Genomic responses to selection for tame/aggressive behaviors in the silver fox (*Vulpes vulpes*)

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Animal domestication efforts have led to a shared spectrum of striking behavioral and morphological changes. To recapitulate this process, silver foxes have been selectively bred for tame and aggressive behaviors for more than 50 generations at the Institute for Cytology and Genetics in Novosibirsk, Russia. To understand the genetic basis and molecular mechanisms underlying the phenotypic changes, we profiled gene expression levels and coding SNP allele frequencies in two brain tissue specimens from 12 aggressive foxes and 12 tame foxes. Expression analysis revealed 146 genes in the prefrontal cortex and 33 genes in the basal forebrain that were differentially expressed, with a 5% false discovery rate (FDR). These candidates include genes in key pathways known to be critical to neurologic processing, including the serotonin and glutamate receptor pathways. In addition, 295 of the 31,000 exonic SNPs show significant allele frequency differences between the tame and aggressive populations (1% FDR), including genes with a role in neural crest cell fate determination.

domestication | selection | fox | transcriptome

Differences in the behavior of domesticated animals from their wild ancestors provide some of the best examples of the influence of genes on behavior (1). Domesticated animals have been selected to be easy to handle, and they generally exhibit reduced aggressiveness and increased social tolerance to both humans and members of their own species (2). Even after the genomes of most domesticated species and their wild ancestral species have been sequenced, identification of genes responsible for these behavioral differences has proven challenging (3–6). The selection for different traits in each of the domesticated animals and the antiquity of the time frame make it difficult to identify which genetic changes are causally responsible for changes in behavior (3, 7, 8).

Unlike species domesticated in the distant past, the silver fox (a coat color variant of the red fox, *Vulpes vulpes*) has been domesticated under controlled farm conditions at the Institute of Cytology and Genetics (ICG) of the Russian Academy of Sciences (9–11). The red fox and the domestic dog (*Canis familiaris*) share a common ancestor just 10 Mya (12), making the fox experiment a model for dog domestication. To test whether selection for behavior was the primary force in the canine domestication process, starting in 1959, Dmitry Belyaev and Lyudmila Trut have been selecting conventional farm-bred foxes against fear and aggression to humans, followed by selection for contact-seeking behavior, which led to the development of a tame strain of foxes (Fig. 1*A*) (9–11). The response to selection was extremely rapid: the first tame animal classified as "elite of domestication" appeared in generation 4, 1.8% of such foxes were observed at generation 6 (4/213), and by generation 45 almost all foxes belonged to that category (11). Foxes from the tame population relate with humans in a positive manner similar to that of friendly dogs (13). They are eager to establish human contact by 1 mo after birth and remain friendly throughout their entire lives (11).

In parallel with selection for tameness, selective breeding for an aggressive response to humans was started in 1970, with the aim of developing a population demonstrating less variation in behavior than conventional foxes (10, 11). This trait also showed a selection response (Fig. 1*A*). The tame and aggressive fox strains were selected solely for specific behavioral traits, and the pedigree information was maintained during the entire breeding program (10, 11). Efforts were made to avoid close inbreeding in these populations, allowing continuous selection for many decades and generations (9–11). The heritability of these behavioral traits has been confirmed in multiple experiments (14–17), making these fox strains a promising model for identifying the genetic basis of tame and aggressive behaviors.

To identify the genetic basis of the behavioral differences between tame and aggressive fox strains, we developed the fox meiotic linkage map, experimentally cross-bred pedigrees, and mapped nine significant and suggestive quantitative trait loci (QTL) for behavioral traits (17–20). Although QTL mapping is a promising strategy for identifying genomic regions implicated in complex traits, this approach alone usually does not allow identification of the causative genes and mutations. In the present study, we analyzed fox brain transcriptomes of 12 aggressive and 12 tame

Significance

The behavior of domesticated animals differs dramatically from that of wild relatives, and the Russian tame fox experiment demonstrated clearly that these changes can occur in just a few generations of selection. Analysis of gene expression in the brains of tame and aggressive foxes from this experiment allows us to ask what brain pathways have been altered by this recent, strong selection. Pathways that impact the function of both serotonergic and glutaminergic neurons were clearly modulated by selection, consistent with the roles of these neurons in learning and memory. Both allele frequency and gene expression changes also implicate genes important in neural crest cell function, supporting a possible role of neural crest cells in the domestication syndrome.

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Data deposition: The data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (accession no. GSE76517).

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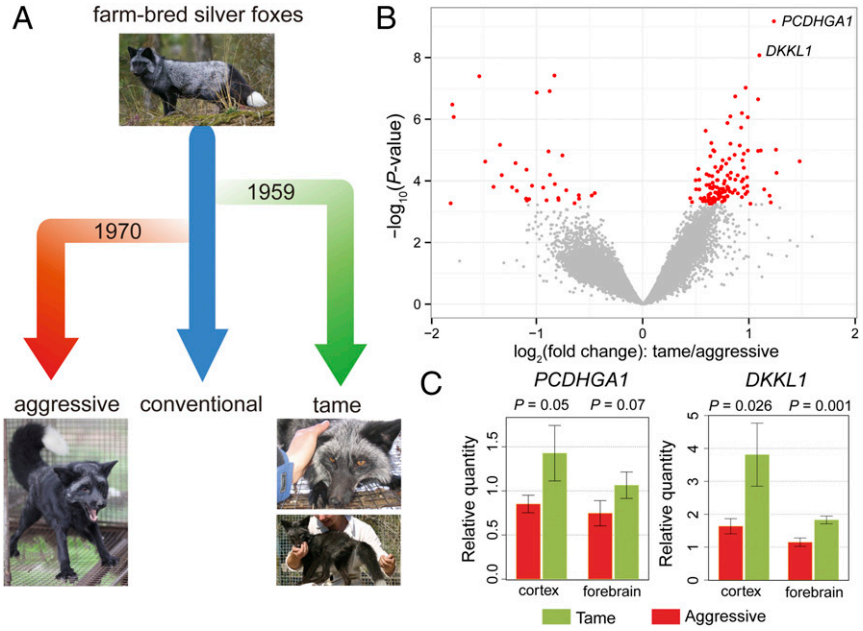

Fig. 1. RNA-seq analysis identified differentially expressed genes in brain tissues between the tame and aggressive fox populations. (*A*) Artificial selection scheme for tameness and aggression in foxes. The conventional population of farm-bred foxes (blue arrow) was a founding population for both tame and aggressive fox populations. The population of conventional farm-bred foxes is still maintained in Novosibirsk. Starting in 1959, the selection experiment for tame foxes has been carried out to recreate the evolution of canine domestication. In 1970, an aggressive population was also selected to compare with the tame population. (*B*) A volcano plot showing differentially expressed genes detected in 12 tame and 12 aggressive fox prefrontal cortex samples. Plotted on the *x*-axis is the log2 fold difference between tame and aggressive samples. Plotted on the *y*-axis is the –log10 (*P* value) calculated with the R package edgeR. Significant differentially expressed genes (FDR <0.05) are indicated in red, and nonsignificant genes are shown in gray. (*C*) Bar plot of qRT-PCR validation results in prefrontal cortex and forebrain samples for the top two significant candidate genes, *PCDHGA1* and *DKKL1*.

1.5-y-old sexually naive males. We evaluated gene expression in two brain regions, the prefrontal cortex and basal forebrain.

The prefrontal cortex is the site of memory and learning. It coordinates a wide range of neural processes and plays a central role in the integration of diverse information needed for complex behaviors (21). The basal forebrain modulates cortical activity and plays an important role in arousal, attention, and decision making (22). RNA-seq analysis of these two brain regions identified significant differences in gene expression between the two fox strains and pinpointed several gene networks that were modified during the course of artificial selection for tame/aggressive behaviors.

Results and Discussion

Gene Expression Profiles in the Brains of Tame and Aggressive Individuals. The profound behavior differences appeared rapidly after selection, and changes in brain gene expression levels might have played an important role in the response. To investigate this, Illumina RNA-seq experiments were performed on brain tissues from 12 aggressive and 12 tame individuals (*SI Appendix*, Figs. S1–S3), including the right prefrontal cortex and right basal forebrain (*SI Appendix*, Fig. S4). These experiments yielded a total of 1.57 billion RNA-seq reads, with an average of 30 million reads per sample (*SI Appendix*, Tables S1 and S2). These reads were aligned to both the fox draft genome scaffolds and de novo brain transcriptome assembly (*Materials and Methods*), producing high-quality read count data on the 48 samples for 12,808 annotated genes in the transcriptome. Among these genes, 146 are differentially expressed in prefrontal cortex of tame and aggressive individuals at a 5% false discovery rate (FDR; q <0.05) (Fig. 1*B* and *SI Appendix*, Fig. S5 and Table S3). In addition, there were 33 differentially expressed genes in the basal forebrain (*SI Appendix*, Fig. S6 and Table S4).

Among these hits, the two most significant genes are *DKKL1* and *PCDHGA1* ($P < 10^{-8}$ in prefrontal cortex and $P < 10^{-11}$ in basal forebrain; Fig. 1*B*), and their up-regulation in tame fox was confirmed using qRT-PCR in the same RNA-seq samples (*SI Appendix*, Fig. S7 and Table S5; *Materials and Methods*). *DKKL1*, Dickkopf-like protein 1, has signal transducer activity and interacts with the noncanonical Wnt pathway. In the mouse brain, *DKKL1* displays region-specific expression, with the highest expression level in the cortical neurons of the adult cortex (7). Little is known about the function of *DKKL1* in the brain except that it bears sequence similarity to *DKK1*, an antagonist of canonical Wnt signaling implicated in a wide spectrum of physiological processes, including neurogenesis, neuronal connectivity, and synapse formation. Overexpression of *DKKL1* in the ventral hippocampus, but not in the prefrontal cortex, has been associated with increased susceptibility to social defeat stress in mice (23). *PCDHGA1*, the Protocadherin Gamma Subfamily A1 gene, encodes a neural cadherin-like cell adhesion protein. Protocadherins are known to play critical roles in the establishment and function of specific cell–cell connections in the brain, such as synapse development (24), as well as dendrite arborization and self-avoidance in the central nervous system (25, 26). *Pcdhga1* expression was down-regulated in a learned helpless rat model, suggesting that its expression might affect behavioral phenotypes (27). Twenty-eight of the 146 significant genes in the prefrontal cortex (*SI Appendix*, Table S6) and 15 of 33 genes in the forebrain (*SI Appendix*, Table S7) are under known fox behavior QTL peaks (17, 20), and more than one-half of these genes overlap with the two significant QTL peaks on fox chromosome 12. The RNA-seq experiments identified 163 differentially expressed genes that might be responsible for the behavioral phenotype changes after selection.

Expression Changes in Serotonin and Glutamate Receptor Signaling Pathways. Previous studies of pathological aggression and anxiety in humans and other animals strongly suggest that genes involved in several neurologic receptor pathways may have altered expression

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levels in tame foxes. Serotonin (5-HT) is a neurotransmitter known to play a role in feelings of contentment/happiness in humans, and deficiency has been linked with many mood disorders, including anxiety and depression (28). Altered expression levels of serotonin receptors have been documented in patients with schizophrenia and bipolar disorder (29). Serotonin and serotonin metabolite (5-HIAA) levels have been found to be significantly elevated in tame foxes compared with aggressive foxes (7), similar to other mammals and invertebrates (19, 20). In this study, we examined genes in the serotonin receptor pathways based on the KEGG database (30, 31) and found significantly differentially expressed genes, including serotonin receptors 5A, 3A, and 7 and a pair of downstream signaling genes: DUSP1 in the cAMP/PKA pathway and AKT1 in the PI3K/AKT pathway (Fig. 2*A* and *SI Appendix*, Figs. S8 and S9). Nearly all the changes are in the direction of increased serotonin signaling in the tame animals.

Besides the critical role of serotonin, dopamine and glutamate also have been linked to aggression (32). In our dataset, no genes in the dopamine receptor pathway were identified as significantly differentially expressed. For the glutamate receptor pathway, the NMDA receptor 2D subunit and downstream signaling genes *ITPR3* and *ADCY7* were significantly up-regulated in the tame

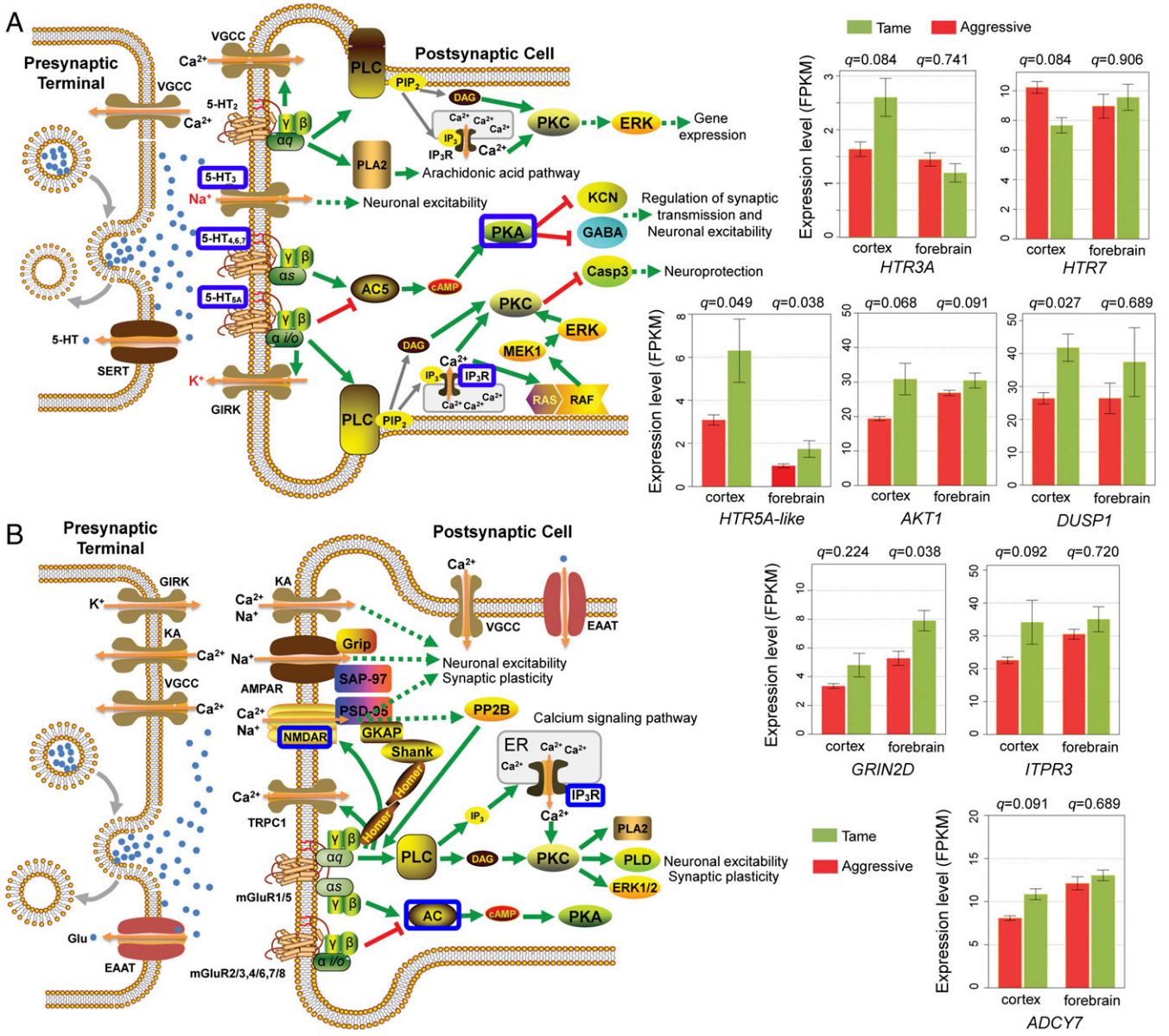

Fig. 2. Genes that are differentially expressed between tame and aggressive fox populations in serotonin and glutamate receptor pathways. Diagrams of a serotonergic synapse (*A*) and a glutamatergic synapse (*B*) show the presynaptic and postsynaptic terminals (adapted from KEGG pathway database). The RNA-seq expression levels in both tissues are plotted in individual bar plots for significantly differentially expressed genes ($q < 0.10$ in at least one tissue) between tame and aggressive foxes. Differentially expressed receptors and genes involved in downstream signaling pathways (assigned by KEGG; *SI Appendix*, Fig. S8) are in blue boxes. (*A*) In tame individuals, serotonin receptors *HTR5A-like* is up-regulated in both tissues. *HTR3A* is up-regulated only in the prefrontal cortex, and *HTR7* is down-regulated in the cortex. *DUSP1* is in the cAMP/PKA pathway (middle right part of the figure), and *AKT1* is a major component of the PI3K/AKT pathway (bottom right of the figure). They are both up-regulated in tame foxes. (*B*) A subclass of glutamate receptors, NMDA receptor 2D (*GRIN2D*; glutamate receptor, ionotropic, *N*-methyl-D-aspartate 2D), and downstream signaling genes *ITPR3* and *ADCY7* (pathways in red boxes in the middle right and bottom right parts of the figure, respectively) are differentially expressed between tame and aggressive foxes, with up-regulation in the tame animals.

animals (Fig. 2*B* and *SI Appendix*, Figs. S8 and S9). NMDA receptors are a subclass of glutamate receptors important for synaptic plasticity, learning, and memory. This pathway also plays a key role in fear conditioning (33). Up-regulation of NMDA signaling might be consistent with increased responsiveness to keepers by the tame foxes. These results suggest that the gene expression response to selection for tameness in silver foxes impacts neurotransmitter receptor pathways and shed light on the biological basis of affiliative and aggressive behaviors by relating to neurologic and pharmacologic correlates with those behaviors.

Allele Frequency Changes During the Selection Process for Tame and Aggressive Behaviors. In addition to the expression response, other genes may manifest changes in coding sequences that could affect protein function. Such genes often show allele frequency changes in their coding SNPs. In the RNA-seq data, we identified 31,025 high-quality exonic SNPs (*Materials and Methods*) and tested allele frequency differences at these positions. Founder effect, inbreeding, and random genetic drift can result in allele frequency changes, and these factors must be controlled to allow accurate assessment of the role of selection. The tame and aggressive fox populations were selected solely for specific behavioral traits, and full pedigree data for the tame (6,670 individuals) and aggressive (1,863 individuals) populations were maintained during the entire breeding program (*SI Appendix*, Figs. S2 and S3) (11).

Using this information, we directly simulated the precise effect of genetic drift and inbreeding on allele frequency changes by "gene dropping", a method that uses the known pedigree structures for an ascertained sample of genotypes drawn from the population (in this case, the 24 RNA-seq individuals) (Fig. 3*A* and *SI Appendix*, Fig. S10). At an adjusted *P* value of 0.01, 295 SNPs in 176 genes had significantly different allele frequencies

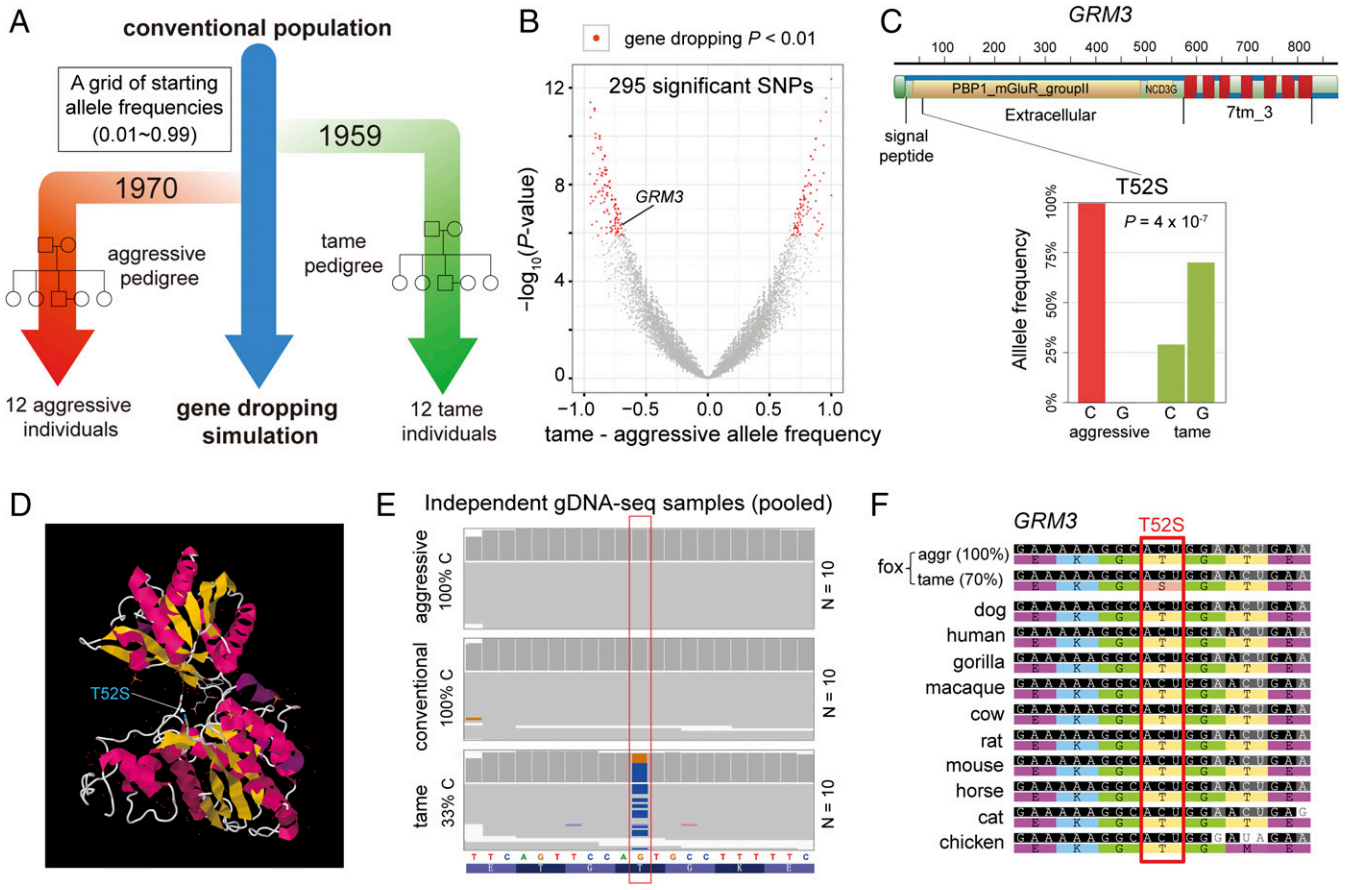

Fig. 3. *GRM3*, a metabotropic glutamate receptor gene with significant allele frequency changes in the tame population. (*A*) Gene dropping simulation scheme to determine the adjusted *P* value under genetic drift, inbreeding, and founder effect. A null distribution assuming no association between SNP genotypes and behavioral phenotypes was generated by simulating all founder genotypes under a grid of starting founder allele frequencies (0.01~0.99 in increments of 0.01). Then alleles were dropped down the observed tame and aggressive pedigree structures (*SI Appendix*, Figs. S2 and S3) based on Mendelian inheritance. This was repeated to produce a null distribution of the magnitude of allele frequency changes. From this, we obtained *P* values for the observed allele frequency difference between tame and aggressive RNA-seq samples. A total of 295 SNPs were significant across all starting allele frequencies at a 1% level based on 10,000 simulations. (*B*) A volcano plot showing allele frequency differences between tame and aggressive RNA-seq samples on the *x*-axis and the –log10 *P* values on the *y*-axis. The 295 significant SNPs are labeled in red. (*C*) *GRM3* (metabotropic glutamate receptor 3) has a C → G nonsynonymous SNP change causing a Thr to Ser missense mutation (T52S). In the RNA-seq data, aggressive foxes have 100% C alleles, and tame foxes only have 30% C alleles ($P = 4 \times 10^{-7}$; adjusted $P < 0.01$). PBP1_mGluR_groupII, ligand-binding domain of the group II metabotropic glutamate receptor; NCD3G, nine cysteines domain of family 3 GPCR; 7tm_3, 7 transmembrane sweet-taste receptor of 3 GCPR. Annotation from RCSB Protein Data Bank (UniProt ID code Q14832). (*D*) Crystal structure of the *GRM3* extracellular region (PDB ID code 3SM9) viewed by jmol software. T52S (labeled in blue) is near the ligand-binding site, suggesting that it might alter the protein function. (*E*) Integrative genomics viewer screen shot at the *GRM3* SNP position in pooled gDNA-seq samples (*SI Appendix*, Figs. S10 and S11). In independently selected gDNA resequencing samples, the tame G allele frequency (67%) is confirmed in the tame population and is missing in the aggressive population. (*F*) The C allele is conserved in dogs, other mammals, and chickens. The tame G allele is the derived allele.

between the tame and aggressive populations (Fig. 3*B* and *SI Appendix*, Table S8), with a mean allele frequency difference of 0.79. Nonsynonymous SNPs were slightly enriched in the significance of allele frequency changes compared with all exonic SNPs (25.9% vs. 23.9%), but the difference did not reach statistical significance (*SI Appendix*, Fig. S11 *C* and *D*). One-third of the 176 significant genes overlapped with significant fox behavior QTL peaks (*SI Appendix*, Table S9).

Ten whole-genome sequences were obtained for each of the tame, aggressive (*SI Appendix*, Figs. S12 and S13), and conventional farm-bred fox populations (34) at 25× coverage per population. These genome sequencing samples were from different individuals, allowing independent cross-validation of allele frequency changes. Overall, the SNP allele frequency changes were significantly correlated (Spearman $\rho = 0.73$; q value < 0.01) between our RNA-seq results and these whole-genome sequences (*SI Appendix*, Fig. S9*B*). Among 176 genes containing SNPs with significant allele frequency differences, 73 are located in the regions highlighted in the genome paper (34). Almost all of these genes ($n = 71$) showed extreme F_{ST} values in comparisons of tame and aggressive foxes from the genome paper (*SI Appendix*, Table S12). *SorCS1*, a transporter important for trafficking AMPA glutamate receptors to the cell surface, is one of the QTL positional candidate genes with decreased heterozygosity and increased divergence between populations that was identified in the analysis of resequenced genomes (34). Six *SorCS1* coding SNPs are among the 295 SNPs with significant tame vs. aggressive allele frequency difference, including the third most significant SNP on the list (*SI Appendix*, Table S8), highlighting the consistency of allele frequency divergence. *SorCS1* is also among the 52 genes under the behavior QTL peaks (*SI Appendix*, Table S9). Despite the consistent allele frequency changes occurring in *SorCS1* due to selection, no changes in expression level were detected, perhaps because the parts of the brain that we used in this study are not among the brain regions showing intense *SorCS1* expression signals in mouse brain (35).

One of the 176 genes exhibiting a significant SNP frequency change is *GRM3*, the metabotropic glutamate receptor 3. This glutamate receptor has been associated with schizophrenia, bipolar, mood disorders, and delayed sexual maturity in human studies (36, 37). In our exonic SNP data, *GRM3* exhibited a C-to-G change, causing a threonine-to-serine missense mutation (T52S) in the coding region, with a C frequency of 100% in the aggressive foxes but only 30% in the tame foxes ($P = 4 \times 10^{-7}$; adjusted $P < 0.01$) (Fig. 3*C*). The altered amino acid is in the extracellular region near the glutamate-binding site, which might affect binding affinity (Fig. 3*D*). The allele frequencies were validated in independently selected tame, aggressive, and unselected individuals (Fig. 3*E* and *SI Appendix*, Figs. S10 and S11). The tame allele (G) is missing in both aggressive and unselected foxes. Evolutionarily, the ligand binding region is highly conserved, with all genome-sequenced mammals and chicken having the C allele (Fig. 3*F*). The increased G allele frequency might be a direct response to the artificial selection for tameness in the farm fox experiment.

Comparative Analysis with Aggressive Rat Selection Experiments and Wild Cat Domestication Revealed Hits on the Same Genes and Gene Families. Our results show that both gene expression and allele frequency responses in the tame foxes occurred in the glutamate receptor signaling pathway; the expression of *GRIN2D* was elevated in tame individuals in the forebrain, and *GRM3* showed significant allele frequency changes. This same pathway also experienced significant changes in both ancient domestication events and recent selection experiments in other mammals. The parallels with the domestic dog are particularly noteworthy, with genes in glutamate receptor signaling (i.e., *GRIA1* and *GRIN2A*) also showing significant changes during the course of domestication (38). Similarly, in domestication of the cat, two glutamate receptor genes, *GRIA1* and *GRIA2* were also found to be under positive selection (39). A recent selective sweep was also found in *GRIK2* in domestic rabbits (6). This convergence of selection signals on glutamate receptor signaling strongly motivates additional experimental confirmation of a functional role for glutamate signaling in behavioral differences of domesticated mammals.

Similarly, genes in the protocadherin family also display both expression and allele frequency changes during selection for tameness in foxes. Three protocadherins, *PCDH9*, *PCDH17*, and *PCDH20* all have multiple SNPs with significant allele frequency changes (adjusted $P < 0.05$) (*SI Appendix*, Table S10). *PCDHGA1*, a protocadherin gamma gene, is the second most significant differentially expressed gene between tame and aggressive fox brains (Fig. 1*B*). Remarkably, another member of the same protocadherin gamma subfamily A, *Pcdhga11,* was among the 20 genes with the highest correlations between gene expression value and tameness score in a F2 rat population (40). Comparative genomic analysis between domestic and wild cats also identified protocadherin A1 and B4 (*PCDHA1* and *PCDHB4*) under the selection peaks (39), suggesting a shared role of protocadherins in tame phenotypes across multiple mammalian species.

Among the candidate domestication genes identified in studies of dogs and other mammals (41, 42), *ITGA6* was also found to have significant allele frequency changes in three informative SNPs in our fox study, which also implicates a role for cell surface adhesion and signaling molecules in behavior. A QTL and transcriptome study using an F2 population of two outbred rat lines selected for tameness and aggression identified four candidate genes for the behavioral difference (40). Five criteria were applied to determine these genes' involvement in tameness. The top two candidate genes, *Gltscr2* and *Lgi4*, were significant for all five criteria, and both have informative SNPs in our fox data. Two synonymous coding SNPs in *Lgi4* both showed significant allele frequency differences at an adjusted $P < 0.05$ (*SI Appendix*, Table S11). Two nonsynonymous and three synonymous SNPs were found in *Gltscr2*, and they were marginally significant, with an allele frequency difference of 0.375 (adjusted $P = 0.10$) (*SI Appendix*, Table S11). In summary, selection for tame/aggressive phenotypes in different mammals can lead to expression and genetic changes in genes in the same pathways (43).

Charles Darwin, along with many others, observed that selection for domestication in mammals often leads to a collection of phenotypes including shortened snout, curly tail, white spotting of fur on the chest, and floppy ears, often referred to as the "domestication syndrome." These features all seem to occur in tissues that are derived from neural crest cells, suggesting that the process of selection for domestication impacts neural crest cell function (44). Intriguingly, several of the genes that manifested significant allele frequency changes in our tame foxes may play a role in neural crest cell fate (45). Wnt signaling plays a key role in initial neutral crest cell differentiation, and both *Wnt3* and *Wnt4* in the fox have more than one SNP with significant allele frequency changes. Protocadherins are also important in neural crest cell function. Direct assessment of whether these genes play roles in neural crest cell function in the fox presents an interesting experimental challenge.

Materials and Methods

More detailed information on the materials and methods used in this study are provided in *SI Appendix, Materials and Methods*. Brain tissue samples were collected from adult foxes maintained at the ICG's experimental farm in Novosibirsk, Russia. The study was approved by the Institutional Animal Care and Use Committees of Cornell University and the University of Illinois at Urbana–Champaign. RNA-seq was performed on total RNA samples extracted from prefrontal cortex and the rostral part of the basal forebrain from 12 foxes in the tame population and 12 foxes in the aggressive population. Genes that were differentially expressed between tame and aggressive individuals in the two brain tissues were detected using the edgeR

package in Bioconductor (46, 47) at a 5% FDR level ($q < 0.05$). Normalization and expression level estimation (fragments per kilobase of transcript per million mapped reads) were also calculated using edgeR. To confirm the RNA-seq results, we performed a qRT-PCR experiment on selected candidate genes in all 48 individual samples with two independent technical replicates (*SI Appendix*, Fig. S7). To estimate allele frequencies in each population, we called 31,025 high-quality exonic SNPs after stringent quality filtering. To determine the statistical significance of the allele frequency differences between the tame and aggressive populations, gene dropping simulations were performed to generate a null distribution assuming no correlation between the SNP and the behavioral trait.

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