Genetic and epigenetic factors of social behavior: comparison of human-raised wolves and dogs living individually or in captive packs (ANN107726)

Background

Our aim was to gain a deeper insight into the role and mechanism of genetic, epigenetic and environmental factors in shaping social behavioral traits. For this purpose, molecular biological analyses were carried out on canine samples taken from behaviorally tested dogs and wolves living under different social circumstances.

Behavior, as all other complex traits, is strongly influenced both by inherited and environmental factors. Variants of genes affecting quality or quantity of protein products required for a functioning nervous system affect behavioral outcomes as well, although the effect of a single specific variant in itself is often minor. Unlike quality, the quantity of proteins does not only depend on DNA sequence variations (i.e. genetic variants), but also on the presence or absence of so-called epigenetic modifications, which are chemical alterations associated to the DNA that leave the primary DNA sequence intact, yet influence gene expression efficiency. Epigenetic marks can be inherited, but at the same time they are sensitive to a wide variety of environmental factors, which can induce dynamic or permanent changes in epigenetic profiles. This, in turn, leads to altered gene expression, as a middle- or long-term adaptive response to the external effects. As such, epigenetic factors represent a key molecular link between genotype and environment. One of the best characterized epigenetic marks is DNA methylation. Extended DNA methylation, especially in promoter region CpG dense areas (so–called CpG islands and shores), generally represses transcription, working in concert with other types of epigenetic marks.

The domestic dog as an animal model for social behavior

We used the dog as an animal model for our work as this species offers several advantages for social behavior related genetic and epigenetic studies. Pet dogs live in a social environment shared with humans. They are pack animals with a complex hierarchical social structure, and several aspects of their social behavior is analogous to that of humans. Furthermore, purebred dogs represent several different, limited diversity gene pools that can facilitate the identification of inherited factors underlying phenotypic variance. Finally, wolf, the wild ancestor to dog, exhibits several characteristic phenotypic, including behavioral, differences from the domestic dog, yet two species are genetically so close to each other that they can interbreed and produce fertile offspring. This implies a major role of differential epigenetic regulation of gene expression, assumedly facilitating phenotype-related epigenetic studies.

Results

Candidate gene analyses

The oxytocin system is considered as a major regulator of social behavior, therefore the analysis of oxytocin receptor (OXTR) variants became a main direction for our investigations. Direct sequencing of protein coding and regulatory regions of the OXTR gene in 40 dogs and 6 wolves revealed 8 common SNPs in the canine OXTR gene that were further analyzed in the course of several behavior association, descriptive genetic and functional studies. Allele frequencies and linkage disequilibrium highly varied between dog breeds, wolf and golden jackal, and the existence of wolf- and dog-characteristic genetic variants was also shown. As expected, OXTR polymorphisms were found to be linked to social behavioral traits, in particular to aspects of human-directed social behavior, e.g. proximity seeking, reaction to a

stranger and human-directed greeting behavior. The latter was shown to be associated with variants in opioid receptor genes as well, in a breed-dependent manner. Further analyses also indicated that OXTR polymorphism related greeting behavior was dependent on the context of previous social experience and also on individual characteristics of the dog. In addition, differences in methylation levels of OXTR promoter CpG sites were also found to be related to social behavioral aspects, e.g. to reaction to a threateningly approaching stranger.

Effects of social environment

Behavior of pet dogs was also analyzed in terms of handling styles of their owners. Dog-owner dyads were investigated in 8 novel developed, standardized test situations with 20 variables, leading to the extraction of 3 owner interaction style factors using exploratory factor analysis. Reaction of dogs to a threateningly approaching stranger appeared to be influenced by one of these factors ("owner warmth"). It was checked whether this relationship could be due to altered OXTR methylation in the dog induced by behavior of the owner, but no association was found between owner interaction styles or personality traits and dog OXTR methylation.

However, in the course of a longitudinal study on a cohort of 14 puppies, where samples were taken from the same individual animals at a regular time interval from the age of 2 weeks to 2 years, it was shown that OXTR promoter methylation levels in dogs increased at the age of 3-6 months, when puppies are most sensitive to social effects. Puppy OXTR methylation levels also appeared to change in the function of social environment, as puppies who remained living as pack dogs had higher methylation values than littermates adopted as pets by families, emphasizing the importance of environment on methylation levels of CpG sites with putative functional potential (manuscript in preparation).

Functional molecular biological studies

In silico analyses on the OXTR polymorphisms identified as common by our group predicted that variants both in the 5' UTR/ promoter region and in the 3' UTR region could have a direct transcription regulatory role. This possibility was tested by functional *in vitro* assays. Regions of interest were cloned into reporter plasmids containing the luciferase gene, OXTR genetic variants were introduced by *in vitro* mutagenesis and the reporter gene was expressed in both neuronal and non-neuronal cell lines. Luciferase reporter assays showed that 1 of the 4 SNPs studied in the 5' UTR/ promoter region significantly increased gene expression, and a GT dinucleotide insertion/ deletion variant coupled with a SNP in the 3' UTR also had prominent gene regulatory effect. Also, *in vitro* methylation of the canine OXTR promoter lead to a marked transcriptional repression.

Inter-population epigenetic comparison studies

In order to gain insight into the potential role of epigenetics in inherited differences between canine populations, comparative inter-species and inter-breed DNA methylation studies were also conducted. First, methylation patterns in promoter regions of 7 genes related to nervous system function and behavior were analyzed in pet dogs of 3 breeds and in human-raised, captive gray wolves. Hierarchical cluster analysis indicated an unambiguous segregation of dog from wolf, and also the complete segregation of one of the dog breeds form the other two breeds, implying that inherited methylation patterns indeed exist between canine populations. However, as keeping conditions differed with the animals' place of residence, it was not clear whether the observations were not, at least partially, due to the effect of differential social environments.

Hence, a second comparative DNA methylation analysis was carried out with the inclusion of a wolf and a mixed-breed dog population, both living at the AT-partner at the Wolf Science Center in captive packs. Methylation levels were measured in promoter regions of 20 genes previously associated with behavioral variance, but not all genes had brain-related functions according to gene ontology term analysis. Dogs and wolves formed separate clusters with this kind of experimental setup again, both when all CpGs measured were included in the analysis and also when only CpGs in genes with high expression levels in the brain were included, but not in the case of the subset of genes with reported brain-related functions. These findings hint on that changes in environment e.g. during the domestication process could have long-term effects on epigenetically inherited gene expression regulation.

Results, specifically according to the tasks presented in the proposal

Task 1

Specific behavioral parameters, including social interaction styles with their owners and with humans in general, of altogether 220 pet Border Collies were tested. Behavior was assessed and videotaped in 15 different standard test situations (e.g. interaction with the owner, interaction with stranger, reaction to novel object and problem solving tasks). Dog handling style of the owners was also recorded in 8 standardized situations. Both dog and owner behavioral outcomes were coded, a random set of the original videos was double-checked by an independent coder naive to the study hypotheses and the obtained data were uploaded in the CSB PHENOTYPE DATABASE. Data of the CSB PHENOTYPE DATABASE were later used for several analyses regarding relationship between owner and dog behavior, dog genotype and dog behavior as well as dog epigenotype and dog behavior.

Task 2

Wolves and dogs living in packs at the Wolf Science Center (14 wolves and 23 dogs) were tested by the so-called “Relationship test”. Dominance-subordinate relationships and affiliation within the packs were recorded based on observations of the animals’ spontaneous interactions, while social support and social orientation between two individuals was tested by subjecting the pair of animals to different potentially stressful episodes (e.g. novel object or human approach).

In addition, 72 pet dogs (19 mix-breed dogs and 15 Border Collies living in single dog households, 23 mix-breed and 15 Border Collies living in multiple dog households) were also tested for their position in hierarchy and for social relationships. These dogs were tested twice with 2 of 3 potential partners: the owner, a dominant conspecific or the most affiliative conspecific they shared a household with. Preliminary results on the comparison of groups in relation to DNA methylation levels in the *OXTR* gene promoter region suggested that differences in rank and social environment could affect social behavioral aspects through influencing DNA methylation levels of regions with gene expression regulation potential. Furthermore, results of an object-choice test conducted on pet dogs suggested that dogs do not understand the referential intention of human pointing gesture but they do take into account the social intention depending on formal social interaction with the human in question.

Owners of the 220 Border Collies included in the CSB PHENOTYPE DATABASE were also tested for 20 variables in 8 standard test situations. Exploratory Factor Analysis revealed 3 factors that can be viewed as analogues to human parenting style dimensions. Results were published and also used for experimental setup in other publications.

Task 3
Buccal samples of the 220 pet Border Collies uploaded in the CSB PHENOTYPE DATABASE as well as samples from another set of 183 pet Border Collies were taken by the AT-partner and transported to the HU-partner. Also, buccal samples were regularly collected for DNA methylation change follow-up from 9 pack dogs living at the Wolf Science Center and their altogether 14 puppies, as well as from 14 adult and 2 cub pack wolves living at the same site. With regard to the 14 puppies born at the Wolf Science Center, buccal samples taken directly before and after undergoing the so-called Strange Situation Test were collected.

Task 4
DNA from buccal samples of the 220 Border Collies included in the CSB PHENOTYPE DATABASE were isolated together with DNA from another 183 pet Border Collies. All DNA samples were analyzed for quality and quantity, with satisfactory results (i.e. with A260/230 and A260/280 ratios in optimal range and concentration generally higher than 20 ng/µl). Saliva samples from several animals also arrived and were processed, with similar results as buccal samples. Data of the isolated DNA samples were recorded and regularly updated when new samples arrived. This also included data on wolf samples and samples of dogs living/ born at the Wolf Science Center.

Task 5
Exploratory investigations identified 8 common polymorphisms in the canine OXTR gene: 1 in the 3' UTR region (19208A/G), 2 in the coding region (rs22927829 in exon 1 and rs8679682 in exon 2) and 5 in the 5' UTR region (−213A/G, −94C/T, −74C/G, −50C/G and rs8679684). These 8 OXTR polymorphisms were measured in the collected Border Collie samples, together with samples from wolves and dogs living in captive packs at the Wolf Science Center as well as from pet dogs of breeds other than Border Collie. All genotype data were recorded in the CSB GENOTYPE DATABASE. This database has a web-based version, where data can be stored and accessed in a password-protected form. This provides a highly reliable data storage as well as easy access of data for the collaborating groups working in the two countries.
Linkage disequilibrium and allele frequency similarities and differences between different canine populations was analyzed. Marked differences regarding allele distributions was observed between dog and wolf in several cases, e.g. the SNP site rs8679682 appeared to be monomorphic for allele C in wolves, while allele T was dominating in dogs. Also, after a second round of exploratory sequencing of the OXTR gene, linkage analyses revealed that a GT ins/del polymorphism in the 3' UTR was in very strong linkage disequilibrium with SNP rs8679682.
Furthermore, social behavior of Border Collie and German Shepherd dogs was assessed in a series of 5 newly developed tests and analyzed in terms of OXTR genetic variations. Several aspects of human-directed social behavior in dogs was found to be influenced by OXTR polymorphisms, including proximity seeking and reaction to strangers.
A pilot study was also carried out with regard to common SNPs in the opioid receptor gene OPRM1 and OPRK1 as well as 3 OXTR SNPs in a small sample set of gray wolves and 3 dog breeds. Results pointed to a relationship between oxytocin and opioid receptor genetic variants and human-directed greeting behavior in canines in a breed-dependent manner.

Task 6
Pyrosequencing following bisulfite treatment was chosen as means of DNA methylation measurement for candidate region analysis on a relatively large sample set. Candidate region

selection was based on the considerations that DNA-methylation related gene expression regulation often occurs at promoter regions, more specifically at so-called CpG island shores in promoter regions. CpG island shores are regions neighboring so-called CpG islands, that is genomic regions extremely rich in CpG sites. A classic definition for a CpG island is a "region of 200 base pairs where the overall GC percentage is above 50% and the observed-to-expected CpG ratio is above 60%". This definition was used for locating CpG islands near possible candidate genes. In the first round, CpG islands were localized for 7 candidate gene promoters (OXTR, COMT, HTR1A, MAOA, SLC6A4, TPH1 and WFS1). The best overlap between localization of the promoter region and a CpG island was observed in the case of OXTR, a gene whose genetic polymorphisms have previously been related to social traits in several studies. Accordingly, PCR reactions producing short enough (up to 400 bp) amplicons suitable for downstream pyrosequencing applications was set up for 3 segments of the canine OXTR promoter. Finally, 4 CpG sites were selected for further investigations on a larger, phenotypically characterized sample. First analyses revealed a relationship between OXTR promoter methylation and dogs' reaction in the Threatening Stranger Test, but not with the social environmental factor owner interaction style. As it was not clear from this experimental setup if OXTR methylation levels were attributable rather to inherited or environmental factors, a longitudinal study was carried out as a following step. Samples taken at regular time intervals from the age of 2 weeks old from altogether 14 puppies born at the Wolf Science Center were measured till the puppies reached adulthood (2 years of age). Statistical analyses of several behavioral and social parameters of the puppies (e.g. adopted as pet dogs or remaining in the Wolf Science Center as captive pack dogs) in terms of DNA methylation profiles are underway. According to preliminary results, OXTR methylation levels increased in 3 out of 4 CpGs investigated at the age of 3–6 months, which is a very sensitive period for social development. Furthermore, pack dogs exhibited higher methylation levels than their siblings adopted by families as pet dogs at 2 CpG sites.

Task 7

Functional *in vitro* studies of regulatory regions of the canine OXTR gene were performed by luciferase reporter assays. 5' UTR was cloned into a pGL3-Basic and 3' UTR into a pMIRluc vector. *In silico* analyses suggested that an SNP in the 5' UTR might disrupt a transcription factor binding site and another possibly interferes with splicing; while a GT ins/del polymorphism in the 3' UTR was predicted to affect the binding of a miRNA. All these potentially gene expression modifying polymorphisms were introduced into the corresponding plasmids. Allele −94T in the 5' UTR caused a marked increase in gene expression, and the GT ins/del polymorphism in the 3'UTR coupled with variants of SNP 18779G/A also had prominent transcription efficiency modulator effect. *In vitro* methylation of the 3' UTR region resulted in a marked drop in reporter gene expression, in harmony with the observations that high promoter methylation levels generally inhibit transcription.

Task 8

All 403 Border Collie samples, as well as samples of all dogs and wolves living or born at the Wolf Science Center were genotyped for the 8 common OXTR SNPs listed above. Results were recorded in the CSB GENOTYPE DATABASE. This password protected database is a house-made, web based application, which ensures that data are always up-to-date and available for both the HU and the AT workgroups. Data can be downloaded in a text file, which can simply be exported to and analyzed in Excel.

Differentially methylated regions identified by the previously outlined exploratory studies were measured in triplicate in samples of the 220 Border Collies included in the CSB

PHENOTYPE DATABASE, as well as in samples taken at different time point from wolves and dogs living or born at the Wolf Science Center for methylation change follow-up.
Data entered to these databases were used in analyses of several publications.

Task 9
Custom-made mass array spectrometry method based EpiTYPER DNA methylation assays were developed for the promoter region of 7 behavior-related genes (OXTR, COMT, HTR1A, MAOA, SLC6A4, TPH1 and WFS1). Methylation levels were measured for a wolf and three dog populations. Hierarchical cluster analysis indicated unambiguous segregation of wolf from dog, and also specific dog bred characteristic differences. As results created a widespread interest in the scientific community, a short review/ commentary type paper was requested by several journals, leading to the publication of a commentary.
For gaining a deeper insight into these observations, further EpiTYPER assays were developed later on. The second round of analyses included 20 genes, with several of them not primarily involved in nervous system function and behavior. DNA methylation levels were measured in one dog and one wolf population only, living under the same environmental conditions. Data evaluation indicated an unambiguous segregation of dog from wolf again, which could be shown also on a smaller subset of genes previously reported to be expressed primarily in the brain, but not on the subset of genes with functions reportedly related to the brain (manuscript in preparation/ under submission).

Task 10
DNA methylation measurement data obtained from the two different EpiTYPER analyses described above were uploaded into the CSB EPIGENOME DATABASE. Only male animals were included in these analyses in order to avoid biases caused by hormonal difference of sexes. The first analysis involved 8 gray wolves, 8 Border Collies, 8 Siberian Huskies and 8 Golden Retrievers. Here, methylation information was gained on altogether 65 so-called CpG units (smallest possible fragments analyzable by the EpiTYPER method) comprising 79 individual CpG sites. The second round of analysis involved 8 gray wolves and 8 mixed-breed dogs living in captive packs at the Wolf Science Center. Here, methylation values were obtained for 117 CpG units comprising 147 individual CpG sites.

Task 11
Combined evaluation of genetics, epigenetics, behavior, and social environment of the 220 Border Collies included in the CSB PHENOTYPE DATABASE resulted so far in three journal publications. In order for a deeper understanding of the results, a longitudinal study on DNA methylation level changes has been carried out on puppies born at the Wolf Science Center. In addition to careful monitoring and recording of changes in their social rank and social environment, these animals have also been subjected to behavioral tests including the so-called Strange Situation Test. A joint HU- and AT-partner journal publication on the results is underway.
Discussions on future collaborative research between the AT- and HU-partners are currently in progress. Possible research topics include investigating the relationship between rank in social hierarchy and DNA methylation levels in wolves living in packs, combined genetic/ epigenetic behavior association studies and analysis of behavior-related gene expression levels in terms of DNA methylation levels in the canine brain.