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ORIGINAL ARTICLE

Differentially expressed genes in hypothalamus in relation to genomic regions under selection in two chicken lines resulting from divergent selection for high or low body weight

Sojeong Ka · Frank W. Albert · D. Michael Denbow · Svante Pääbo · Paul B. Siegel · Leif Andersson · Finn Hallböök

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Abstract Long-term divergent selection for low or high body weight from the same founder population has generated two extremely divergent lines of chickens, the high- (HWS) and low-weight (LWS) selected lines. At selection age (56 days), the lines differ by more than nine times in body weight. The HWS line chickens are compulsive feeders, whereas in the LWS line, some individuals are anorexic and others have very low appetite. Previous studies have implicated the central nervous system and particularly the hypothalamus in these behavioural differences. Here, we compared the mRNA expression in hypothalamus tissue from chickens on day 4 post-hatch using oligonucleotide arrays and found that the divergent selection had resulted in minor but multiple expression differences. Differentially expressed genes were enriched in processes 'DNA metabolism, repair, induction of apoptosis and metabolism'. Several differentially expressed genes participate in the regulation of neuronal plasticity and development, including apoptosis, or are neurotransmittor receptor subtypes. Less change was seen when comparing

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S. Ka · F. Hallböök
Department of Neuroscience, Uppsala University,
Uppsala, Sweden

F. W. Albert · S. Pääbo
Department of Evolutionary Genetics,
Max Planck Institute for Evolutionary Anthropology,
Leipzig, Germany

D. M. Denbow · P. B. Siegel
Department of Animal and Poultry Sciences,
Virginia Polytechnic Institute and State University,
Blacksburg, VA, USA

S. Pääbo
Department of Immunology, Genetics and Pathology,
Uppsala University,
Uppsala, Sweden

L. Andersson
Science for Life Laboratory, Department of Medical Biochemistry and Microbiology, Uppsala University,
Uppsala, Sweden

L. Andersson
Science for Life Laboratory, Animal Breeding and Genetics,
Swedish University of Agricultural Sciences,
Uppsala, Sweden

F. Hallböök (✉)
Department of Neuroscience,
Unit for Developmental Neuroscience, BMC,
Box 593, 751 24 Uppsala, Sweden
e-mail: Finn.Hallbook@neuro.uu.se

Present Address:
F. W. Albert
Lewis-Sigler Institute for Integrative Genomics,
Princeton University,
Princeton, NJ, USA

Present Address:
S. Ka
Integrative Omics Research Centre,
Korea Research Institute of Bioscience and Biotechnology,
Yuseong, Daejeon 305-806, South Korea

hypothalamic neuropeptide mediators of appetite such as the melanocortin receptors. The genomic locations of these differentially expressed genes were then compared to the locations of growth QTLs and to a genome-wide map of chromosomal regions that have been under divergent selection between the lines. The results indicate which differentially expressed hypothalamic genes have responded to the divergent selection and that the results predict that it is more likely to find causative genes among these most differentially expressed genes. Because of such differential gene expression in hypothalamus, the lines may adapt behaviourally different particularly to the post-hatch situation when independent feeding to obtain energy is established.

Keywords Anorexia · Body weight · Chicken · Divergent selection · Feeding behaviour · Gene expression · Hypothalamus

Introduction

For most of their history, domestic chicken populations have been bred for two main purposes, egg and meat production. A strong human-driven selection for specific traits has, during the domestication process, produced breeds with a range of morphological, physiological and behavioural phenotypic characters. As an example of the remarkable change that can be caused by artificial selection, in this work we have studied two lines of chickens, established by divergent selection for body weight at 56 days of age for 50 generations from a founder population of White Plymouth Rock chickens [1–3]. The two populations, the high-weight (HWS) and the low-weight (LWS) selection lines, show more than a 9-fold difference in body weight at 56 days. Several metabolic, endocrine, immunological and behavioural differences between lines have evolved during this long-term selection experiment although they were not directly selected for [4–6]. Among the notable correlated responses to the selection for differential growth were differences in feeding behaviour and food consumption. While the HWS chickens are hyperphagic and compulsive feeders and accumulate fat, the LWS chickens have a very low appetite and tend to be extremely lean and anorexic, even when fed ad libitum. In some generations, up to one fifth of the LWS chicks do not survive the first 2 weeks post-hatch because they never start to eat. This particular feature seems to have a genetic component because increased mortality during the first 2 weeks is more common among progeny from certain families [2]. A neural component and involvement of hypothalamus function in the establishment of the phenotypes was implied after electrolytic lesions of the ventromedial hypothalamus. The lesion had no effect on feed intake in the HWS birds implying that they already carry a genetic defect in hypothalamic regulation of feed intake. The LWS chickens gained weight after a similar lesion [7]. The lines also responded with different feeding behaviours to central stimulation of α-melanocyte stimulating hormone [8] or neuropeptide S [9], which further support a neural involvement in the phenotypic differences.

A large intercross of the HWS and LWS generation 41 lines has previously been constructed and used for quantitative trait locus (QTL) mapping, showing a complex genetic basis for the differential growth. Thirteen loci affecting growth were detected [10–12] and it has been demonstrated that epistatic interactions between many QTLs have contributed significantly to the selection response in these lines [13]. A recent genome-wide analysis of 60,000 single nucleotide polymorphisms in the lines identified a large number of chromosomal regions that have been divergently selected for during 50 generations after the start of the divergent selection [14]. The results are accordant with that the phenotypic difference is a result of selection at multiple loci across the genome [14]. Genome-scale expression analysis provides complementary information to uncover pathways underlying these complex growth traits. In this study, we therefore performed gene expression profiling of hypothalamus from the HWS and LWS lines by using oligonucleotide Affymetrix microarrays, with the aim to identify and describe genes and gene networks that are under alternate regulation in these two lines. The genomic locations of these differentially expressed genes were then compared to the locations of the growth QTLs and to the genome-wide map of chromosomal regions that have been under divergent selection. The results indicate which differentially expressed genes in the hypothalamus have responded to the divergent selection in these lines.

Materials and methods

Animals and tissues

The LWS and HWS lines were established from a common founder population generated by crosses among seven inbred lines of White Plymouth Rock chicken. The lines have been maintained as closed populations by continuous selection for high or low body weight at 56 days of age. Feed and water were supplied ad libitum. Descriptions of the selection programme and various correlated responses to the selection within the lines are provided elsewhere [2, 3, 10].

Among the LWS chickens, depending on generation, 2–20% was severely anorexic and did not survive the post-hatch period; some survived but did not lay eggs, while still

others reproduced normally. We observed that this variation within the LWS line probably has a genetic component. However, the breeding protocol will select against early mortality or non-fertility and if not taken into account during sampling, this bias may artificially reduce observed gene expression differences that may otherwise exist between the lines. Therefore, at generation 46, two subsequent hatches were produced from the same parental matings. A first hatch identified LWS matings, which had progeny with increased early mortality. Only these families with increased early mortality were used for brain dissection in the second hatch. Hypothalamic tissue was dissected from chicks 4 days post-hatch from consecutive 50 μm cryosections throughout the diencephalon. The early age was chosen to avoid secondary influences by the nutritional conditions of older animals and to ensure that the hatchlings overall survived the start of the post-hatch development. Tissue from ten successfully dissected chicks from each line was collected. The sex of the chicks was determined by the expression of the female-specific W-chromosome-linked protein kinase C inhibitor-8 (Supplementary information Fig. S1). Among the 20 chicks, seven from each HWS and LWS line were females and three were males. We here present the female data only. Animal procedures were performed according to the National Research Council publication and Guide for care and use of laboratory animals and were approved by the Virginia Tech Institutional Animal Care and Use Committee.

RNA preparation and array experiments

Total RNA was isolated with Trizol (Invitrogen Corporation, Carlsbad, CA, USA) and purified with RNeasy mini kit (Qiagen, Valencia, USA). The quality of the RNA was checked with an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, USA) and the RNA samples were frozen at −70°C until used. Five microgrammes of the total RNA samples from each individual was used for analysis with GeneChip Chicken Genome Array with 37,703 probe sets (Affymetrix Ltd., High Wycombe, UK), following the manufacturer's instructions. HWS and LWS samples were processed together throughout the entire experiments to avoid potential experimental batch effects. The quality of RNA and the hybridizations were tested by the signal ratio by 5′- and 3′-probes for the same transcript.

Array data analysis

The raw intensity values were background corrected, log2 transformed and quantile normalised using the Robust Multi-array Analysis [15]. All probes in the analysis were determined as either present (expressed) or absent (not-expressed) using the detection calling function in the statistical algorithm MAS5 in the Affymetrix expression console software [16]. Probes were included for analysis if present in at least five individuals per group and tested for differential expression (DE) using one-way analysis of variance (ANOVA) resulting in a 'first' list of present DE probes based on the complete affymetrix probe set (Supplementary information Table S1). This complete probe set contained multiple probes for single genes as well as probes for genes that were absent from the chick genome assembly (WashUniv2.1 May06 assembly, http://genome.ucsc.edu) [17]. In order to obtain one data point for one target gene, probe sets were listed according to chromosomal position and only those probes in an annotated gene present in the genome assembly (WashUniv2.1 May06 assembly) were retained. By doing this, multiple probe sets or a subset of probes in a probe set was assigned to single genes. This procedure resulted in 7,522 genes that were detected as present in at least five animals of both lines. Using this set, a 'second' list of DE genes was generated using one-way analysis of variance (Supplementary information Table S2). False discovery rates were estimated using permutation tests. One thousand permutations were conducted, each corresponding to a random assignment of the chicken to either the HWS or the LWS line (Supplementary information Table S3). To assess and visualise the individual variation in each line, pair-wise global gene expression distances between individuals were calculated as the mean square difference across all expressed genes, and a multidimensional scaling graph using two dimensions was plotted (Fig. 1a). Functional enrichment tests of differentially expressed vs all expressed genes were performed with the software 'FUNC' [18]. Gene Ontology (GO) annotation for all the genes on the chip was downloaded from Ensmart, and GO annotation as well as the ANOVA p value for differential expression was fed into FUNC.

The chromosomal location of the DE genes was compared to previously identified QTL regions for growth [10, 11] or to regions with significantly different allele frequency, which has been under divergent selection in the lines [14]. The locations and number of genes in QTL regions were retained from the Ensmart (http://www.ensembl.org/Multi/martview) by listing all genes within each QTL region. χ^2 tests were used to test if the overlap of the locations of DE genes with the QTL regions or with regions under directional selection exceeded that expected by chance.

Quantitative reverse transcriptase polymerase chain reaction

Complementary DNA (cDNA) synthesis from total RNA and two-step qPCR were performed as described [19]. Briefly, quantitative reverse transcriptase polymerase chain

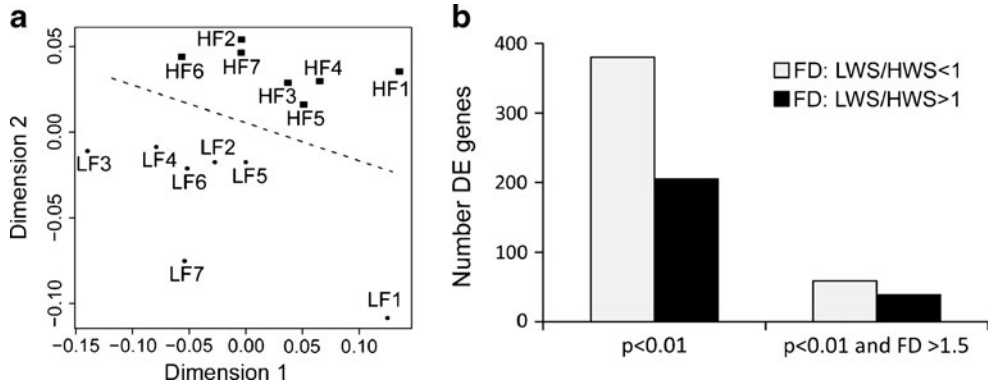

Fig. 1 Individual and line variation of differentially expressed (DE) genes. **a** Plot showing variation of the relative mRNA expression based on the complete array data for each individual female. The average square distances (expression level) were calculated for all gene pairings, and the multidimensional scaling (MDS) in two dimensions was applied. *HF* HWS female (*dots*), *LF* LWS female (*squares*); *numbers* indicate the individual chicken. **b** Number of DE genes that had higher expression in HWS than in LWS (*white bars*) and vice versa (*black bars*). *Bars* $p<0.01$ represent all DE genes with $p<0.01$ (585 genes) and *bars* $p<0.01$, FD>1.5 include the 97 top DE genes with more than 1.5-fold difference (FD)

reaction (qRT-PCR) was performed using the iQ SYBR Green supermix (Bio-Rad Laboratories, Inc., CA, USA) in combination with MyiQ Single-Colour Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). Primers were designed with Primer Express 1.5 (Applied Biosystems, Carlsbad, CA, USA) software (Supplementary information Table S3). Chicken ß-actin and glyceraldehyde 3-phosphate dehydrogenasewere used as references. Twenty genes were selected from the most differentially expressed genes for validation purposes of the array data. The collected data were normalised against Ct values of the reference and, subsequently, the relative mRNA expression levels of the test genes were determined in comparison with the average of 4-day-old HWS male expression levels. Unpaired Student *t* test with GraphPad Prism 3.03 (GraphPad Software, San Diego, CA, USA) was used.

Results

Differential gene expression in hypothalamus

The result of the array analysis of DE transcripts was compiled into two tables of data that were sorted by fold expression difference and statistical significance. One table contained the results based on Standard Affymetrix probe set annotations and the other contained the results based on our reassignment of individual array probes to annotated genes present in the chicken genome assembly (Supplementary information Tables S1 and S2). First, we assessed the variation of hypothalamus mRNA expression levels by plotting the relative differences expressed as the average square distances for all gene pairings from each female individual. The samples separated into two distinct groups according to their respective line (Fig. 1a). Permutation analysis confirmed that the number of DE genes at different statistical thresholds was much higher than randomly expected (Supplementary information Table S4). ANOVA was used to determine what probes or genes could be regarded as DE. At $p<0.01$, 585 DE genes were identified with false discovery rates less than 5.6%, of which the majority (380 DE genes) had expression levels that were higher in the HWS than in the LWS chickens (Fig. 1b). The fold expression differences were relatively low. Ninety-seven DE genes had a fold expression difference more than 1.5 ($p<0.01$), and these genes were denoted top-score DE genes (listed in supplementary information Table S5). Since our probe reassignment often grouped multiple Affymetrix probe sets into a single ‘gene’, the probe set list was longer than the gene list. Two thousand sixty-eight probe sets were DE when the statistical threshold was set to $p<0.01$ (not shown) and 383 probe sets ($p<0.001$) are listed in supplementary information Table S1. More than two probe sets represented one gene for the majority of the top-score genes. In the gene list, probes for sequences that were not present in the genome assembly were excluded.

Validation of the array result by qRT-PCR

We selected 20 genes among the 97 top-score DE genes to represent both genes with high and low fold difference DE and analysed the expression in hypothalamus from the two lines using qRT-PCR. Of these, 19 showed that the differential expression was confirmed (Supplementary information Table S5). The validated genes were among the 97 most DE genes and this may have introduced a bias. The frequency of false positive genes may be higher if genes with less DE would have been analysed. The fold

difference was on average 13% larger when analysed by using qRT-PCR than by the array analysis, indicating that the array results may have underestimated the DE.

Classification of differentially expressed genes

Three approaches were used to classify the DE genes and to find pathways subjected to change during the selection: (1) analysis of enriched GO terms and processes among all present genes using the software package FUNC [18], (2) manual characterization of the top DE genes and (3) examination of phenotypes of knock-out mice for mouse orthologues of the top DE genes.

(1) The statistically supported enriched GO terms for 'biological processes', 'molecular functions' and 'cellular components' are listed in Table 1.
(2) The cellular and physiological functions of the 97 top-score DE genes are listed in supplementary information Table S6. Genes involved in multiple functions were assigned to multiple categories with 27 genes involved in cell adhesion and signalling, 28 in neuronal plasticity and actin cytoskeletal processes in the nervous system, 13 in cell cycle regulation, 12 in channels/intra-cellular transport and 12 in DNA, amino acid and lipid metabolism. Six of the 12 metabolism genes were associated to lipid metabolism. Five of these were higher in the HWS than the LWS chicks. Eight protein modification genes, seven transcriptional regulation genes and six RNA processing genes were also altered. There were 23 genes that had no conclusive information about gene functions and could not be classified.
(3) The phenotypes of more than 50 existing knock-out or transgenic mice for the 97 top DE genes were examined. Twenty-seven have been reported to give a clear phenotype that is relevant to hypothalamic functions and to the phenotype differences between the lines. A majority of those 27 knock-out mice exhibited abnormalities during nervous system development or neurological behaviours with 15 directly related to growth and feeding behaviours (Supplementary information Table S7).

Table 1 Altered pathways as shown by the FUNC analysis

Gene Ontology annotation	Gene Ontology ID	*p* value
Biological process		
DNA metabolic process	GO:0006259	<0.001
DNA repair	GO:0006281	0.001
Negative regulation of catalytic activity	GO:0043086	0.005
Actin polymerizing activity	GO:0030041	0.009
Induction of apoptosis	GO:0006917	0.016
Leukocyte differentiation	GO:0002521	0.027
Lymphocyte-mediated immunity	GO:0002449	0.028
Response to biotic stimulus	GO:0009607	0.034
Metabolic process	GO:0008152	0.041
Regulation of MAP kinase activity	GO:0043405	0.044
DNA recombination	GO:0006310	0.049
Molecular function		
Enzyme inhibitor activity	GO:0004857	0.022
DNA binding	GO:0003677	0.036
Ligase activity	GO:0016874	0.038
ATP binding	GO:0005524	0.039
Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	GO:0016818	0.042
Cellular component		
Vacuole	GO:0005773	0.027
Nucleoplasm	GO:0005654	0.045

FUNC a software package for detecting significant associations between gene sets and ontological annotations [18]

Localization of DE genes related to QTLs for growth and to genomic regions under divergent selection

The chromosomal localization of 585 DE genes was compared to QTL regions for growth in the lines (Supplementary information Tables S1, S2, S6) [10–12]. The potential enrichment of DE genes in QTL regions was calculated by comparing the fraction of all present genes with the fraction DE genes in the QTL regions. In all, 7,522 genes were classified as present in the samples and of those, 15.8% (1,187 genes) was positioned in QTL regions. Among the 585 DE genes, 16.9% (99 genes) was positioned in QTLs, which is not more than expected by chance (χ^2, P=0.5). When only the 97 top DE genes were considered, 22.7% (22 genes) were in QTL regions, a suggestive (but not-significant) enrichment (χ^2, P=0.06). We then plotted the chromosomal localization of the 97 top DE genes on to the map of genomic regions with significantly different allele frequency between lines (the complete map is found in supplementary Fig. S2 in ref. [14]). Genes that fell into regions that have been under divergent selection were noted (Supplementary Table S6). The fraction of the total genome with significantly different allele frequency was estimated by summing up all regions on all chromosomes that showed a significantly different allele frequency at generation 40 between the lines. By such approach, 18.7% (193,000 Mb) of the genome (1,031,000 Mb) was estimated to have significantly different allele frequency. Among the 97 top DE genes, 40

(41.3%) were located in genomic regions that had a significantly different allele frequency, which is much more than what was expected by chance (χ^2, $P<0.001$). An example of such a region on the distal chromosome 4p, overlapping with a QTL for growth (*growth6*) is shown in Fig. 2.

Relative expression of candidate genes

The canonical hypothalamic mediators of feeding behaviour such as pro-opiomelanocortin (POMC) and melanocortin receptors did not specifically show up in the array results. A few candidate genes including the leptin receptor, neuropeptide Y, POMC, agouti-related protein, melanocortin-3, -4 and -5 receptors, brain-derived neurotrophic factor and tropomyosin receptor kinase B (TrkB) were specifically checked. The array probe sets for the leptin receptor, agouti-related protein, melanocortin-3 and -5 receptors were not retained as present genes and POMC, melanocortin-4 receptor, brain-derived neurotrophic factor and TrkB were not identified as DE genes in the array result. Neuropeptide Y was differentially expressed (1.22-fold up-regulation in HWS chicks) in the array data. We validated the results by qRT-PCR and confirmed DE for neuropeptide Y in females but not males, as well as the absence of DE for brain-derived neurotrophic factor, TrkB, POMC and melanocortin-4 receptor (Fig. 3a, b, e, f, h). Agouti-related protein and melanocortin-5 receptor were not differentially expressed. The leptin receptor and melanocortin-3 receptor were significantly differentially expressed but only in one sex (Fig. 3c, d, g, i). The differential expression of dopamine D1B receptor, caspase-3 and phosphatase and tensin homologue (PTEN) was confirmed (Fig. 3j–l).

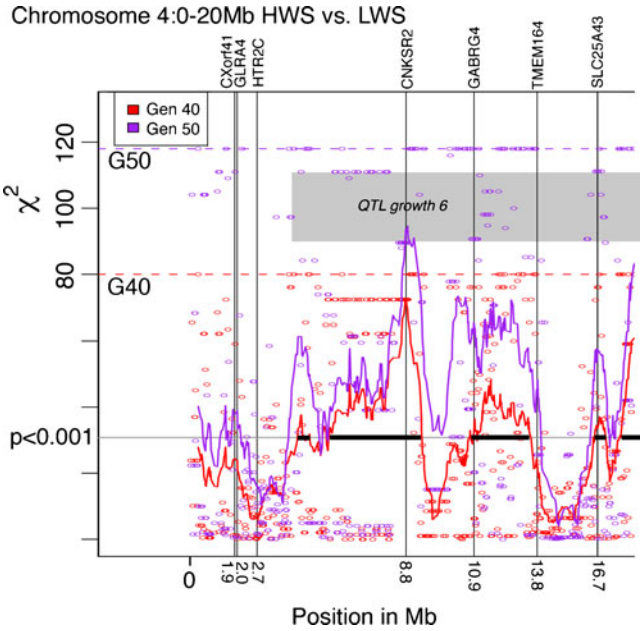

Fig. 2 DE genes on chromosome 4:0–20 Mb in relation to allele frequency differences of HWS and LWS lines and to QTL growth6 region. The graph is adapted from Supporting Fig. S2 in Johansson et al. [11] and illustrates the location of differentially expressed genes in relation to differences of single nucleotide polymorphism allele frequency between HWS and LWS lines along the distal p-arm of chromosome 4. The nucleotide polymorphism allele frequency for two time points during the selection scheme, generation 40 and 50, is shown. The result for individual single nucleotide polymorphisms shown as *circles* and the average allele frequency in a sliding window of 20 markers are shown as a *red line* for generation 40 (*G40*) and as a purple line for generation 50 (*G50*). The *dashed red* and *purple lines* indicate the maximum values obtained when a SNP is fixed for different alleles in the high and low line (80 and 118, respectively). The *grey line* indicates the Bonferroni corrected significance level at $p<0.001$. The localization of DE genes is indicated by vertical lines with position and gene names. The *growth6* QTL [12, 13] region is marked in *grey* and the regions with significant allele differences at generation 40 is marked with a *black horizontal bar*

Discussion

Global mRNA expression was analysed in hypothalamus obtained from females of two selected lines of chickens, which have been established by long-term divergent selection for high and low body weight. We used Affymetrix oligonucleotide microarrays to assess gene expression profiles of chicks 4 days post-hatch. Differences in hypothalamic gene expression between these lines were moderate and less than 1.5-fold for the majority of DE genes, despite extreme phenotypic differences. Several minor but significant expression differences for genes belonging to networks related to regulation of cell cycle, DNA repair and cell death, lymphocyte-mediated immunity, mitogen-activated protein kinase signalling as well as to neuronal plasticity including actin polymerisation were identified. Differences were also found for genes that are involved in regulation of catalytic activity and certain metabolic processes confirming that aspects of differential growth regulation could also be detected in these neural tissues. The results did not identify any general changes in known hypothalamic mediators of appetite regulation such as the melanocortin receptors that would explain the different behaviours. We recently performed a gene expression study of a larger brain region with diencephalon and brain stem including the hypothalamus from these two lines of chicken [20], and the results from both studies were consistent in many aspects in that more genes were upregulated in the HWS than the LWS line and that several genes involved in neuronal plasticity were identified.

Oligonucleotide array analysis has an increased risk to detect sequence polymorphisms in the probe regions instead of true differential expression [21] and domestic

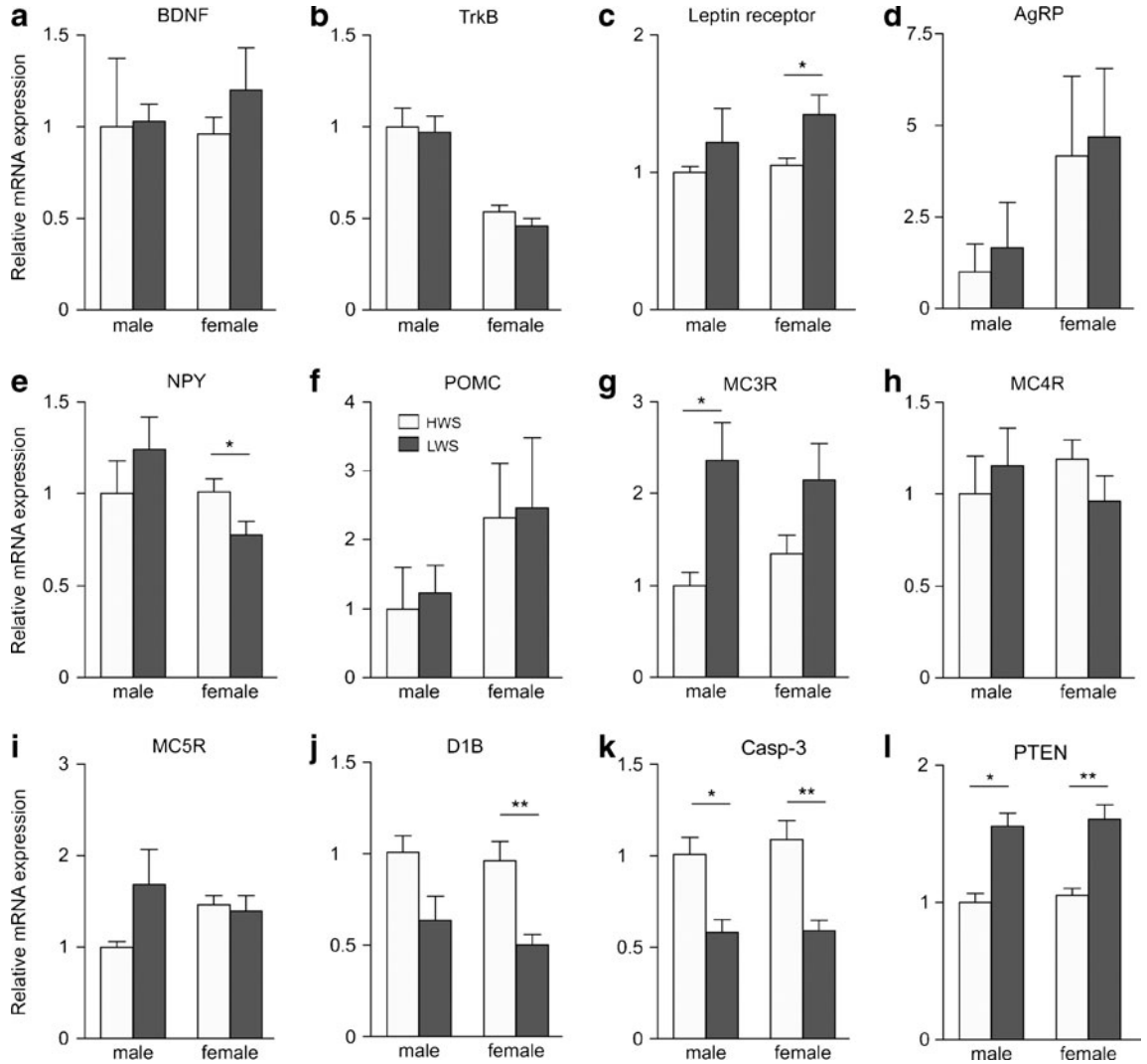

Fig. 3 Relative mRNA expression of candidate genes in hypothalamus from HWS and LWS lines. The relative mRNA expression was analysed using qRT-PCR. **a** Brain-derived neurotrophic factor (BDNF). **b** The BDNF receptor TrkB. **c** Leptin receptor. **d** Agouti-related protein (AgRP). **e** Neuropeptide Y (NPY). **f** Pro-opiomelanocortin (POMC). **g** Melanocortin-3 receptor (MC3R). **h** Melanocortin-4 receptor (MC4R). **i** Melanocortin-5 receptor (MC5R). **j** Dopamin D1B receptor. **k** Caspase-3. **l** Phosphatase and tensin homologue (PTEN). *White bars* represent HWS and *black bars* represent LWS line. Unpaired *t* test was used for statistical analysis ($n=3$ in each line for male and $n=7$ in each line for female). *$p<0.05$, mean±SEM

chickens have a high frequency of sequence polymorphisms [22]. Probe sets with opposite differential expressions for one and the same gene were seen for two genes among the highest ranked sets. When the probe sets were combined, the two genes were excluded because of low statistical support. Sequencing revealed the presence of sequence polymorphisms in the probe region for both genes and differential expression could not be verified by qRT-PCR (data not shown). Hence, for those genes the array DE signal likely was the result of polymorphisms rather than from different mRNA levels. By using the list of annotated genes with representations by more than one probe for many top-list genes, the occurrence of such false positive DE sequences was decreased. However, this restricted the analysis to annotated genes present in the genome assembly. Among the DE probes that were excluded by this procedure were these related to endogenous avian leucosis virus. Previously, we reported that endogenous avian leucosis virus sequences are differentially expressed in central nervous and peripheral tissues in these lines [20] and that high endogenous avian leucosis virus expression is associated to the development of a low body weight in LWS pullets [19]. When we manually examined probes for endogenous avian leucosis virus in the current array results, we found them to be DE (not shown), in line with our previous observations.

Neuronal development, growth and plasticity

The regulation of actin filament dynamics is instrumental in neuronal development and plasticity [23], and several of the top-list DE transcripts were associated with neuronal plasticity in one or several aspects (see also supplementary Table S6). There was also a significant over-representation of DE genes classified as 'Regulation of mitogen-activated protein kinase activity' and 'actin polymerisation'. Rho-type Ras-related GTPases are master regulators of actin polymerisation during dendrite morphogenesis in neuronal plasticity and are regulated by the mitogen-activated protein kinase pathway [24]. The Rho-activating protein RhoGAP plays an important regulatory role in this process. Both RhoGTPase and RhoGAP mRNA were differentially expressed. Animals with changed levels of RhoGAP suffer from abnormal actin polymerisation and maturation of spines and dendrites that leads to progressive deficits in different behavioural tasks [25]. These results are consistent with the hypothesis that LWS and HWS lines are equipped with neuronal plasticity systems that are slightly different and suggest that the two lines are set in different developmental trajectories gradually leading to increasingly different hypothalamic functions. This hypothesis was suggested from results of our previous study using a brain region containing hypothalamus [20] and was supported by the exclusively hypothalamic tissue used in the current study. Neuronal plasticity is dependent on a large number of genes that are dynamically regulated. Therefore, multiple minor genetic alterations with additive or synergistic effects may result in extensive behavioural modifications. The hypothesis suggests that the two lines will deal differently with the transition after hatch, from yolk as the internal source of energy, to external sources of food for energy, gained by feeding. It has been shown, in rodents, that the homeostatic regulation of energy balance changes dramatically during the first 3 weeks post-natally and that the hypothalamic network undergoes concomitant structural and neurochemical changes [26].

Naturally occurring neuronal death is an inherent part of many structural changes that occur during nervous system development and is also a component of neuronal plasticity [27]. Caspase-dependent cell death has been shown to occur and to participate in hypothalamic development [28]. Caspase-3, the major executioner caspase in the apoptosis cascade, was differentially expressed and 'Induction of apoptosis' was one of the significantly enriched biological processes with DE genes. Recently, a causal link between caspase-3 and long terminal depression, one of the major mechanisms in neuronal plasticity was established [29]. Furthermore, PTEN, an important regulator of both apoptosis and cell proliferation by means of its regulation of the Akt/Protein kinase B signalling pathway, was also differentially expressed (Fig. 3l, Supplementary information Tables S5, S6). 'Lymphocyte mediated immunity', 'Leukocyte differentiation', 'DNA repair', 'DNA recombination' and 'DNA metabolism' imply that cells are exposed to damage or degeneration that are also related to cell death and inflammation processes (Table 1). The LWS individuals in this study were selected to have increased early post-hatch mortality in similarity to mice homozygous for the anorexia (*anx*) mutation, which have poor food intake and die by 3 to 5 weeks after birth. Expression analysis implies that degeneration of hypothalamic arcuate neurones is a primary event in that anorectic phenotype [30] with increased cell death and inflammation during the degenerative process [31].

Among the top 97 DE genes, receptor subunits for serotonin (*HTR2C*), dopamine (*DRD5*, D1B), glycin (*GLRA4*) and GABA (*GBRAB2*, *GBRG4*/ε) were identified, implying not only structural neuroplastic properties but also different neurochemical properties of the hypothalamic networks in these two lines. Serotonin has been associated with changes in human body mass index and serum leptin [32] and the serotonin receptor-2C subtype is involved in the influence of serotonin on food intake [33]. The dopamine receptor D5/D1B is a susceptibility locus for attention deficit disorder with hyperactivity in humans. GABA, the major inhibitory transmitter in the brain, goes through a transitory phase of excitation during development. The excitatory phase promotes neuronal growth and integration into circuits [34]. GABA receptors, including the *GBRAB2* and *GBRG4*/ε, are also known mediators of neuronal progenitor cell development [35].

Knock-out or transgenic mice for at least 25 of the top DE genes possessed phenotypes related to developmental neurological and behavioural deficits, fatness or increased body weight, changed feeding behaviour or altered growth. Several of these mice have abnormalities in heart rate/blood pressure as well as reproduction and this is consistent with functions regulated by the autonomic nervous system [36]. The HWS and LWS chicken lines have a documented dysfunction of the autonomic nervous system [5, 6, 37].

Trans-regulation or *cis*-acting regulatory elements

The phenotypic differences that evolved during the 40–50 generations of divergent selection reflect the underlying changes in gene regulation and can be monitored by differential gene expression, as presented here. When all 585 DE genes were taken into account, there was no significant enrichment of DE genes in QTL regions for growth. However, when only the top DE genes were considered, the fraction of DE genes in QTLs increased markedly. Moreover, when comparing the genomic distribution of DE genes to the genome-wide analysis data of

chromosomal regions that have been under divergent selection in the lines [14], a significantly higher proportion of the genes was found in regions that have been under divergent selection than what would have been expected by chance. Several of these genes also overlap with the QTLs for growth, as illustrated by the distal part of the p-arm of chromosome 4. While such genes are excellent candidates for being causative of the growth difference, causation cannot at present be formally confirmed. The distal part of chick chromosome 4p hosts the QTL *growth6* and contains a high proportion of regions under divergent selection with the DE genes CNKSR2, GABRG4, SLC25A43 and additional genes in the close neighborhood (Fig. 2 and supportive Table S6). The DE genes that reside in genomic regions without a significant difference in allele frequencies between HWS and LWS are likely to contain identical or similar *cis*-acting transcriptional regulatory elements. Their differential expression is therefore likely to be a result of gene regulation in *trans* by influence via other gene products. Hence, such DE genes will not be causatively involved in the phenotype. Even though the clear majority of the DE genes is regulated in *trans*, the present data imply that the top DE genes are enriched for being located in regions under divergent selection. Such genes are more likely to have line specific sequence polymorphisms in *cis*-acting elements and are thus more likely to be causative for the growth phenotypes.

Hypothalamic neuropeptide mediators

The melanocortin circuit in the hypothalamus is one of the important and well-studied pathways that regulate feeding behaviours. Gene expression analysis of hypothalamus from fasting and feeding chickens support such role for POMC in the chicken hypothalamus [38]. Only neuropeptide Y of the canonical hypothalamic mediators of feeding behaviour, POMC, agouti-related protein, melanocortin-3, -4, -5 receptors and the leptin receptor were found in the array results. When the result was verified by qRT-PCR, the leptin receptor and neuropeptide Y were differentially expressed in females and melanocortin-3 receptor in males (Fig. 3). These differences were small, which explained why the genes did not appear in the array results. Melanocortin receptor accessory protein 2 (*MRAP2*) was identified as a DE gene indicating possible alterations downstream of MC receptor signalling. It is expressed in hypothalamic neurones where melanocortin-3 and -4 receptors are expressed [39, 40] and modulates the responsiveness of the receptors to the ligands [41] and thus represents the underlying modulatory or plastic system in these cells. We conclude that the expression of genes in the melanocortin circuit was not changed systematically at this age in response to the selection.

Several aspects of the results reported here are consistent with those from our previous cDNA microarray study of these lines [20] and with an array study of hypothalamus from two other divergently selected fat and lean chicken lines [42]. Although the experimental designs, tissues and animals were different, similarities between lines were seen: (1) only moderate DE with more genes with higher expression levels in brains of large/fat than in the small/lean chicken lines and (2) only minor alterations in the melanocortin system expression were observed. In addition, our results identified the genes that are functional in neuronal development, degeneration and plasticity. Thus, we conclude that it is unlikely that the long-term divergent selection for 8-week body weight resulting in large differences in food intake have led to an accumulation of *cis*-acting nucleotide plymorphisms in the melanocortin system. However, the results suggest that there is an increased representation of *cis*-acting polymorphisms in genes that are differentially expressed in the hypothalamus of these chicken lines. If these data may be extrapolated to humans, we speculate that the post-natal development would be of particular interest to target if the eating behaviours are to be manipulated.

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