Brain neurotransmitters and hippocampal proteome in pigs under stress and intrauterine growth restriction

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FATTENING PIGS ARE SUBJECTED TO SEVERAL TYPES OF STRESS

SLAUGHTERHOUSE

HOUSING

TRANSPORT

ENVIRONMENTAL ENRICHMENT
Glucocorticoids (cortisol) (catecholamines, others, ...)

LIVER

ADIPOSE TISSUE

LIPID MOBILIZATION

STRESS

ROS, OTHER SOURCES OF OXIDATIVE STRESS

Oxidation markers

Antioxidant enzymes

Innate immune system involvement

Cell damage

Acute phase proteins

STRESS BIOMARKERS (IN PLASMA)
BUT WE ALL KNOW THAT STRESS AND WELFARE ARE MAINLY A “BRAIN ISSUE”

THE PIG MAY BE A GOOD MODEL FOR HUMANS:
- The pig brain is large
- The pig brain is more similar to the human brain (i.e. It has convolutions)
COMMUNICATION BETWEEN BRAIN AREAS IS CARRIED OUT BY CHEMICAL NEUROTRANSMITTERS

NORADRENALINE

DOPAMINE

SEROTONIN (5-HYDROXYTRYPTAMINE)

By CYL - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=18074053
### Noradrenalin pathways
- Stress

### Dopamine pathways
- Reward (motivation)
- Pleasure
- Motor functions
- Compulsion
- Perseveration

### Serotonin pathways
- Mood
- Memory processing
- Sleep
- Cognition

<table>
<thead>
<tr>
<th>Amygdala</th>
<th>PFC</th>
<th>Hippocampus</th>
<th>Striatum</th>
<th>Hypothalamus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emotions</td>
<td>Cognitive behaviour</td>
<td>Memory</td>
<td>Reward</td>
<td>Connection with the organism</td>
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<tr>
<td>Fear</td>
<td>Emotion-behaviour</td>
<td>Spatial coding</td>
<td>Addiction</td>
<td>Neuroendocrine signalling</td>
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<tr>
<td>Stress</td>
<td>Decision-making</td>
<td>Stress</td>
<td></td>
<td>Stress</td>
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<tr>
<td>Decision-making</td>
<td>Social interactions</td>
<td></td>
<td></td>
<td>Appetite</td>
</tr>
<tr>
<td>Memory</td>
<td>Stress</td>
<td></td>
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</tbody>
</table>
HOW ARE THE NEUROTRANSMITTERS SYNTHESIZED AND DEGRADED?

PRECURSOR OF DOPAMINE AND NORADRENALINE IS THE AMINOACID TYROSINE
PRECURSOR OF SEROTONIN IS THE AMINOACID TRYPTOPHAN

HPLC system

NA, DA, DOPAC, HVA cathecholamines

5-HT, 5-HIAA indoleamines
DIFFERENT BRAIN AREAS ARE CHARACTERIZED BY THEIR NEUROTRANSMITTER PROFILE

<table>
<thead>
<tr>
<th>Variables</th>
<th>Loadings</th>
</tr>
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<tbody>
<tr>
<td>NA</td>
<td>0.23</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>0.22</td>
</tr>
<tr>
<td>DOPAC</td>
<td>0.90</td>
</tr>
<tr>
<td>DA</td>
<td>0.95</td>
</tr>
<tr>
<td>HVA</td>
<td>0.93</td>
</tr>
<tr>
<td>5-HIAA</td>
<td>0.82</td>
</tr>
<tr>
<td>5-HT</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Principal component 1 vs. Principal component 2

- PFC
- AMY
- HC
- HPT

Loadings:
- PFC
- AMY
- HC
- HPT
PROTEOMICS

TWO MAIN STRATEGIES

Proteins

Gel-based approach

Separation on 2D electrophoresis

Digestion

Peptides

Gel-free approach

Digestion

Peptides

Chromatographic separation

Mass spectrometry

Data analysis

Protein identification and quantification
LONG TERM EFFECTS OF GROUP MIXING AND MANAGEMENT

Biochemical parameters

<table>
<thead>
<tr>
<th>Date</th>
<th>Transport to the farm</th>
<th>Mixing of animals</th>
<th>Sample collection</th>
<th>Start of handling treatment</th>
<th>Sample collection</th>
<th>Slaughterhouse</th>
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<td>May 22</td>
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</tr>
</tbody>
</table>

### Table 3

Brain serotonin (5-HT) concentration (ng/g tissue) in the prefrontal cortex and amygdala of pigs subjected to PH or NH.

<table>
<thead>
<tr>
<th>Brain area</th>
<th>NH Mean</th>
<th>NH SE</th>
<th>PH Mean</th>
<th>PH SE</th>
<th>P</th>
<th>Handling*Time</th>
<th>Time</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prefrontal cortex</td>
<td>200.45</td>
<td>12.90</td>
<td>229.18</td>
<td>10.19</td>
<td>0.093</td>
<td>0.410</td>
<td>0.410</td>
<td>0.826</td>
</tr>
<tr>
<td>Amygdala</td>
<td>683.86</td>
<td>22.46</td>
<td>627.61</td>
<td>20.90</td>
<td>0.073</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


In collaboration with Dr Antonio Velarde (IRTA)
DIGE: Differential labeling gel electrophoresis

Etan DIGE is the only system with a pooled internal standard for every spot on every gel.
LONG TERM EFFECTS OF GROUP MIXING AND MANAGEMENT

DIGE:
1180 spots analyzed
54 differential proteins
in the comparison t2 vs t0

LONG TERM EFFECTS OF GROUP MIXING AND MANAGEMENT

CONCLUSIONS

Stress biomarkers as hair cortisol indicate that the stress degree decreases throughout the time in farm.
Mild indications of the benefits of positive handling.

In the proteomic approach, many of the identified proteins are targets of GCs and, hence, indicate that changes in the PBMC proteome mirror the variations of endogenous cortisol and the degree of stress, since they vary concomitantly with hair cortisol and APPs.

Taken together, these findings suggest that changes in the PBMC proteome may be sensitive indicators of animal stress.

In collaboration with Dr Antonio Velarde (IRTA)

# ENVIRONMENTAL ENRICHMENT

## Biochemical serum stress markers

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Post-treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (U/mL)</td>
<td>Enriched</td>
<td>2.34 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>Barren</td>
<td>3.41 ± 0.61</td>
</tr>
<tr>
<td>Lactate (mM)</td>
<td>Enriched</td>
<td>89.33 ± 2.79 *</td>
</tr>
<tr>
<td></td>
<td>Barren</td>
<td>99.60 ± 2.79 *</td>
</tr>
<tr>
<td>Cortisol (ng/mL)</td>
<td>Enriched</td>
<td>16.77 ± 2.03 *</td>
</tr>
<tr>
<td></td>
<td>Barren</td>
<td>26.42 ± 3.38 *</td>
</tr>
<tr>
<td>Haptoglobin (mg/mL)</td>
<td>Enriched</td>
<td>0.37 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>Barren</td>
<td>0.46 ± 0.10</td>
</tr>
</tbody>
</table>

PIGS IN BARREN CONDITIONS SHOW A HIGHER DEGREE OF STRESS

ENVIRONMENTAL ENRICHMENT

MANY EFFECTS ON NEUROTRANSMISSION IN PFC, AMYGDALA, HYPOTHALAMUS AND STRIATUM

PIGS IN BARREN CONDITIONS “FEEL” “LESS REWARDED”

THE HIPPOCAMPUS

Involved in:
- Memory processes
- Spatial coding
- Learning capacities
- Motor abilities
- Stress

Hippocampus and seahorse
Professor Laszlo Seress- CC BY-SA 3.0,
https://commons.wikimedia.org/w/index.php?curid=9451294

Drawing by Camillo Golgi of a hippocampus
stained using the silver nitrate method
Public Domain,
https://commons.wikimedia.org/w/index.php?curid=391548
PROTEOMIC ANALYSIS OF THE HIPPOCAMPUS: iTRAQ 8-plex

Isobaric tag for relative and absolute quantitation

**Samples**
- Reduction
- Alkylation
- Trypsinization

**iTRAQ labeling**
- 113 label
- 114 label
- 115 label
- 116 label
- 117 label
- 118 label
- 119 label
- 121 label

**Number of samples:**
- 20 barren
- 20 enriched
- 8 control samples
- 6 iTRAQ labeling reactions

**Peptide analysis on Orbitrap Fusion Lumos Tribrid**

**Combine**

**iTRAQ reporter ions**

**Identify**

**Quantify**
GENE ONTOLOGY ANALYSIS (Panther)

MOLECULAR FUNCTION

15649 peptides
2418 proteins

59 differentially abundant proteins (>1.3-fold; p<0.05)

- 2% Binding proteins
- 22% Structural proteins
- 31% Catalytic activity
- 45% Transport

<table>
<thead>
<tr>
<th>Uniprot</th>
<th>Identification</th>
<th>Fold change</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1SIS9</td>
<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10</td>
<td>1.50</td>
<td>up</td>
</tr>
<tr>
<td>F1SL07</td>
<td>NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9</td>
<td>1.39</td>
<td>up</td>
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<tr>
<td>I3LDC1</td>
<td>Succinate dehydrogenase [ubiquinone] iron-sulfur subunit</td>
<td>1.44</td>
<td>up</td>
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<tr>
<td>I3LQ34</td>
<td>Mitochondrial import receptor subunit TOM70</td>
<td>1.67</td>
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<tr>
<td>A0A0B8RT</td>
<td>Lysophospholipase I</td>
<td>0.52</td>
<td>down</td>
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<tr>
<td>A8U4R4</td>
<td>Transketolase</td>
<td>0.79</td>
<td>down</td>
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<tr>
<td>F1RWM4</td>
<td>Protein phosphatase 1 regulatory subunit 1B</td>
<td>1.92</td>
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<tr>
<td>F1SB62</td>
<td>Acetyl-CoA acetyltransferase</td>
<td>0.72</td>
<td>down</td>
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<tr>
<td>F1SEN4</td>
<td>Adipogenesis regulatory factor</td>
<td>0.68</td>
<td>down</td>
</tr>
<tr>
<td>F1SUH8</td>
<td>V-type proton ATPase proteolipid subunit</td>
<td>0.47</td>
<td>down</td>
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<tr>
<td>I3L656</td>
<td>ADP-sugar pyrophosphatase</td>
<td>0.68</td>
<td>down</td>
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<tr>
<td>K7GQV5</td>
<td>Maleylacetoacetate isomerase</td>
<td>0.75</td>
<td>down</td>
</tr>
<tr>
<td>F1RFF5</td>
<td>Protein NipSnap homolog 1</td>
<td>1.51</td>
<td>up</td>
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<tr>
<td>F1RG61</td>
<td>TBC1 domain family member 10B</td>
<td>1.42</td>
<td>up</td>
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<tr>
<td>I3LSU1</td>
<td>Non-POU domain-containing octamer-binding protein</td>
<td>1.42</td>
<td>up</td>
</tr>
<tr>
<td>I3LUP6</td>
<td>Nucleophosmin</td>
<td>1.54</td>
<td>up</td>
</tr>
<tr>
<td>P04574</td>
<td>Calpain small subunit 1</td>
<td>1.34</td>
<td>up</td>
</tr>
<tr>
<td>P63246</td>
<td>Receptor of activated protein C kinase 1</td>
<td>1.51</td>
<td>up</td>
</tr>
<tr>
<td>A5GFR8</td>
<td>Breast carcinoma amplified sequence 1</td>
<td>0.63</td>
<td>down</td>
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<tr>
<td>A0A0AM0</td>
<td>Immunoglobulin lambda-like polypeptide 5</td>
<td>0.59</td>
<td>down</td>
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<tr>
<td>Y58</td>
<td>IgG heavy chain</td>
<td>0.61</td>
<td>down</td>
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<tr>
<td>I3LEH4</td>
<td>Amine oxidase [flavin-containing]</td>
<td>1.46</td>
<td>up</td>
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<tr>
<td>I3LKS6</td>
<td>Dihydropteridine reductase</td>
<td>0.79</td>
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<tr>
<td>I3LCN6</td>
<td>Transcriptional activator protein Pur-alpha</td>
<td>1.33</td>
<td>up</td>
</tr>
</tbody>
</table>

**Catalytic activity**

- Ligase
- Oxidoreductase
- Transferase
- Regulator
- Hydrolase
- Lyase
PATHWAY ANALYSIS WITH REACTOME

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

THE “ENRICHED” HIPPOCAMPUS POTENTIALLY HAS:

- Increased capacity for protein synthesis
- Increased capacity for axonal / dendrite transport
- Increased oxidative phosphorylation (ATP)

CONCLUSIONS

PIGS IN BARREN CONDITIONS SHOW A HIGHER DEGREE OF STRESS

PIGS IN BARREN CONDITIONS “FEEL” “LESS REWARDED”

INTRAUTERINE GROWTH RESTRICTION (IUGR)

• IUGR is due to nutritional or placental conditions in the mother, which restrain the availability of nutrients and/or oxygen to the foetus.

• IUGR provokes the birth of low birthweight offspring (LBW)

• Asymmetric foetal development

• Brain sparing (but not normal SNC development)

• Porcine model for IUGR: nutritional restriction of the mothers during the last two thirds of pregnancy, multiparous, LBW and NBW piglets from the same litter
INTRAUTERINE GROWTH RESTRICTION (IUGR)

Medical problems associated to intrauterine exposure to undernutrition

Potential prevention: supplementation of the maternal diet with antioxidants

Reproduction (2014) 148 R111–R120
Objective 1: analyze the effects of supplementation of the maternal diet with the antioxidant hydroxytyrosol (HTX) on the SNC of the offspring.

Experimental design:
- 51 pregnant sows
- 2/3 of gestation: 50% caloric restriction
- 100 d foetuses
- 1 month piglets
- 6 month pigs
- Control: without HTX
- HTX: with antioxidant

In collaboration with Dr Antonio González-Bulnes (INIA, Madrid)

Yeste et al. Polyphenols and IUGR Pregnancies: Effects of the Antioxidant Hydroxytyrosol on Brain Neurochemistry and Development in a Porcine Model. Antioxidants 2021, 10, 884
INTRAUTERINE GROWTH RESTRICTION (IUGR)

The effects of the supplementation of the maternal diet with HTX are only visible in 100-days foetuses, but not postnatally.

Most probable, the continuous presence of HTX is needed to maintain its effects on the brain.

Yeste et al. Polyphenols and IUGR Pregnancies: Effects of the Antioxidant Hydroxytyrosol on Brain Neurochemistry and Development in a Porcine Model. Antioxidants 2021, 10, 884
In CA1, 100-day-old fetuses in the HTX-group showed a higher number of mature neuronal cells (immunopositive to NeuN), whereas the immunostaining of immature neurons (immunopositive to DCX) was lower, indicating that HTX induced a faster neuron differentiation process in this layer.

A similar but milder effect was observed in the GD

The effects of the supplementation of the maternal diet with HTX are only visible in 100-days foetuses, but not postnatally.
PROTEOMIC ANALYSIS OF THE HIPPOCAMPUS: TMT 10-plex

Tandem mass tagging

<table>
<thead>
<tr>
<th>Reporter ions</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
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<tr>
<td>126</td>
<td>CH.1</td>
<td>CM.3</td>
</tr>
<tr>
<td>127N</td>
<td>CM.5</td>
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<td>127C</td>
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<td>CH.5</td>
</tr>
<tr>
<td>131</td>
<td>TH.3</td>
<td>TM.5</td>
</tr>
</tbody>
</table>

Number of samples:
- 10 control
- 10 HTX
- Males and Females

2 TMT reactions

TMT labelling

Peptide analysis on Orbitrap Fusion Lumos Tribrid

Combine

TMT reporter ions

Identify (Proteome discoverer)

Quantify

11 DAPs

3 more abundant in HTX
8 more abundant in Ctrl
FC ≥ 1.5  p < 0.5
PROTEOMIC ANALYSIS OF THE HIPPOCAMPUS: TMT 10-plex

Tandem mass tagging

Upregulated proteins in the HTX group are involved in detoxification, cell protection and sinapsis formation, suggesting a potential mechanism for the neuroprotective effects of HTX

Downregulated proteins in the HTX group are involved in protein synthesis, transcriptional regulation and fatty acid degradation

3 more abundant in HTX: ECHDC1 (Ethylmalonyl-CoA- decarboxylase), TXNDC5 (Thioredoxin5), NRGN (Neurogranin)

8 more abundant in Ctrl: 4 ribosomal proteins (RPL7, RPL7A, RPL36, FAU), HSPE1, TARS1 (Thr-tRNA synthase), RBMX (RNA binding protein), HADHA (3-hydroxyacyl-CoA dehydrogenase)
AND... WHAT HAPPENS WITH IUGR?

Objective 2: Since the effects of HTX were only visible in 100-days foetuses, an analysis was performed on the effects of IUGR (LBW, NBW)

The differences observed between NBW and LBW Control groups, disappear when comparing HTX-treated groups

Supplementation of the mother’s diet with HTX is able to reverse the deleterious effects of IUGR

Yeste et al., Polyphenols and IUGR Pregnancies: Intrauterine Growth Restriction and Hydroxytyrosol Affect the Development and Neurotransmitter Profile of the Hippocampus in a Pig Model. Antioxidants 2021, 10, 1505
AND... WHAT HAPPENS WITH IUGR?

Neurotransmitter profile: There were almost no differences between NBW and LBW animals, but there is an interaction between treatment and bodyweight in the hippocampal concentration of 5-HT (and total indoleamines).

Again, the differences observed between NBW and LBW in the Ctrl group disappeared in the HTX group.

Yeste et al., Polyphenols and IUGR Pregnancies: Intrauterine Growth Restriction and Hydroxytyrosol Affect the Development and Neurotransmitter Profile of the Hippocampus in a Pig Model. Antioxidants 2021, 10, 1505
GENERAL CONCLUSIONS

✓ Pigs are a good model for human pathologies and conditions, due to their similar physiology, anatomy and development

✓ Pigs are a good model to study the CNS, because the pig’s brain is large, gyrencephalic and has a development rate similar to the human brain

✓ Stress and welfare can be approached with traditional techniques quantifying biomarkers (stress hormones, acute phase proteins) in serum/plasma and other sample types (hair, PBMCs,....)

✓ The neurotransmitter profile in several brain areas can detect subtle changes caused by the environmental conditions

✓ Proteomic approaches are useful to provide new perspectives and interpretation of stressful conditions
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INIA. Antonio Gonzalez-Bulnes
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FESTA DE SANT ANTONI
CALDES DE MONTBUI