794. Distinctive gene-expression profiles characterise tail-biting precursors in pigs under dietary protein restriction

L. Couteller[1,2], L. Roch[2,3] and C. Kasper[2*]

[1]École Supérieure d'Agricultures, 55 Rue Rabelais, 49007 Angers, France; [2]Agroscope, Tioleyre 4, 1725 Posieux, Switzerland; [3]Animal Welfare Division, Veterinary Public Health Institute, University of Bern, Länggassstrasse 120, 3012 Bern, Switzerland; claudia.kasper@agroscope.admin.ch

Abstract

Tail biting is a common problem in pig production, but genetic studies are impeded by the difficulty of observing it. It is a broader phenomenon that begins before lesions manifest. Therefore, precursors of tail biting, such as oral and nasal manipulation of conspecifics, should be studied. However, the molecular physiology underlying those behaviours is not well understood. Here we show that gene expression profiles in the hypothalamus of 73 non-tail-docked pigs fed a protein-reduced diet differed between individuals showing abnormal and normal behaviours. We found that differences in the expression of genes involved in neurotransmission, notably in dopamine, serotonin and GABA, and G-protein coupled signalling, energy metabolism and appetite. Our results demonstrate that abnormal behaviours have distinct signatures in the brain, which could be partly caused by genomic variation. These insights can lead to a better understanding of the biological mechanisms involved and thus may ultimately inform genomic selection programmes.

Introduction

Damaging behaviours such as tail biting are a common problem in pig production, compromising animal welfare and causing economic losses. A previous study found low to zero heritability for tail biting (Breuer *et al.*, 2003). Genetic studies are impeded by the enormously complex phenotyping, because tail biting often cannot be observed directly. Tail biting is thought to be a broader phenomenon, and behavioural problems caused by various stressors begin before escalating into damaging behaviour where serious injuries occur (Valros, 2018). Therefore, it useful to study the precursors of tail biting, which are behaviours that are termed 'abnormal' such as belly nosing, ear biting and 'tail-in-mouth' (Brunberg *et al.*, 2011) to gain insights into the molecular physiology of tail biting.

Poor sanitary conditions in combination with dietary protein reduction increase the prevalence of tail biting (Van der Meer *et al.*, 2017). Since restricting the protein content in the diet is a promising way to reduce nitrogen emissions in pig manure, it is important to investigate whether this measure can lead to a reduction in animal welfare. Even if essential amino acids are substituted, the overall reduction in dietary protein could lead to an increase of abnormal behaviours due to neurotransmitter system dysfunction because of the reduced availability of amino acids required for neurotransmitter synthesis. Pigs differ in their ability to utilise dietary proteins, which has been shown to be heritable (Kasper *et al.*, 2020). Therefore, we expect that individuals are not equally affected by protein reduction. Here, we investigate whether, under protein-restricted feeding with essential amino-acid substitution, gene expression profiles reflect differences in behaviour. Furthermore, we explore the genes and their functions as well as the pathways that are affected by abnormal behaviours.

Materials & methods

Animals. Ninety-five non-tail-docked pigs were reared on a diet formulated to contain 80% of crude protein of the recommendation. Between day 98 and 115 after birth, all instances of nasal and oral manipulation behaviours towards conspecifics (biting, seizing and nosing), classified as 'abnormal' behaviours, were recorded during five-min observations at four occasions. The protein efficiency of each pig was calculated

R.F. Veerkamp and Y. de Haas (eds)

DOI: 10.3920/978-90-8686-940-4_794, © L. Couteller *et al.* 2022

as the amount of protein in the carcass after slaughter divided by the amount of protein ingested. The effect of protein efficiency and sex on abnormal behaviours was analysed with generalized mixed-effects models with a Poisson and binomial family. The individual ID as well as the sire ID were added as random effects to correct for multiple observations and the family structure.

Brain extractions, RNA preparation and sequencing. The brains of the remaining 94 pigs (one died before slaughter) were extracted around 20:33±01:50 min after the start of exsanguination. The hypothalamus was dissected, immediately frozen in liquid nitrogen and stored at -80 °C until RNA extraction. The brains of the first eight pigs and further six samples were discarded as the training set for the sampling procedure and because of bad sampling quality. RNA extraction and sequencing of 80 hypothalamus samples (from 38 females and 35 males) were carried out by an external provider (Alithea Genomics, Lausanne, Switzerland). RNA-seq was done using the BRB-seq method (Bulk RNA Barcoding and sequencing, Alpern *et al.*, 2019), in which samples are multiplexed after the first reverse-transcription step.

Differential gene expression and network analysis. We removed genes with zero variance, genes with zero counts in more than 90% of samples and genes with a low expression (i.e. less than 10 counts) and samples with sequencing depth of <1M. We applied a multiple regression design, which included manipulation behaviour towards conspecifics, protein efficiency and sex, to control for additional variance, in DESeq2 (Love *et al.*, 2014). *P*-Values and fold-changes of the differential gene expression analysis were computed using the negative binomial GLM fitting and Wald statistics. GO-term enrichment of differentially expressed genes (DEG) was performed in ToppGene Suite (https://toppgene.cchmc.org). Weighted gene co-expression network analysis in WGCNA (Langfelder and Horvath, 2008) was carried out on the filtered read count matrix after variance-stabilizing transformation. To divide the co-expressed genes into modules, we chose a maximum block size of 10,000, a soft power threshold of 10 and 'signed' as the topological overlap matrix type. The modules enriched for DEG related to abnormal behaviour were determined with least-square linear models, an empirical Bayes moderation of standard errors in the R package limma (Ritchie *et al.*, 2015). The same procedure for GO-term enrichment as described above was applied to the genes contained in the significant modules. All *P*-values were corrected with a Benjamini-Hochberg FDR (a=0.05).

Results & discussion

Behavioural observations. Only three pigs were involved in tail biting (3.2%, two biters, one individual was both biter and victim). Even though the majority of pigs performed nasal or oral manipulation behaviour towards conspecifics, 16 pigs (17%) did not show these behaviours in any of the four observations. The number of abnormal behaviours was not influenced by protein efficiency or sex (GLMM with Poisson family: P=0.194 for protein efficiency, P=0.836 for sex), nor was the probability to engage in those behaviours (GLMM with binomial family: P=0.190 for protein efficiency, P=0.672 for sex).

Differential gene expression. Sequencing produced a read count matrix of 31,908 genes by 80 individuals. Filtering resulted in the removal of 13,775 genes. Sequencing depths ranged from 0.56M to 3.57 M reads per sample (1,774,168±649,509 reads). Seven individuals were removed because their sequencing depth was <1M reads. We found 23 genes differentially expressed between behaviourally abnormal and normal pigs ($P \leq 0.05$), of which 17 were up-regulated in abnormally behaving pigs compared to normally behaving ones and six genes were down-regulated (Table 1). In the set of DEG, 23 GO terms were enriched, most notably pertaining to G protein-coupled activities, amino acid binding, neurotransmitter and synaptic vesicle transport. The heterotrimeric G-protein signalling pathway-Gq alpha and Go alpha mediated pathway and the 5HT2 type receptor mediated signalling pathway were also significantly enriched.

Table 1. Significantly differentially expressed genes across behavioural groups of pigs.

Gene	base mean[1]	log2-fold change	SE	*P*-value	adj. *P*-value[2]
TNNT1	24.94	1.80	0.52	6.7E-06	0.019
RGS16	228.38	1.86	0.60	1.5E-05	0.032
PRKCG	133.18	1.01	0.32	3.5E-05	0.041
PRKCH[3]	50.38	1.40	0.48	4.6E-05	0.041
RADIL	49.86	0.57	0.17	3.4E-05	0.041
PLXDC1	112.50	1.53	0.52	3.6E-05	0.041
SPOCK1	242.23	0.55	0.18	4.5E-05	0.041
KITLG	79.64	1.42	0.50	4.6E-05	0.041
NTNG1	177.89	1.12	0.39	7.0E-05	0.043
SIN3B	128.42	0.30	0.10	6.2E-05	0.043
GRM8	27.03	0.67	0.22	7.1E-05	0.043
AK8	21.96	0.78	0.25	6.3E-05	0.043
RIMS3	130.90	0.02	0.05	7.3E-05	0.043
GRM5	120.07	0.46	0.15	7.8E-05	0.044
GABRD	35.91	1.48	0.55	9.1E-05	0.047
PCP4	2,017.17	1.40	0.53	9.3E-05	0.047
NEXN	171.30	1.38	0.56	1.0E-04	0.048
CD44	19.34	-0.96	0.24	2.5E-06	0.019
PMP2	29.05	-1.32	0.34	5.5E-06	0.019
APLNR	47.80	-1.47	0.39	7.3E-06	0.019
DDC	51.37	-1.10	0.31	3.7E-05	0.041
CKK	69.87	-1.09	0.34	7.2E-05	0.043
SLC18A2	66.41	-1.31	0.41	1.0E-04	0.048

[1] Mean expression across samples.

[2] Hochberg-Benjamini adjusted FDR.

[3] Originally unknown gene in the Sscrofa11.1 assembly. A search for orthologs in the Expression Atlas (https://www.ebi.ac.uk/gxa/home) yielded PRKCHA.

Weighted gene co-expression network analysis. The genes were divided into 20 modules, of which only one was significantly enriched for DEG related to manipulation behaviour (logFC=0.11, t=3.08, adj. *P*=0.04). This module contained 30 genes, which were down-regulated in abnormally behaving relative to normally behaving pigs (Figure 1). Only three genes in the module overlapped with the list of significant DEG (*CCK*, *SLC18A2* and *DDC*). Among the genes contained in the module were six transcription factors (*NR2F2*, *FOXA1* and *FOXA2*, *EN1* and *EN2*, and *LMX1B*). Eight genes were involved in neurotransmitter metabolism (*DRD2*, *CHRNB3, CHRNA6, SLC6A3. SLC18A2, DDC*, *ALDH1A1* and *TH*). Three genes coded for proteins involved in the regulation of feed intake and energy metabolism (*RET*, *CCK* and *INS*). The other genes were related to cell migration (*PRPRU* and CD164), G-protein-coupled receptors (*GPR26* and *TRPC6*), dopaminergic neuron differentiation (*RSPO2*), regulating mitochondrial Ca2+ uptake in neurons (*PDZD8*), synaptic vesicle formation (*CPLX2*) and genes with other functions (*SLC10A4*, *ZAR1, LRRC3B, GSTO1, SFRP4* and *MXRA7*). We found 71 GO terms significantly enriched, most notably dopamine and catecholamine binding, neurotransmitter transport, neurotransmitter metabolic processes, synaptic vesicle transport, neuron generation and differentiation, behaviour and response to drugs.

Conclusions

As expected, tail biting was very rare. Classifying animals into groups based on their oral and nasal manipulation behaviour towards conspecifics (i.e. abnormal behaviour) is therefore useful and offers

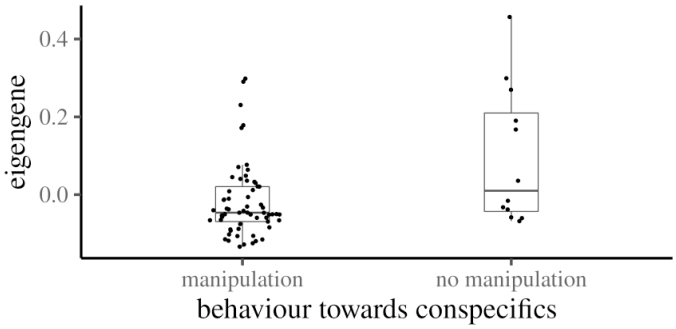

Figure 1. Eigengene values in the module that is significantly enriched for genes related to manipulation behaviour.

interesting insights. Here, we found evidence that abnormal behaviour in pigs is associated with gene expression profiles that are implicated in neuropsychiatric disorders in humans. Genes related to the synthesis, synaptic transmission and uptake of neurotransmitters, notably GABA, glutamate, dopamine, acetylcholine and serotonin, were differentially expressed in pigs showing abnormal behaviour. This was corroborated with the findings from pathway enrichment analysis. Interestingly, some of the DEG have been associated with energy metabolism, feed intake and appetite. Especially in the context of protein-efficiency studies, this warrants further investigation. As of now, it is unclear whether the differences in expression found in this study are caused by genetic variants, DNA methylation or other modifications, or short-term environmental influences. A better knowledge of these causes could inform genomic selection programs and allow the inclusion of welfare in the breeding goals.

References

Alpern, D, Gardeux, V, Russeil, J, Mangeat, B, Meireles-Filho, ACA, *et al.* (2019) - Genome Biol 20:71. https://doi.org/10.1186/s13059-019-1671-x

Breuer, K, Sutcliffe, MEM, Mercer, JT, Rance, KA, Beattie, *et al.* (2003) - Appl Anim Behav Sci 84:59–74. https://doi.org/10.1016/S0168-1591(03)00147-3

Brunberg, E, Jensen, P, Isaksson, A, Keeling, LJ (2013) - Genes Brain Behav 12:275–281. https://doi.org/10.1111/gbb.12002

Brunberg, E, Wallenbeck, A, Keeling, LJ (2011) - Appl Anim Behav Sci 133:18–25. https://doi.org/10.1016/j.applanim.2011.04.019

Kasper C, Ruiz-Ascacibar I, Stoll P, and Bee G (2020) J Anim Breed Genet 137(6):545-558. https://doi.org/10.1111/jbg.12472

Langfelder, P, Horvath, S (2008) - BMC Bioinformatics 9:559. https://doi.org/10.1186/1471-2105-9-559

Love, MI, Huber, W, Anders, S (2014) - Genome Biol 15:550–550. https://doi.org/10.1186/s13059-014-0550-8

Ritchie, ME, Phipson, B, Wu, D., Hu, Y., Law, C.W. *et al.* (2015) - Nucleic Acids Res 43:e47. https://doi.org/10.1093/nar/gkv007

Valros, A (2018). Chapter 5 - Tail biting, In: Špinka, M. (Ed.), Advances in Pig Welfare, Herd and Flock Welfare. Woodhead Publishing, pp. 137–166. https://doi.org/10.1016/B978-0-08-101012-9.00004-6

van der Meer, Y, Gerrits, WJJ, Jansman, AJM, Kemp, B, Bolhuis, JE (2017) – PLoS ONE 12:e0174688. https://doi.org/10.1371/journal.pone.0174688