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

Anna Bassols

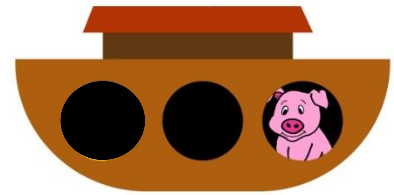
Departament de Bioquímica i Biologia Molecular
Facultat de Veterinària
Universitat Autònoma de Barcelona
Spain

UAB
Universitat Autònoma de Barcelona

FATTENING PIGS ARE SUBJECTED TO SEVERAL TYPES OF STRESS

SLAUGHTERHOUSE

ENVIRONMENTAL ENRICHMENT



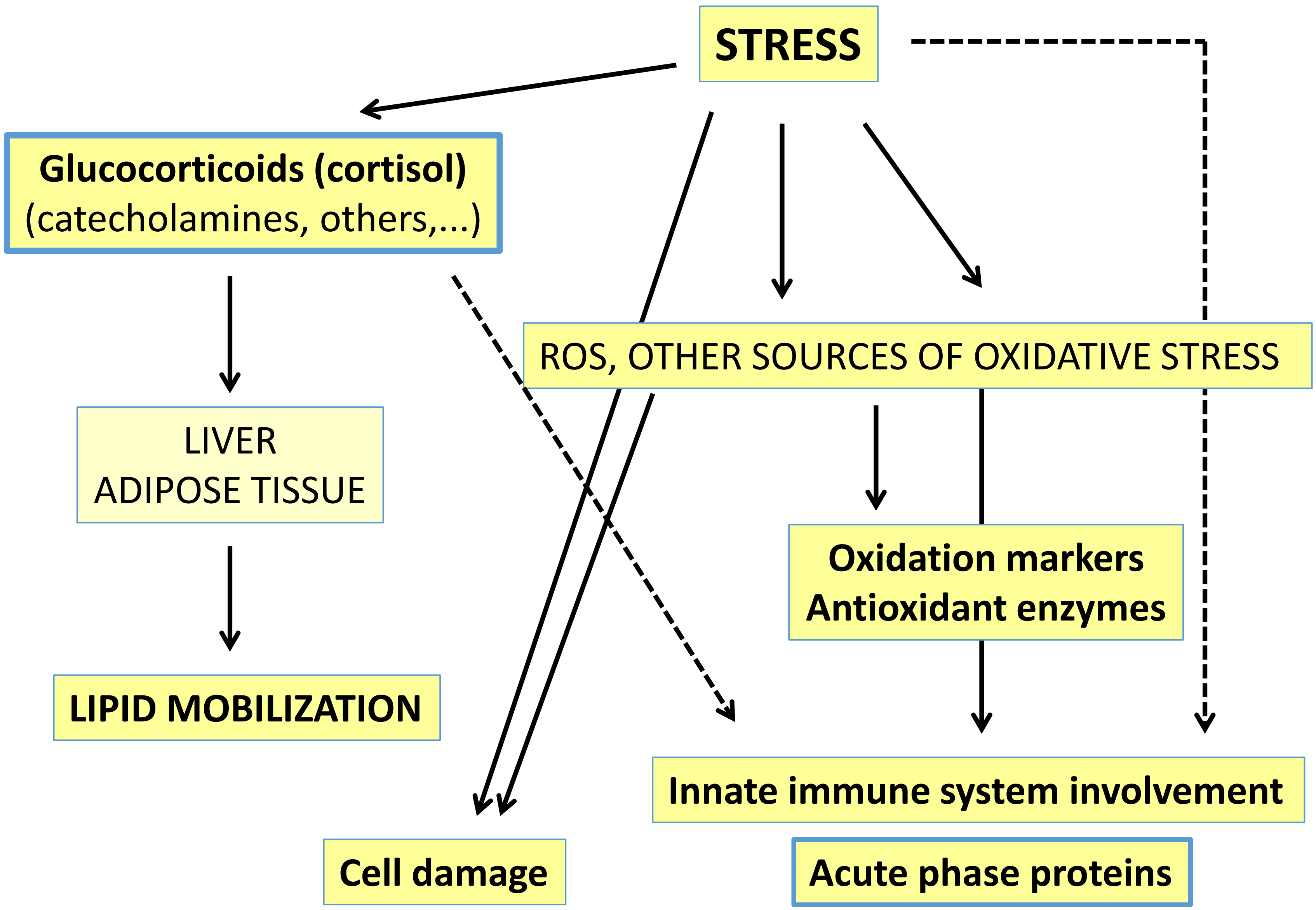
2

HOUSING

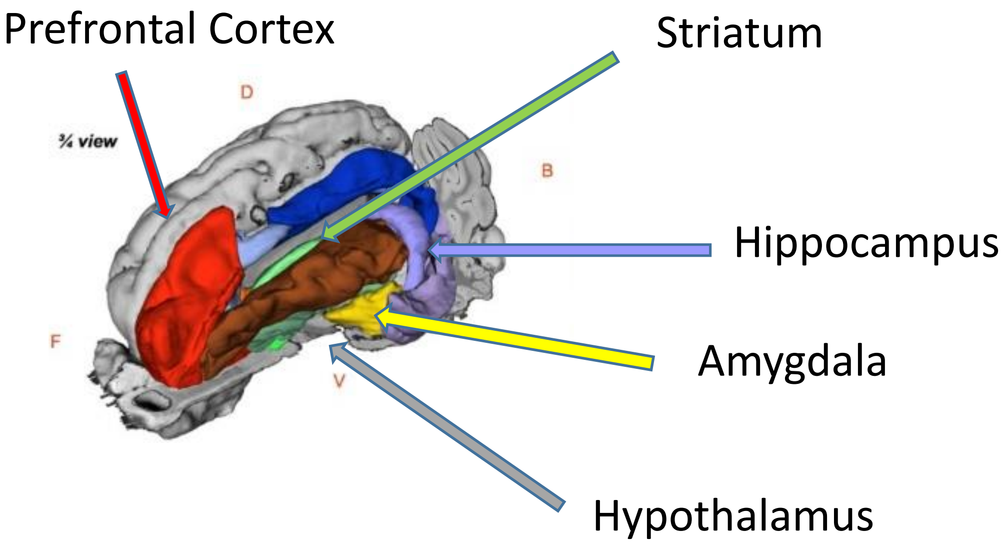
1

TRANSPORT

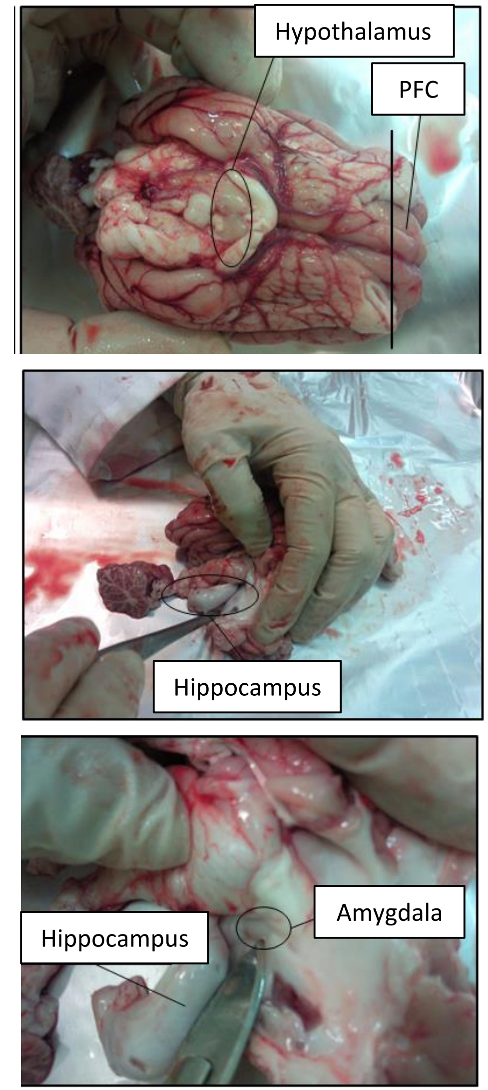
STRESS BIOMARKERS (IN PLASMA)



BUT WE ALL KNOW THAT STRESS AND WELFARE ARE MAINLY A “BRAIN ISSUE”



Saikali et al. (2010)

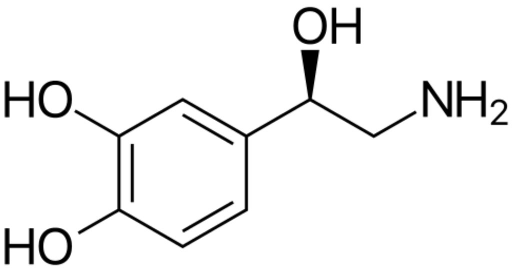


THE PIG MAY BE A GOOD MODEL FOR HUMANS:

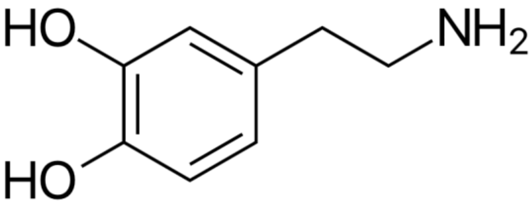
- The pig brain is large
- The pig brain is more similar to the human brain (i.e. It has convolutions)

COMMUNICATION BETWEEN BRAIN AREAS IS CARRIED OUT BY CHEMICAL NEUROTRANSMITTERS

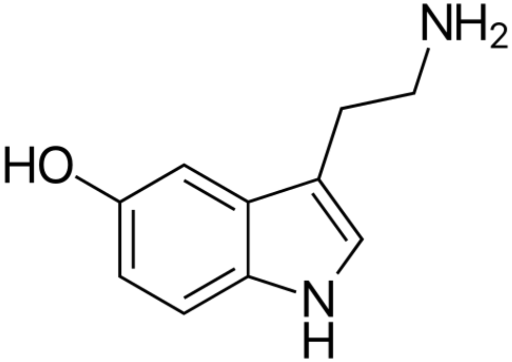
NORADRENALINE



DOPAMINE



SEROTONIN (5-HYDROXYTRYPTAMINE)

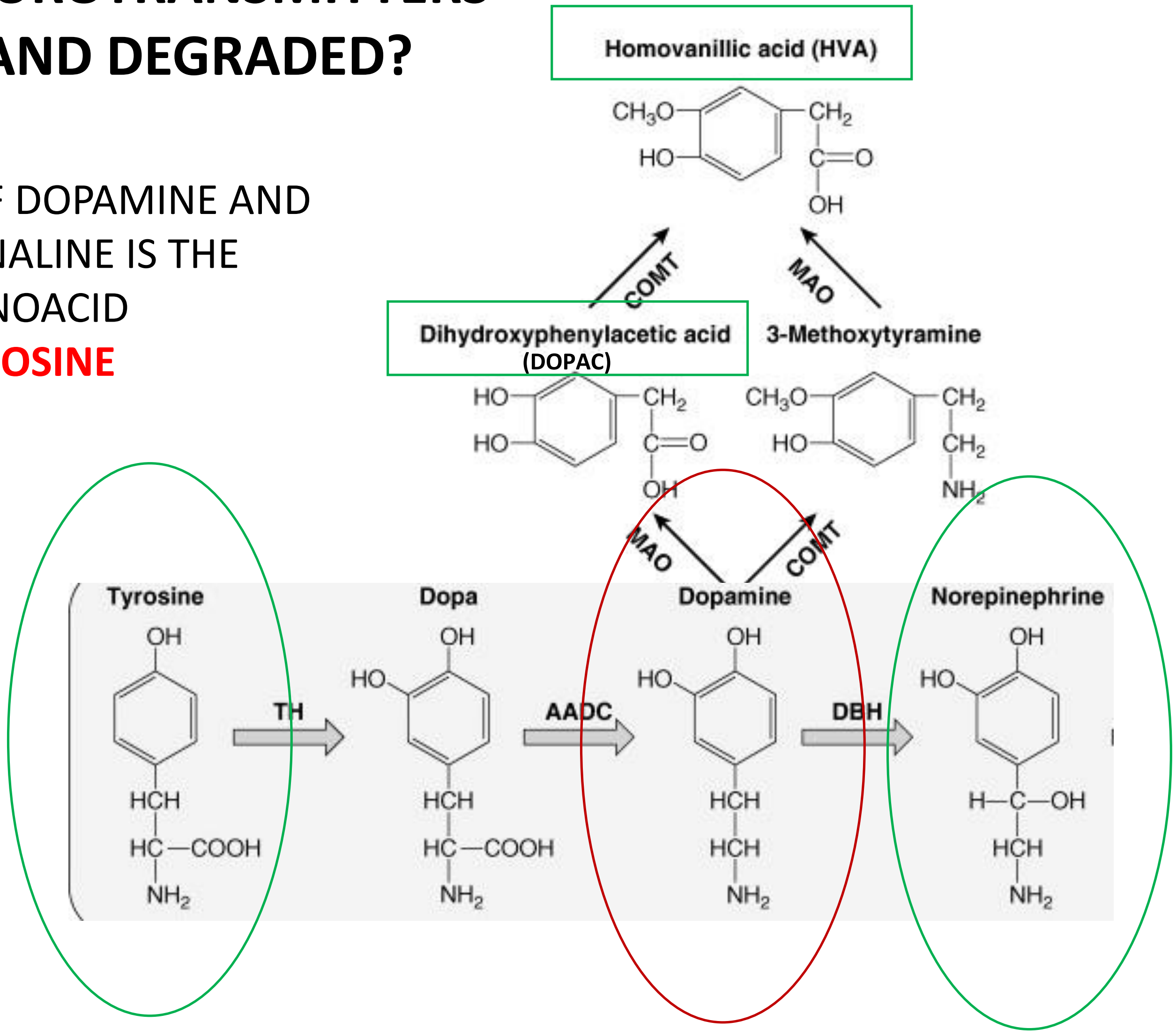


Noradrenalin pathways	Dopamine patways	Serotonin pathways
Stress	Reward (motivation) Pleasure Motor functions Compulsion Perseveration	Mood Memory processing Sleep Cognition

Amygdala	PFC	Hippocampus	Striatum	Hypothalamus
Emotions Fear Stress Decision-making Memory	Cognitive behaviour Decision-making Social interactions Stress	Memory Spatial coding Stress	Reward Addiction	Connection with the organism Neuroendocrine signalling Stress Appetite

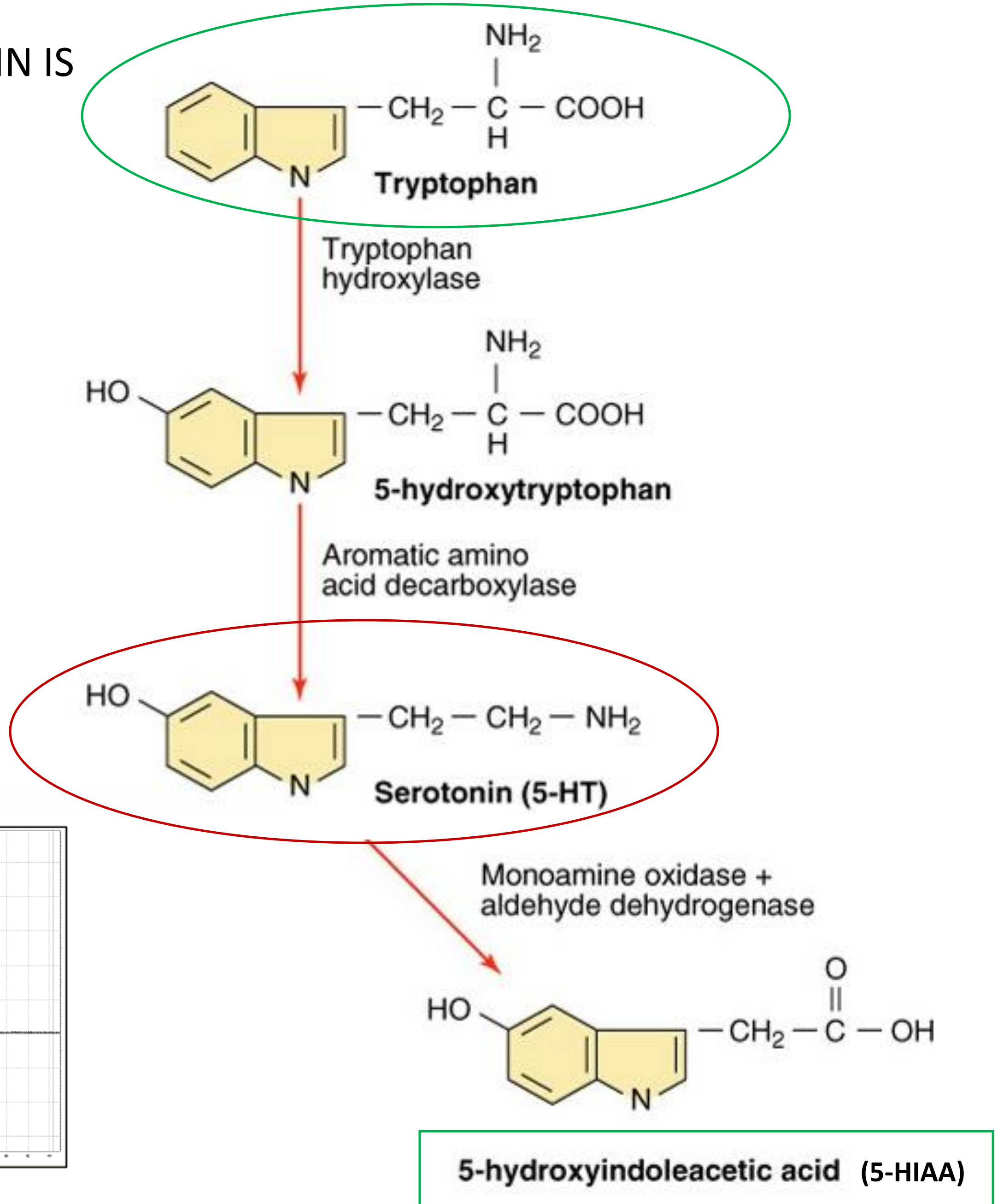
HOW ARE THE NEUROTRANSMITTERS SYNTHESIZED AND DEGRADED?

PRECURSOR OF DOPAMINE AND NORADRENALINE IS THE AMINOACID **TYROSINE**

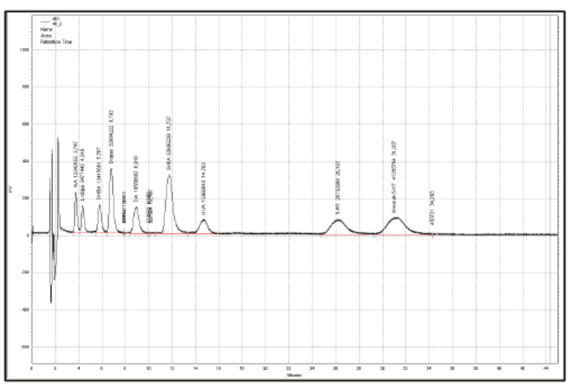


TH = Tyrosine hydroxylase
AADC = Aromatic L-amino acid decarboxylase
DBH = Dopamine β-hydroxylase
PNMT = Phenylethanolamine N-methyltransferase
MAO = Monoamine oxidase
COMT = Catechol-*O*-methyltranferase

PRECURSOR OF SEROTONIN IS
THE AMINOACID
TRYPTOPHAN



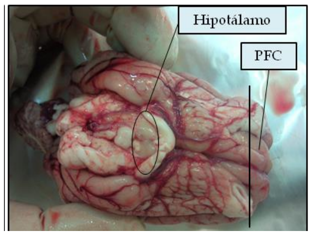
HPLC system

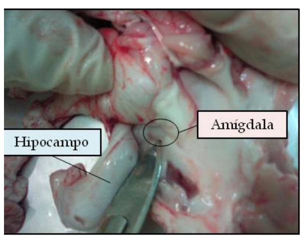


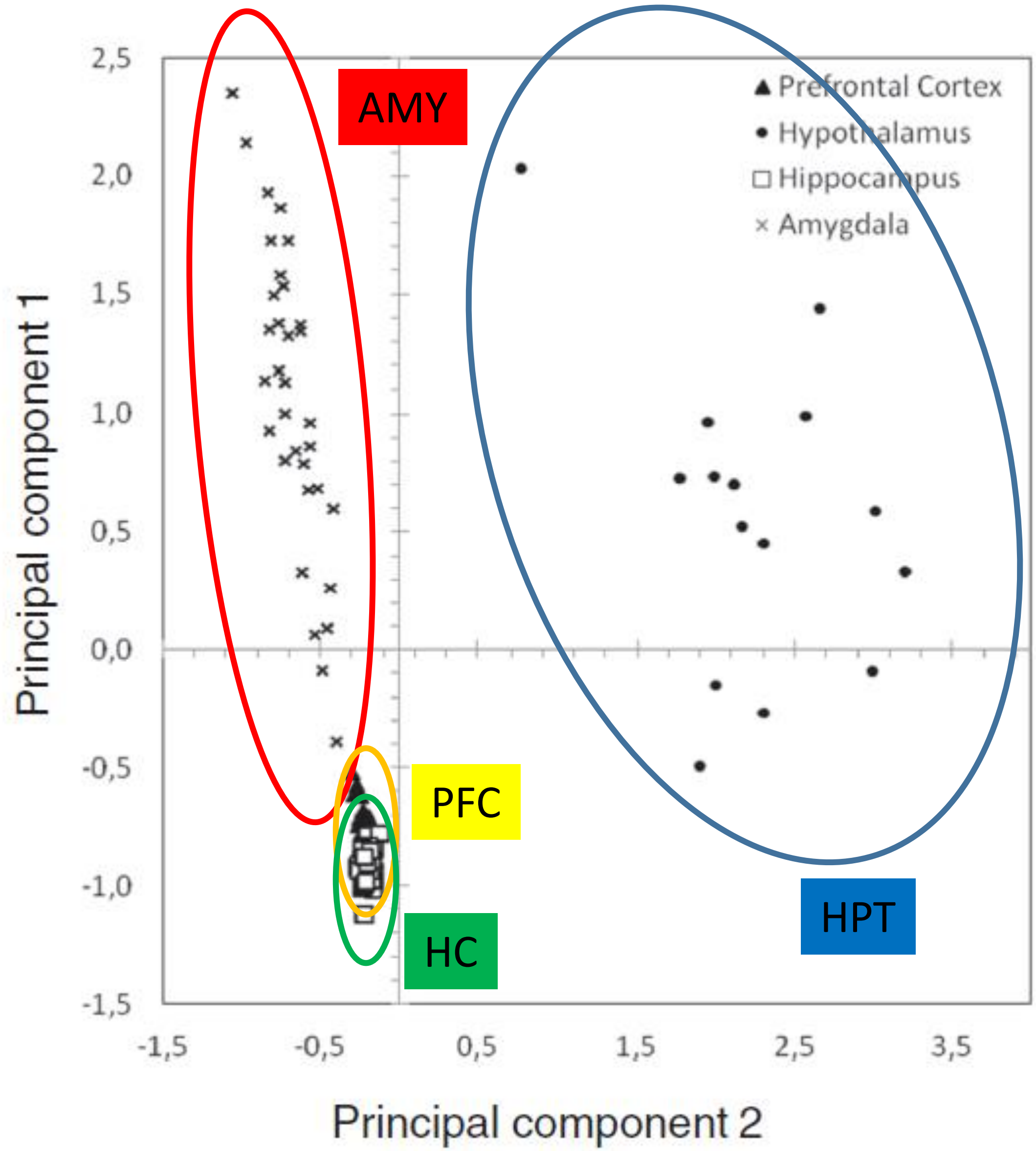
NA, DA, DOPAC, HVA
cathecholamines

5-HT, 5-HIAA
indoleamines

DIFFERENT BRAIN AREAS ARE CHARACTERIZED BY THEIR NEUROTRANSMITTER PROFILE

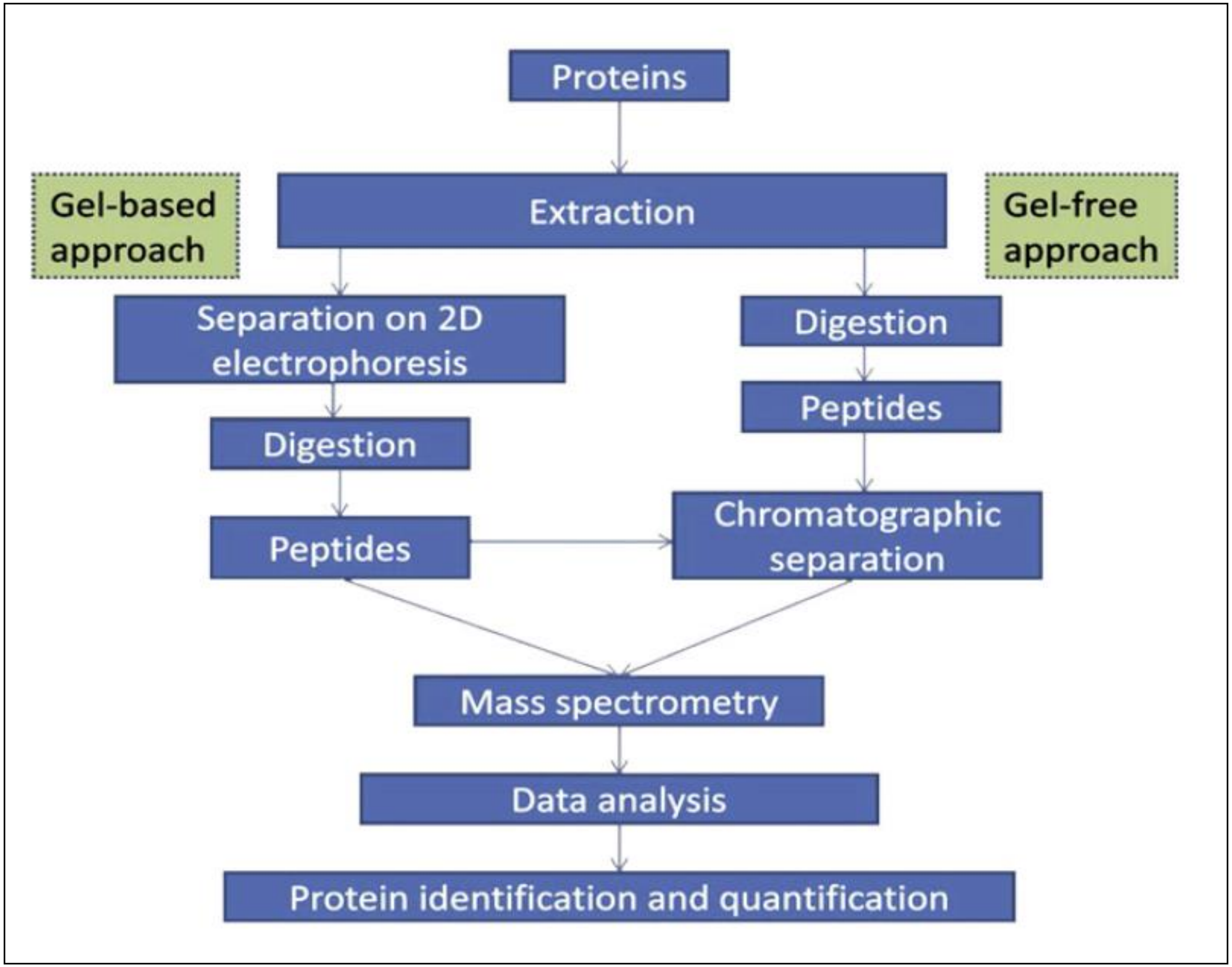






Variables	Loadings	
NA	0.23	**0.97**
L-DOPA	0.22	**0.97**
DOPAC	**0.90**	0.28
DA	**0.95**	0.11
HVA	**0.93**	0.18
5-HIAA	**0.82**	0.46
5-HT	**0.91**	0.26

PROTEOMICS



TWO MAIN STRATEGIES

1 LONG TERM EFFECTS OF GROUP MIXING AND MANAGEMENT

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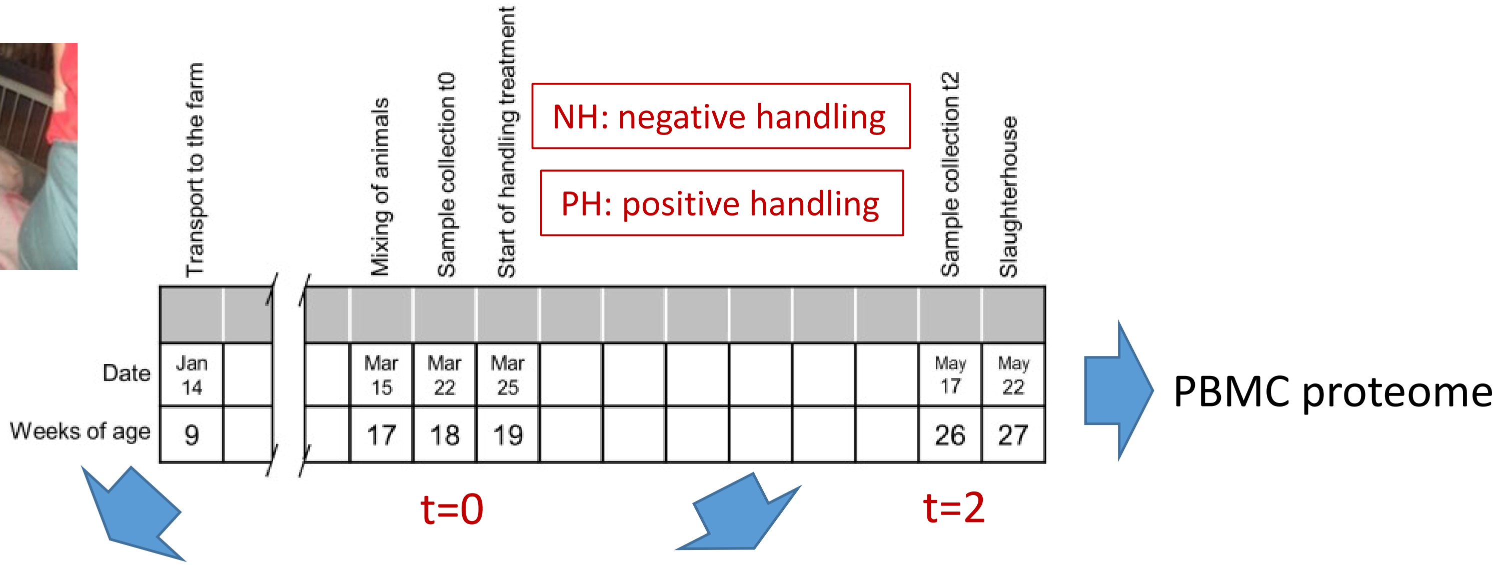


Biochemical parameters

	t 0				t 2				Handling	Time	Handling*Time
	NH		PH		NH		PH				
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	P	P	P
Hair Cortisol (pg/mg)	24.70	2.05	19.80	1.35	20.80	2.00	17.61	1.53	0.208	0.007	0.852
Serum Cortisol (ng/mL)	23.50	2.22	17.57	1.77	24.21	2.09	21.20	2.09	0.094	0.252	0.410
Saliva Cortisol (ng/mL)	3.47	0.30	4.25	0.57	3.57	0.21	3.60	0.27	0.604	0.172	0.826
Haptoglobin (mg/mL)	0.64	0.07	0.86	0.07	0.39	0.06	0.43	0.04	0.166	<0.0001	0.047

Brain neurotransmitters

Table 3

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

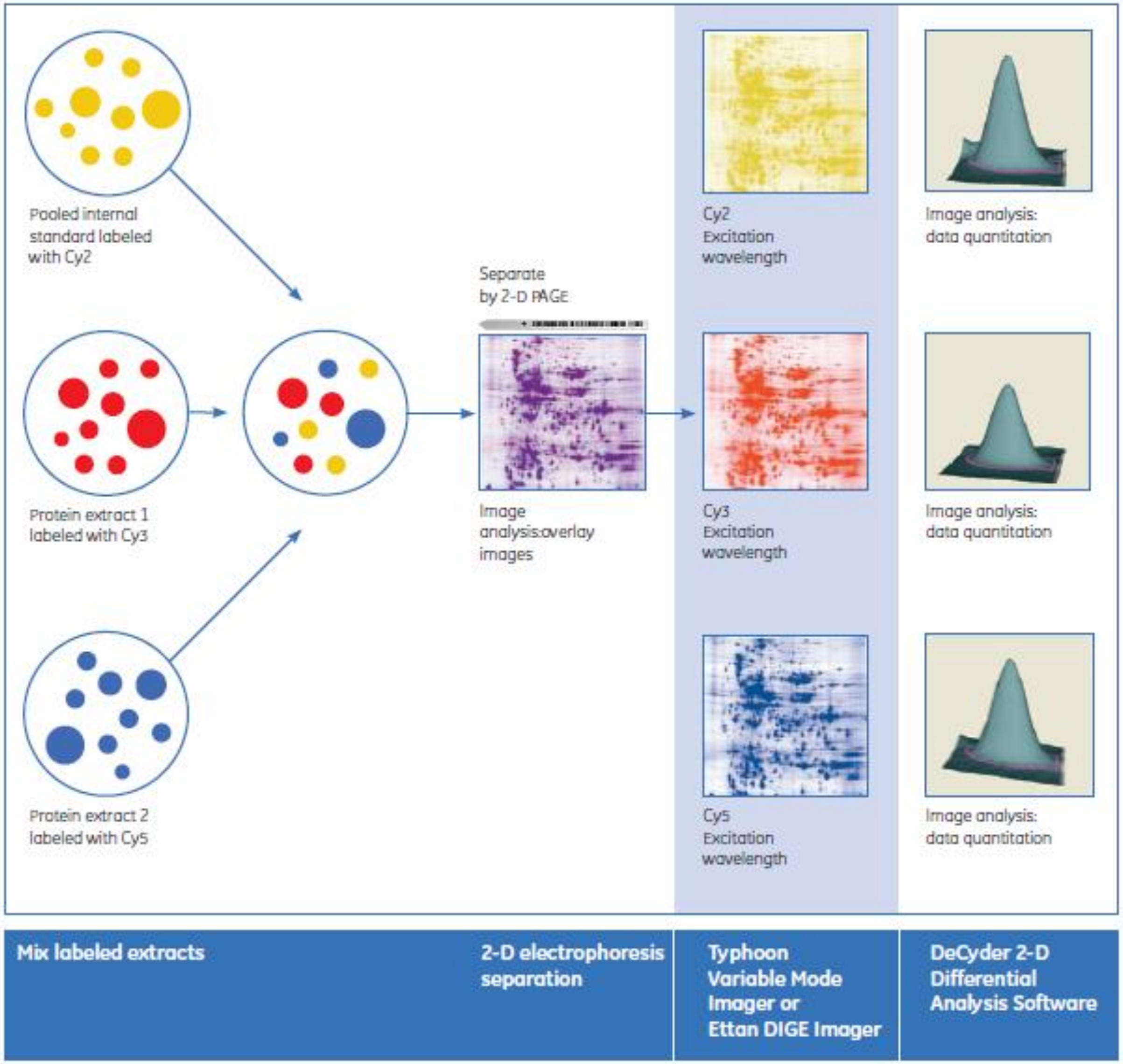
Brain area	*NH*		*PH*		*P*
	Mean	*SE*	*Mean*	*SE*	
Prefrontal cortex	200.45	12.90	229.18	10.19	0.093
Amygdala	683.86	22.46	627.61	20.90	0.073

In collaboration with Dr Antonio Velarde (IRTA)

Valent et al., Effects on pig immunophysiology, PBMC proteome and brain neurotransmitters caused by group mixing stress and human-animal relationship. PLoS One. 2017; 12(5): e0176928.

DIGE: Differential labeling gel electrophoresis

Ettan DIGE is the only system with a pooled internal standard for every spot on every gel



1 LONG TERM EFFECTS OF GROUP MIXING AND MANAGEMENT

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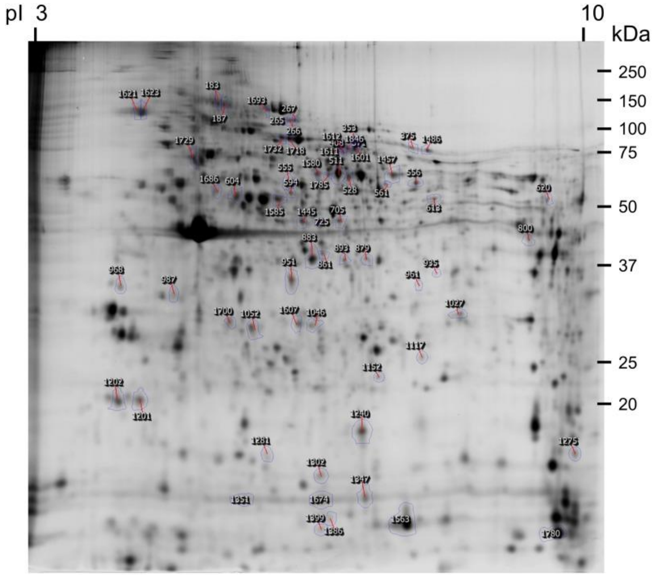
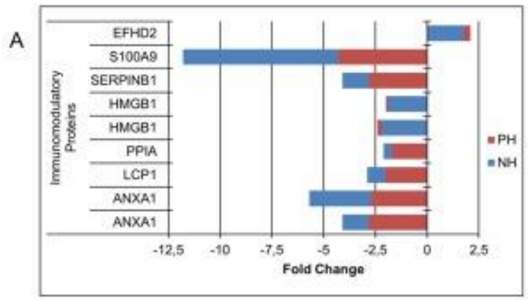
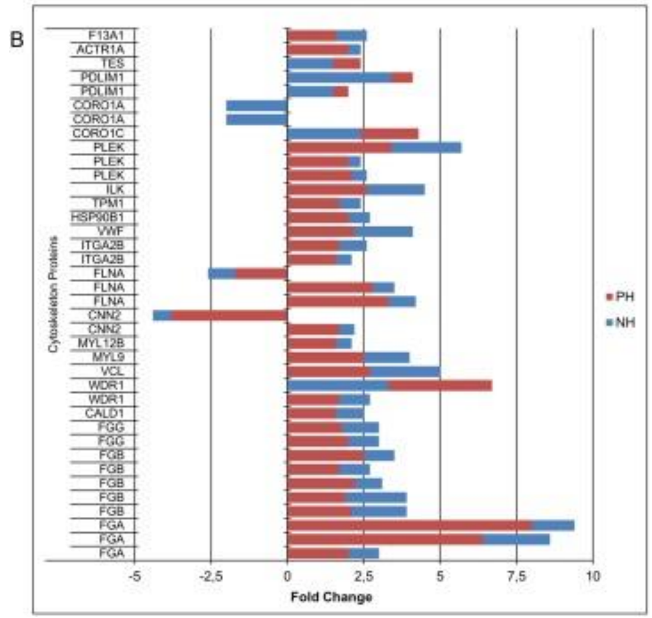
PBMC proteome



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

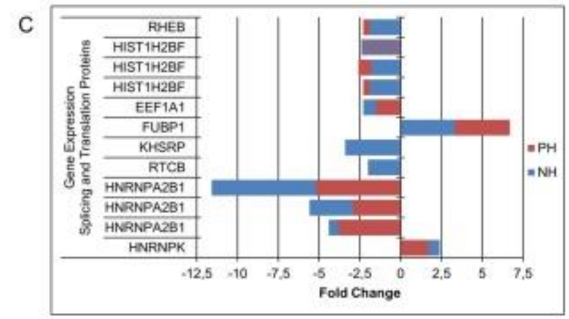
Immunomodulatory

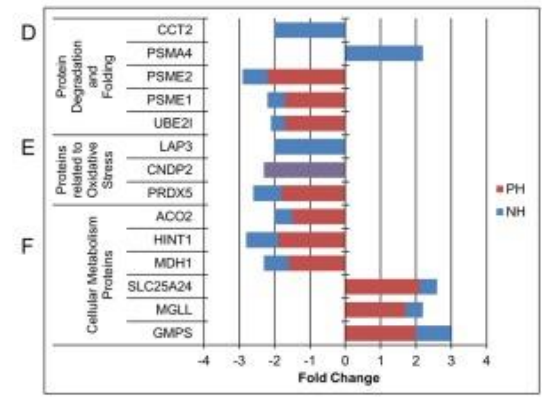




Cytoskeletal

Gene expression

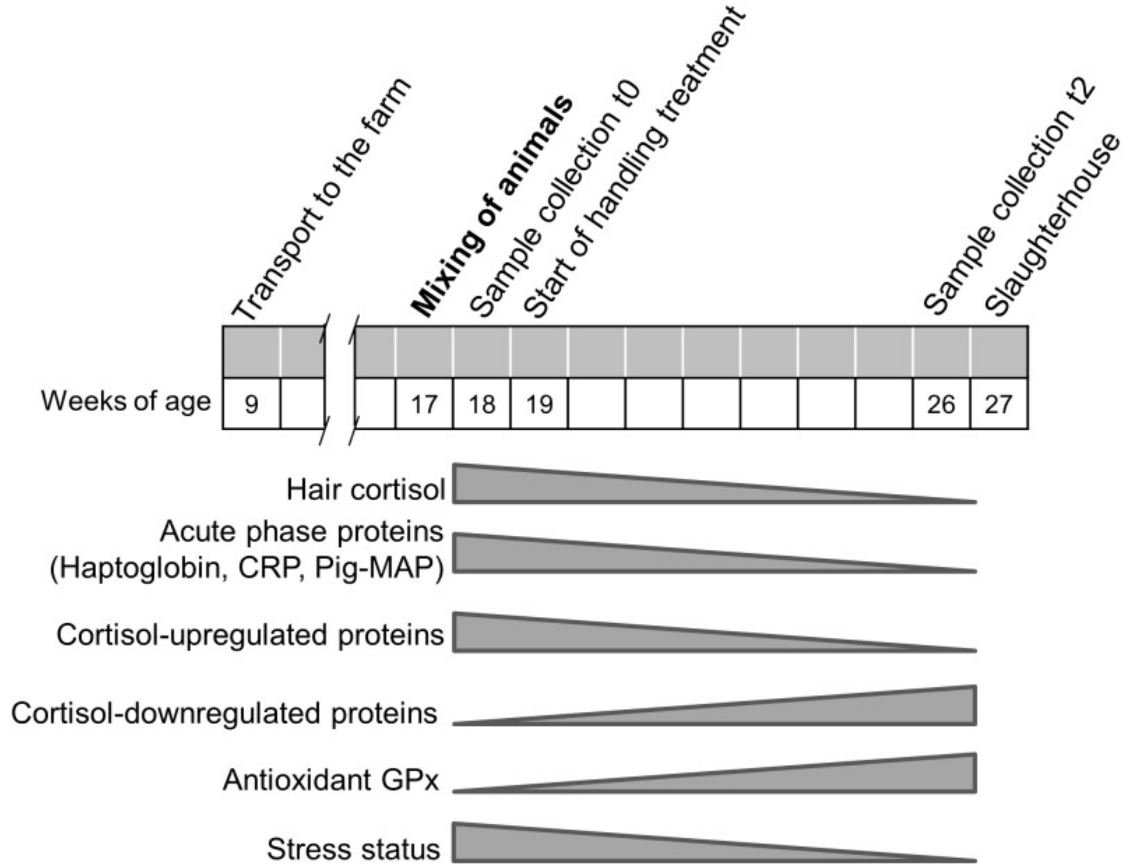




Oxidative stress

Valent et al., Effects on pig immunophysiology, PBMC proteome and brain neurotransmitters caused by group mixing stress and human-animal relationship. PLoS One. 2017; 12(5): e0176928.

1 LONG TERM EFFECTS OF GROUP MIXING AND MANAGEMENT



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1 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.

Valent et al., Effects on pig immunophysiology, PBMC proteome and brain neurotransmitters caused by group mixing stress and human-animal relationship. PLoS One. 2017; 12(5): e0176928.

2 ENVIRONMENTAL ENRICHMENT

44 piglets
♀

11x2 piglets
BARREN

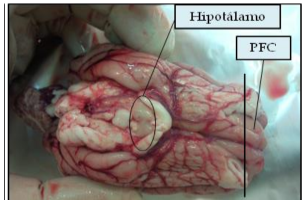
Control conditions:
0,7 m^2/pig
slat

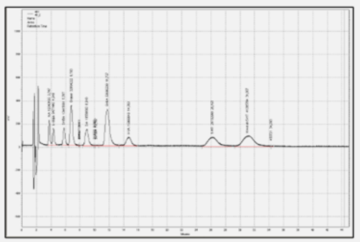
11x2 piglets
ENRICHED

Enriched conditions:
1,2 m^2/pig
straw

4 weeks

Transport to the slaughterhouse





Arroyo et al. Neurobiology of environmental enrichment in pigs: changes in monoaminergic neurotransmitters in several brain areas and in the hippocampal proteome. J Proteomics 2020 Oct 30;229:103943

In collaboration with Dr Antonio Velarde (IRTA)

2 **ENVIRONMENTAL ENRICHMENT**

Biochemical serum stress markers

Parameter	Treatment	Post-treatment
CK (U/mL)	Enriched	2,34 ± 0,29
	Barren	3,41 ± 0,61
Lactate (mM)	Enriched	89,33 ± 2,79 *
	Barren	99,60 ± 2,79 *
Cortisol (ng/mL)	Enriched	16,77 ± 2,03 *
	Barren	26,42 ± 3,38 *
Haptoglobin (mg/mL	Enriched	0,37 ± 0,05
	Barren	0,46 ± 0,10

PIGS IN BARREN CONDITIONS SHOW A HIGHER DEGREE OF STRESS

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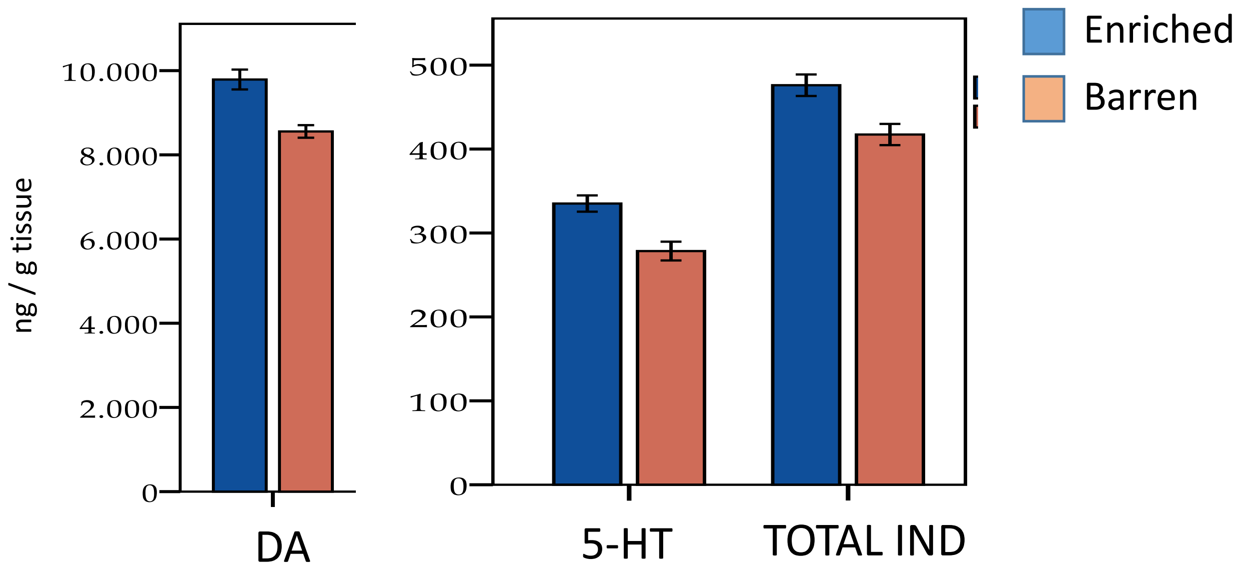
2 ENVIRONMENTAL ENRICHMENT

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

STRIATUM



↓DA

↓5-HT

PIGS IN BARREN CONDITIONS “FEEL” “LESS REWARDED”

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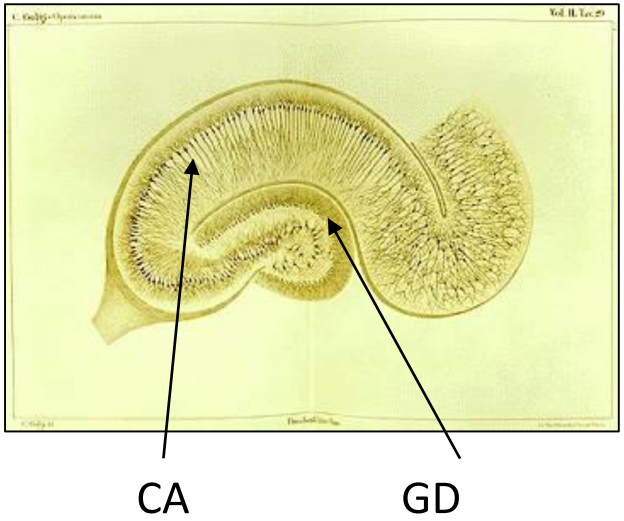
THE HIPPOCAMPUS

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

Hippocampus and seahorse



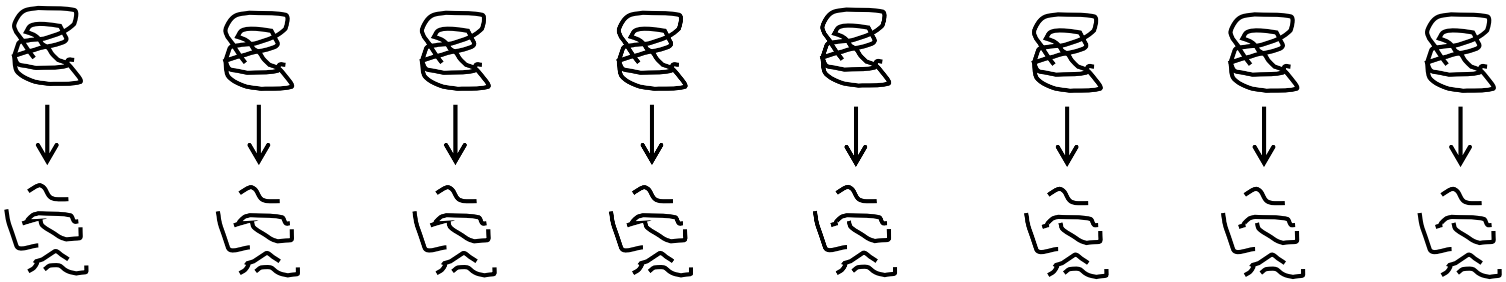
Drawing by Camillo Golgi of a hippocampus stained using the silver nitrate method

PROTEOMIC ANALYSIS OF THE HIPPOCAMPUS: iTRAQ 8-plex

Isobaric tag for relative and absolute quantitation

Samples

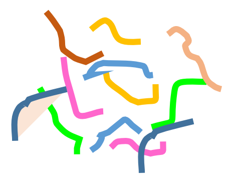
Reduction
Alkylation
Trypsinization



iTRAQ labeling

113 label	114 label	115 label	116 label	117 label	118 label	119 label	121 label

combine



Number of samples:
20 barren
20 enriched
8 control samples

6 iTRAQ labeling reactions

Peptide analysis on Orbitrap Fusion Lumos Tribrid

iTRAQ reporter ions

Identify

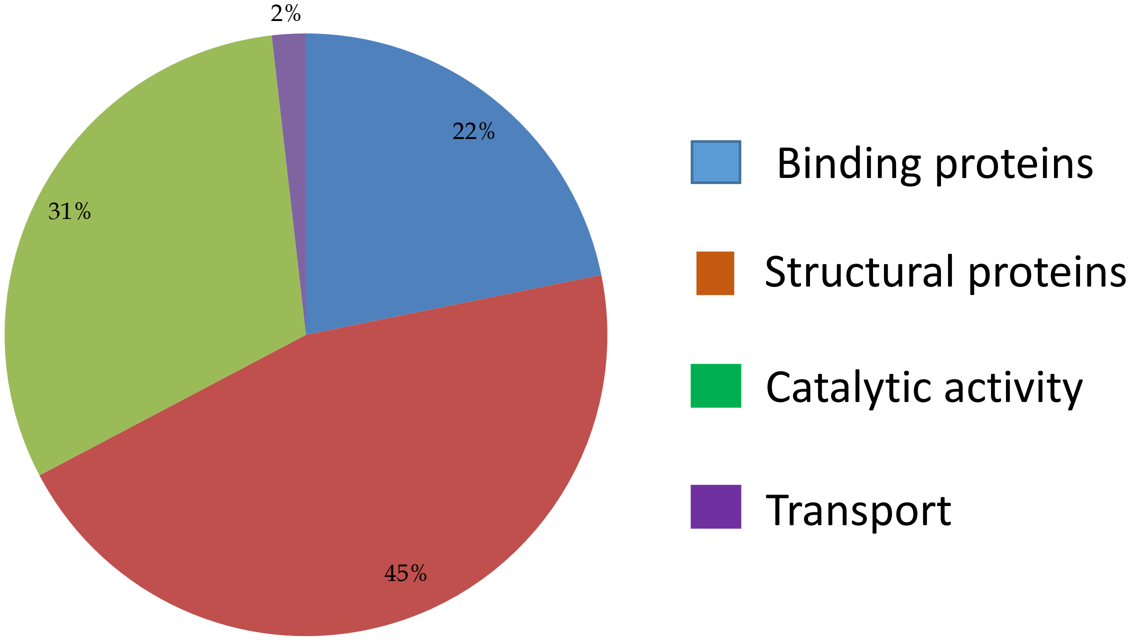
Quantify

GENE ONTOLOGY ANALYSIS (Panther)

MOLECULAR FUNCTION

15649 peptides
2418 proteins

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



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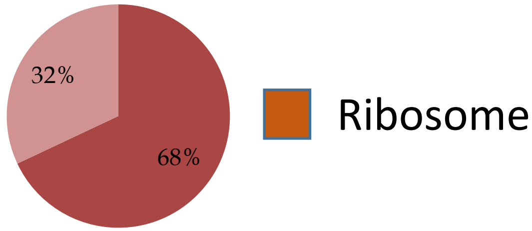
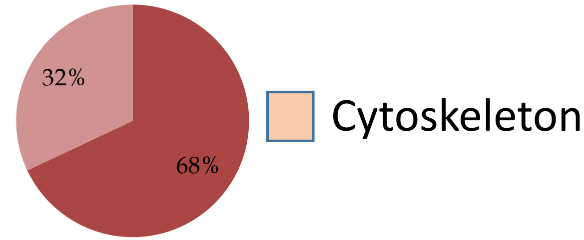
Structural components



Uniprot	Identification	Fold change E vs B	
A0A0B8RT95	Ribosomal protein L4	1,50	up
A1XQU3	60S ribosomal protein L14	1,55	up
A1XQU9	40S ribosomal protein S20	1,66	up
B0FWK5	Ribosomal protein L5	1,58	up
F1RQ91	40S ribosomal protein S4	1,54	up
F1S2E5	40S ribosomal protein S24	1,81	up
F1SEG5	40S ribosomal protein S16	1,72	up
F2Z512	40S ribosomal protein S23	1,49	up
F2Z522	60S ribosomal protein L23a	1,69	up
F2Z5G8	40S ribosomal protein S25	1,59	up
F2Z5Q6	40S ribosomal protein S6	1,58	up
I3L5B2	40S ribosomal protein S7	1,47	up
I3L6F1	60S ribosomal protein L18	1,66	up
I3LBH4	60S ribosomal protein L12	1,45	up
I3LJ87	40S ribosomal protein S2	1,44	up
P46405	40S ribosomal protein S12	1,60	up
P62901	60S ribosomal protein L31	1,72	up
P67985	60S ribosomal protein L22	1,62	up
Q29194	Ribosomal protein S2 (Fragment)	1,65	up
Q4GWZ2	40S ribosomal protein SA	1,94	up
Q6QAS9	60S ribosomal protein L7	1,93	up
Q95281	60S ribosomal protein L29	1,77	up
F1RGD9	Histidine--tRNA ligase	0,73	down
I3L8P7	Phenylalanine--tRNA ligase beta	1,33	up

Structural components



Uniprot	Identification	Fold change E vs B	
F1SNK9	ELAV-like protein	1,81	*up*
F1S6M7	Tubulin beta-3 chain	1,40	*up*
F2Z571	Tubulin beta-4B chain	1,61	*up*
F2Z5K5	Tubulin beta-4A chain	1,44	*up*
F2Z5S8	Tubulin alpha-4A chain	1,44	*up*
P02550	Tubulin alpha-1A chain	1,40	*up*
P02554	Tubulin beta chain	1,40	*up*
Q2HPK3	Tubulin alpha-3 chain (Fragment)	1,53	*up*
F1SSA6	Myosin-10	1,43	*up*
I3LNV3	Isoform 4 of Unconventional myosin-XVIIIa	2,47	*up*

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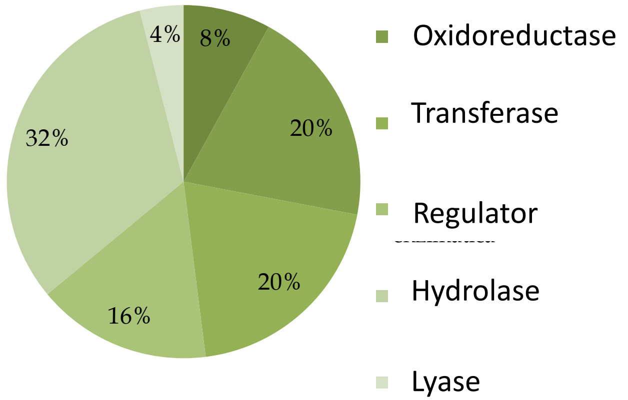
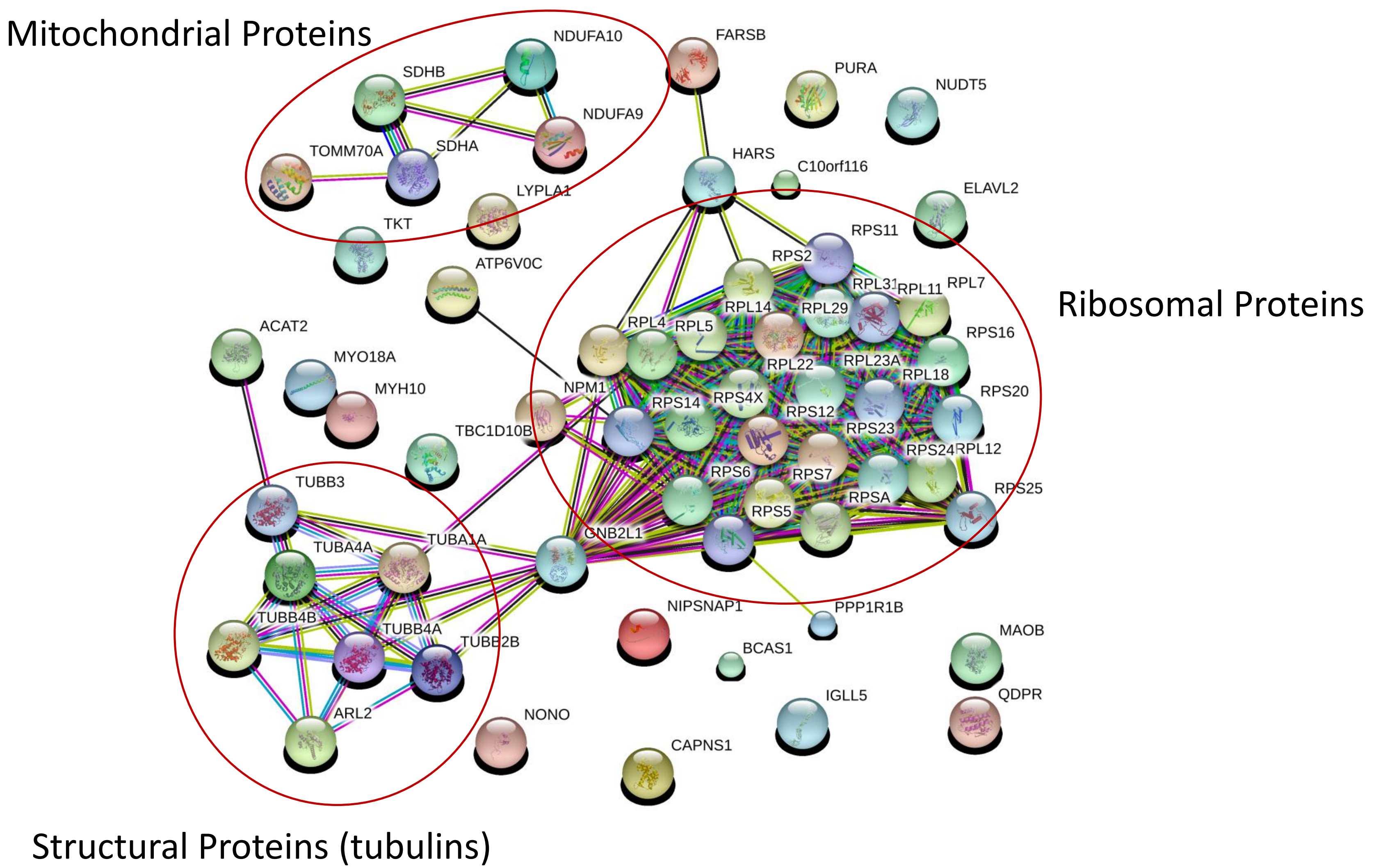
Uniprot	Identification	Fold change E vs B	
F1SIS9	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10	1,50	*up*
F1SL07	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9	1,39	*up*
I3LDC1	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit	1,44	*up*
I3LQ34	Mitochondrial import receptor subunit TOM70	1,67	*up*
A0A0B8RTH9	Lysophospholipase I	0,52	*down*
A8U4R4	Transketolase	0,79	*down*
F1RWM4	Protein phosphatase 1 regulatory subunit 1B	1,92	*up*
F1SB62	Acetyl-CoA acetyltransferase	0,72	*down*
F1SEN4	Adipogenesis regulatory factor	0,68	*down*
F1SUH8	V-type proton ATPase proteolipid subunit	0,47	*down*
I3L656	ADP-sugar pyrophosphatase	0,68	*down*
K7GQV5	Maleylacetoacetate isomerase	0,75	*down*
F1RFF5	Protein NipSnap homolog 1	1,51	*up*
F1RG61	TBC1 domain family member 10B	1,42	*up*
I3LSU1	Non-POU domain-containing octamer-binding protein	1,42	*up*
I3LUP6	Nucleophosmin	1,54	*up*
P04574	Calpain small subunit 1	1,34	*up*
P63246	Receptor of activated protein C kinase 1	1,51	*up*
A5GFR8	Breast carcinoma amplified sequence 1	0,63	*down*
A0A0A0MY58	Immunoglobulin lambda-like polypeptide 5	0,59	*down*
L8B180	IgG heavy chain	0,61	*down*
I3LEH4	Amine oxidase [flavin-containing]	1,46	*up*
I3LKS6	Dihydropteridine reductase	0,79	*down*
I3LCN6	Transcriptional activator protein Pur-alpha	1,33	*up*

Catalytic activity



NETWORK ANALYSIS BY STRING



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PATHWAY ANALYSIS WITH REACTOME

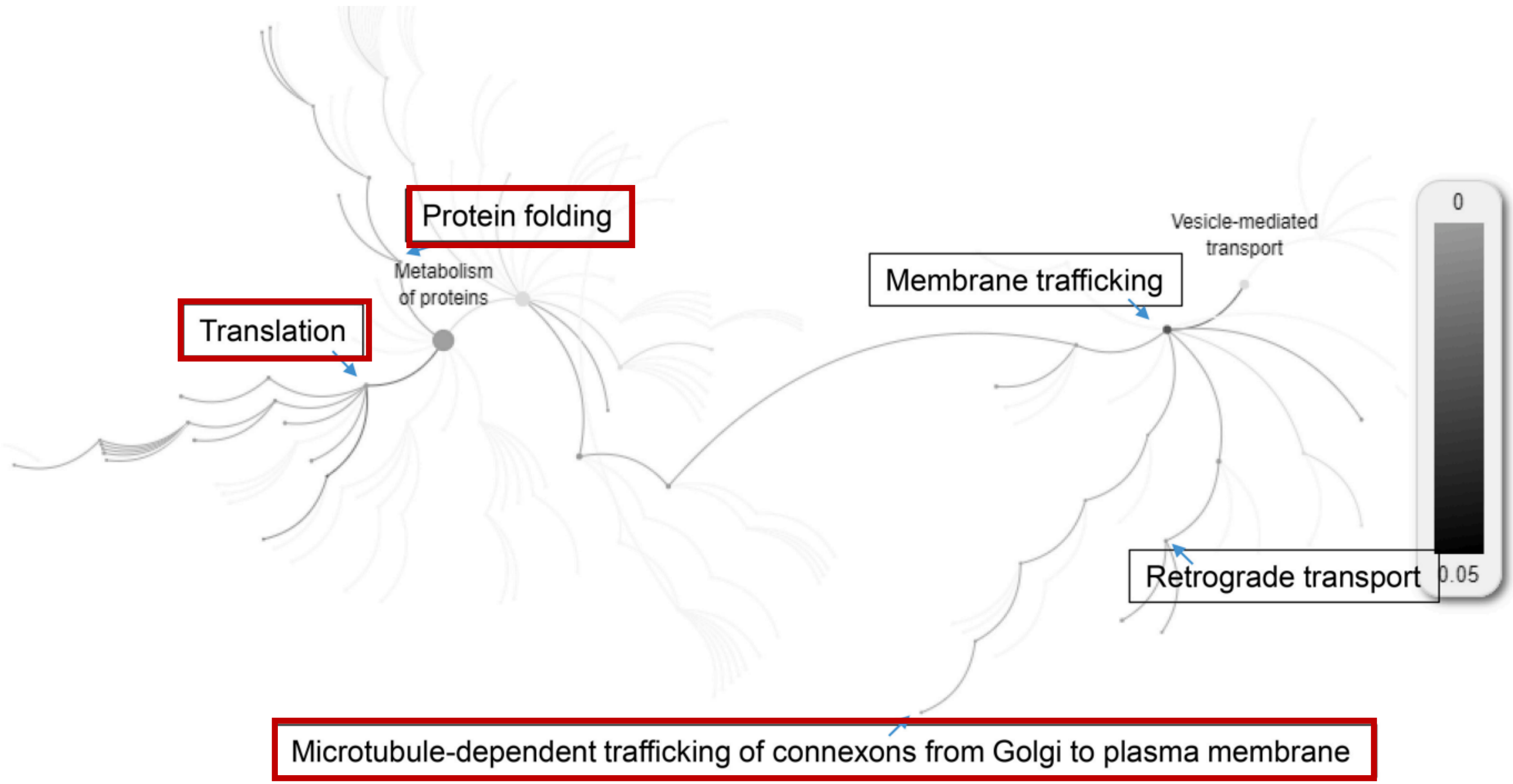

Fig. 4. Reactome diagram of Metabolism of proteins and Vesicle-mediated transport pathways in the hippocampus of pigs raised in EE-conditions with over-represented reactions highlighted in black.

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CONCLUSIONS

PIGS IN BARREN CONDITIONS SHOW A HIGHER DEGREE OF STRESS

PIGS IN BARREN CONDITIONS “FEEL” “LESS REWARDED”

THE “ENRICHED” HIPPOCAMPUS POTENTIALLY HAS:

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

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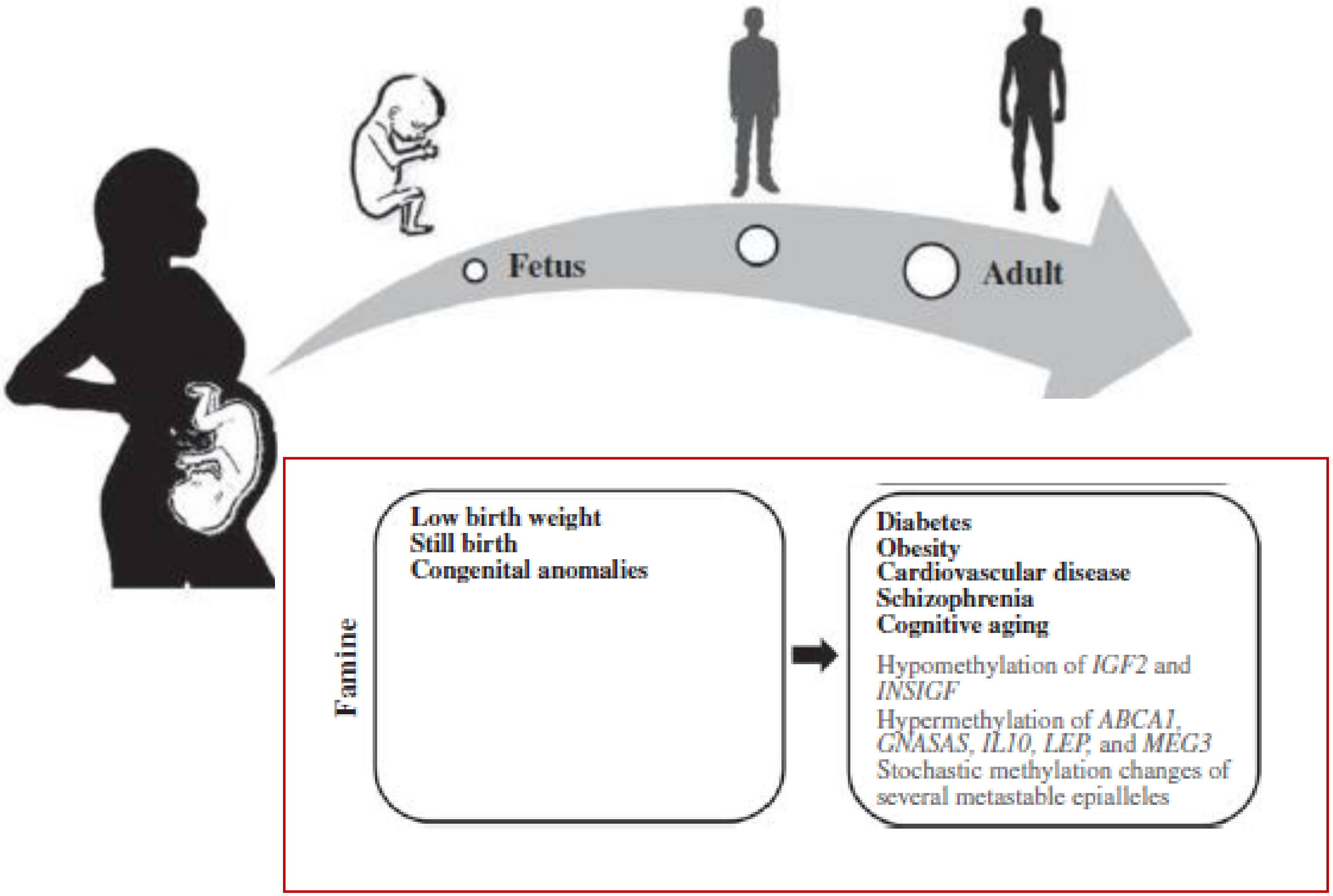
3 INTRAUTERINE GROWTH RESTRICTION (IUGR)

UAB

- IUGR is due to nutritional o placentary 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

3 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

3 INTRAUTERINE GROWTH RESTRICTION (IUGR)

UAB

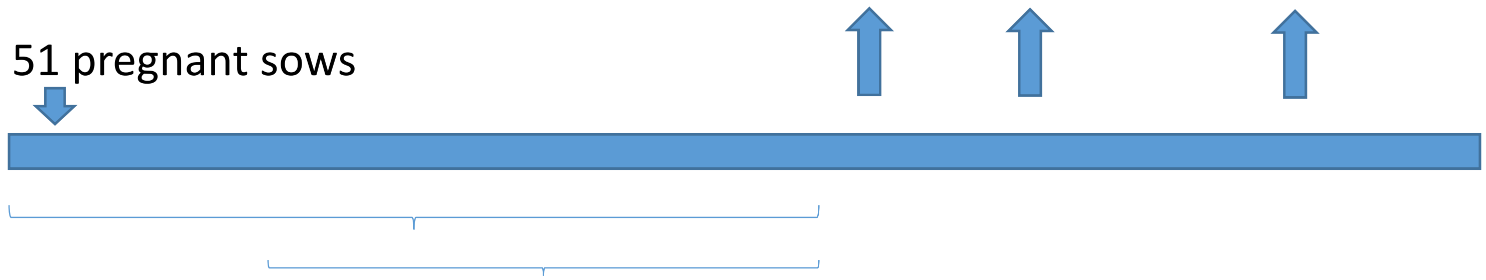
Objective 1: analyze the effects of supplementation of the maternal diet with the antioxidant hydroxytyrosol (HTX) on the SNC of the offspring

Amygdala
Hippocampus
Prefrontal Cortex

Experimental design:

100 d foetuses | 1 month piglets | 6 month pigs

51 pregnant sows



2/3 of gestation: 50% caloric restriction

Control: without HTX
HTX: with antioxidant

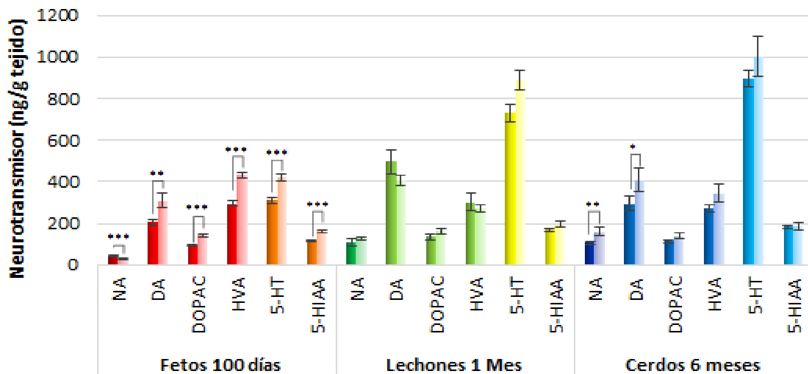
In collaboration with Dr Antonio González-Bulnes (INIA, Madrid)

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

3 INTRAUTERINE GROWTH RESTRICTION (IUGR)

UAB

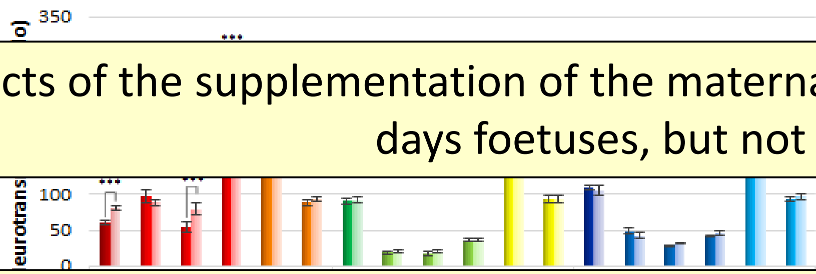
Neurotransmitter profile



Dark bars: Control

Light bars: HTX

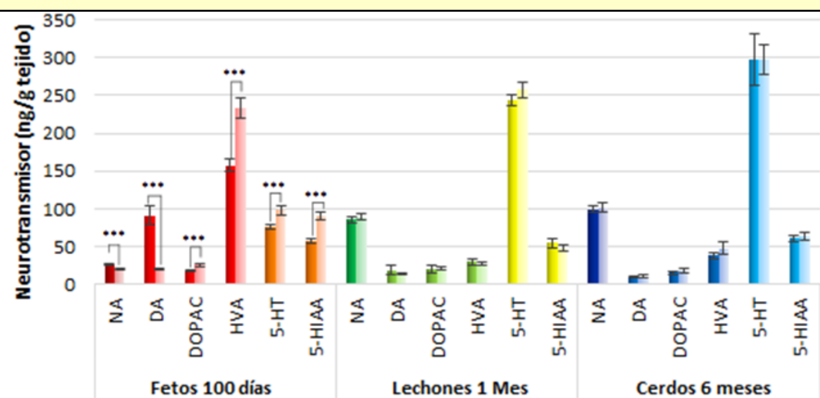
AMYGDALA



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

HIPPOCAMPUS

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

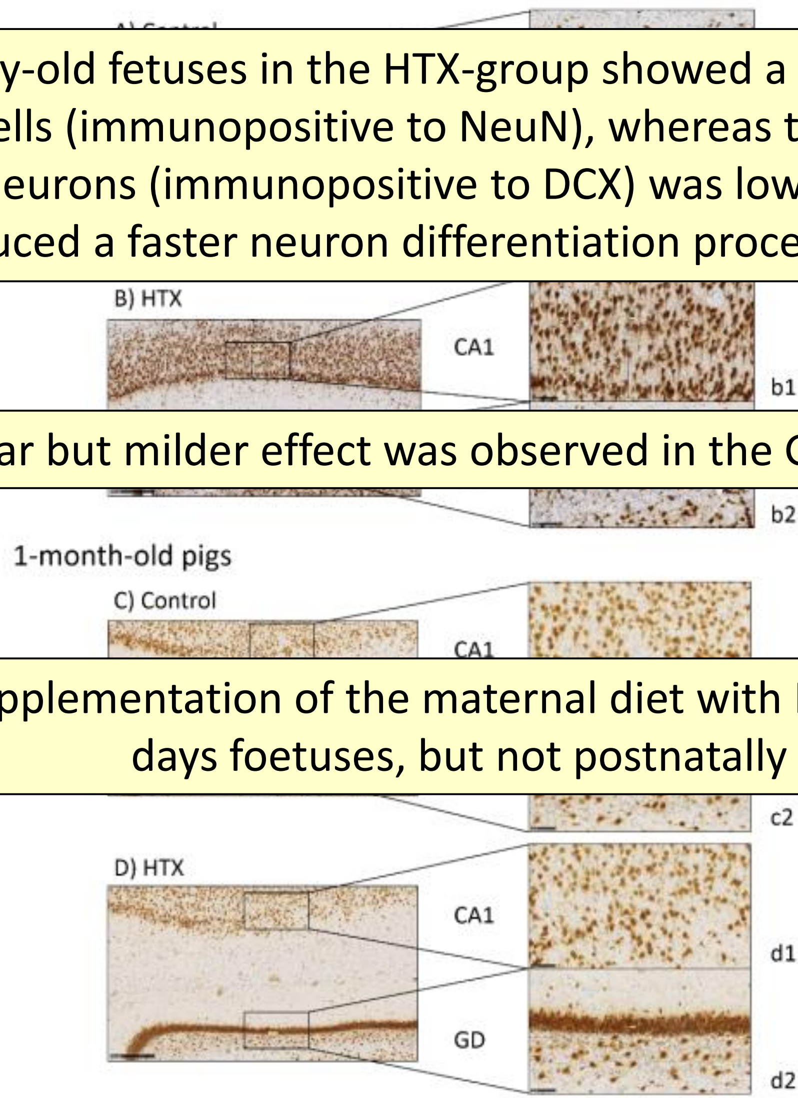


PREFRONTAL CORTEX

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

3 INTRAUTERINE GROWTH RESTRICTION (IUGR)

Imm... ochemistry of the hippocampus



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.

ns

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

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

PROTEOMIC ANALYSIS OF THE HIPPOCAMPUS: TMT 10-plex

Tandem mass tagging

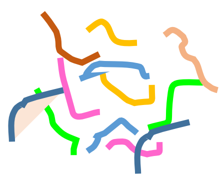
TMT labelling

Reporter ions	Experiment 1	Experiment 2
126	CH.1	CM.3
127N	CM.5	TH.2
127C	CH.2	TM.3
128N	TM.1	CH.4
128C	TH.5	CM.2
129N	CH.3	TM.4
129C	CM.4	TH.1
130N	TH.4	CM.1
130C	TM.2	CH.5
131	TH.3	TM.5

Number of samples:
10 control
10 HTX
Males and Females

2 TMT reactions

combine



Peptide analysis on Orbitrap Fusion Lumos Tribrid

TMT reporter ions

Identify
(Proteome discoverer)

Quantify

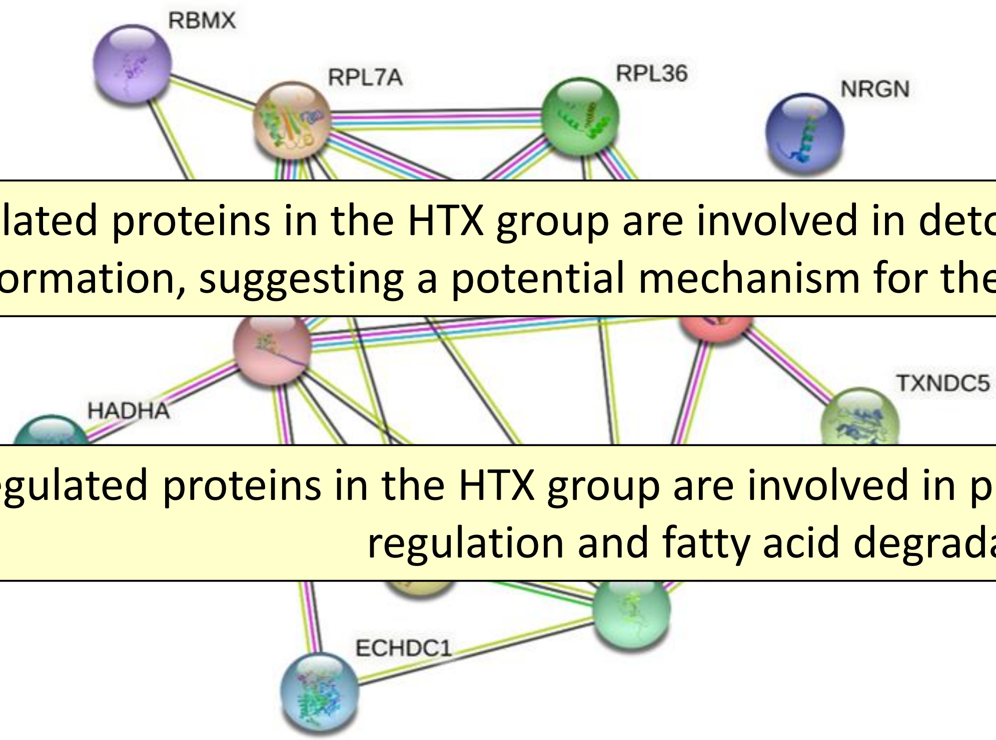
11 DAPs

3 more abundant in HTX
8 more abundant in Ctrl
FC $\geq$ 1,5 $p < 0,5$

PROTEOMIC ANALYSIS OF THE HIPPOCAMPUS: TMT 10-plex

Tandem mass tagging

11 DAPs



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)

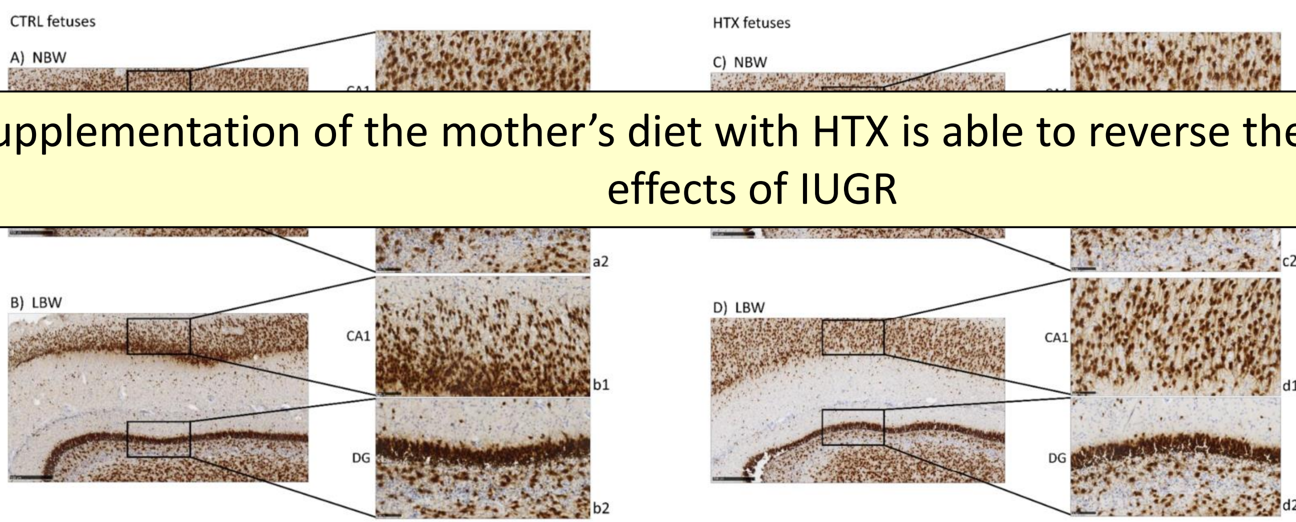
8 more abundant in Ctrl: 4 ribosonal proteins (RPL7, RPL7A, RPL36, FAU), HSPE1, TARS1 (Thr-tRNA synthase), RBMX (RNA binding protein), HADHA (3-hydroxyacyl-CoA dehydrogenase)

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)

			NBW	LBW	*p*-Values Treatment	Body Weight	Interaction
CA1	Cell Count	CTRL	143.06 ± 9.02^{Aa}	87.25 ± 11.40^{Ab}	<0.001	0.004	0.035
		HTX	170.86 ± 9.69^{Ba}	162.00 ± 6.04^{Ba}			
	Mean size (μm^2)	CTRL	1352.44 ± 247.19^{Aa}	2961.48 ± 676.06^{Ab}	<0.001	0.013	0.015
		HTX	817.85 ± 61.87^{Aa}	834.88 ± 44.94^{Ba}			
	Area (%)	CTRL	33.48 ± 1.29^{Aa}	41.63 ± 2.76^{Ab}	<0.001	0.007	0.033
		HTX	30.07 ± 0.99^{Aa}	31.07 ± 1.14^{Ba}			
DG	Cell Count	CTRL	79.91 ± 2.25^{Aa}	79.33 ± 4.55^{Aa}	0.045	0.831	0.719
		HTX	86.50 ± 3.64^{Aa}	88.75 ± 6.03^{Aa}			
		CTRL	1449.37 ± 51.72^{Aa}	1620.75 ± 116.92^{Aa}			

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

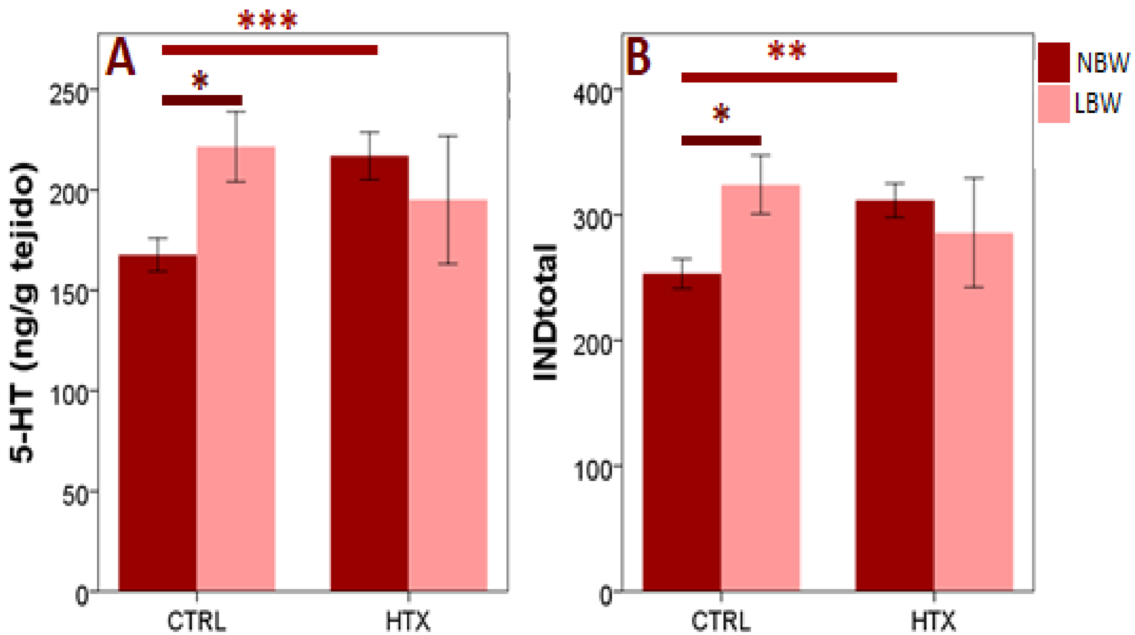


Supplementation of the mother's diet with HTX is able to reverse the deleterious effects of IUGR

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

3 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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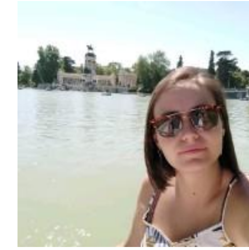
DANIEL VALENT



NATALIA YESTE

Departament de Bioquímica i Biologia Molecular. UAB. **Laura Arroyo, Daniel Valent, Natalia Yeste**
Servei de Bioquímica Clínica Veterinària. Facultat de Veterinària. UAB. **Raquel Pato, Raquel Peña, Yolanda Saco**
Institut de Neurociències (INc, UAB). **Josefa Sabrià, Susana Benitez**
IRTA. **Antonio Velarde, Ricard Carreras**
INIA. **Antonio Gonzalez-Bulnes**
Facultat de Veterinària. UAB. **Martí Pumarola**
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