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Research report

From wild wolf to domestic dog: gene expression changes in the brain ☆

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Abstract

Despite the relatively recent divergence time between domestic dogs (*Canis familiaris*) and gray wolves (*Canis lupus*), the two species show remarkable behavioral differences. Since dogs and wolves are nearly identical at the level of DNA sequence, we hypothesize that the two species may differ in patterns of gene expression.

We compare gene expression patterns in dogs, wolves and a close relative, the coyote (*Canis latrans*), in three parts of the brain: hypothalamus, amygdala and frontal cortex, with microarray technology. Additionally, we identify genes with region-specific expression patterns in all three species. Among the wild canids, the hypothalamus has a highly conserved expression profile. This contrasts with a marked divergence in domestic dogs. Real-time PCR experiments confirm the altered expression of two neuropeptides, *CALCB* and *NPY*. Our results suggest that strong selection on dogs for behavior during domestication may have resulted in modifications of mRNA expression patterns in a few hypothalamic genes with multiple functions. This study indicates that rapid changes in brain gene expression may not be exclusive to the development of human brains. Instead, they may provide a common mechanism for rapid adaptive changes during speciation, particularly in cases that present strong selective pressures on behavioral characters.

Theme: Neural basis of behavior
Topic: Neuroethology

Keywords: DNA microarrays; Gene expression variation; Domestication; Brain evolution; Speciation

1. Introduction

Rapid differentiation and speciation is known to occur in some situations such as island colonization and domestication. The genetic mechanism by which this type of punctuated diversification occurs has been long and hotly debated. One theory, which accounts for the limited amount of mutations in functional genes occurring during rapid differentiation, suggests that changes in regulatory regions could be a major source of variation [8]. Subsequently, it has been postulated that changes in the mechanisms controlling gene expression may be more important for biological differences between species than structural changes in gene products [23]. Several studies have indicated that natural genetic variation can cause significant differences in gene expression [7,10,11,33,35]. Some of this genetic variation could be responsible for behavioral changes. For example, it has been shown that altered gene expression of a steroid hormone receptor in the brain may explain the evolution of novel social behavior in closely related whiptail lizards [48]. Comparisons of mRNA levels in primates show that the rate of evolutionary change of gene expression has been accelerated in the human brain, suggesting that altered gene expression may be important

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for behavioral differences between human and great apes [14,16].

The domestic dog (*Canis familiaris*) originated from the domestication of wild gray wolves (*Canis lupus*) at least 15,000 years ago [36,39,46]. From an evolutionary point of view, this divergence is very recent, and the genome sequence of dogs and wolves is almost identical [46]. The behavioral and morphological differences between dogs and wolves is the result of strong human-mediated selection for desired behavioral traits, morphological characters, and/or the ability to learn and perform different tasks [19,32]. This short evolutionary history coupled with strong selection and a known, extant progenitor species makes this system ideal for exploring genetic mechanisms of speciation. Since selection for behavioral traits likely started earlier than selection for morphological traits (by the preferential breeding of docile and tame animals), we expect the expression differences between dogs and wolves to be particularly pronounced in brain, especially in tissues involved in emotion and behavior. Consequently, we predict that the domestication process resulted in large changes in gene expression patterns in the brains of dogs as compared to the patterns in their wild ancestors, which should be more conserved. A comparison of gene expression patterns in dogs, wolves and coyotes (*Canis latrans*), who probably diverged from the wolf lineage around 2 million years ago [47], should enable us to differentiate neutral change accumulated over time from the effect of selection.

To compare expression patterns, we evaluated mRNA expression levels of 7762 genes in dogs, wolves, and coyotes in three regions of the brain: the frontal lobe, the amygdala and the hypothalamus. These regions were selected because of their involvement in cognition and/or emotion [12,27,28,31,41]. A recent draft of the dog genome sequence indicates that more than 18,000 annotated human genes may exist as orthologs in dogs [24]. Messenger RNA was extracted from post mortem autopsies and hybridized to human microarrays, including clones with an average 88% sequence identity to dog cDNA sequences, and the global gene expression pattern was compared among species and brain regions. For selected genes, the expression profiles obtained by microarray analyses were confirmed by real-time PCR. We show that the hypothalamus has a highly conserved expression profile among the wild canids that contrasts with a marked divergence in domestic dogs. Our results suggest that strong selection for behavior in dogs during domestication resulted in modifications of mRNA expression patterns in a few hypothalamic genes with multiple functions.

2. Materials and methods

2.1. Tissue samples and RNA isolation

Post mortem brain tissue samples were extracted from 10 dogs (3 Labrador retrievers and 7 German shepherds) from Sweden, 10 coyotes from Texas, USA, and 5 gray wolves (3 from Sweden, 1 from Spain and 1 from Canada). All animals died for reasons other than their participation in this study. During autopsy, 0.1–0.5 g from each tissue (amygdala, frontal lobe and hypothalamus) were collected and frozen on dry ice, followed by storage at −70 °C. Cortex samples from three additional dogs were collected to be used as a reference pool. Tissues were homogenized and total RNA was prepared as previously described [9].

2.2. RNA quantification

RNA concentration was estimated with RiboGreen RNA Quantitation Reagent Kit (Molecular Probes, Sweden). The relative fluorescence was measured using the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). Each sample was measured three times and the results were averaged.

2.3. Sequence comparisons

A crude estimate of the sequence similarities between human and dog mRNA was obtained by BLAST comparisons of all available dog mRNA sequences (19799 sequences, 26 Nov. 2002) against the sequence of the human cDNA-clones attached to the array. Only one hit per query was recorded (highest score). The homology of aligned fragments with at least 100 bp in length (1175 sequences) ranged between 78% and 100%, with an average of 88%. Due to the similarity between human and dog sequences, we concluded that human arrays could be used for cross-hybridization experiments with dog, wolf, and coyote mRNA. For selected genes, the results from the arrays were confirmed by real-time PCR using dog sequences.

2.4. Microarray hybridizations

In a first set of microarray experiments, we searched for brain region-specific differences in mRNA expression. We compared the variation between different regions of the brain and identified genes which showed a region-specific expression pattern in all three species (domestic dog, gray wolf and coyote). For these experiments, we made nine RNA pools from amygdala, frontal lobe and hypothalamus obtained from each of the three species. Each pool consisted of equal amounts of total RNA from each animal. Pooling of the biological replicates was necessary because of the limited amount of mRNA extracted from hypothalamus and amygdala. Pooling of samples also reduced the cost of the microarray experiments. Inter-individual differences were later assessed for selected genes using real-time PCR.

The total RNA pool was used to prepare mRNA and 250 ng of each mRNA were used for a reverse transcription reaction with the MICROMAX™ TSA™ Labeling and Detection Kit (NEN® Life Science Products). The c-DNA

products of each transcription reaction were labelled with Cy5 and hybridized with the reverse transcription products from a reference mRNA pool, which had been labelled with Cy3. In other words, each of the nine cDNA pools was hybridized with the same "reference" cDNA pool resulting in a total of nine microarray experiments. This "common reference design" allowed for indirect comparisons of gene expression between the nine arrays (three species × three regions) [49].

In a second set of microarray experiments, we searched for species-specific differences in gene expression within each brain region. The same nine cDNA pools described above were used for these experiments but a different design was utilized for the microarray hybridizations to increase the power to detect differences among the species. For each of the three regions, the c-DNA pool from each of the three species was pair-wise hybridized with each of the other species, allowing for a direct inter-species comparison [49]. Each hybridization was repeated twice, with the dye assignment reversed in the second hybridization (dye swap pairs), resulting in a total of 18 arrays (6 arrays for each region).

Samples were hybridized to cDNA arrays (Wallenberg Consortium North, Uppsala University, http://www.genpat.uu.se/wcn/uppsala.html) with 7762 clones (Research Genetics) each spotted twice on every array. Hybridizations were performed at 60 °C (region-specific experiments) or 55 °C (species-specific experiments) for 16 h, otherwise following manufacturer's protocols (NEN® Life Science Products).

2.5. Image processing

The microarrays were scanned at 10 μm resolution using a GenePix 4000B scanner (Axon Instruments). Spots on the resulting images were quantified with the software package GenePix Pro 3.0 (Axon Instruments). The mean intensity of the Cy5-labelled sample (R) and the Cy3-labelled reference (G) was used to calculate the log transformed ratio between the sample and the reference for each spot: $M = \log_2 (R/G)$.

2.6. Real-time PCR assay

Quantification of mRNA levels in brain autopsies was performed using a fluorogenic 5′ nuclease assay ("TaqMan") as we described previously [9]. We prepared 25-μl reactions using a SYBR®Green PCR Core Reagents kit (Applied Biosystems). The use of 96-well replicate plates allowed for independent analysis of all samples from all individuals. Real-time PCR was done for each individual sample independently without prior pooling. The sequences of the human clones for *AQP4*, *CALCB*, *CRYM*, and *NPY*, present in the arrays were used for BLAST searches, and the dog homologous sequence was used to design primers for real-time PCR. Primers were also designed for the dog reference genes *GAPD* (Glyceraldehyde-3-phosphate dehydrogenase) and *ACTB* (Beta-actin). The PCR reactions were run on an ABI PRISM® 7000 Sequence Detector System (Applied Biosystems).

2.7. Data analysis

We used a robust scatter plot smoother (Proc Loess, SAS v8.2) to perform a sub-array intensity-dependent normalization of M, with the smoothing parameter set to 40% [50]. In the experiments performed to search for region-specific differences, each species and brain region combination was only represented by one array, and thus we had to perform a between slide scaling to avoid systematic errors due to differences in the spread of M between microarrays [50]. All spots with a mean spot intensity below the local median background were excluded from the analysis. We used the average M from the two replicate cDNA spots on each array for further analysis. In the region-specific experiments, a total of 4082 genes were analyzed, of which 2471 were found on all nine arrays. In the experiments designed to search for species-specific differences, a total of 7259 genes were analyzed, of which 6365 where found on all 18 arrays.

To identify differentially expressed genes with respect to species or regions, we used ANOVA models (Proc GLM, SAS v8.2). In the analysis of the region-specific experiments, the factors "species" and "region" were included in the model, and genes showing evidence of differential expression (p_{species} or $p_{\text{region}} \leq 0.01$) were identified. In the analysis of the species-specific experiments, we included the factors "species" and the interaction "species*region" in the model, and we identified genes with strong evidence for differential expression (p_{species} or $p_{\text{interaction}} \leq 0.001$). Due to the different experimental design, the statistical model for the species-specific experiments was parameterized as a regression model [49].

The real-time PCR data was analyzed with a mixed ANCOVA model (Proc GLM, SAS v8.2). Species effects were assessed using the between-animal variability whereas the region effects and the interaction between region and species were assessed using the variability of samples within animal. The expression of the two reference genes *ACTB* and *GAPD* were included as covariates in the model [6].

3. Results

3.1. Brain region-specific differences in gene expression

In a first set of microarray experiments, we compared the variation between different regions of the brain and identified genes which showed a region-specific expression pattern in all three species (domestic dog, gray wolf and coyote). The experiments indicated differential expression with respect to brain region for 156 genes (ANOVA, $p \leq 0.01$). This number of genes is four times higher than the 41 false positive signals expected under the null hypothesis when the significance level is set at $p = 0.01$ and $n_{\text{genes}} = 4082$. Thirty-five of these genes could be classified

as being region-specific in the sense that the expression level in one region differed at least 2-fold from the expression level in the other two regions (see online supplemental material Table 1). A majority of these genes showed an enriched (9 genes) or decreased (21 genes) expression in the hypothalamus as compared to the other two tissues. Only two genes showed frontal lobe-specific expression (1 up-regulated, 1 down-regulated), and two genes showed an enriched expression in the amygdala.

To examine the relationship between hypothalamus-specific expression and biological function, we classified the region-specific genes into functional categories using Gene Ontology (GO) [3]. We then compared the proportion of hypothalamus-specific genes in each functional category (22 genes were successfully classified) with the proportion in each category of all genes detected in the array (4080 genes). According to this classification, genes involved in transmission of nerve impulse (cell–cell signalling) and transport were over-represented in the hypothalamus-specific gene set. Thus, there were five genes classified in the transmission of nerve impulses category (only one was expected in a random sample of 22 genes) and nine genes classified in the transport category (three were expected) (see online supplemental material Table 1 for a complete classification).

To display the major patterns in gene expression across the nine arrays, the expression of the 156 selected genes was studied with principal component analysis of the covariance matrix. The first four principal components (PCs) revealed systematic expression differences between regions or species, and together accounted for 95% of the total variation in gene expression over the nine arrays. The first PC, accounting for 68% of the variation, separated the expression profile of hypothalamus from that of the other two regions ($p < 0.0001$, Fig. 1A). The second (PC2) accounted for an additional 15% of the total variation in gene expression, and separated the expression profiles of the amygdala from that of the frontal lobe ($p < 0.0001$, Fig. 1B). The third and fourth PCs reflected differences in gene expression between the three species and accounted for only 8% and 4% of the expression variation.

The results from the first set of microarray experiments demonstrated that the expression profile of the hypothalamus was clearly different from that of the frontal lobe and the amygdala, and that the variation in gene expression between the three brain regions was substantially larger than the variation between species.

3.2. Gene expression differences between coyotes, wolves and dogs

To detect expression differences between species within each brain region, we designed a second set of microarray experiments with a higher statistical power (see Materials and methods). A total of 114 genes with strong evidence for differential inter-species expression in at least one of the three tissues were identified (ANOVA, $p \leqq 0.001$). This number is eight times higher than the number of false positive signals expected under the null hypothesis when $p = 0.001$, $n_{genes} = 7259$ and $n_{tests} = 2$.

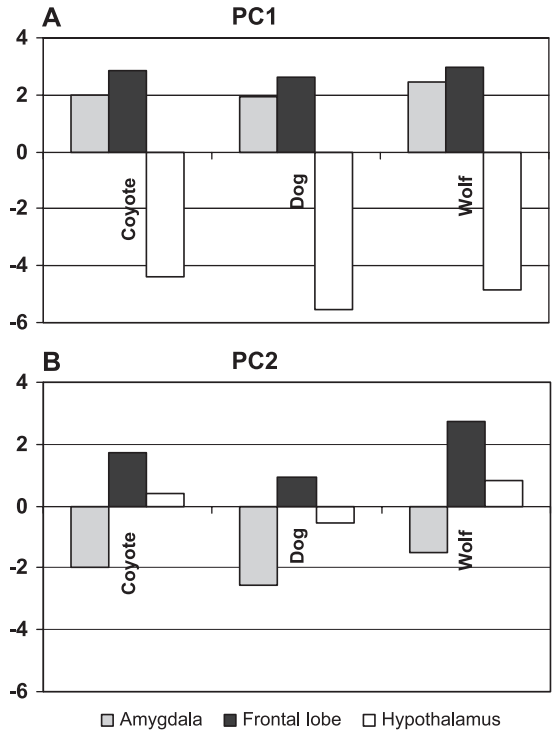

Fig. 1. Patterns of brain gene expression studied by principal component analysis. (A) The loadings for the first principal component, PC1, separate the expression profile of hypothalamus from that of the other two tissues ($p < 0.0001$). (B) PC2 separates the expression profiles of the amygdala from that of the frontal lobe ($p < 0.0001$).

First, we compared the average inter-species expression differences in each tissue for the 114 selected genes with that for all expressed genes (Fig. 2A). For each tissue, the average expression differences between species for the 114 selected genes were significantly larger than the expression difference for all genes, which provides an estimate of the experimental background noise level. In the amygdala and the frontal lobe, the average expression differences of the selected genes were 29% and 32%, respectively, and similar among the three species. In contrast, the average expression difference in the hypothalamus was 19%, with a difference between wolves and coyotes of only 13%, slightly higher than the background noise level (9%). Thus, mRNA expression patterns are clearly more conserved in the hypothalamus than in the other two regions, particularly between coyotes and wolves. The expression profile of dogs, on the other hand, had clearly diverged in the hypothalamus from those of the other two species. The average expression difference between dogs and wolves reached 24% in the hypothalamus, and it was 22% between dogs and coyotes.

Thirty-six of the 114 genes could be classified as showing species-specific expression in at least one tissue, in the sense

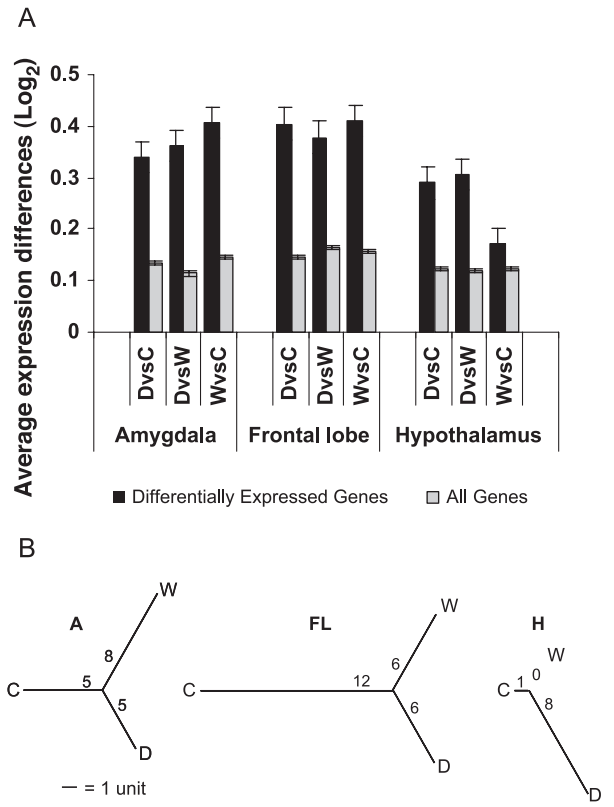

Fig. 2. Mean mRNA expression differences between coyotes, dogs and wolves. (A) Average pairwise expression differences (and 95% confidence intervals) for 114 genes with significant ($p < 0.001$) differences between species are shown by the black bars. Gray bars indicate the average expression of all genes for each comparison (7259 genes; see text), which is an estimation of the random noise level for each experiment. C = coyote, W = wolf and D = dog. (B) Relative extent of expression changes between species for amygdala (A), frontal lobe (FL) and hypothalamus (H). Thirty-six genes were classified as species-specific in at least one tissue, and their expression differences were used to construct the trees. The branch lengths correspond to the expression differences on a $\log_2$-scale summed over genes that show species-specific differences. One unit refers to a 2-fold expression change. The number of species-specific genes is indicated next to each branch. C = coyote, W = wolf and D = dog.

that the gene expression in one species was at least 1.5-fold higher or lower than in the other two species and the expression difference between these two was under the 1.5-fold threshold (Table 2, supplemental online data). To examine the relationship between species-specific expression and biological function, we compared the proportion of species-specific genes in each functional category (25 genes were successfully classified with Gene Ontology) with the proportion in each category of all genes detected by the array (7259 genes). These comparisons revealed that genes involved in cell–cell signalling and neurogenesis (organogenesis) were over-represented in the species-specific gene set. Six of the 25 species-specific genes fell in the neurogenesis category (only two were expected in a random sample of 25 genes), and four genes fell in the cell–cell signalling category (only one was expected) (see online supplemental information Table 2 for complete classification).

In the hypothalamus, eight genes (with a total expression distance of 7.76, 95% confidence interval CI: 6.71–8.81) were specific in expression for the dog lineage as compared to only one gene showing a coyote-specific expression (expression distance: 0.89, 95% CI: 0.06–1.73), and no gene showed a wolf-specific expression (Fig. 2B). Seven of the dog specific genes showed an enriched expression in the hypothalamus and only one (*NPY*) showed a decreased expression (online supplemental information Table 2).

In the frontal lobe, there were 12 genes with coyote-specific expression as compared to six genes in each wolves and dogs, resulting in a total expression distance that was twice as large in coyotes (12.85, 95% CI: 11.44–14.25) than in wolves (5.87, 95% CI: 4.69–7.06) or dogs (5.50, 95% CI: 4.74–6.27). Interestingly, three out of seven GO-classified genes with coyote-specific expression in the frontal lobe were involved in neurogenesis, namely: *SNCA*, *MOPB* and *DPYSL2*. Two additional genes with decreased expression in coyote frontal lobe, *MAG* and *CSRP1*, may also be involved in neuronal development, although these genes have not yet been assigned to any GO-classes [17,42].

In the amygdala, the differences in expression distance were similar among the three lineages, with eight genes showing wolf-specific expression and five genes being specifically expressed in each coyote and dog. Consequently, differences in the resulting expression distances were relatively small and similar across species. For wolves, the expression distance was 7.35 (95% CI 6.02–8.68), for coyotes it was 5.32 (95% CI 4.23–6.40) and for dogs it was 4.50 (95% CI 3.66–5.35).

3.3. Gene expression determined by real-time quantitative PCR

The expression levels for four genes were determined separately in each individual and tissue by real-time PCR to assess the importance of inter-individual differences as compared to differences between species. This approach allowed us to confirm the results of the arrays by an independent method. We selected one gene with hypothalamus-specific expression, *AQP4* (aquaporin 4), which also showed a 2-fold expression difference between wolves and dogs across all three tissues ($p = 0.02$, data not shown), and two neuropeptide genes with dog-specific expression in the hypothalamus, *CALCB* (calcitonin-related polypeptide, beta) and *NPY* (neuropeptide Y). We added a forth gene, *CRYM* (crystalline, mu), which showed 2-fold expression differences between all three regions ($p = 0.04$), and a 2-fold decreased expression in dog hypothalamus ($p = 0.003$). *CRYM* did not meet the significance threshold we used to select region- or species-specific genes but it was tested to evaluate the sensitivity of our approach.

The major expression patterns from the arrays were confirmed by real-time PCR (Fig. 3). *AQP4* had a clearly enriched expression in the hypothalamus, with mRNA levels being 2.6- and 3.7-fold higher in the hypothalamus

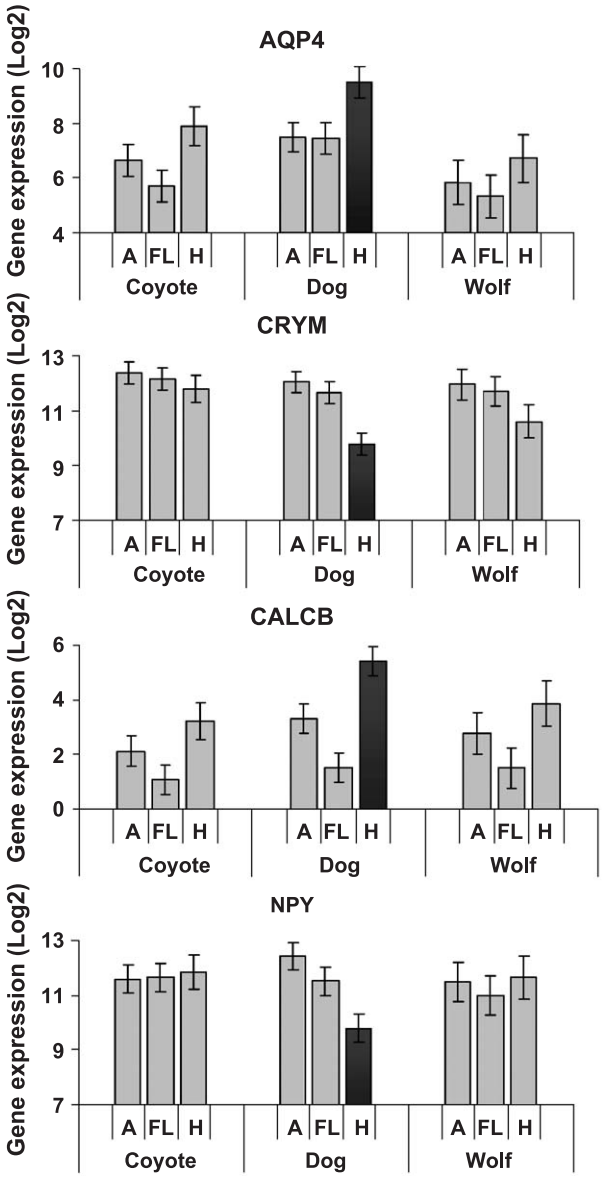

Fig. 3. Mean expression levels (and 95% confidence intervals) for *AQP4*, *CALCB*, *CRYM* and *NPY* determined by real-time PCR for 10 coyotes, 5 wolves and 10 dogs in three brain tissues (A = amygdala, FL = frontal lobe and H = hypothalamus). Values have been adjusted for the expression levels of two reference genes. Black bars indicate expression values for dog hypothalamus. *AQP4* and *CALCB* show an increased expression in dog hypothalamus while *CRYM* and *NPY* were down-regulated in this tissue.

than in the amygdala and the frontal lobe, respectively ($p < 0.0001$). *CRYM* had an enriched expression in the amygdala, and a decreased expression in the hypothalamus ($p < 0.0001$). However, the expression difference between the amygdala and the hypothalamus was somewhat lower than expected from the array. In addition to confirming the array results, the real-time PCR experiments revealed the weakly expressed *CALCB* to have clearly tissue-specific expression, having an enriched expression in the hypothalamus and a decreased expression in the frontal lobe ($p < 0.0001$).

The differences among species (averaged over all tissues) were significant for three genes, with $p = 0.004$ for *AQP4*, $p < 0.0001$ for *CRYM*, and $p = 0.002$ for *CALCB*. For *AQP4*, the expression difference between dog and wolf was consistent across all tissues with the expression being 4-fold higher in dog than in wolf ($p < 0.004$). For *CRYM* and *CALCB*, the species differences varied slightly with tissue (as indicated by a significant interaction between the factors species and brain region, $p = 0.01$ and $p = 0.04$, respectively). Dogs showed a lower level of *CRYM* and a higher level of *CALCB* expression in the hypothalamus, and a modest enrichment of the *CALCB* expression in the amygdala. For *NPY*, only small expression differences were observed between species averaged over all tissues. However, the expression of *NPY* was clearly decreased in dog hypothalamus by a factor of 3.6 (compared to wolf) and 4.1 (compared to coyote), whereas the expression tended to be enriched in dog amygdala ($p_{\text{interaction}} < 0.0001$).

These results suggest that the expression patterns observed in the arrays indeed correspond to species-specific differences in the canids. Moreover, for the selected genes, the average expression differences between the three species are significant compared to the inter-individual variation within each species.

4. Discussion

4.1. Experimental strategy

We have compared gene expression patterns in hypothalamus, amygdala and frontal cortex obtained from dogs, wolves and coyotes. Our strategy included hybridization of pooled mRNA samples on microarrays containing human cDNA clones, and then confirmation of results using real-time PCR on each individual separately.

While pooling samples from individuals is not likely to have a large effect on comparisons of global gene expression profiles, it increases the risk for individual genes to be identified as consistently differentially expressed between groups when in fact the difference is due to one or a few animals showing a remarkably distinct response, or lack of response [18]. However, the four genes analyzed on each individual separately by real-time PCR indicated that the expression differences between the groups were large (and significant) compared to the differences between individuals for these four genes. This suggests that a significant proportion of the expression differences between dogs and other canids are not due to inter-individual differences.

We carried out hybridizations at temperatures 5–10 °C below the manufacturer's recommendations, to allow for successful cross-hybridization between mRNA from canids and the human cDNA clones present on the microarrays. Thus, it is possible that genes with high sequence similarity

to the listed genes contributed to the obtained signals. Therefore, our lists of differentially expressed genes should be interpreted with some caution. Nevertheless, the expression patterns of four genes that were differentially expressed on the array (>2-fold) could be confirmed with real-time PCR (with canidae-specific primers), showing that we were able to correctly determine the expression levels of the homologous genes in canids.

4.2. Brain region-specific differences in gene expression

We have shown here that the variation in gene expression between different brain regions was substantially larger than the variation between species. This was evident from the principal component analysis that indicated that 83% of the total variation in expression levels was explained by tissue differences while only 12% was explained by species differences. A similar pattern has been found in a comparison of four brain regions in two strains of mice, where the number of genes that showed differential expression with respect to brain regions was approximately three times higher than the number of genes that showed expression differences between the strains [38]. In the case presented here, we analyzed differences between species instead of closely related strains, and expected the variation between the species to be larger than between strains. Still, the variation between species only accounted for a small fraction of the total variation. Our results indicate that high-resolution strategies are needed to investigate differences in mRNA expression between closely related species.

The hypothalamus was the most differentiated brain region, with a high proportion of genes being down regulated. Similarly, Bonaventure et al. [5] found that the expression profiles of two divisions of the hypothalamic paraventricular nucleus were clearly separated from those of five other nuclei in brains of Wistar rats. Our results suggest that there may be large differences in gene pathways related to nerve impulse transmission and transport between the hypothalamus and other regions in the brain.

4.3. Gene expression in the frontal lobe parallels evolutionary distance

If the differences in gene expression levels between species paralleled evolutionary distance, we would expect coyotes to display the most divergent expression pattern among the three species. This was observed in the expression patterns of species-specific genes in the frontal lobe (Fig. 2B), where the number of genes with coyote specific expression differences was twice as large as the number of genes showing dog- or wolf-specific expression changes. Thus, the divergence in gene expression distances in the frontal lobe corresponded better to the known evolutionary distances between the species than for the other investigated tissues.

4.4. Dog domestication and changes in gene expression in the hypothalamus

The hypothalamus is an ancient structure that is involved in many behavioral responses essential to survival [26]. This brain region forms a crucial node to link exploratory behavior and specific emotional, endocrinological and autonomic responses of the organism [22]. The structure and function of this tissue has probably been highly conserved throughout mammalian evolution, since even a tiny lesion can produce dramatic and often fatal disruptions of widely dispersed bodily functions [4]. The ontology is also well conserved, and this structure is produced early during embryonal development [29]. The two wild species in our study, the gray wolf and the coyote, had a very conserved pattern of gene expression in this tissue in spite of having diverged millions of years ago [47]. In contrast with this, the pattern of gene expression in the hypothalamus of domestic dogs has diverged markedly in an evolutionarily short time. This suggests that the domestication process of dogs has greatly accelerated the rate of divergence in gene expression in the hypothalamus.

However, it is difficult to estimate the proportion of expression differences that are due to genetic variation and the proportion of differences due to environmental differences. To date, there are only a few studies that have tried to estimate the genetic contribution to natural variation in gene expression: in mice, approximately 25% of genes showing differential expression in liver tissue could be linked to a genetic marker [40], whereas the percentage of genes that displayed segregating transcription levels in yeast, range between 25% and 33% [7,51]. For genes that were differentially expressed in human lymphoblastoid cells, 29% had a detectable genetic component [40]. Thus, it is possible to speculate that at least a similar portion of the observed differences in hypothalamic gene expression between dog and its wild relatives may reflect genetic changes. The hypothalamus is involved in modulating neuroendocrine function in response to environmental changes, and differences in hypothalamic gene expression may thus partly reflect differences in availability of shelter, food and water between dog and its free-living relatives. Moreover, the biological clock is controlled through interactions among transcription regulatory proteins (e.g. Refs. [15,34,52]), and we cannot exclude that some of the observed differences in gene expression, especially in the hypothalamus, are due to systematic differences in the time of day when the animals died.

Two neuropeptides, *NPY* and *CALCB*, showed a dog-specific expression pattern in the hypothalamus, as quantified by microarray analysis and real-time PCR. *NPY* and *CALCB* are widely expressed in the mammalian brain and are co-localized and released with classical neurotransmitters [1,2,20]. Both peptides have been implicated in energy control and feeding behavior of mammals, linked to the hypothalamus [25,37], and in the neuroendocrine stress

response linked to the *HPA* axis [21]. It has also been proposed that *NPY* and *CALCB* play a role in behavior, such as anxiety and depression [30,43]. The observed expression differences in the two neuropeptides may have wide implications for multiple brain functions in domestic dogs.

A long-term study on farmed silver foxes (*Vulpes vulpes*) in which animals were selected for non-aggressive behavior towards man for more than 40 generations resulted in silver foxes that were docile and friendly towards people. The selection for tame foxes also produced a diversity of morphological and behavioral changes similar to those observed in many dogs, as compared to wolves (e.g. curly tails and pedant ears) and a "dog-like" loss of the seasonal reproductive pattern [44,45]. Changes in hormone levels related to the pituitary–adrenal function were observed, suggesting that tame foxes had decreased activity in their *HPA* axis. Several hypothalamic peptides, including *NPY* and *CALCB* have been reported to modulate the activity of the *HPA* axis [13,37] and they may thus be involved in the changes produced during the taming of foxes. Similarly, during the early phases of dog domestication, the most likely target of selection may also have been tame behavior. Selection for tameness in these foxes and during dog domestication may have had similar effect on the hypothalamic function.

5. Conclusions

Domestication has led to dramatic changes in dogs as compared to their ancestors. Domestic dogs were (and still are) subject to selective forces very different from those in wolves. Our results suggest that changes in the level of expression in a limited number of genes in the hypothalamus may be responsible for changes in the regulation of multiple brain functions. However, the observed changes in expression could also be the result of environment and life history instead of just genetics. To determine which expression changes have a significant genetic component, further research comparing wild and domestic or tame conspecifics bred under identical condition should be carried out.

Similarly to what happens in dogs, changes in the environment and in the selection regime may affect the patterns of gene expression in other species, leading to rapid differentiation of populations. This could thus be a mechanism of population and species phenotypic divergence that would have a faster effect than the accumulation of point mutations in structural genes.

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