M ethylenediaminetetraacetate (EDTA) and kept frozen at -80°C until use. After centrifugation at 12000 x g for 10 min at 4 °C, the concentration of catecholamines (NA, DA, DOPAC and HVA) and indoleamines (5-HT and 5-HIAA) were determined in 20 µL aliquots using HPLC (Elite LaCHrom, Merck, Hitachi, Japan) equipped with a Chromolith Rp-18e 100 x 4.6 mm column (Merck KgaA, Darmstadt, Germany) with electrochemical detection (ESA Coulochem II 5200, Bedford, MA). The mobile phase consisted of 0.5 M citrate buffer pH 2.8, 0.05 mM EDTA, 1.2 mM sodium octyl sulphate (SOS) and 1 % acetonitrile. The applied voltage was set at 400 mV and the flow rate was 1 mL/min [48]. Validation of the methodology is described in Arroyo et al. [43]. The internal control DHBA allowed the comparison between runs. Dopaminergic total system (DA-system) and serotonergic total system (5-HT-system) are calculated as the sum of all metabolites in the pathway (DA, DOPAC and DA; and 5-HT and 5-HIAA; respectively).

Proteomic Analysis by Isobaric tag for relative and absolute quantitation (iTRAQ)

Hippocampal extracts from 20 animals from the BE group and 20 animals from the EE group (10 from each pen) were used for iTRAQ analysis. Brain extracts (see above) were treated as follows: 85 µg of total protein in a total volume of 50 µL were reduced with 1.3 µL of 200 mM tris (2-carboxyethyl) phosphine (TCEP) (final concentration 50 mM) at 35°C for 60 min, and sulfhydryl groups were alkylated using iodoacetamide (IAA) to a final concentration of 20 mM. The excess of IAA was eliminated by incubating with 5mM TCEP for 1h at 35°C. To decrease the urea concentration, 250 µL of 0.5M triethyl ammonium carbonate (TEAB) was added and then proteins were subjected to trypsin digestion (1:33 w/w trypsin:protein) for 20 hours at 37°C. Protein digestion was stopped by adding 0,1% formic acid (final concentration). Peptides were desalted with PolyLC tips C18 (PolyLC Inc, Columbia, MD, USA), dried by vacuum centrifugation and reconstituted in 30 µL 0f 500 mM TEAB.

Peptide samples were differentially labelled with iTRAQ® Reagents 8-plex according to the manufacturer's protocol (AB Sciex, Framingham, MA). An internal pool, formed by all the samples, was also labelled and used as control. Six reactions were performed to accommodate all samples. The experimental design for the iTRAQ labelling is shown in Supplementary Table S1.

Nanoliquid chromatography electrospray ionization tandem mass spectrometry (nanoLC-ESI-MS/MS)

After labelling, samples were combined, desalted, dried and resuspended in 5% acetonitrile, 1% formic acid prior to MS analysis. The peptide mixture was analysed using an Orbitrap Fusion Lumos Tribrid mass spectrometer coupled to a nano-UPLC system (EASY-nanoLC 1000 liquid chromatograph). Peptides were loaded directly onto the analytical column and were separated by reversed-phase chromatography using a 50-cm colum (EASY-Spray; 75-µm ID, PepMap RSLC C18, 2-µm particles, 45°C). Chromatographic gradients started at 97% buffer A (0,1% formic acid in H2O) and 3% buffer B (0.1% formic acid in acetonitrile) with a flow rate of 300 nl/min and gradually increased to 35% buffer B in 270 min and then to 50% buffer B in 5 min. After each analysis, the column was washed for 10 min with 5% buffer A and 95% buffer B.

The mass spectrometer was operated in positive ionization mode with an EASY-Spray nanosource with spray voltage set at 2.4 kV and source temperature at 275 °C. Internal mass calibration is using with lock mass m/z 445.12003. All data were acquired with Xcalibur software v3.0.63. The mass spectrometer was operated in a data-dependent acquisition (DDA) mode. In each data collection cycle, one full MS scan (400-1600 m/z) was acquired in the Orbitrap (1.2 x 105 resolution setting and automatic gain control (AGC) of 2 x 105). The following MS2-MS3 analysis was conducted with a top speed approach. The most abundant ions were selected for fragmentation by collision induced dissociation (CID). CID was performed with a collision energy of 35%, 0.25 activation Q, an AGC target of 1 x 104, an isolation window of 0.7 Da, a maximum ion accumulation time of 50 ms and turbo ion scan rate. Previously analyzed precursor ions were dynamically excluded for 30 s. For the MS3 analyses for iTRAQ quantification, multiple fragment ions from the previous MS2 scan (SPS ions) were coselected and fragmented by HCD using a 65 % collision energy and a precursor

isolation window of 2 Da. Reporter ions were detected using the Orbitrap with a resolution of 30,000, an AGC of 1 x 105 and a maximum ion accumulation time of 120 ms. RF Lens were tuned to 30%. Minimal signal required to trigger MS to MS/MS switch was set to 5,000. The mass spectrometer was working in positive polarity mode and singly charge state precursors were rejected for fragmentation.

Database searching

Database searches were performed with Proteome Discoverer v2.1.0.81 software (Thermo Scientific) using Sequest HT search engine and Uniprot *Sus scrofa* 2016_08 and contaminants databases. Search was run against targeted and decoy database to determine the false discovery rate (FDR). Search parameters included trypsin, allowing for two missed cleavage sites, carbamidomethyl in cysteine and iTRAQ 8plex peptide N-terminus as static modification and iTRAQ 8plex in K/Y, methionine oxidation and acetylation in protein N-terminus as dynamic modifications. Peptide mass tolerance was 10 ppm and the MS/MS tolerance was 0.6. Peptides with a q-value lower than 0.1 and a FDR < 1% were considered as positive identifications with a high confidence level.

Quantitative analysis

iTRAQ reporter ions intensities were used for protein quantification. Unique + razor peptides (peptides that are not shared between different protein groups) were considered for further quantitative and statistical analysis. Within each iTRAQ experiment, peptide quantitation was normalized by summing the abundance values for each channel over all peptides identified within an experiment and then the channel with the highest total abundance was taken as a reference and all abundance values corrected in all other channels by a constant factor per channel, so that at the end the total abundance is the same for all channels. Protein quantitation was done by summing all peptide normalized intensities for a given protein. Normalization across each of the six 8plex experiments was done using quantile normalization [49].

DanteR [50] (Pacific Northwest National Laboratory) was used to pre-process, visualize data (boxplots and principal component analysis) and perform relative quantification of proteins labelled with iTRAQ.

Gene Ontology and Bioinformatic analysis

For protein names and Gene Ontology (GO) classifications, PANTHER version 14.1 software (http://pantherdb.org/) was used together with the UniProt databases (http://www.uniprot.org/) [51]. Complete GO and GO slims were run. GO slims are cut-down versions of the GO ontologies containing a subset of the terms in the whole GO. They give a broad overview of the ontology content, but excluding the details of the specific fine grained terms (gene.ontology.org).

For pathway analysis, the Reactome platform version 67 was used (https://reactome.org/) [52], as well as the Kegg Mapper tool version 4.0 (https://www.genome.jp/kegg/mapper.html) [53]. For protein interaction network analyses, identified proteins were analysed with STRING version 10 (http://string-db.org/).

Statistical analysis

Statistical analysis was performed in SPSS 22.0 software (IBM, Chicago, IL, USA). Normality test of data and residuals was performed for each measure. Whenever possible, data were log transformed to correct the distribution and hence permit use of parametric statistics. Normally distributed measures were analysed using the t-Student parametric test. The significance level was established at $P < 0.05$ and a tendency was considered at $0.05 \leq P \leq 0.1$. Descriptive data are presented with the means and the standard error (mean ± SE).

The Statistical Analyses System (SAS v9.4; software SAS Institute Inc., Cary, NC; 2002±2008) was used to analyse serum biochemistry and NT data. Descriptive data is presented with the means and the standard error and the significance level was established at $P < 0.05$ and a tendency was considered at $0.05 < P < 0.1$.

226 Shapiro-Wilk normality test of data and residuals was performed for each measure. Whenever possible, data
227 was log transformed to correct the distribution.

228 The MIXED procedure with repeated measures analysis was performed for biochemical data. The full
229 factorial model includes time (pre-treatment and post-treatment) as within-subject factor, environmental
230 conditions (BE or EE) as between-subject factor and their interaction. Pig was introduced as the
231 experimental unit and the housing pen as a random effect nested within the two handling treatments.

232 MIXED procedure with Tukey adjustment was performed for NT (and oxidative markers data). Each pig was
233 introduced as the experimental unit, treatment (BE or EE) as fixed effect and the housing pen as a random
234 effect nested within the two handling treatments.

235

236 For iTRAQ analysis, two-way analysis of variance (ANOVA) was performed at protein level using a linear
237 model. Peptides were ordered using median and minimum number of peptides was set to 1 and maximum
238 to 50. Weighting function was used to allow data variability to depend on data value. Factors considered for
239 the two-way ANOVA were: the comparison we are interested in (BE and EE) as a first factor and each pen
240 (Ea, Eb, Ba, Bb) as a second factor, in order to minimize experimental bias and to ensure that there was no
241 pen effect. Finally, p-values were adjusted for multiple testing using the Benjamini & Hochberg FDR
242 correction. Differential expressed proteins were determined using an adjusted p-value cutoff of 0.05 and a
243 fold change lower than 0.8 (down) or higher than 1.25 (up).

244

245

246 **RESULTS**

247

248 **Serum biochemistry**

249 Biochemical parameters were determined in serum before starting the treatment and at the end of the
250 experiment, and results are shown in Table 1. The muscular marker CK, cholesterol and haptoglobin
251 decreased with time without effect of the environmental treatment. Lactate decreased only in the EE group.
252 The antioxidant enzyme GPx increased its concentration in both treatments with a significant interaction of
253 time*treatment, whereas SOD was not affected by time nor treatment. The stress hormone cortisol
254 decreased with time only in the EE group.

255

256 **Brain monoamine NT profiles in PFC, HC, Amy, HPT and Str**

257 The concentrations of brain monoamines and their metabolites in PFC, HC, Amy, HPT and Str are presented
258 in Table 2.

259 Important differences are observed in the noradrenergic system in PFC and Amy, since an increase in NA is
260 observed in the BE group, whereas it is not altered in the EE group.

261 Housing conditions has a significant effect on dopaminergic system in all regions. In general, BE conditions
262 provoke an increase in the DA pathways in all areas, except in Str, where there is a decrease. In Amy, an
263 increase in DA and in its metabolites is observed, as well as in total DA-system. In the PFC, the increase was
264 shown in the dopaminergic metabolites, as well as in total DA-system, but not in DA, the actual NT. In HC, no
265 differences are visible except for a tendency to increase in DA. In HT, only a tendency to increase is observed
266 in DA and L-DOPA.

267 The serotoninergic system is markedly altered in the PFC and HPT, with an increase in 5-HT and total
268 indoleamines, and Str, with a decrease in 5-HT and total IND.

269

270 **Proteomic analysis of the HC in barren and enriched environments**

271 A total of 63097 peptide spectrum matches corresponding to 15649 peptides and 2418 proteins were
272 identified in the iTRAQ analysis. Uncharacterized proteins were identified by homology (>98%) with other

mammalian databases. Finally, 2304 proteins were identified and quantified. Table 3 lists the 56 differential proteins identified between the EE and BE groups. From these, 46 proteins were upregulated whereas 10 proteins were downregulated. Complete results for the proteomic analysis are given in Supplementary Table S2.

Gene Ontology (GO) analysis is shown in Fig 1 and Supplementary Figure S1. The GO analysis of the differentially abundant proteins identified in EE and BE groups clearly revealed three main GO groups according to their molecular function (Figure 1A): structural proteins (GO:0005198, 35.4%); binding proteins (GO: 0005488, 36.9%) and catalytic activity (GO:0003824, 23.1%). Structural proteins were mainly ribosomal proteins (GO:0003735, 17 proteins, 100% of hits). The binding proteins included 12 proteins corresponding to the heterocyclic compound binding category (GO:1901363). Proteins with catalytic activity (GO:0003824) included transferases (7 proteins, GO:0016787), oxidoreductases (4 proteins, GO:0016491), and hydrolases (4 proteins, GO:0016740) amongst others.

According to biological processes (Figure 1B), 22 proteins were in the category of metabolic processes (GO:0008152), mostly in the metabolism of organic substances (19 proteins, GO:0071704). Twenty proteins were involved in cellular processes (GO:0009987), mostly metabolism (7 proteins, GO:0044237), microtubule-based processes (6 proteins, GO:0007017), cell cycle (6 proteins, GO:0007049) and organization of cellular components (5 proteins, GO:0016043). Finally, the 7 proteins involved in the organization or biogenesis of cellular components (GO:0071840) were related to ribosomes (GO:0044085 and GO:0016043).

A complete list of GO terms is shown in Supplementary Table S3.

Pathway analysis

The KEGG Mapper analysis (Supplementary Table S4) indicated that Ribosome was the most relevant pathway with 21 proteins corresponding to the large (10 proteins) and small (11 proteins) ribosome subunits. Metabolic pathways (10 proteins), especially oxidative phosphorylation (4 proteins) were also highlighted. Structural proteins appeared as Cytoskeleton proteins (6 proteins, tubulins and myosins); chromosome-associated proteins

298 (6 proteins, tubulins and others); exosome-associated proteins (6, proteins, tubulins and others). Other
299 pathways appeared related to transcription and translation (mRNA biogenesis, amino acid-related enzymes,
300 spliceosome, tRNA biogenesis). Finally, some regulatory proteins were also identified (protein phosphatase-
301 associated proteins, peptidases and GTP-binding proteins).

302 Pathway analysis with Reactome showed that the main nodes were "Metabolism of proteins" (mostly pathways
303 related with Translation and Protein Folding); "Metabolism of RNA"; "Vesicle-mediated transport";
304 "Metabolism" (specially Oxidative Phosphorylation and Amino Acid Metabolism); "Developmental biology"
305 (specially Axon Guidance); and "Neuronal system" (specially Neurotransmission) (Supplementary Table S5).

306 Finally, network analysis with STRING showed the existence of three main nodes. The most relevant is
307 composed by the ribosomal proteins, whereas two minor but relevant nodes are cytoskeleton proteins and
308 mitochondrial proteins (Figure 2).

309

310 **DISCUSSION**

311 In the present work, changes in serum biochemical parameters related to stress and welfare have been
312 measured as well as some actions on the central nervous system in pigs subjected to EE conditions. The
313 study of the brain function has been focused on two central aspects: first, the alterations of the
314 monoaminergic NT systems in several brain areas related to stress, memory, mood and reward and,
315 secondly, the changes in the proteome of the hippocampus, a brain area related to memory, spatial
316 cognition, fear and affective processes. This work complements the behavioural study performed in these
317 same animals which demonstrated that EE increases the qualitative behaviour assessment scores and a
318 lower number of wounds in the carcass [47].

319 Several serum biochemical parameters have been determined as suitable biomarkers for the several
320 components of stress and welfare. For example, CK, lactate, haptoglobin and cortisol are all indicators of
321 physical and/or psychological stress: pigs living together in a closed space may suffer injuries (CK being the
322 biomarker), have a subclinical inflammatory status due to injuries (indicated by haptoglobin), and be

323 submitted to a social stress (indicated by cortisol and probably by lactate and Hp [55,56]. All four mentioned
324 parameters decrease at the end of the treatment, in BE as well as in EE conditions. This is probably due to a
325 better adaptation of pigs to the farm and to the caretakers. Nevertheless, there is an interaction between
326 time and treatment for cortisol and lactate, which are lower in the EE group, suggesting that the adaptation
327 is better when animals are living in better conditions. Bonferroni adjustments for pairwise comparisons
328 showed a statistical difference between pre- and post-treatment values for lactate and cortisol only in the EE
329 group (P<0,001 and P=0,031, respectively) but not in the BE group. A decrease of serum cholesterol at the end
330 of both treatments probably also indicates a better adaptation to the farm since altered lipid metabolism has
331 been also associated to physiological stress, likely as a consequence of the lipolytic activity of cortisol. Here there
332 is also an interaction between time and treatment, with lower cholesterol values in EE conditions. Finally, GPx
333 and SOD are antioxidant enzymes which are considered part of the defences of the individual against oxidative
334 stress. The increase at the end of the treatment indicates that these defences are more developed at this time,
335 maybe associated to the older age of the pigs. Altogether, the biochemical results indicate that pigs get used to
336 their environment after some time, but that the adaptation is easier when they are living in EE conditions.
337 Comparable results were found by us in a study leading with outdoors or indoors rearing of pigs and their
338 response to road transport [44].

339 Our results on chemical neurotransmission also indicate that the EE provides a less stressful environment to
340 the pigs. EE provokes large differences in the three analysed NT systems (noradrenergic, dopaminergic and
341 serotoninergic) to a greater or lesser extent in all five brain areas under study. The most affected system was
342 the dopaminergic pathway, since the concentration of DA and/or its metabolites was lower in Amy, PFC, HPT
343 and HC (tendency) in pigs raised in EE, indicating a lower degree of stress, since high DA levels in several brain
344 areas have been related to maintained stress [24]. Our results also indicate that pigs raised in EE cope better
345 with the slaughterhouse stress, with a lower anxiogenic reaction than pigs raised in BE. Our previous results
346 [44] comparing NT levels in PFC and HC in pigs raised outdoors (a condition that provides pigs with an EE
347 [57]), and their response to road transport also indicate that pigs raised outdoors may cope better with the
348 stress associated to management (if the degree of stress is not very high). Similar results have been reported

349 in rats housed in EE conditions that showed a lower mesocortical DA reactivity in front of stressful stimuli
350 [26,30]. Since it has been proposed that Amy is involved in the regulation of the DA pathway in mesocortical
351 areas, the lower activity of the DA system in the Amy may be the mechanism by which EE-induced changes
352 lead to a lower reactivity of the DA system and a better response to stress factors.

353 Noradrenergic pathways were higher in Amy and PFC in BE conditions. NA neurons in the *locus coeruleus* are
354 the principal system involved in the stress response, including social stress, and they project to regions as
355 the Amy and the HC [24,58,59]. High levels of NA are associated with the initial fight or flight response, with
356 abnormal responses to stress and anxiety [60]. Thus, higher NA concentrations in Amy and PFC in BE can
357 lead to a disturbed response to a stress situation.

358 On the contrary, our results showed that the DA concentration was lower in the Str in BE conditions.
359 Similarly, rats subjected to several types of stress show lower activity of the DA pathway in the Str [61],
360 suggesting that indeed living in a BE is associated to a higher stress response. The Str is a critical component
361 of the motor and reward systems, and coordinates multiple aspects of cognition, including motor-planning,
362 motivation, reinforcement, and reward perception [21], suggesting that it may link the increased explorer
363 ability of individuals raised in EE conditions [62].

364 Finally, the serotoninergic pathway was altered in the HPT, PFC and Str: 5-HT and total IND in the HPT and
365 PFC were lower in EE conditions, whereas they were slightly but significantly higher in the Str. A decrease in
366 hypothalamic 5-HT has been also described in EE-housed mice [63].

367 In conclusion, our results on NTs indicate that the catecholaminergic systems are the most relevant in EE,
368 supporting the same conclusion described in rodents [13,17,27,64]. Our results suggest that pigs raised in BE
369 conditions may suffer an anxiety-like status and that, in front of a stressful event such as the arrival at the
370 slaughterhouse, stunning and slaughtering, undergo a higher response to these stressors.

371

372 On the other hand, several studies have shown significant changes at cellular, molecular and behavioural levels,
373 particularly in the hippocampus of rodents as a result of animals living in an enriched environment. Adult

374 neurogenesis, more dendrites per neuron, an increase in total area of synaptic contacts and enhanced long term
375 potentiation (LTP) amplitude have been found in enriched rats [20,65]. To provide new clues into the
376 mechanisms of environment-dependent plasticity of the brain, the proteome of the HC was analysed in pigs
377 raised in EE and BE using the iTRAQ quantitative approach. The experimental design in the iTRAQ experiment
378 was aimed to avoid any bias and to obtain reliable results by using 20 samples from each condition. The analysis
379 of the differential proteins by network and pathway analysis yielded clear results. First of all, 22 ribosomal
380 proteins corresponding to the 40S and 60S subunits are upregulated in EE-housed pigs, together with other
381 proteins involved in protein translation, as FARS (Phenylalanine-tRNA ligase). GNB2L1 and NPM1 are also linked
382 to translation and heavily connected to the ribosome in the network analysis. GNB2L1 contributes to cap-
383 dependent translation and found associated to huntingtin in the brain [66]. Nucleophosmin (NPM1) is involved
384 in diverse cellular processes such as ribosome biogenesis, cell proliferation and genomic stability, and it binds
385 ribosome presumably to drive ribosome nuclear export, being present in neurons [67]. Other upregulated
386 proteins are binding proteins as NONO “Non-POU domain-containing octamer-binding protein” and ELAV-like
387 protein. NONO is involved in transcription and splicing and may act as an RNA binding proteins involved in
388 mRNA localization and translation in neurons [73]. ELAV-like protein is a RNA-binding protein found un neural
389 cells that binds the 3´-UTR to control mRNA degradation of genes like FOS (Ma, 1996). PUR-alpha (PURA) is a
390 DNA binding protein involved in replication with neurological functions [69]. Figure 3, based in the Reactome
391 pathway analysis, shows that all cytoplasmic translation-related mechanistic stages are overrepresented in the
392 analysis. Altogether, the upregulation of these proteins is probably an indication of the neurogenesis and higher
393 dendrite density associated to EE [70,71]. Dendrites are the main target of synaptic afferents from other
394 neurons and they are rich in ribosomes and cytoskeletal proteins that reflect their function in reception and
395 processing of the information from other neurons [21,72]. Supporting our findings, it has been previously shown
396 that, in rodents, EE increases the number of ribosomes and synapsis in the HC dendrites, as well as their density,
397 whereas a decrease in the number of ribosomes or alterations in ribosomal proteins are associated to
398 depression and deficit in neuronal development [20,32,73,74].

399 The other main group of hippocampal proteins upregulated in EE are cytoskeletal proteins, specifically several
400 tubulins of the alfa and beta types, main components of microtubules, as well as myosin X and XVIII.
401 Microtubules form the longitudinal structure of axons and dendrites, and participate in the protein transport
402 along axons from the soma to the cell periphery and in the formation of secretory vesicles. Both, "Metabolism of
403 proteins" and "Vesicle-mediated transport", are overrepresented in pathway analysis (Figure 4). The increase in
404 these proteins is probably associated to the higher dendritic arborisation and spine density in EE, already
405 supported by the increase in ribosomal proteins, as mentioned above. It is especially interesting that the post-
406 chaperonin tubulin folding pathway is overrepresented in EE conditions, in contrast to the general chaperonin-
407 mediated protein folding, as visualized after Reactome pathway analysis (Supplementary Figure S2). Besides
408 tubulins and myosins, other microtubule-associated regulatory proteins are also differentially abundant. Thus,
409 ARL2 (ADP-ribosylation factor-like protein 2), a monomeric G-protein able to bind the GTP-tubulin thus
410 modulating microtubule dynamics [75], is downregulated in EE. On the other side, NIPSNAP1, exclusively
411 expressed in neurons and localized in the postsynaptic density fraction of synapses and associated with several
412 neuronal diseases [76], and TBC1D10B (TBC1 domain family member 10B), a GTPase activating protein involved
413 in vesicle fusion and retrograde transport [77] are also upregulated in EE. Calpain-small subunit, also
414 upregulated, has been involved in cytoskeletal organization and synaptic plasticity [78]. Altogether, our findings
415 again support the changes in HC plasticity associated to EE conditions.

416 The increased protein synthesis and higher dynamics of axons and dendrites would require a high amount of
417 ATP. The hippocampal cells from pigs raised under EE appear to have a higher efficiency in ATP synthesis, since
418 components of the mitochondrial respiratory chain as NDUFA9 and NDUFA10 (subunits of the NADH:ubiquinone
419 oxireductase) and SDH (succinate dehydrogenase) have been found upregulated.

420 Finally, two enzymes involved in monoamine synthesis have been identified: QPDR (Dihydropteridine
421 reductase), downregulated in EE, which produces tetrahydrobiopterin, a cofactor for Tyrosine and Tryptophan
422 hydroxylases, the regulatory enzymes for catecholamine and indoleamine synthesis; and MAOB
423 (monoaminooxidase B), upregulated in EE, which is involved in the degradation of these NTs and it is found

upregulated in EE. Both events may explain the lower DA levels observed in the HC of pigs raised under EE conditions.

In conclusion and considering all approaches, the proteomic results indicate that pigs under EE conditions show higher abundance of proteins in the HC compatible with increased capacity for protein synthesis, axonal/dendrite transport and increased oxidative energy metabolism. Furthermore, the variation in NT concentration and the serum biochemistry may indicate a lower response to stress in pigs housed in enriched conditions, suggesting that these animals have a better welfare than pigs in barren conditions. The same animals involved in the present study were subjected to behavioural studies, that indicated that indeed the EE pigs had better welfare scores and lower number of skin lesions on the carcass than pigs raised in BE conditions [47].

Legends to the figures

Figure 1. Functional classification of differentially abundant proteins identified in pigs raised in EE or BE conditions by Slim-GO analysis. (A) Molecular function ontologies. (B) Biological process ontologies. The most represented categories, the number of hits in each GO category (#) and the percentage versus the total number of hits (%) are shown. The upper panel represents the main GO classification for molecular function (A) or biological process (B). Lower panels indicate the GO subcategories for the most important GOs. Only GO categories with more than 5% of hits are shown. Complete data are presented in Supplementary Figure S1.

Figure 2. Network analysis by STRING of differentially abundant proteins in the hippocampus of pigs subjected to a barren environment (BE) or enriched environment (EE). Different colours of the lines represent the types of evidence for association: Cyan line: database; Pink line: experimental; Green line: gene neighbourhood evidence; Red line: gene fusion evidence; Blue line: gene co-occurrence evidence; Yellow line, text mining evidence; Black line, co-expression evidence and Grey line: protein homology.

Figure 3. Scheme of the Reactome pathway analysis for Translation (R-HSA-72766.4) indicating the contribution of cytoplasmic translation-associated stages. Yellow colour in boxes indicate the proportion of proteins identified in relation to the total number of proteins in the pathway.

Figure 4. Reactome diagram of Metabolism of proteins and Vesicle-mediated transport pathways in the hippocampus of pigs raised in EE-conditions with overrepresented reactions highlighted in black.

Supplementary material

Supplementary Figure S1: Functional classification of differentially abundant proteins identified in pigs raised in EE or BE conditions by Slim-GO analysis. (Tab 1A) Molecular function ontologies. (Tab 1B) Biological process ontologies. The most represented categories, the number of hits in each GO category (#) and the percentage versus the total number of hits (%) are shown. The upper panel represents the main GO classification for molecular function (Tab 1A) or biological process (Tab 1B). Lower panels indicate the GO subcategories for the most important GOs. Only GO categories with more than 5% of hits are shown.

Supplementary Figure S2: Display of the Reactome pathway analysis for Protein folding (R-HSA-391251.1) indicating the involvement of chaperonin-mediated protein folding (red) and post-chaperonin tubulin folding pathway (blue). Green/brown colour in boxes indicate the proportion of proteins identified in relation to the total number of proteins in the process.

Supplementary Table S1: Experimental design for iTRAQ labelling for individual samples from pigs housed in environmental enrichment (EE) or barren conditions (BE).

Supplementary Table S2: Complete list of proteomic identification of proteins, peptide groups and peptide spectrum matches (PSM) with all data on normalization, replicates and statistical procedures.

Supplementary Table S3: PANTHER GO-Slim and Complete GO analysis of biological process, cellular components and molecular functions of the differential proteins in the hippocampus of pigs housed under environmental enrichment (EE) or barren conditions (BE).

Supplementary Table S4: KEGG Mapper Search Result of the differential proteins in the hippocampus of pigs housed under environmental enrichment (EE) or barren conditions (BE).

Supplementary Table S5: Reactome Pathway Analysis of the differential proteins in the hippocampus of pigs housed under environmental enrichment (EE) or barren conditions (BE).

Author Contributions

Study design: AB and AV. Experimental work: LA, DV, RP, RC y conduct: CC and XH. Data analysis: LA and AB. Data interpretation: LA and AB. Drafting manuscript: LA and AB. Revising and approving manuscript content: DV, RC, RP, JS, AV.

Acknowledgements

We are grateful to Susana Benítez (Departament de Bioquímica i Biologia Molecular, Facultat de Medicina, UAB) for their help with the HPLC analysis, and to Dr Marina Gay and Dr Marta Vilaseca for their help with the proteomic analysis (Institut de Recerca Biomèdica, IRB, Barcelona, Spain). This work was supported by grant AGL2015-68463-C2-2-P from the Spanish Ministerio de Economía y Competitividad. Part of the funding was financed by the FEDER program from the European Union.

Conflict of Interest

The authors declare no conflict of interest.

Table 1: Serum biochemical parameters in pigs housed in barren (BE) or enriched (EE) environments. Blood samples were obtained one week before beginning both treatments ("Pre-treatment", 14 weeks old) and at the end of the eight weeks treatment ("Post-treatment", 22 weeks old).

Parameter	**Treatment**	**Sample**		***P* value**		
		Pre-treatment	*Post-treatment*	*Treatment*	*Time*	*Time* treatment*
CK (U/mL)	*EE*	6.02 ± 1.58	2.34 ± 0.29	0.417	**0.001**	0.770
	BE	7.73 ± 1.37	3.41 ± 0.61			
Cholesterol (mg/dL)	*EE*	103.58 ± 4.26	89.33 ± 2.79	0.998	**0.001**	0.059
	BE	103.59 ± 2.62	99.60 ± 2.07			
Lactate (mmol/L)	*EE*	5.08 ± 0.45a	2.75 ± 0.34b	0.431	**0.002**	**0.044**
	BE	4.58 ± 0.43a	4.03 ± 0.45a			
Hp (mg/mL)	*EE*	0.82 ± 0.13	0.37 ± 0.05	0.561	**<0.001**	0.316
	BE	0.73 ± 0.10	0.46 ± 0.10			
GPx (U/mL)	*EE*	3.58 ± 0.16a	5.40 ± 0.19b	0.242	**<0.001**	**0.032**
	BE	3.82 ± 0.12a	6.09 ± 0.21b			
SOD (U/mL)	*EE*	0.32 ± 0.05	0.26 ± 0.03	0.931	0.224	0.518
	BE	0.32 ± 0.03	0.30 ± 0.04			
Cortisol (ng/mL)	*EE*	24.27 ± 2.79a	16.77 ± 2.03b	0.573	0.249	**0.049**
	BE	24.69 ± 3.45a	26.42 ± 3.38a			

CK: creatine kinase; Hp: haptoglobin; GPx: glutathione peroxidise; SOD: superoxide dismutase

496 Table 2. Brain neurotransmitters in pigs housed in barren (BE) or enriched (EE) environments

	Neurotransmitter (ng/g tissue)	Housing		*P* value
		Enriched (EE)	Barren (BE)	
Amygdala	NA	333.17 ± 15.20	427.52 ± 15.48	**0.040**
	DA	520.50 ± 39.56	656.07 ± 42.74	**0.026**
	DOPAC	190.87 ± 11.11	280.79 ± 11.01	**0.001**
	HVA	610.50 ± 30.77	797.11 ± 27.33	**0.047**
	DA*total*	1289.91 ± 69.22	1720.14 ± 68.02	**0.008**
	5-HT	900.88 ± 57.65	1241.65 ± 124.77	0.512
	5-HIAA	205.30 ± 10.81	367.53 ± 41.66	0.216
	IND*total*	1106.18 ± 64.81	1609.18 ± 164.57	0.435
PFC	NA	123.91 ± 3.03	177.41 ± 7.64	**0.020**
	DA	41.78 ± 2.32	48.56 ± 2.97	0.349
	DOPAC	16.32 ± 1.07	28.04 ± 2.26	**0.009**
	HVA	79.48 ± 5.35	121.34 ± 5.87	**0.021**
	DA*total*	137.66 ± 8.05	192.48 ± 10.10	**0.040**
	5-HT	108.20 ± 16.13	246.29 ± 29.30	**0.001**
	5-HIAA	50.89 ± 2.09	114.16 ± 8.67	**0.001**
	IND*total*	159.62 ± 16.38	360.45 ± 37.35	**0.001**
Hippocampus	NA	177.69 ± 5.46	232.37 ± 19.97	0.545
	DA	29.66 ± 1.76	48.76 ± 3.08	*0.085*
	DOPAC	67.29 ± 1.92	90.87 ± 5.82	0.440
	HVA	81.77 ± 4.90	141.00 ± 13.85	0.161
	DA*total*	178.72 ± 7.21	283.68 ± 22.52	0.320
	5-HT	401.24 ± 24.51	497.51 ± 46.96	0.681
	5-HIAA	119.30 ± 3.89	192.50 ± 15.22	0.298
	IND*total*	520.54 ± 27	690.01 ± 61.42	0.542
Hypothalamus	NA	3470.85 ± 249.62	3696.79 ± 201.14	0.557
	L-DOPA	1016.32 ± 63.17	1203.79 ± 66.64	*0.096*
	DA	361.03 ± 25.27	601.58 ± 42.56	*0.054*
	DOPAC	1098.54 ± 42.95	1248.98 ± 57.60	0.438
	HVA	852.93 ± 36.13	990.34 ± 49.50	0.447
	DA*total*	2312.51 ± 59.63	2840.90 ± 129.91	0.389
	5-HT	1069.28 ± 71.93	1532.47 ± 89.99	**0.001**
	5-HIAA	528.96 ± 28.35	659.64 ± 26.71	0.100
	IND*total*	1572.74 ± 87.80	2192.11 ± 108.06	**0.003**
Striatum	NA	1568.22 ± 121.26	1931.11 ± 122.93	0.170
	L-DOPA	329.32 ± 20.42	300.64 ± 17.18	0.669
	DA	9789.16 ± 235.76	8555.41 ± 149.35	**< 0.001**
	DOPAC	1761.72 ± 48.82	1721.06 ± 56.96	0.802
	HVA	6497.82 ± 211.32	6199.65 ± 242.90	0.615
	DA*total*	18445.53 ± 430.63	17179.41 ± 381.53	0.240
	5-HT	327.89 ± 10.81	278.46 ± 11.19	**0.003**
	5-HIAA	144.58 ± 4.40	138.86 ± 4.75	0.592
	IND*total*	472.46 ± 12.49	417.32 ± 12.62	**0.012**

497 Table 3: Differentially abundant proteins in the hippocampus of pigs housed in enriched (EE) versus barren
498 (BE) environments

Protein accession	Gene name	String node	Protein description	log2(FC) EE vs BE
A0A0B8RT95	RPL4	RPL4	Ribosomal protein L4	0.589
A1XQU3	RPL14	RPL14	60S ribosomal protein L14	0.631
A1XQU9	RPS20	RPS21	40S ribosomal protein S20	0.730
B0FWK5	RPL5	RPL6	Ribosomal protein L5	0.657
F1RQ91	RPS4	RPS4X	40S ribosomal protein S4	0.623
F1S2E5	RPS24	RPS24	40S ribosomal protein S24	0.859
F1SEG5	RPS16	RPS16	40S ribosomal protein S16	0.783
F2Z512	RPS23	RPS23	40S ribosomal protein S23	0.575
F2Z522	RPL23A	RPL23A	60S ribosomal protein L23a	0.759
F2Z5G8	RPS25	RPS25	40S ribosomal protein S25	0.670
F2Z5Q6	RPS6	RPS6	40S ribosomal protein S6	0.657
I3L5B2	RPS7	RPS7	40S ribosomal protein S7	0.560
I3L6F1	RPL18	RPL18	60S ribosomal protein L18	0.728
I3LBH4	RPL12	RPL12	60S ribosomal protein L12	0.540
I3LJ87	RPS2	RPS2	40S ribosomal protein S2	0.524
P46405	RPS12	RPS12	40S ribosomal protein S12	0.679
P62901	RPL31	RPL31	60S ribosomal protein L31	0.781
P67985	RPL22	RPL22	60S ribosomal protein L22	0.692
Q29194	RPS2	RPS3	Ribosomal protein S2 (Fragment)	0.722
Q4GWZ2	RPSA	RPSA	40S ribosomal protein SA	0.958
Q6QAS9	RPL7	RPL7	60S ribosomal protein L7 (Fragment)	0.947
Q95281	RPL29	RPL29	60S ribosomal protein L29	0.821
I3L8P7	FARSB	FARSB	Phenylalanine--tRNA ligase beta subunit	0.411
I3LSU1	NONO	NONO	Non-POU domain-containing octamer-binding protein	0.505
I3LCN6	PURA	PURA	Transcriptional activator protein Pur-alpha	0.416
F1SNK9	ELAVL2	ELAVL3	ELAV-like protein	0.859
I3LUP6	NPM1	NPM2	Nucleophosmin	0.620
F1S6M7	CDCBM	ENSG00000258947	Tubulin beta-3 chain	0.482
F2Z571	TUBB4B	TUBB4B	Tubulin beta-4B chain	0.688
F2Z5K5	TUBB4A	TUBB4A	Tubulin beta-4A chain	0.523
F2Z5S8	TUBA4A	TUBA4A	Tubulin alpha-4A chain	0.523
P02550	TUBA1A	TUBA1A	Tubulin alpha-1A chain	0.487
P02554	TUBB2N	TUBB2N	Tubulin beta chain	0.484
Q2HPK3	TUBA3A	TUBA3A	Tubulin alpha-3 chain (Fragment)	0.610
F1SSA6	MYH10	MYH11	Myosin-10	0.512
I3LNV3	MYO18A	MYO18A	Isoform 4 of Unconventional myosin-XVIIIa	1.304
P04574	CAPNS1	CAPNS2	Calpain small subunit 1	0.427
F1RFF5	THOC5	GBAS	Protein NipSnap homolog 1	0.590
F1RG61	TBC1D10B	TBC1D10B	TBC1 domain family member 10B	0.507
F1SIS9	NDUFA10	NDUFA10	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10	0.589
F1SL07	NDUFA9	NDUFA9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9	0.477
I3LDC1	SDHB	SDHB	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	0.525

I3LQ34	TOMM70A	TOMM70A	Mitochondrial import receptor subunit TOM70	0.738
F1RWM4	PPP1R1B	PPP1R1B	Protein phosphatase 1 regulatory subunit 1B	0.943
P63246	RACK1	GNB2L1	Receptor of activated protein C kinase 1	0.593
I3LEH4	MAOB	MAOB	Amine oxidase [flavin-containing]	0.551
F1RGD9	HARS	HARS	Histidine--tRNA ligase	-0,45
F1RQS8	ARL2	ARL3	ADP-ribosylation factor-like protein 2	-0,533
A0A0B8RTH9	LYPLA1	LYPLA2	Lysophospholipase I	-0,932
A8U4R4	TKT	TKT	Transketolase	-0,341
I3L656	NUDT5	NUDT6	ADP-sugar pyrophosphatase	-0,554
F1SB62	ACAT2	ACAT3	Acetyl-CoA acetyltransferase	-0,472
F1SEN4	C10orf116	ADIRF	Adipogenesis regulatory factor	-0,559
F1SUH8	ATP6V0C	ATP6V0C	V-type proton ATPase proteolipid subunit	-1,101
K7GQV5	GSTZ1	GSTZ2	Maleylacetoacetate isomerase	-0,422
I3LKS6	QDPR	QDPR	Dihydropteridine reductase	-0,345

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