Lessons from the canine *Oxtr* gene: populations, variants and functional aspects

Running title: Lessons from canine *Oxtr* variants

Key words: oxytocin receptor, canine, dog breed, wolf, polymorphism, microsatellite, linkage, neighbor joining, gene expression, splicing, behavior, domestication

Melinda Bence[1,2,*], Peter Marx[3], Eszter Szantai[1,#], Eniko Kubinyi[2,4], Zsolt Ronai[1], Zsofia Banlaki[1]

[1] Department of Medical Chemistry, Molecular Biology and Pathobiochemistry, Semmelweis University, Budapest, Hungary

[2] Comparative Ethology Research Group, Hungarian Academy of Sciences, Budapest, Hungary

[3] Department of Measurement and Information Systems, Budapest University of Technology and Economics, Budapest, Hungary

[4] Department of Ethology, Eotvos Lorand University, Budapest, Hungary

* Current address: Institute of Genetics, Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary

[#] Current address: Institute of Molecular Biology and Genetics, Biomedical Sciences Research Centre Alexander Fleming, Athens, Greece

Correspondence to: Zsofia Banlaki; Tuzolto utca 37-47, Budapest 1094, Hungary; telephone: (36 1) 266 26 15; fax: (36 1) 266 38 02; e-mail: banlaki.zsofia@med.semmelweis-univ.hu

Date of submission: December 3rd 2015

Abstract

Oxytocin receptor (OXTR) acts as a key behavioral modulator of the central nervous system, affecting social behavior, stress, affiliation and cognitive functions. Variants of the *Oxtr* gene are known to influence behavior both in animals and humans, however, canine *Oxtr* polymorphisms are less characterized in terms of possible relevance to function, selection criteria in breeding and domestication. In this report, we provide a detailed characterization of common variants of the canine *Oxtr* gene. In particular, (1) novel polymorphisms were identified by direct sequencing of wolf and dog samples, (2) allelic distributions and pairwise linkage disequilibrium patterns of several canine populations were compared, (3) neighbor joining tree based on common SNPs was

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/gbb.12356

constructed, (4) mRNA expression features were assessed, (5) a novel splice variant was detected and (6) *in vitro* functional assays were performed. Results indicate marked differences regarding *Oxtr* variations between purebred dogs of different breeds, free-ranging dog populations, wolf subspecies and golden jackals. This, together with existence of explicitly dog specific alleles and data obtained from the neighbor joining tree implies that *Oxtr* could indeed have been a target gene during domestication and selection for human preferred aspects of temperament and social behavior. This assumption is further supported by the present observations on gene expression patterns within the brain and luciferase reporter experiments, providing a molecular level link between certain canine *Oxtr* polymorphisms and differences in nervous system function and behavior.

Introduction

The domestic dog (*Canis lupus familiaris*) can be regarded as the most unique domesticated animal from several aspects. Apart from being the only large carnivore ever to have been domesticated (Wayne and vonHoldt, 2012), it is also the first domesticated species (Freedman et al., 2014, Pang et al., 2009, Ovodov et al., 2011, Sablin and Khlopachev, 2002) that has got subjected to the probably largest-scale – and still ongoing – genetic experiment in human history (Parker et al., 2004, Akey et al., 2010). Breeding practices have generated an extraordinary richness both in morphology and temperament (Drake and Klingenberg, 2010, Wayne, 1986). For millennia, trait selection was mostly driven by practical reasons, creating an already great variety of phenotypes, which was boosted by the foundation of breed clubs aiming at a strong artificial selection for desired traits (Parker et al., 2004, Akey et al., 2010).

Singular skills of individual breeds, however, can hardly provide a full explanation to how and why the domestic dog has gained its special place in human society; valued in modern times principally as companion instead of servant and worker. Most probably, what lies at the very core of this phenomenon is rather similarity and only to a lesser extent divergence. Similarity between individual dogs across breeds, and, even more importantly, similarity between dog and man in terms of social behavior, cognition and communication (Udell and Wynne, 2008, Topal et al., 2009). Several observations underpin the parallelism between social-cognitive skills of dogs and human infants, and evidence also suggests that dogs share a similar attitude to their owners as young human children to their parents (Horn et al., 2013). Moreover, it has recently been shown that mechanism of processing emotional auditory information in the brain is alike in dog and man (Andics et al., 2014). Efficient inter-specific communication and an at least as strong bonding to humans than to conspecifics seem to be inherited features critical in differentiating the domesticated dog from wolves (Viranyi et al., 2008, Udell et al., 2008, Persson et al., 2015, Miklosi and Topal, 2013). Nevertheless, the questions remain: which pathway of the nervous system could mostly be affected, and do behavioral differences characteristic to breeds or individual dogs reflect variations within this system?

Given its key role in social behavior, affiliation and memory, the oxytocin system seems to be a perfect candidate for looking for an answer. Higher levels of oxytocin in cerebrospinal fluid, plasma and urine have been linked to positive social emotions in a variety of mammals (Nakajima et al., 2014, Zelkowitz et al., 2014, Odendaal and Meintjes, 2003, Mitsui et al., 2011, Winslow et al., 2003, Haller et al., 1996, Insel and Hulihan, 1995, Parr et al., 2013), and polymorphisms of the human oxytocin receptor gene (*Oxtr*) have been found in association with a wide range of social-cognitive skills (Slane et al., 2014, Creswell et al., 2014, Buffone and Poulin, 2014, Wade et al., 2014, Peltola et al., 2014, Saphire-Bernstein et al., 2011, Rodrigues et al., 2009, Lucht et al., 2009, Bakermans-Kranenburg and van Ijzendoorn, 2008, Tost et al., 2010, Connelly et al., 2014). Our group has previously demonstrated that SNPs in the canine *Oxtr* gene are associated with social behavior in different dog breeds (Kis et al., 2014). These observations raise the possibility that the domestication process of dogs might have involved changes in the *Oxtr* gene influencing their behavior.

In the present study, we analyzed allelic distribution and linkage of novel and previously identified canine *Oxtr* polymorphisms in a large population involving nearly 700 dogs from 10 different breeds and also 42 wolves. Genetic distance based on the SNPs investigated was also assessed by applying neighbor joining (NJ) method. Finally, we performed molecular analyses to explore features of mRNA expression and functional effects of identified *Oxtr* variants were also investigated.

Materials and methods

Ethics statement

According to the operative law of Hungary (Act XXVIII of 1998 on the protection and welfare of animals), non-invasive sample taking and tissue preparations from animals euthanized for purposes other than research are not animal experiments and require no ethics license. All owners volunteered to participate and approved of the genetic analyses of their animals.

Animals involved

A total of 689 purebred family dogs (Beagle, N=70; Border Collie, N=144; German Shepherd, N=128; Golden Retriever, N=43; Groenendael, N=22; Hungarian Vizsla, N=32; Labrador Retriever, N=49; Malinois, N=40; Siberian Husky, N=138; Tervueren, N=23); 42 wolves (34 Eurasian gray, 6 North American timber, 2 Alaskan), 6 golden jackals (*Canis aureus*), 8 dingos (*Canis lupus dingo*) and 45 Asian street dogs (pariah dog) were involved in the genetic analyses.

Brain samples from 3 male beagle dogs euthanized on the owner's request were obtained at the Department of Anatomy and Histology, Faculty of Veterinary Science, Szent István University, Budapest, Hungary.

Buccal sample collection and DNA isolation

Buccal cells were collected by scraping the inner cheek with cotton-tipped collection swabs. Genomic DNA was isolated by a traditional, salting-out procedure (Boor et al., 2002).

Sequencing and Genotyping

Nucleotide order of protein coding sequence as well as of 582 bp of 5' UTR and 585 bp of 3' UTR was determined as described earlier in 6 wolves (2 Eurasian gray and 4 North American timber) in addition to 15 previously investigated dogs (3-3 Beagles, Border Collies, German Shepherds, Golden Retrievers and Siberian Huskies) (Kis et al., 2014). 3' UTR was also sequenced in 25 additional dogs (5-5 Hungarian Vizslas and Labrador Retrievers as well as 3-3 Beagles, Border Collies, German Shepherds, Golden Retrievers and Siberian Huskies).

Genotyping of −213A/G, 19208A/G (previously called −212A/G and 19131A/G, respectively) and rs8679684 was as described formerly in (Kis et al., 2014). SNPs −50C/G, −74C/G and rs8679682 were genotyped by PCR-RFLP method using the 5' CCA TTG GAA TCC GCC CCC T 3' Fw (forward) and 5' CAC CAC CAG GTC GGC TAT G 3' Rv (reverse) primers for both −50C/G and −74C/G and the 5' GAA AGG CCA TTC TCA GGA AA 3' Fw and 5' CCC CCA TCA TCT TCT ACC A 3' Rv primer for rs8679682. Thermocycler was set to 40 cycles [94°C 1 min, 56°C 30 sec and 72°C 1 min]. PCR products were digested for 3 h at 37°C (−50C/G and rs8679682) or 60°C (−74C/G) with 0.5 U/µl AvaII (−50C/G), BsiEI (−74C/G) or PshAi (rs8679682) (New England Biolabs, Ipswich, Massachusetts), 1x BSA and 1x NEB4 buffer. The fragments were run on 1.5% SeaKem® - 2% MetaPhor™ composite agarose (Lonza, Basel, Switzerland) (−50C/G and −74C/G) or 2.5% SeaKem® agarose (rs8679682) gel.

The −94T/C, rs22927829 and 18575C/T SNPs were genotyped by allele specific amplification. Primer sequences were: (1) −94T/C: 5' CCA TTG GAA TCC GCC CCC T 3' (Fw), 5' CAC CAC CAG GTC GGC TAT G 3' (Rv), 5' CCG ATC TGC TGG TCC CGA 3' (T specific), 5' CCG ATC TGC TGG TCC CGG 3' (C specific); (2) rs22927829: 5' CAT GTT CGC CTC CAC CTA CC 3' (Fw), 5' GCC CCG CTC GCT ACC TT 3' (Rv), 5' CAC CGT GAA GAT GAC CTT CAT A 3' (A specific), CAC CGT GAA GAT GAC CTT CAT T (T specific) and (3) 18575C/T: 5' GAA AGG CCA TTC TCA GGA AA 3' (Fw), 5' CCC CCA TCA TCT TCT ACC A 3' (Rv), 5' CCT TCA GGT AGC TGG AGG 3' (C specific) and 5' CCT TCA GGT AGC TGG AGA 3' (T specific). Thermocycler was programmed at 95°C for 15 min, followed by 40 cycles of 94°C 1 min, 60°C (−94T/C and rs22927829) or 50°C (18575C/T) 30 sec, 72°C 1 min, and a final extension at 72°C for 10 min. The PCR products were run in 2.5% (−94T/C and rs22927829) or 1.5% (18575C/T) SeaKem® agarose gel.

Reverse transcriptase (RT)-PCR analysis and real-time PCR

Total RNA from brain was isolated by RNeasy kit (Qiagen, Valencia, California) and DNaseI treated (Thermo Fisher Scientific, Waltham, Massachusetts). 1 µg RNA was reverse-transcribed with recombinant moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies, Grand Island, New York) using random primers in 20 µL volume.

Oxtr mRNA expression was assessed by quantitative real-time PCR in 15 µL final volume containing 1 µL cDNA, 1× SYBR Green qPCR master mix (Life Technologies, Grand Island, New York), and 1-1 uM Fw (5' TCT TCG TGC AGA TGT GGA GC 3') and Rv (5' GCC CGT GAA GAG CAT GTA GA 3') primer. Primers were specific for different exons in order to prevent amplification from genomic DNA. Expression of the dog *Hprt* mRNA were detected with TaqMan assay in 25 µL final volume containing 0.2 µL cDNA, 1× ABI PCR master mix, gene-specific TaqMan primers and FAM-labeled probe (Life Technologies Grand Island, New York , #4331182). Denaturation at 95°C for 10 min was followed by 40 thermocycles (65°C, 15 sec and 60°C, 1 min). Reactions were performed in triplicate in an ABI 7300 Real-Time PCR System (Applied Biosystems, Foster City, California). Expression levels of *Oxtr* gene were first normalized to the *Hprt* internal control gene and then to expression levels measured in the prefrontal cortex. Results were expressed as fold changes calculated with the formula $2^{-\Delta\Delta Ct}$.

To analyze expression and splicing of the 5' and 3' UTR of canine *Oxtr* mRNA, 1µl cDNA was PCR amplified with the primer pairs used for sequencing.

Plasmid constructs

A 1713 bp stretch of the *Oxtr* promoter / 5' UTR region spanning SNPs −213A/G, −94T/C, −74C/G, and −50C/G was PCR amplified using the 5' GTT **AAG CTT** GCA CGC AAA GAG GCA GAA ATG GC 3' Fw and 5' CCA GGA AGA GGA TGA GGC ACA GC 3' Rv primers (bold letters indicate a HindIII recognition site). The PCR product was digested with HindIII and NcoI, gel extracted and ligated into a pGL3-Basic Vector (Promega, Madison, Wisconsin). In the original construct, DNA was homozygous for the alleles −213A, −94C, −74C, and −50G. The −213G, −94T, −74G, and −50C variants were introduced by the QuikChange Site Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, California) using the primer pairs Fw 5' CCG GGA GGC G**G**C GGC GCC CCT 3' and Rv 5' AGG GGC GCC G**C**C GCC TCC CGG 3'; Fw 5' GAT CCG TTC CAG G**T**C GGG ACC AGC AGA 3' and Rv 5' TCT GCT GGT CCC G**A**C CTG GAA CGG ATC 3'; Fw 5' AGC AGA TCG GCC **G**CC TCG GAG TCT C 3' and Rv 5' GAG ACT CCG AGG **C**GG CCG ATC TGC T 3' and Fw 5' GGG AGC GGA C**C**C CCC GCC GCG 3' and Rv 5' CGC GGC GGG G**G**G TCC GCT CCC 3'**,** respectively (bold underlined letters indicate the introduced mutations).

In the case of the 3' UTR constructs, a 609 bp long PCR product starting right after the stop codon was generated using the 5' TTT ACG CGT CCA GGC TGG GCC AGG 3' Fw and 5' GCT **AAG CTT** AGG AAT CCT CTG TTC CCA GG 3' Rv primers (bold letters indicate a HindIII recognition site). The PCR product was digested with MluI and HindIII, purified and cloned into a pMIR-Report Vector (Life Technologies, Grand Island, New York). The original clone was heterozygous for the CLF20OXTR1 microsatellite variants short/A and long/G. Short/G and long/A constructs were generated by site-directed mutagenesis (see above) using the primer pairs Fw 5' CCT TGG TGG CTG TGT GC**G** TGT GTG TGT GTA TAA GG 3' and Rv 5' CCT TAT ACA CAC ACA CA**C** GCA CAC AGC CAC CAA GG 3' and Fw 5' CCT TGG TGG CTG TGT GC**A** TGT GTG TGT GTG TAT AA 3' and Rv 5' TTA TAC ACA CAC ACA CA**T** GCA CAC AGC CAC CAA GG 3', respectively (bold underlined letters indicate the introduced mutations). All constructs were confirmed by direct sequencing (Microsynth Austria Gmbh, Vienna, Austria).

Cell transfection and luciferase assays

SK-N-FI (ATCC® CRL-2142™) neuroblastoma and human embryonic kidney HEK293 cell lines were cultured in DMEM High Glucose Medium (Life Technologies, Grand Island, New York) containing 10% fetal bovine serum (Lonza, Basel, Switzerland) and supplemented with 1% non-essential amino acids (Life Technologies, Grand Island, New York) for SK-N-FI cells. Transfection was performed in 24-well plate with 0.8 μg of 5' UTR or 0.05 μg of 3' UTR construct and 0.4 μg of β-galactosidase vector (Ambion) for SK-NF-I cells and with 0.4 μg of 5' UTR or 0.05 μg of 3' UTR construct and 0.2 μg of β-galactosidase vector for HEK293 using 2.0 μl Lipofectamine® 2000 (Life Technologies, Grand Island, New York) per well. Cells were harvested 48 (SK-N-FI) or 24 hours (HEK293) after transfection and subjected to three freeze-thaw cycles. Cell lysates were centrifuged and the supernatants were kept for luciferase measurement. Luminescence for luciferase as well as optical density for β-galactosidase (representing transfection efficiency) was assessed by a Varioskan Flash Multimode Reader. Luciferase activity was normalized by β-galactosidase activities. All experiments were carried out in triplicates and performed on three independent occasions.

Statistical and computer analyses

Statistical analyses were carried out using GraphPad Prism 5.03 for Windows (GraphPad Software, San Diego, California; www.graphpad.com). One-way ANOVA followed by Tukey-Kramer test was used for assessing differences between normalized luciferase reporter data.

Pairwise linkage disequilibrium (LD) in terms of Lewontin's disequilibrium coefficient D' and correlation coefficient r^2 was calculated by Haploview 4.2 program (Barrett et al., 2005).

Population structure of the given breeds was inferred using the software tool Structure (Pritchard et al., 2000, Hubisz et al., 2009), a Bayesian model-based MCMC method. The admixture model with correlated allele frequencies was used. First a parameter sweep was carried out on K (values of K ranging from 2 to 20) with 10.000 steps for burn-in and 50.000 for sampling. Each run was repeated 20 times. After evaluating the results using Evanno's method (Evanno et al., 2005, Earl and von Holdt, 2012) together with statistics on log-likelihood of the data with given K, the most likely K was rerun with 10.000 burn-in and 100.000 sampling steps for validating the results. For neighbor joining tree construction, Phylip package was used (Felsenstein, 2005).

Results

Novel polymorphisms in the canine *Oxtr* gene

To identify novel polymorphisms in the canine *Oxtr* gene, the protein coding and the surrounding regulatory regions were determined by direct sequencing in two Eurasian gray and four North American timber wolves as well as 25 additional dogs to our previous study (Kis et al., 2014). Sequence alignment identified 3 novel polymorphic sites: the −74C/G and 18575C/T SNPs as well as a microsatellite marker at the 18772-18792 position here designated as CLF20OXTR1 (where CLF stands for *Canis lupus familiaris* and 20 for chromosome 20) (all positions are given relative to ATG of canine *Oxtr* mRNA version HM856596.1). Of these, the −74C/G variation is located in the 5' UTR while the 18575C/T is found in the protein coding region and causes a serine to phenylalanine residue change at amino acid position 343 (S343F). The microsatellite marker located in the 3' flanking region consisted of 9 or 10 repeats of a 2 bp module and in addition contained another novel SNP (18779G/A). All wolves sequenced were homozygous for the short (9 repeats) microsatellite version containing G at position 18779 (short/G), while among dogs, the most characteristic variant was long/G, but the putatively ancient short/G as well as short/A (but not long/A) variants were also observed (with relative frequencies 0.52, 0.22 and 0.34, respectively). Sequencing data also demonstrated that the number of repeats is in complete linkage disequilibrium with the rs8679682 SNP in all animals studied. According to our results, the short (9 repeats) allele of the microsatellite contains the C allele of the rs8679682 SNP whereas the long (10 repeats) variant carries a T at this site.

Genetic study of the identified *Oxtr* polymorphisms

Genotyping studies of the 18575C/T SNP involving 19 dogs of different breeds (Border Collie, German Shepherd, Groenendael, Malinois, Siberian Husky, Tervueren) and 40 wolves (34 Eurasian gray, 4 North American timber, 2 Alaskan) in addition to those sequenced revealed that all tested North American timber and Alaskan wolves were homozygous for the T allele. However, this variant was absent from both the investigated Eurasian wolves and all of the dogs.

The novel -74C/G SNP and seven additional canine *Oxtr* polymorphisms deposited in public databases (dbSNP, Ensembl, DoGSD (Bai et al., 2015)) or described in (Kis et al., 2014): −50C/G, −94T/C, −213A/G (formerly: −212A/G), rs22927829, rs8679682 rs8679684 and 19208A/G (formerly: 19131A/G) were genotyped in a larger population involving nearly 700 dogs from 10 breeds and 40 wolves. Our results demonstrated that these SNPs are polymorphic in canines, however, allele frequencies showed marked differences between both species/ subspecies (Fig. 1) and purebred dog breeds (Fig. 2). To compare allele frequency distributions for the 8 polymorphisms, chi square probes were applied. Difference among dog breeds was significant in the case of all SNPs investigated (Table S1). Furthermore, in the case of the −50C/G and rs8679682 SNPs there was greater difference in allele frequencies between dog and wolf than among breeds. These two SNPs were further investigated in other canines involving 6 golden jackals, 6 Australian dingos, 38 Asian street dogs (pariah dog) and 6 Czechoslovakian Wolfdogs (Table S2). Results indicate that regarding SNP −50C/G, allele C is more frequent in all populations investigated (including golden jackals) with the only exception of wolves. SNP site rs8679682 was monomorphic for allele C both in wolves and golden jackals, while in dogs (including the free-ranging Australian dingo and the Asian street dog pariah)

allele T was dominating. In fact, the only dog breed analyzed in this study apparently monomorphic for the ancient allele C was the Czechoslovakian Wolfdog.

Levels of linkage disequilibrium between the examined *Oxtr* SNPs were also determined in all canine populations consisting of at least 20 individuals (Fig. S1 & S2). Only SNPs with major allele frequency (MAF)>0.05 (with respect to the population in question) were analyzed. All dog breeds and wolves possessed SNP pairs which were in significant LD (D'>0.8). However, the extent of LD was highly different between dog breeds and wolf. Differences could be observed even between dogs of the same breed originating from different countries.

Neighbor joining tree

Neighbor joining tree consisting of the above mentioned 8 common *Oxtr* SNPs was assembled based on animals with complete genotype information (total, N=273; Beagle, N=17; Border Collie, N=81; German Shepherd, N=21; Golden Retriever, N=22; Groenendael, N=8; N=22, Hungarian Vizsla, N=8; Malinois, N=17; Siberian Husky, N=24; Tervueren, N=15; Asian street dog (pariah), N=4; dingo, N=3; wolf, N=22; golden jackal, N=4). According to results of log-likelihood statistics and Evanno's method, optimal K value was estimated to be K=4 (Fig. 3). The most probable population structure is shown in Fig. 4.

Although not all breeds could unambiguously be assigned to a specific branch, clear clustering patterns emerged in several cases. Wolves and golden jackals, the two wild, non-dog canine populations investigated here, clustered together and segregated from both of the free-ranging populations descended from dogs: the Australian dingo and the semi-domesticated Asian street dogs (pariahs). Representatives of the two long-haired Belgian Shepherd varieties (Groenendael and Tervueren), differing from each other essentially in coat color only, presented themselves mainly on the same branch. Malinois, the third Belgian Shepherd variety studied, however, appeared rather on another branch containing the temperamentally similar German Shepherd. Border Collies mostly segregated both from the wolf/ jackal and the Belgian/ German Shepherd groups (Fig. 4).

MRNA expression and splicing analysis

Quantitative real-time PCR was performed to assess expressional differences of *Oxtr* mRNA between three regions of the dog brain: prefrontal cortex, amygdala and hippocampus. PCR product quality check by agarose gel electrophoresis showed no aspecific band or genomic DNA contamination (Fig S3). Expression levels were lowest in the prefrontal cortex and highest in the hippocampus. As compared to that observed in the prefrontal cortex, mRNA levels in the amygdala and the hippocampus showed a 2.8 and 16.4 fold increase, respectively (Fig. 5).

In order to verify that the investigated polymorphisms are indeed located on mRNA transcripts, cDNA were amplified by the same primer pairs as used for sequencing. Gel electrophoresis of the corresponding PCR products showed clear and specific bands matching the length of the expected PCR products. Direct sequencing of gel extracted bands confirmed that the amplicons arose from *Oxtr*, indicating that the genetic variants studied are indeed part of the mRNA (Fig. S4). In the case of 5' UTR, our results also gave evidence of the existence of a predicted but experimentally not yet confirmed alternative intron located −513 to −95 bp upstream from ATG (chromosome 20 genomic coordinates 9359034-9359451 according to genome annotation CanFam3.1:CM000020.3) (Fig. S5).

Molecular study of *Oxtr* variants with functional relevance

In order to explore whether polymorphisms located within putative gene regulatory regions (e.g. variations possibly affecting transcription factor binding, splicing or miRNA binding), luciferase reporter measurements were performed in two different cell lines (SK-NF-I and HEK293). Possible role of frequent polymorphisms in the canine *Oxtr* 5' UTR was investigated using firefly luciferase plasmid constructs containing all four SNP sites identified within this region. The reference construct (5' UTR-AH) possessed alleles (−213A; −94C; −74C; and −50G) characteristic of the Eurasian gray wolf, thus presumably representing the ancestral state prior to domestication. Mean relative luciferase activity values as normalized to β-galactosidase activities are shown in Fig. 6. Independent of the cell line used, neither −213A/G, −74C/G or −50C/G polymorphisms had an effect on gene expression as compared to 5' UTR-AH, however, the −94T variant caused a spectacular increase in luciferase activity (ANOVA: $F_{4,10}=37.98$, $p<0.0001$ (SK-NF-I) and ANOVA $F_{4,10}=39.48$, $p<0.0001$ (HEK293); remaining highly significant after Tukey-Kramer posthoc test).

Possible gene regulatory effect of variants of the microsatellite marker CLF20OXTR1, predicted by miRBase to affect a binding site for the miRNA cfa-miR574, was also investigated. Segments of the 3' UTR harboring either the long or short version of CLF20OXTR1 were cloned downstream of the firefly luciferase gene and alleles of the SNP 18779G/A were introduced by site-directed mutagenesis. Mean luciferase activities did not differ between the constructs long/G and short/A, neither between short/G and long/A. However, short/G and long/A haplotypes showed dramatically higher levels of gene expression than long/G and short/A (ANOVA: $F_{3,8}=30.45$, $p<0.0001$ (SK-NF-I) and ANOVA $F_{3,8}=40.70$, $p<0.0001$ (HEK293); remaining highly significant after Tukey-Kramer posthoc test) (Fig. 7).

Discussion

The domestic dog offers an unrivaled opportunity for investigating characteristics of genes involved in complex traits (Parker, 2012, Boyko, 2011). Though studies highlighting the advantages of the dog as a genetic model system mostly focus on its usefulness for mapping complex disease loci (Parker et al., 2004, Shearin and Ostrander, 2010, Dodman et al., 2010), underlying features make this species equally suitable for investigating genetics of normal-range traits such as behavior (Vaysse et al., 2011). Both the event of domestication and the establishment of breeds with strict mating control caused severe population bottlenecks (Cruz et al., 2008, Wayne and Ostrander, 2007) leading to extensive linkage disequilibrium and breed-specific enrichment of rare allelic variants, which facilitate identification and functional characterization of genes involved in complex traits (Sutter et al., 2004). Moreover, domestication process itself always involves a strong artificial selection for desirable behavioral traits such as tameness, manifesting among others in an altered pattern of expression of brain-related genes (Li et al., 2013, Saetre et al., 2004, Lindberg et al., 2005, Kukekova et al., 2011, Natt et al., 2012, Albert et al., 2012). Investigating genes involved in behavior in purebred dogs and their ancestor, the gray wolf, is thus especially intriguing.

Here we present the results of detailed descriptive and functional analyses of the canine *Oxtr* gene, a key modulator in social behavior (Kumsta and Heinrichs, 2013). Genotypic data of 8 common SNPs indicate considerable variation in allelic frequencies and level of linkage disequilibrium among different canine populations, including geographically distinct populations of the same breed. This is in consistency with the literature on broader genetic regions (Sutter et al., 2004, Stern et al., 2013, Quignon et al., 2007) and most probably reflects gene pool differences, founder effect and genetic drift. Allelic distributions of individual loci overlap between breeds and breed groups, however, characteristic differences also exist, possibly reflecting that *Oxtr* could indeed have been a selection target. Especially intriguing was the discovery of a SNP site (rs8679682) being polymorphic in dogs but not in wolves, as domesticated populations generally derive from a founder population with a limited number of individuals, resulting in a loss and not gain of genetic diversity. This possibly indicates that allele T emerged shortly after the event of domestication and became fixed in various breeds. Alternatively, allele T could be a remnant of a substantially greater genetic diversity of ancient wolves, that went through a severe population bottleneck after their divergence from the domestic dog (Freedman et al., 2014). It is currently unknown whether the spread of allele C in the Czechoslovakian Wolfdog, a wolf-resembling hybrid breed resulting from a recent cross between gray wolves and German Shepherd dogs, is due simply to founder effect or a product of artificial selection directed to favor wolf-like features, which would suggest that either this or a nearby polymorphism exerts influence on behavioral traits. Be that as it may, an interesting aspect of this SNP is that it seems to be in strong linkage disequilibrium with length variants of microsatellite marker CLF20OXTR1 located in 3'UTR, which is here shown to influence transcription efficiency. The discovery that all North-American wolves involved in this study were homozygous for an allele (18575T) totally absent from both Eurasian wolf and dog populations also fits in our current knowledge on domestication. Although it is argued whether or not domestication was a single and monocentric event, it is widely agreed that dogs are descendants of wolves living in the Eurasian continent (Thalmann et al., 2013, Ding et al., 2012, Savolainen et al., 2002).

Neighbor joining tree based on *Oxtr* SNPs partly reflects both phylogeny/ breeding history and temperamental similarity, nevertheless, not even the wolf ancestor did completely segregate from dog. This underlines that even if these SNPs were important in selection for human-preferred behavior, they could only be a piece of the genetic puzzle, just as it can be expected for a complex trait. Alternatively, it might reflect that the domestic dog still shares essential common features with

the gray wolf. This would be in harmony with the virtually full similarity between their genomic DNA sequences, naturally-occurring hybridization events (Vila and Wayne, 1999, Kopaliani et al., 2014) and recurrent, though rare, reports of pet wolves with dog-like affiliation towards humans. Taken all round, human-preferred behavioral traits characteristic to dogs might not have been achieved purely by domestication, and it might not be a product of mere chance that of all wild animals, it was the gray wolf whose descendants are now considered as "man's best friend". Similarly, the fact that several breeds are distributed across distinct branches might reflect that polymorphisms of this gene exert their primary influence on behavioral traits different from those characteristic to specific breeds or breed groups. Former observations also indicate that personality dimensions likely to be influenced by *Oxtr* variations, such as sociability and aggressiveness, are general to the dog as species and are not exclusive to dog breeds (Svartberg and Forkman, 2002). Evidence also suggests that basic temperamental traits of dogs reflect rather recent than historical selection factors, including selection for working, pet and show dogs. (Svartberg, 2006).

Molecular studies further support the functional-behavioral relevance of *Oxtr*. The observation that *Oxtr* is abundantly expressed in the hippocampus, a brain region crucial for cognition, memory, stress and social behavior (Kesner et al., 2004, Rubin et al., 2014), in itself underpins the biological basis for its role in behavior. In addition, allele T of SNP −94T/C apparently substantially enhances transcription. Given the presented results on transcript variants and the database annotation discrepancies for this genomic region, the mechanism possibly lies in alternative splicing. This would not be the first reported case of an exonic SNP affecting splicing (Pagani and Baralle, 2004, Crotti and Horowitz, 2009) and, consequently, gene expression efficiency (Beer and Sahin-Toth, 2014). Furthermore, here we demonstrate that variations of the microsatellite CLF20OXTR1 in the 3' UTR also interfere with gene expression. The putatively most ancient variant for which all wolves sequenced were homozygous, i.e. short version containing G at position 18779 (short/G), promotes a significantly higher level of transcription than any of the other two variants (short/A and long/G) observed in dogs. It is noteworthy that SNP site rs8679682, which was formerly found to be related to temperamental traits in dogs (Kis et al., 2014), is in full LD with the length of CLF20OXTR1.

In summary, our results are in accordance with those in the literature regarding the influence of *Oxtr* on socio-cognitive skills (Gimpl and Fahrenholz, 2001, Carter, 2014). However, it seems that the observed distribution of *Oxtr* variants indicates *Oxtr*-driven variance in temperament is mostly independent of breeds. Though historical remnants of domestication can be traced in the case of certain polymorphisms, it is not unequivocal whether these slight differences were drivers or mere byproducts of domestication. According to our current knowledge, all truly wolf-specific *Oxtr* alleles constitute a single haplotype (rs8679682C/ 18779A/ short version CLF20OXTR1) that both involves functional variants and also affects behavior, but the very same haplotype is also frequent in dogs. On the whole, it seems that though *Oxtr* variants contributing to behavioral traits desirable by dog owners do exist, selection for those is challenging. The reason behind might be fairly complex, including incomplete penetrance, differences in expressivity, varying levels of linkage with other functional polymorphisms not identified in this study and also interplay with variants of non-linked genes such as those of the dopaminergic system (Ito et al., 2004, Lit et al., 2013).

Naturally, a more in-depth study of both *Oxtr* and other behavior-related canine genes would be necessary before drawing far-reaching conclusions. Thought-provoking though our data are, it must not be forgotten that full sequencing of regulatory regions, as well as the very same regions investigated here on a larger sample would possibly reveal further polymorphisms, whose detailed analysis could show a different picture. Mechanism of gene regulatory effect of SNP −94T/C and microsatellite CLF20OXTR1 variants would require further investigation, and possible functional

relevance of polymorphisms not analyzed in this study should also be examined. Despite these limitations, the results presented here bring us a step forward to understanding the role of *Oxtr* variations in canine behavior and selective breeding.

Acknowledgments

This work has been supported by grants to Maria Sasvari-Szekely (OTKA ANN 107726), Zsofia Viranyi (FWF I 1271-B24 and WWTF CS11-026), Adam Miklosi (MTA 01 031, Bolyai Foundation) and Eniko Kubinyi (OTKA K84036). The authors declare no conflict of interest.

References

Akey, J. M., Ruhe, A. L., Akey, D. T., Wong, A. K., Connelly, C. F., Madeoy, J., et al. (2010) Tracking footprints of artificial selection in the dog genome. *Proc Natl Acad Sci U S A* **107**, 1160-5.

Albert, F. W., Somel, M., Carneiro, M., Aximu-Petri, A., Halbwax, M., Thalmann, O., et al. (2012) A comparison of brain gene expression levels in domesticated and wild animals. *PLoS Genet* **8**, e1002962.

Andics, A., Gacsi, M., Farago, T., Kis, A.,Miklosi, A. (2014) Voice-sensitive regions in the dog and human brain are revealed by comparative fMRI. *Curr Biol* **24**, 574-8.

Bai, B., Zhao, W. M., Tang, B. X., Wang, Y. Q., Wang, L., Zhang, Z., et al. (2015) DoGSD: the dog and wolf genome SNP database. *Nucleic Acids Res* **43**, D777-83.

Bakermans-Kranenburg, M. J.,van Ijzendoorn, M. H. (2008) Oxytocin receptor (OXTR) and serotonin transporter (5-HTT) genes associated with observed parenting. *Soc Cogn Affect Neurosci* **3**, 128-34.

Barrett, J. C., Fry, B., Maller, J.,Daly, M. J. (2005) Haploview: analysis and visualization of LD and haplotype maps. *Bioinformatics* **21**, 263-5.

Beer, S.,Sahin-Toth, M. (2014) Exonic variants affecting pre-mRNA splicing add to genetic burden in chronic pancreatitis. *Gut* **63**, 860-1.

Boor, K., Ronai, Z., Nemoda, Z., Gaszner, P., Sasvari-Szekely, M., Guttman, A., et al. (2002) Noninvasive genotyping of dopamine receptor D4 (DRD4) using nanograms of DNA from substance-dependent patients. *Curr Med Chem* **9**, 793-7.

Boyko, A. R. (2011) The domestic dog: man's best friend in the genomic era. *Genome Biol* **12**, 216.

Buffone, A. E.,Poulin, M. J. (2014) Empathy, target distress, and neurohormone genes interact to predict aggression for others-even without provocation. *Pers Soc Psychol Bull* **40**, 1406-22.

Carter, C. S. (2014) Oxytocin pathways and the evolution of human behavior. *Annu Rev Psychol* **65**, 17-39.

Connelly, J. J., Golding, J., Gregory, S. P., Ring, S. M., Davis, J. M., Davey Smith, G., et al. (2014) Personality, behavior and environmental features associated with OXTR genetic variants in British mothers. *PLoS One* **9**, e90465.

Creswell, K. G., Wright, A. G., Troxel, W. M., Ferrell, R. E., Flory, J. D.,Manuck, S. B. (2014) OXTR polymorphism predicts social relationships through its effects on social temperament. *Soc Cogn Affect Neurosci*.

Crotti, L. B.,Horowitz, D. S. (2009) Exon sequences at the splice junctions affect splicing fidelity and alternative splicing. *Proc Natl Acad Sci U S A* **106**, 18954-9.

Cruz, F., Vila, C.,Webster, M. T. (2008) The legacy of domestication: accumulation of deleterious mutations in the dog genome. *Mol Biol Evol* **25**, 2331-6.

Ding, Z. L., Oskarsson, M., Ardalan, A., Angleby, H., Dahlgren, L. G., Tepeli, C., et al. (2012) Origins of domestic dog in southern East Asia is supported by analysis of Y-chromosome DNA. *Heredity (Edinb)* **108**, 507-14.

Dodman, N. H., Karlsson, E. K., Moon-Fanelli, A., Galdzicka, M., Perloski, M., Shuster, L., et al. (2010) A canine chromosome 7 locus confers compulsive disorder susceptibility. *Mol Psychiatry* **15**, 8-10.

Drake, A. G.,Klingenberg, C. P. (2010) Large-scale diversification of skull shape in domestic dogs: disparity and modularity. *Am Nat* **175**, 289-301.

Earl, D. A.,von Holdt, B. M. (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genet Resour* **4**, 359-361.

Evanno, G., Regnaut, S.,Goudet, J. (2005) Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Mol Ecol* **14**, 2611-20.

Felsenstein, J. (2005) PHYLIP (Phylogeny Inference Package) version 3.6. *Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.*

Freedman, A. H., Gronau, I., Schweizer, R. M., Ortega-Del Vecchyo, D., Han, E., Silva, P. M., et al. (2014) Genome sequencing highlights the dynamic early history of dogs. *PLoS Genet* **10**, e1004016.

Gimpl, G.,Fahrenholz, F. (2001) The oxytocin receptor system: structure, function, and regulation. *Physiol Rev* **81**, 629-83.

Haller, J., Makara, G. B., Barna, I., Kovacs, K., Nagy, J.,Vecsernyes, M. (1996) Compression of the pituitary stalk elicits chronic increases in CSF vasopressin, oxytocin as well as in social investigation and aggressiveness. *J Neuroendocrinol* **8**, 361-5.

Horn, L., Huber, L.,Range, F. (2013) The importance of the secure base effect for domestic dogs - evidence from a manipulative problem-solving task. *PLoS One* **8**, e65296.

Hubisz, M. J., Falush, D., Stephens, M.,Pritchard, J. K. (2009) Inferring weak population structure with the assistance of sample group information. *Mol Ecol Resour* **9**, 1322-32.

Insel, T. R.,Hulihan, T. J. (1995) A gender-specific mechanism for pair bonding: oxytocin and partner preference formation in monogamous voles. *Behav Neurosci* **109**, 782-9.

Ito, H., Nara, H., Inoue-Murayama, M., Shimada, M. K., Koshimura, A., Ueda, Y., et al. (2004) Allele frequency distribution of the canine dopamine receptor D4 gene exon III and I in 23 breeds. *J Vet Med Sci* **66**, 815-20.

Kesner, R. P., Lee, I.,Gilbert, P. (2004) A behavioral assessment of hippocampal function based on a subregional analysis. *Rev Neurosci* **15**, 333-51.

Kis, A., Bence, M., Lakatos, G., Pergel, E., Turcsan, B., Pluijmakers, J., et al. (2014) Oxytocin receptor gene polymorphisms are associated with human directed social behavior in dogs (Canis familiaris). *PLoS One* **9**, e83993.

Kopaliani, N., Shakarashvili, M., Gurielidze, Z., Qurkhuli, T.,Tarkhnishvili, D. (2014) Gene flow between wolf and shepherd dog populations in Georgia (Caucasus). *J Hered* **105**, 345-53.

Kukekova, A. V., Johnson, J. L., Teiling, C., Li, L., Oskina, I. N., Kharlamova, A. V., et al. (2011) Sequence comparison of prefrontal cortical brain transcriptome from a tame and an aggressive silver fox (Vulpes vulpes). *BMC Genomics* **12**, 482.

Kumsta, R.,Heinrichs, M. (2013) Oxytocin, stress and social behavior: neurogenetics of the human oxytocin system. *Curr Opin Neurobiol* **23**, 11-6.

Li, Y., Vonholdt, B. M., Reynolds, A., Boyko, A. R., Wayne, R. K., Wu, D. D., et al. (2013) Artificial selection on brain-expressed genes during the domestication of dog. *Mol Biol Evol* **30**, 1867-76.

Lindberg, J., Bjornerfeldt, S., Saetre, P., Svartberg, K., Seehuus, B., Bakken, M., et al. (2005) Selection for tameness has changed brain gene expression in silver foxes. *Curr Biol* **15**, R915-6.

Lit, L., Belanger, J. M., Boehm, D., Lybarger, N.,Oberbauer, A. M. (2013) Differences in behavior and activity associated with a poly(a) expansion in the dopamine transporter in Belgian Malinois. *PLoS One* **8**, e82948.

Lucht, M. J., Barnow, S., Sonnenfeld, C., Rosenberger, A., Grabe, H. J., Schroeder, W., et al. (2009) Associations between the oxytocin receptor gene (OXTR) and affect, loneliness and intelligence in normal subjects. *Prog Neuropsychopharmacol Biol Psychiatry* **33**, 860-6.

Miklosi, A.,Topal, J. (2013) What does it take to become 'best friends'? Evolutionary changes in canine social competence. *Trends Cogn Sci* **17**, 287-94.

Mitsui, S., Yamamoto, M., Nagasawa, M., Mogi, K., Kikusui, T., Ohtani, N., et al. (2011) Urinary oxytocin as a noninvasive biomarker of positive emotion in dogs. *Horm Behav* **60**, 239-43.

Nakajima, M., Gorlich, A.,Heintz, N. (2014) Oxytocin Modulates Female Sociosexual Behavior through a Specific Class of Prefrontal Cortical Interneurons. *Cell* **159**, 295-305.

Natt, D., Rubin, C. J., Wright, D., Johnsson, M., Belteky, J., Andersson, L., et al. (2012) Heritable genome-wide variation of gene expression and promoter methylation between wild and domesticated chickens. *BMC Genomics* **13**, 59.

Odendaal, J. S.,Meintjes, R. A. (2003) Neurophysiological correlates of affiliative behaviour between humans and dogs. *Vet J* **165**, 296-301.

Ovodov, N. D., Crockford, S. J., Kuzmin, Y. V., Higham, T. F., Hodgins, G. W.,van der Plicht, J. (2011) A 33,000-year-old incipient dog from the Altai Mountains of Siberia: evidence of the earliest domestication disrupted by the Last Glacial Maximum. *PLoS One* **6**, e22821.

Pagani, F.,Baralle, F. E. (2004) Genomic variants in exons and introns: identifying the splicing spoilers. *Nat Rev Genet* **5**, 389-96.

Pang, J. F., Kluetsch, C., Zou, X. J., Zhang, A. B., Luo, L. Y., Angleby, H., et al. (2009) mtDNA data indicate a single origin for dogs south of Yangtze River, less than 16,300 years ago, from numerous wolves. *Mol Biol Evol* **26**, 2849-64.

Parker, H. G. (2012) Genomic analyses of modern dog breeds. *Mamm Genome* **23**, 19-27.

Parker, H. G., Kim, L. V., Sutter, N. B., Carlson, S., Lorentzen, T. D., Malek, T. B., et al. (2004) Genetic structure of the purebred domestic dog. *Science* **304**, 1160-4.

Parr, L. A., Modi, M., Siebert, E.,Young, L. J. (2013) Intranasal oxytocin selectively attenuates rhesus monkeys' attention to negative facial expressions. *Psychoneuroendocrinology* **38**, 1748-56.

Peltola, M. J., Yrttiaho, S., Puura, K., Proverbio, A. M., Mononen, N., Lehtimaki, T., et al. (2014) Motherhood and oxytocin receptor genetic variation are associated with selective changes in electrocortical responses to infant facial expressions. *Emotion* **14**, 469-77.

Persson, M. E., Roth, L. S., Johnsson, M., Wright, D.,Jensen, P. (2015) Human-directed social behaviour in dogs shows significant heritability. *Genes Brain Behav* **14**, 337-44.

Pritchard, J. K., Stephens, M.,Donnelly, P. (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-59.

Quignon, P., Herbin, L., Cadieu, E., Kirkness, E. F., Hedan, B., Mosher, D. S., et al. (2007) Canine population structure: assessment and impact of intra-breed stratification on SNP-based association studies. *PLoS One* **2**, e1324.

Rodrigues, S. M., Saslow, L. R., Garcia, N., John, O. P.,Keltner, D. (2009) Oxytocin receptor genetic variation relates to empathy and stress reactivity in humans. *Proc Natl Acad Sci U S A* **106**, 21437-41.

Rubin, R. D., Watson, P. D., Duff, M. C.,Cohen, N. J. (2014) The role of the hippocampus in flexible cognition and social behavior. *Front Hum Neurosci* **8**, 742.

Sablin, M. V.,Khlopachev, G. A. (2002) The Earliest Ice Age Dogs: Evidence from Eliseevichi 1. *Curr Anthropol* **43**, 795-799.

Saetre, P., Lindberg, J., Leonard, J. A., Olsson, K., Pettersson, U., Ellegren, H., et al. (2004) From wild wolf to domestic dog: gene expression changes in the brain. *Brain Res Mol Brain Res* **126**, 198-206.

Saphire-Bernstein, S., Way, B. M., Kim, H. S., Sherman, D. K.,Taylor, S. E. (2011) Oxytocin receptor gene (OXTR) is related to psychological resources. *Proc Natl Acad Sci U S A* **108**, 15118-22.

Savolainen, P., Zhang, Y. P., Luo, J., Lundeberg, J.,Leitner, T. (2002) Genetic evidence for an East Asian origin of domestic dogs. *Science* **298**, 1610-3.

Shearin, A. L.,Ostrander, E. A. (2010) Leading the way: canine models of genomics and disease. *Dis Model Mech* **3**, 27-34.

Slane, M. M., Lusk, L. G., Boomer, K. B., Hare, A. E., King, M. K.,Evans, D. W. (2014) Social cognition, face processing, and oxytocin receptor single nucleotide polymorphisms in typically developing children. *Dev Cogn Neurosci* **9**, 160-71.

Stern, J. A., White, S. N.,Meurs, K. M. (2013) Extent of linkage disequilibrium in large-breed dogs: chromosomal and breed variation. *Mamm Genome* **24**, 409-15.

Sutter, N. B., Eberle, M. A., Parker, H. G., Pullar, B. J., Kirkness, E. F., Kruglyak, L., et al. (2004) Extensive and breed-specific linkage disequilibrium in Canis familiaris. *Genome Res* **14**, 2388-96.

Svartberg, K. (2006) Breed-typical behavior in dogs - Historical remnants or recent constructs? *Appl Anim Behav Sci* **96**, 293-313.

Svartberg, K.,Forkman, B. (2002) Personality traits in the domestic dog *(Canis familliaris)*. *Appl Anim Behav Sci* **79**, 133-157.

Thalmann, O., Shapiro, B., Cui, P., Schuenemann, V. J., Sawyer, S. K., Greenfield, D. L., et al. (2013) Complete mitochondrial genomes of ancient canids suggest a European origin of domestic dogs. *Science* **342**, 871-4.

Topal, J., Gergely, G., Erdohegyi, A., Csibra, G.,Miklosi, A. (2009) Differential sensitivity to human communication in dogs, wolves, and human infants. *Science* **325**, 1269-72.

Tost, H., Kolachana, B., Hakimi, S., Lemaitre, H., Verchinski, B. A., Mattay, V. S., et al. (2010) A common allele in the oxytocin receptor gene (OXTR) impacts prosocial temperament and human hypothalamic-limbic structure and function. *Proc Natl Acad Sci U S A* **107**, 13936-41.

Udell, M. A., Giglio, R. F.,Wynne, C. D. (2008) Domestic dogs (Canis familiaris) use human gestures but not nonhuman tokens to find hidden food. *J Comp Psychol* **122**, 84-93.

Udell, M. A.,Wynne, C. D. (2008) A review of domestic dogs' (Canis familiaris) human-like behaviors: or why behavior analysts should stop worrying and love their dogs. *J Exp Anal Behav* **89**, 247-61.

Vaysse, A., Ratnakumar, A., Derrien, T., Axelsson, E., Rosengren Pielberg, G., Sigurdsson, S., et al. (2011) Identification of genomic regions associated with phenotypic variation between dog breeds using selection mapping. *PLoS Genet* **7**, e1002316.

Vila, C.,Wayne, R. K. (1999) Hybridization between Wolves and Dogs. *Conserv Biol* **13**, 195-198.

Viranyi, Z., Gacsi, M., Kubinyi, E., Topal, J., Belenyi, B., Ujfalussy, D., et al. (2008) Comprehension of human pointing gestures in young human-reared wolves (Canis lupus) and dogs (Canis familiaris). *Anim Cogn* **11**, 373-87.

Wade, M., Hoffmann, T. J., Wigg, K.,Jenkins, J. M. (2014) Association between the oxytocin receptor (OXTR) gene and children's social cognition at 18 months. *Genes Brain Behav* **13**, 603-10.

Wayne, R. K. (1986) Limb morphology of domestic and wild canids: the influence of development on morphologic change. *J Morphol* **187**, 301-19.

Wayne, R. K.,Ostrander, E. A. (2007) Lessons learned from the dog genome. *Trends Genet* **23**, 557-67.

Wayne, R. K.,vonHoldt, B. M. (2012) Evolutionary genomics of dog domestication. *Mamm Genome* **23**, 3-18.

Winslow, J. T., Noble, P. L., Lyons, C. K., Sterk, S. M.,Insel, T. R. (2003) Rearing effects on cerebrospinal fluid oxytocin concentration and social buffering in rhesus monkeys. *Neuropsychopharmacology* **28**, 910-8.

Zelkowitz, P., Gold, I., Feeley, N., Hayton, B., Carter, C. S., Tulandi, T., et al. (2014) Psychosocial stress moderates the relationships between oxytocin, perinatal depression, and maternal behavior. *Horm Behav* **66**, 351-60.

Figure legends

Figure 1

Allele frequencies of the 8 identified common *Oxtr* SNPs in four different canine species.

Number of individual animals investigated were as follows: wolf: N=45; dog: N=659 (consisting of 10 different breeds); dingo: N=8; golden jackal: N=6.

Figure 2

Allele frequencies of the 8 identified common *Oxtr* SNPs in different dog breeds.

Number of individual animals investigated were as follows: Beagle N=65; Border Collie: N=142; German Shepherd N=126; Golden Retriever: N=41; Groenendael N=23; Hungarian Vizsla N=32; Labrador Retriever: N=47; Malinois N=40; Siberian Husky N=126; Tervueren N=24. As a reference, data for wolf (N=45) are indicated as well.

Figure 3

Identifying the optimal K value for canine *Oxtr* SNP-based neighbor joining tree.

Data gained from animals successfully genotyped for all 8 common *Oxtr* SNPs investigated (N=273) are indicated. Upper panel shows results obtained by the maximum likelihood method while bottom panel that by Evanno's method (Evanno *et al.* 2005).

Figure 4

Neighbor joining tree based on 8 common canine *Oxtr* SNPs.

Only animals with complete genotype data (N=273) were considered. (A) Genetic distance and relationship between inferred clusters for K=4. (B) Average membership coefficients for different canine populations. Values greater than 0.5 are highlighted. Cluster numbering is as indicated in (A).

Figure 5

Expression of the canine *Oxtr* mRNA in different brain areas.

Relative mRNA expression levels (arbitrary units) are shown.

Figure 6

Gene expression regulatory effect of four common SNPs (−213A/G; −94T/C; −74C/G; and −50C/G) in the canine *Oxtr* 5' UTR as compared to the wolf-specific, putative ancestral variants (5' UTR-AH).

SK-NF-I neuroblastoma cells (A) and HEK293 human embryonic kidney cells (B) were transfected with constructs containing wolf-specific alleles (5' UTR-AH: −213A, −94C, −74C, and −50G) and constructs where one each SNP sites were mutagenized (to −213G, −94T, −74G, and −50C), respectively.

Relative luciferase intensities (mean ± SEM) normalized to control β-galactosidase activities from three independent experiments are shown. ANOVA indicated statistically significant difference in luciferase activity when assessing the construct involving allele −94T ($p<0.0001$) in the case of both cell lines.

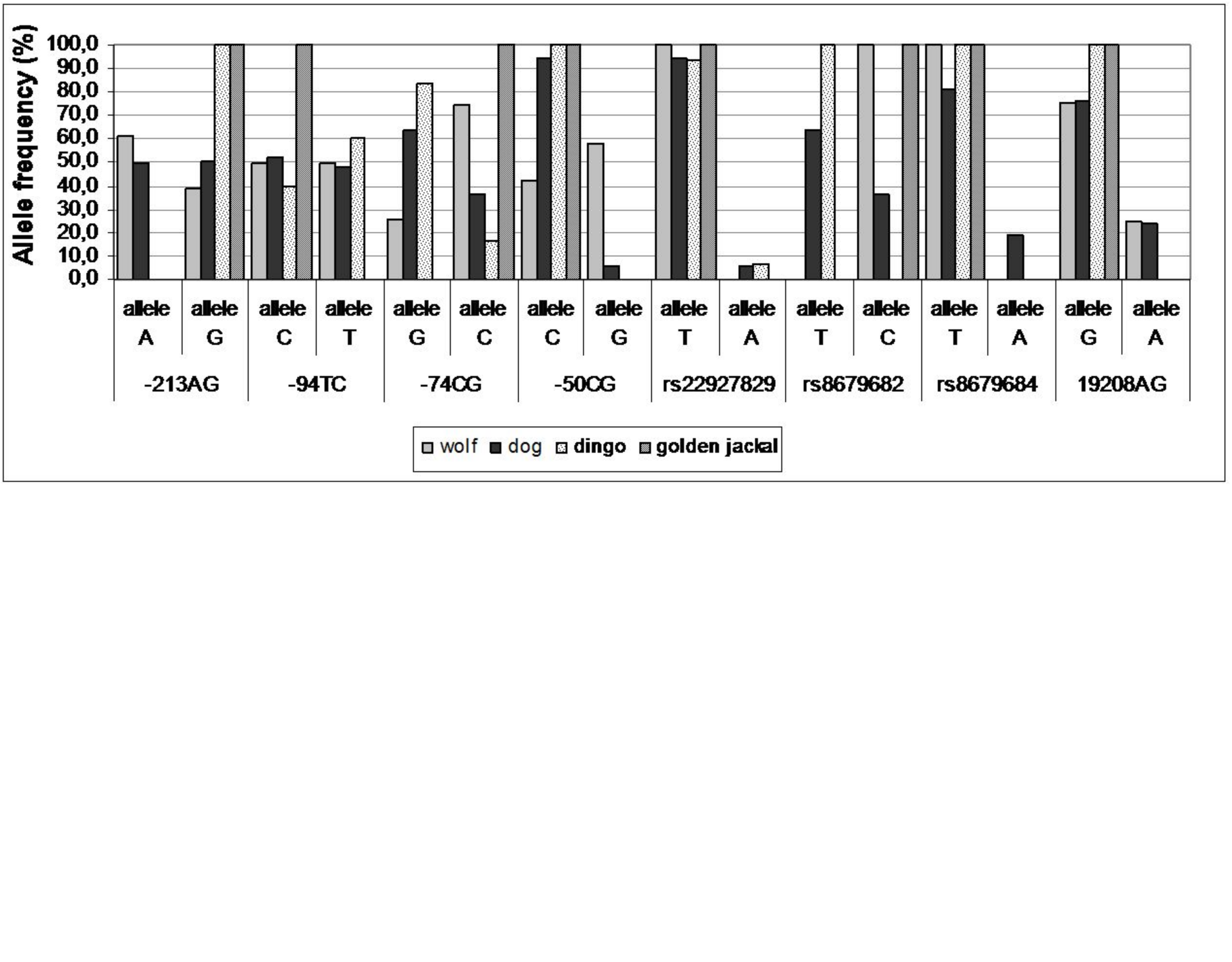
Figure 7

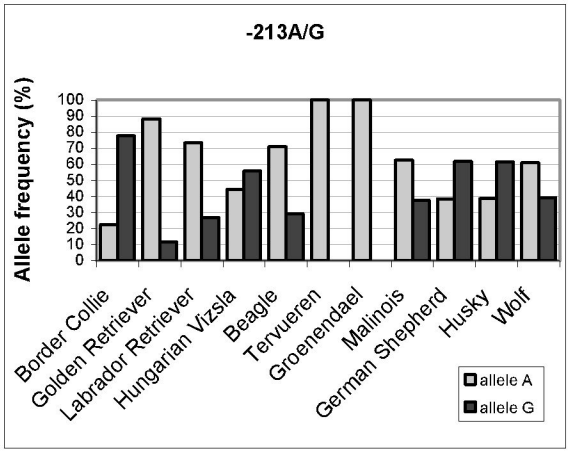
Gene expression regulatory effect of variants of the microsatellite marker designated here as CLF20OXTR1 located in the canine *Oxtr* 3' UTR.

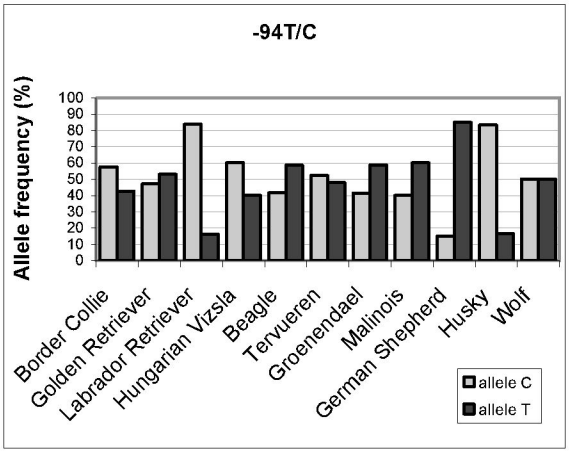
SK-NF-I neuroblastoma cells (A) and HEK293 human embryonic kidney cells (B) were transfected with constructs containing possible variants of the microsatellite CLF20OXTR1. Short/G: 9 GT dinucleotide repeats combined with SNP allele 18779G; short/A: 9 repeats combined with 18779A; long/G: 10 repeats combined with 18779G and long/A: 10 repeats combined with 18779A (the latter variant seems not to occur in nature). Relative luciferase intensities (mean ± SEM) normalized to control β-galactosidase activities from three independent experiments are shown. ANOVA indicated statistically significant difference in luciferase activity between both long/G and short/A vs. both short/G and long/A ($p<0.0001$) in the case of both cell lines.

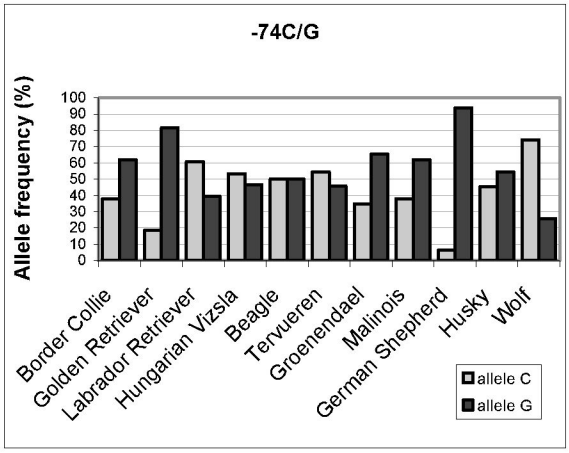
eTOC text

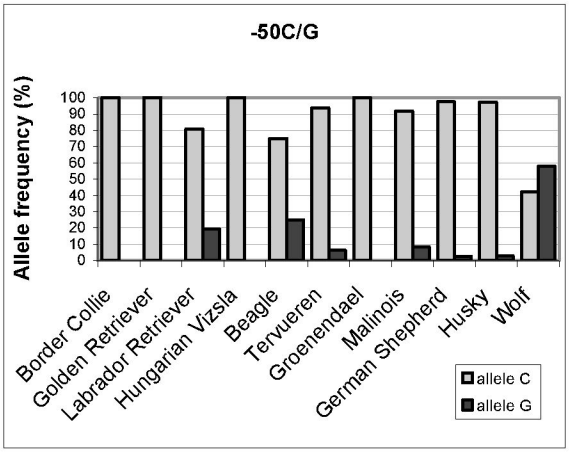
Figure 4: Neighbor-joining tree supports the hypothesis that Oxtr variants could have been a selection target during canine domestication.

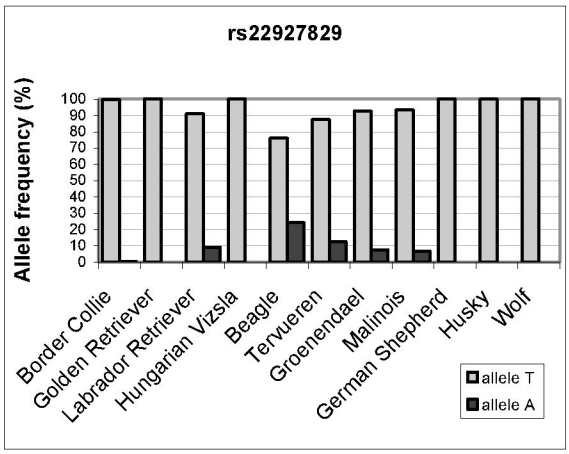


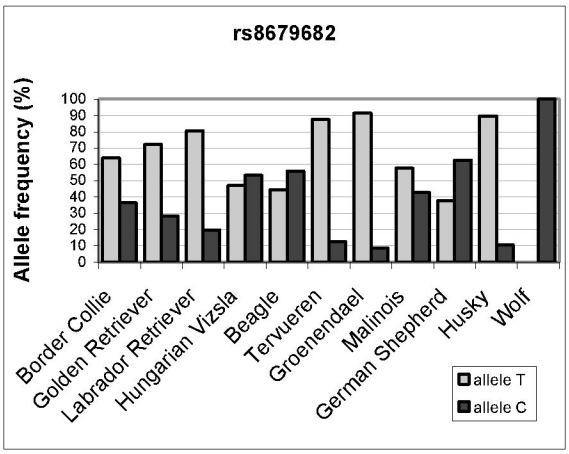


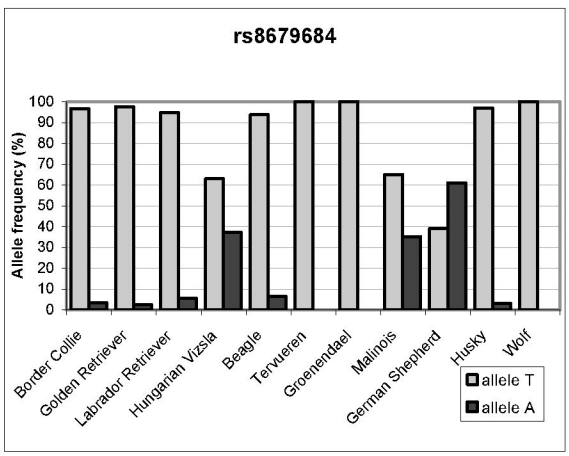


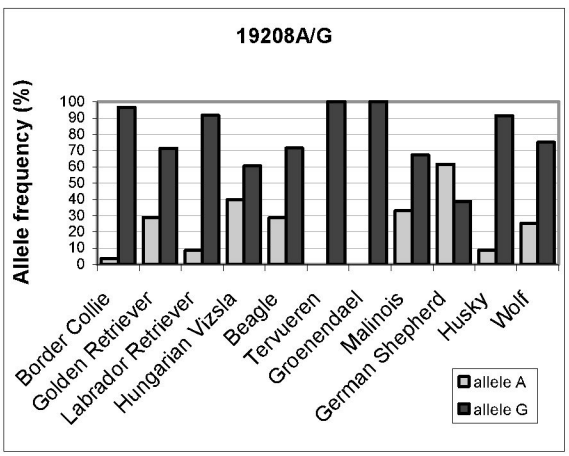


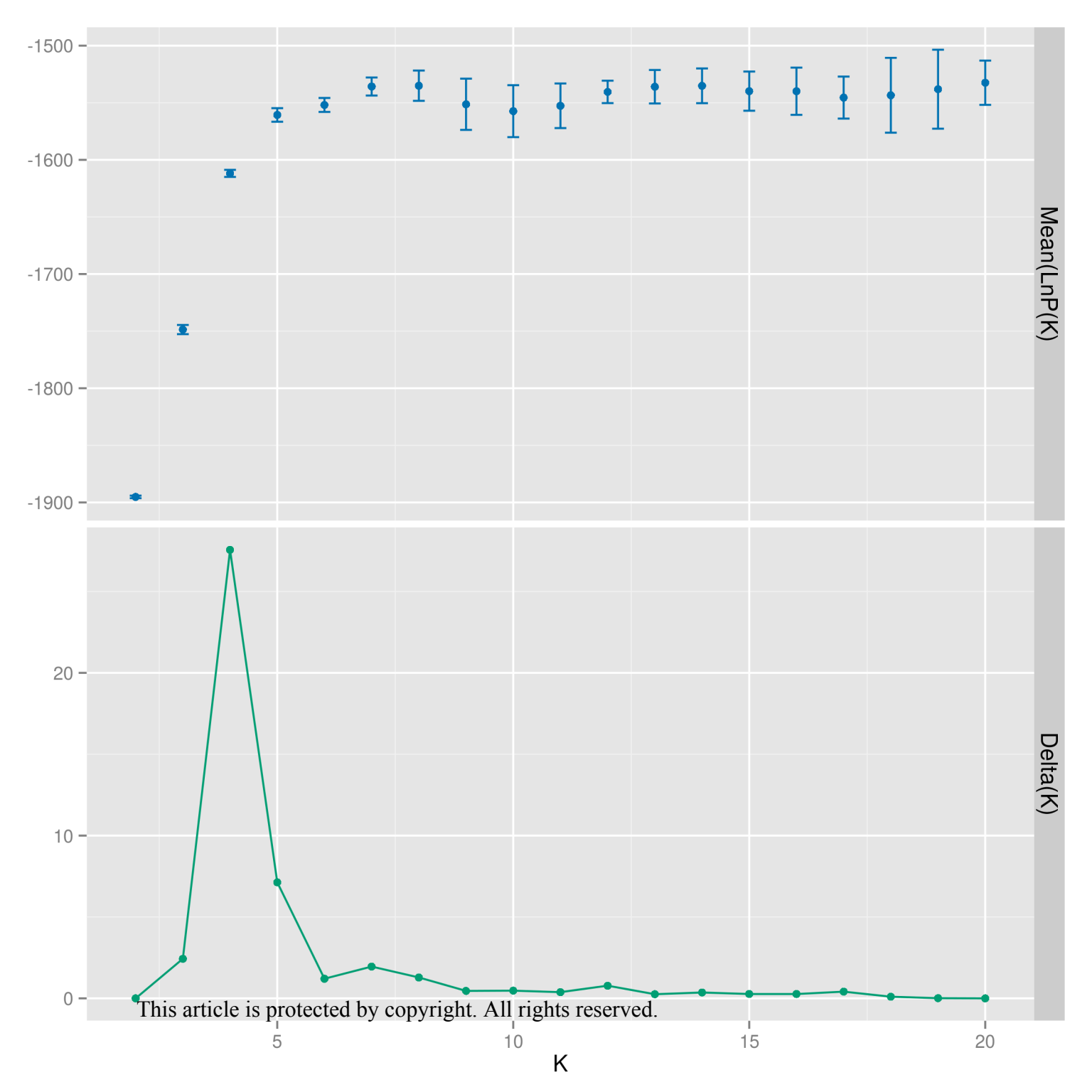




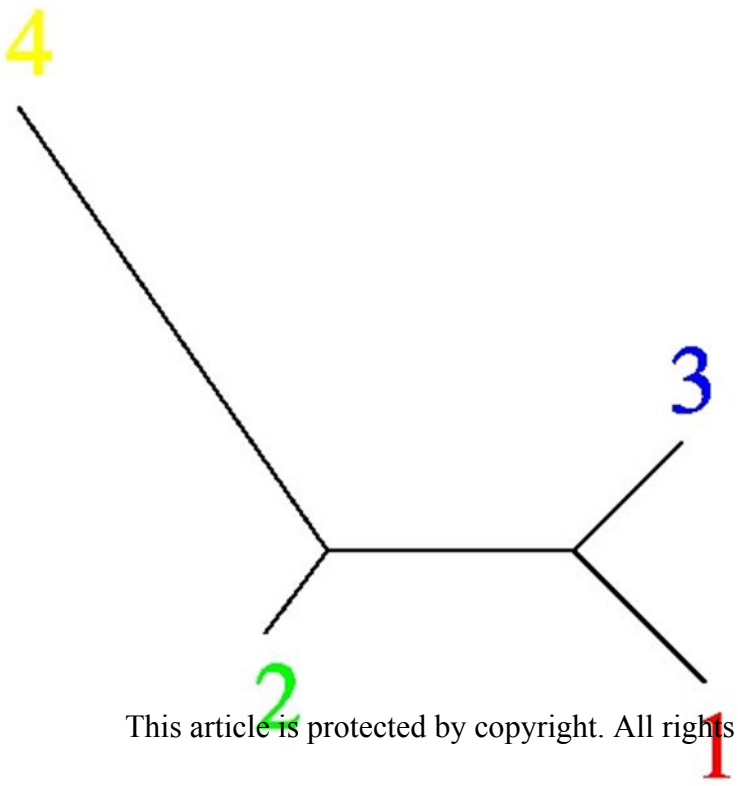






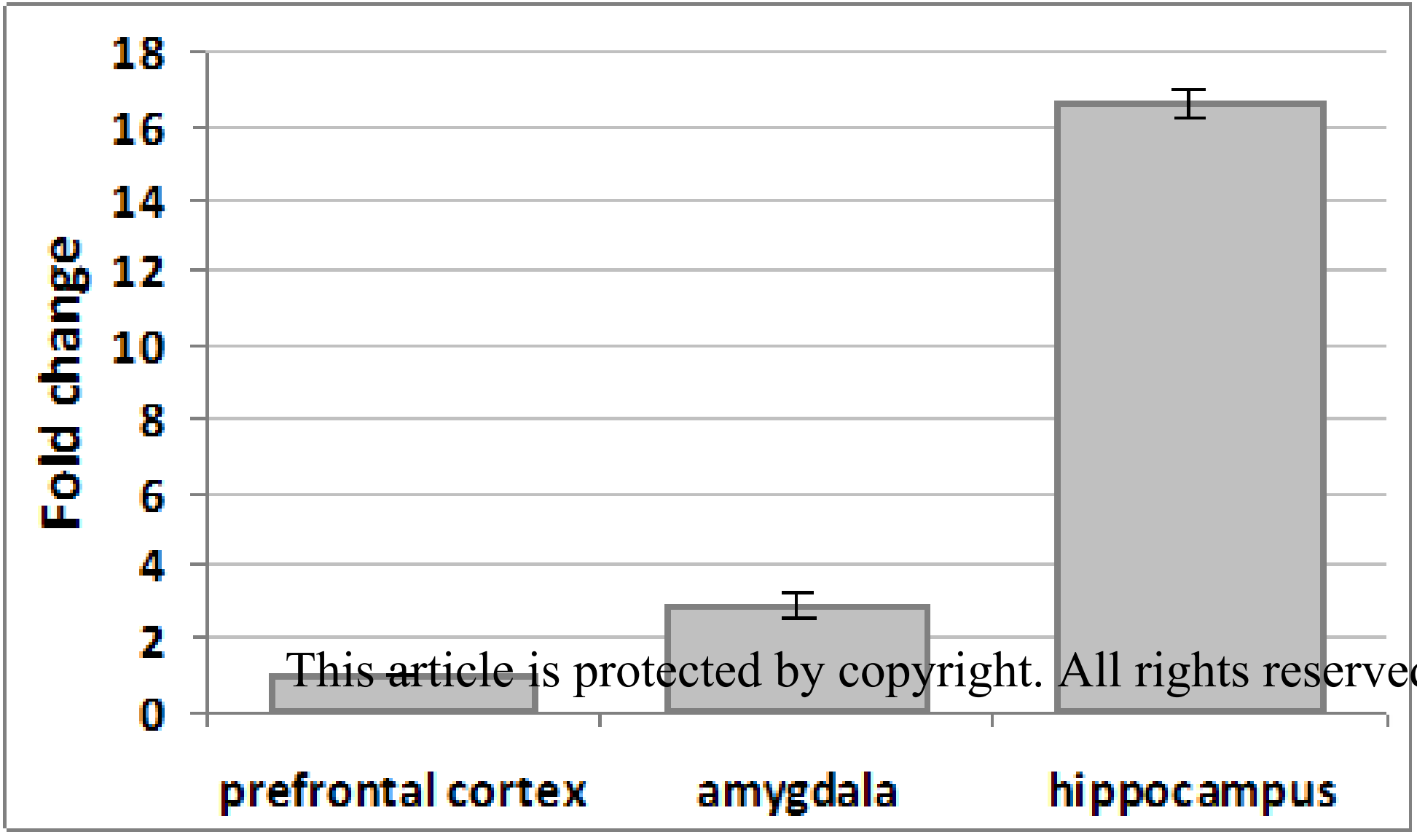


a

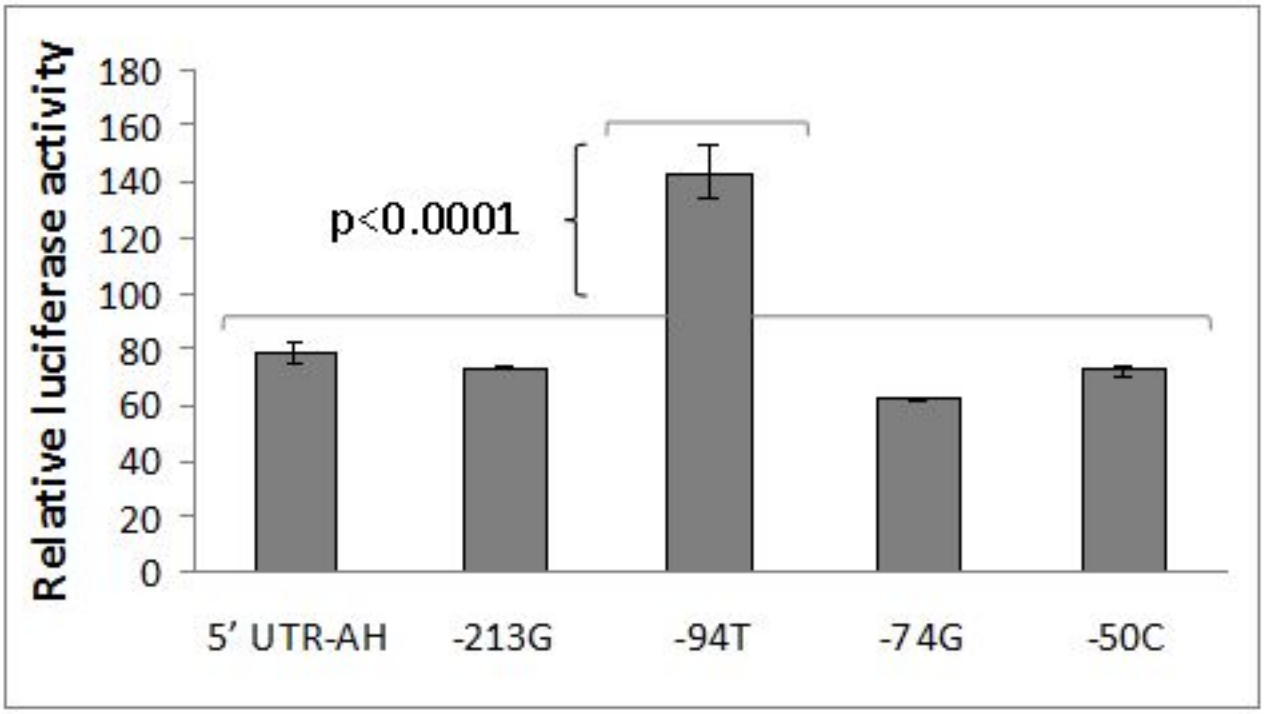


b

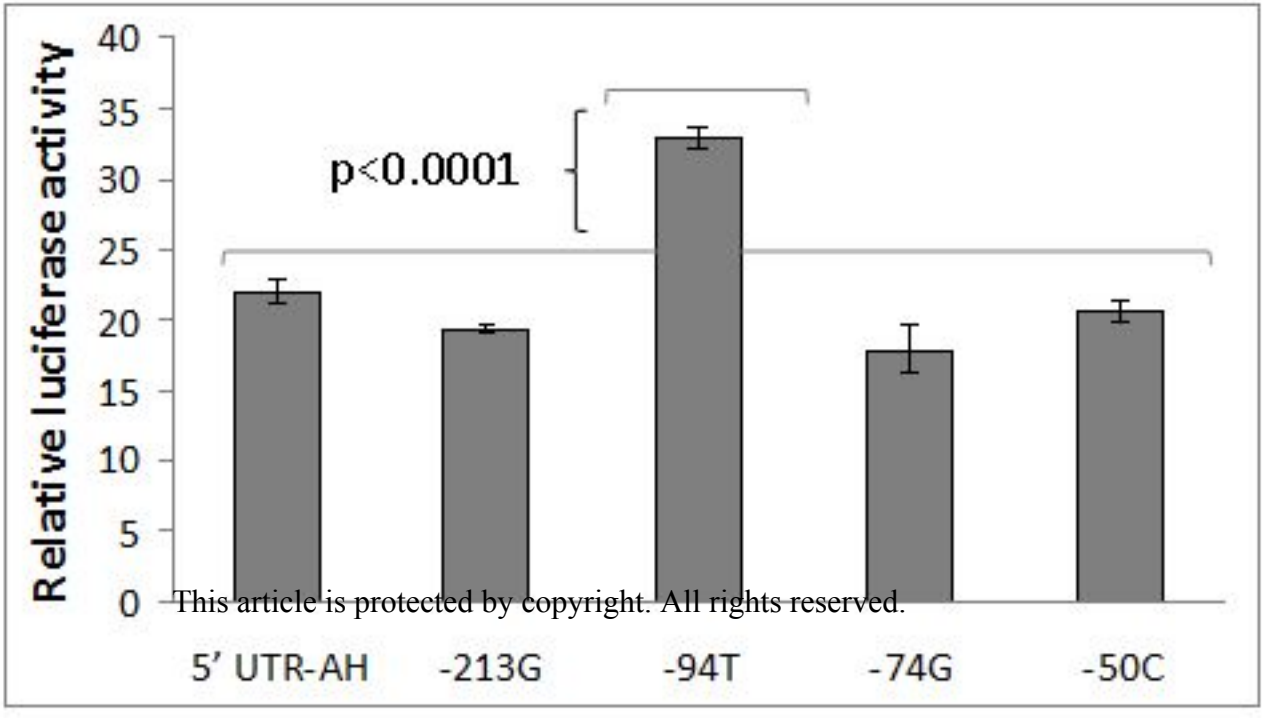
Population	Number of individuals	Inferred clusters			
		1	2	3	4
Wolf	22	0.062	0.061	0.063	0.814
Golden Jackal	4	0.015	0.025	0.282	0.678
Dingo	3	0.015	0.060	0.910	0.016
Pariah dog	4	0.049	0.633	0.177	0.142
Siberian Husky	24	0.088	0.412	0.342	0.158
Tervueren	15	0.020	0.792	0.029	0.159
Groenendael	8	0.114	0.787	0.058	0.041
Malinois	17	0.449	0.377	0.05	0.124
German Shepherd	21	0.810	0.017	0.161	0.012
Border Collie	81	0.064	0.245	0.567	0.124
Golden Retriever	22	0.36	0.495	0.09	0.054
Labrador Retriever	27	0.146	0.396	0.093	0.364
Hungarian Vizsla	8	0.337	0.283	0.123	0.258
Beagle	17	0.269	0.102	0.119	0.509



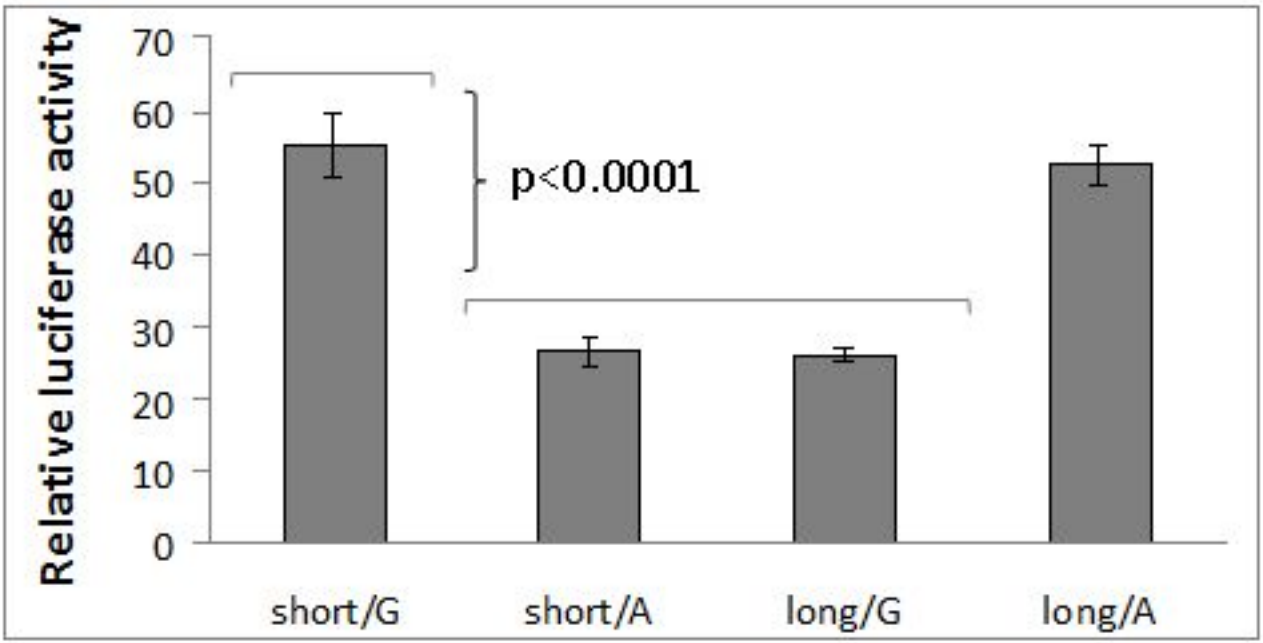
a



b



a



b