NOCT, a potential domestication gene impacting circadian rhythm and behaviors during dog domestication

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ABSTRACT

As the only domesticated carnivore, how proto-domestic canids first adapted to human society at the onset of domestication remains a central enigma in the history of animal domestication. While previous studies have extensively characterized the behavioral transformation during domestication, the circadian shift from the crepuscular rhythms in grey wolves to diurnal patterns in dogs has received limited attention, despite its critical role in adapting to human activity cycles. Here we investigated the highly diverged genes associated with circadian system reorganization, a potential domestication trait preceding the conscious human selection. Our findings identified *NOCT* as a candidate domestication gene with distinct indel frequency shifts from grey wolves to dogs. Functional analyses revealed that reduced *NOCT* induced circadian rhythm disruption, accompanied with enhanced myelin sheath formation in the prefrontal cortex. These physiological alterations led to profound behavioral changes, including prolonged exploratory locomotion, heightened fear/anxiety response, and

improved short-term memory. Collectively, these behavioral changes provide empirical support for the self-domestication hypothesis, suggesting how ancient scavenging wolves may have gradually adapted to human environments. Our study provides new insights into the evolutionary mechanisms driving the successful domestication of the sole carnivore companion species.

Keywords: Dog; Domestication; Circadian rhythm; Behaviors

INTRODUCTION

Dogs (*Canis lupus familiaris*) are the sole carnivore species to have undergone successful domestication, tracing back to grey wolves (*Canis lupus lupus*) during the Upper Paleolithic period (Bergström et al., 2020; Frantz et al., 2016; Freedman & Wayne, 2017; Wang et al., 2016). They experienced remarkable behavioral transformation from fear-aggressiveness to tameness during the initial stage (Wang et al., 2014), accompanied by the development of specialized socio-cognitive adaptations for human interaction (Hare et al., 2002; Topál et al., 2009) Massive studies have revealed rapid evolution of numerous genes involved in nervous system (Li et al., 2013; Pendleton et al., 2018; Sutter & Ostrander, 2004; Zhang et al., 2020), which underlie the behavioral transformation and subsequent breeding process. These candidate genes should be selected gradually during the domestication, which remain a controversial question about the primary target that was selected in the initial stage.

Besides the prominent behavioral changes observed in dogs, their circadian rhythm also differ distinctly from those of other canine species. Notably, wild canids --- including red foxes, arctic foxes, arctic wolves, and grey wolves --- exhibit predominantly nocturnal or crepuscular activity patterns (Campbell & Tobler, 1984; Gittleman, 1986), whereas dogs uniquely display a classic diurnal rhythm. This chronobiological reorganization may represent an evolutionary compromise to synchronize with human activity cycles (Bódizs et al., 2020), potentially enhancing alertness to facilitate socio-ecological adaptation with greater attention to human cues (Kortekaas & Kotrschal, 2019). However, compared to other domestication traits like behavioral transformation, this circadian change has been largely overlooked. One of our studies, based on microarrays with very limited single nucleotide polymorphisms (SNPs), identified a circadian clock gene *NOCT* (*CCRN4L*) that exhibited extreme allele frequency differentiation between wolves and non-breed dogs (Li et al., 2013). *NOCT* is a clock-controlled widely expressed gene involved in development, adipogenesis, lipid metabolism, inflammation, osteogenesis, and obesity (Kulshrestha & Devkar, 2023). However, the underlying genetic basis and subsequent impact of this circadian change remains largely unknown.

Notably, circadian rhythm shift from nocturnal to diurnal patterns in laboratory rats has been evidenced with increased activities (Stryjek et al., 2013), which was a hypothesized prerequisite that enhanced the first interaction between wolves and humans (Trut et al., 2009). Furthermore, emerging evidence positioned circadian regulation as a central modulator of neuroplasticity and cognitive functions (Gerstner & Yin, 2010; Lehr et al., 2021; Reid et al., 2011; Walker

& Stickgold, 2006) --- key determinants of dog social specialization (Hare et al., 2002; Topál et al., 2009). Collectively, these studies provide a promising alternative for decoding the evolutionary enigma of successful canine domestication in the perspective of circadian system reorganization.

Nearly all previous studies on dog domestication that mentioned above focus on SNPs. While several researches have explored indels and/or structure variations associated with morphylogical traits (Axelsson et al., 2013; Meadows et al., 2023; Plassais et al., 2019; Serres-Armero et al., 2021), no study have specifically investigated indels associated with circadian changes. Considering the less impact of SNPs on biological functions relative to indels (Kronenberg et al., 2018), we integrated 365 genomes from globally distributed canine individuals to identify any chronotype-associated indels that diverged specifically in dogs.

Population comparison identified *NOCT* as a promising target associated with circadian rhythm that may be selected during primary domestication. Complemented this genomic analysis with transcriptomic and behavioral analyses, we investigated how *NOCT* expression reduction influenced circadian rhythm, locomotion, fear response, and memory, which are hypothesized core features during the domestication onset.

Collectively, understanding the pleiotropic mechanisms, through which *NOCT*-mediated chronobiological reorganization reshaped proto-domestic canid behavior, will deepen our understanding about the genetic changes occurred during dog initial domestication and provide more clues on the evolutionary mechanisms driving the successful domestication of the sole carnivore.

MATERIALS AND METHODS

Canid genomic analysis

Samples collection: A total of 381 canid genomes were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/sra/). After quality filtration, 365 genomes were remained, including 35 grey wolves, 5 outgroups of domestic dogs (3 Coyote/1 golden/1 Andean fox), and 325 domestic dog samples (Supplementary Table S1).

Raw Data Quality Control: First, check the file format and basic statistical information of the original data. Next, use FastQC to assess sequence quality, including the distribution of base quality, sequence length, and base content. Then, employ Cutadapt to remove adapter sequences and low-quality reads. Subsequently, utilize Picard to detect and handle duplicate sequences. Finally, align the data to the reference genome as required.

Read alignment and InDels calling: All clean reads were aligned to the dog reference genome (CanFam3.1; GCA_000002285.2) using BWA (v.0.7.17, settings: mem -t 4 -k 32 -M -R). The duplicate reads were removed using Picard Tools (v.1.119). InDels were detected using GATK (v.4.0) with HaplotypeCaller method. We filtered the raw InDels using following criteria: (1) QD<2.0 || FS>200.0 || InbreedingCoeff<-0.8 || ReadPosRankSum<-20.0 || SOR>10.0; (2) Biallelic sites; (3) Overall depth (for all individuals) was>1/3× and <3×; (4) sites with missing rate<20%; (5) Individual with

variant missing rate<10%. Finally, the clean InDels were annotated by ANNOVAR.

Population Structural analysis

For the phylogenetic analyses, we first calculated the p-distance matrix using VCF2Dis (https://github.com/BGI-shenzhen/VCF2Dis), the NJ tree was built using R packages ape and visualized by Newick Utilities packages. We performed principle component analysis (PCA) analyses using PLINK (v.1.9, parameters: --bfile pca --pca 3 --chr-set 90). ADMIXTURE (v.1.3) was used to inferred population genetic structure.

Selective sweep analysis

We calculated the genome-wide distribution of F_{ST} and nucleotide diversity θπ with a sliding-window approach (20 kb windows and 10 kb steps) using VCFtools (v.0.1.17). θπ ratio was estimated as the quotient of wolf_θπ divided by dog_θπ. The windows with top 5% values of log2(θπ ratio) and F_{ST} were considered as putative selection targets and visualized in the R package. GO and KEGG analysis using the KOBAS and DAVID.

Canid transcriptome analysis

The genomic expression data from 10 different dog tissues (Affymetrix Canine Version 2.0 array) were downloaded from Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) with accession number GSE20113. According to the chip annotation, probes that might hybridize to multiple loci across the genome were discarded. The expression value of each gene was averaged across four biological replicates. The RNA-seq data from the frontal cortex of the wolf and the dog were from Albert et al (Albert et al., 2012). Tophat (Trapnell et al.(Trapnell et al., 2009)) and Cufflinks (Trapnell et al.(Trapnell et al., 2010)) were used to assemble transcripts and calculate the expression value of genes.

Functional validation of candidate gene

Animal Ethics

This study strictly adhered to national and international ethical guidelines for the use of laboratory animals. All experimental procedures involving animals were reviewed and approved by Institutional Animal Care and Use Committee, Yunnan University (IACUC, YNU), Protocol Approval Number: Yuncae20200302.

Animal model

Transgenic mouse model: The *NOCT* conditional knockout transgenic mouse model was designed and delivered by Shanghai Model Organisms Center, Inc. Using CRISPR/Cas9 gene editing technology, a conditional knockout transgenic mouse model was constructed by targeting the Noct-201 (ENSMUST00000023849.14) exon 3 of the *NOCT* gene in the fertilized eggs of mice through homologous recombination. Specifically, Cas9 mRNA and gRNA were obtained through in vitro transcription, and a donor vector containing a 2.9 kb 5' homologous arm, a 1.6 kb flox region, and a 3.6 kb 3' homologous arm was constructed by In-Fusion cloning. The Cas9 mRNA, gRNA, and donor vector were microinjected into the fertilized eggs of C57BL/6J mice to obtain the homologous recombinant mice. Finally, after mating with the Cre recombinase-expressing mice, the flox region of the flox homozygous and Cre-positive mice was knocked out, resulting in functional deletion of the target gene in specific tissues and cell types.

AAV virus model: The AAV viral vector (CAP.B10) for *NOCT* gene interference was commissioned by Obio Technology (Shanghai) Co, Ltd. The viral target site was consistent with the design plan of the transgenic mouse, which was on exon 3 of the *NOCT*-201 transcript. After intravenous injection of AAV, the virus specifically bound to the mouse brain tissue, reducing the expression level of *NOCT* gene, thereby obtaining the desired mouse model. The model design targeted the shRNA interference fragment of Mouse *NOCT*. Through molecular biological methods, the interference fragment was constructed into an adeno-associated virus vector, which could achieve interference with *NOCT* gene expression. The vector could also package adeno-associated virus to interfere with *NOCT* gene expression at the animal level. The viral vector used green fluorescent signal.

The mice were housed under a standardized 12:12-hour light-dark cycle (lights on at 8 AM, corresponding to zeitgeber time ZT0). Behavioral assays were mainly conducted at 9-12 AM (ZT1-4), with a few supplementary sessions before 6 PM (ZT10). To control for potential circadian confounders, experimental and control groups were tested in parallel using independent equipment, ensuring temporal matching across groups.

For molecular analyses, tissues were collected synchronously within the time frame of 9-12 AM (ZT1-4) to minimize variability associated with circadian fluctuations in gene expression and protein level. Mice were euthanized via cervical dislocation (approved by the institutional animal ethics committee) to minimize tissue stress and RNA degradation. Immediately after dissection, cerebral frontal cortex tissues were immersed in RNAlater ™ (Thermo Fisher Scientific) to stabilize RNA. Samples were stored at 4℃ overnight to allow complete penetration of the preservation solution, then transferred to -80℃ for long-term storage until RNA extraction.

Quantitative real-time PCR

Quantitative real-time PCR (RT-PCR) of mice frontal cortex was performed using a TB Green Premix Ex Taq Ⅱ (Takara, Japan) according to the manufacturer's instructions and results were analyzed using the QuantStudio 5 system (ABI, USA) with the two-step method. We used β-actin as a reference gene and quantified the expression of target genes using the $2^{-\Delta\Delta CT}$ method. Primer sequences used in this study include 5' CGGTGCTCGGATCTCG 3' and 5' TGCTGTTGACGGTCTTGG 3' for *NOCT* (Primers spanned exon 1 and exon 2 regions); and 5' CGTTGACATCCGTAAAGACC 3' and 5' AACAGTCCGCCTAGAAGCAC 3' for β-actin. The PCR conditions are as follows: 10 min at 95°C for DNA denaturation, followed by target cDNA amplification (40 cycles at 95°C for 10s and 30s at 60°C).

Western blot

Whole brains were firstly extracted from the mice. The cerebral frontal cortex region was then carefully dissected, homogenized in lysis buffer RIPA (Sigma, USA) containing phenylmethylsulfonyl fluoride (PMSF) (Thermo, USA) for protein extraction and centrifuged at 10 000×*g* for 10 min at 4°C. The supernatant was removed and stored at −80°C for future use.

Quantification of total protein was conducted using Enhanced BCA Protein Assay Kit (Beyotime, China). Samples were denatured in boiling water for 10 min in sample buffer (Takara, Japan). A total of 20μg of protein from each sample was separated using 15% sodium dodecyl-sulfate

polyacrylamide gel electrophoresis (SDS-PAGE). The resolved proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Amersham, England), which was then blocked with 5% mil in Tris-buffered saline with 0.1% Tween-20. Primary antibodies, including anti-*NOCT* (~48 Kda) (1:1000; Abcam, UK) (rabbit-derived), and secondary antibodies goat anti-rabbit (1:1000, Beyotime, China), were used to detect the corresponding proteins.

Observation of mouse rhythm

When mice step into adolescent period (6-week/18-month), they were separately left through the whole experimental period. Upon arrival in the vivarium, the mice were left undisturbed for one week. Then use a camera to record all mouse movements (5 male young and 5 male old), including sleep time, activity time, and rest time, and make statistics. There were two different lying behavioral conditions: (1) inactive wakefulness: animal is lying, head in an upward position with eyes opened, (2) resting: animal is lying, head in downward position with eyes mainly closed. We judged the former as wakeful condition and the later as sleep condition, according to previous judgement (Kortekaas & Kotrschal, 2019).

The design of behavioral experiments

When mice step into adolescent period, they were separately left through the whole experimental period. Upon arrival in the vivarium, the mice were left undisturbed for one week. They were then gently handled on three consecutive days. The mice were tested in the EPM for 5 min, and the following day they were tested in the OFT for 30 min (Knight et al., 2021). (Experiments were independently conducted in female and male mice.)

Behavioral experiments

Open Field Test: Increased diurnally exploratory locomotion likely enhanced the primary interactions between ancient humans and scavenging wolves under the self-domestication hypothesis (Hewson, 2003; Li et al., 2014; Tancredi & Cardinali, 2023). We then assessed whether there were changes of diurnally exploratory activities via Open Field Test.

The open field apparatus consisted of a clear, open plexiglas box (50cm long × 50 cm wide × 50cm high, Shanghai Xinruan Informatlon Technology Co. Ltd, shanghai, China) fitted with an overhead camera and photo beams to record movements. We divided the open field apparatus into 9 areas, 8 corner areas and 1 central area. Each mouse was placed in the central area of the opening device to start the experiment. Total distance traveled and average moving velocity were quantified over a 30-min period by SuperMaze. The experiment was conducted at room temperature during the daytime (9:00-12:00) ($NOCT^{flox/flox}$: experimental group (males n=17, females n=17), $NOCT^{wt}$: control group (males n=8, females n=8); $NOCT^{AAV}$: experimental group (males n=11, females n=11), $NOCT^{ctrl}$: control group (males n=10, females n=10)).

Elevated Plus Maze: Weakened fear response could shorten the distance from human presence and was assumed to be the prerequisite of dog domestication under the tame-selection hypothesis (Belyaev, 1969; Trut et al., 2009). We then assessed whether there were changes of fear-related behaviors via the Elevated Plus Maze that relied upon the unconditioned fear of heights/open spaces (File et al., 1993; Handley & Mithani, 1984; Hilton et al., 2023; Mendes-Gomes et

al., 2011; Walf & Frye, 2007), despite mixed anxiety-like behavior.

The elevated plus maze consists of two open arms (35cm long x 5cm wide) and two closed arms (35cm long x 5cm wide) extending 50 cm above ground from the central platform (5cm long x 5cm wide) (Shanghai Xinruan Informatlon Technology Co. Ltd, shanghai, China). Each mouse was placed in the center of the elevated plus maze central platform. The mice were placed alone in a central square and allowed to explore freely for five minutes. Data on the animals' movements were recorded using an overhead camera. The experiment was conducted at room temperature during the daytime (9:00-12:00) ($NOCT^{flox/flox}$: experimental group (males n=11, females n=11), $NOCT^{wt}$: control group (males n=9, females n=9). $NOCT^{AAV}$: experimental group (males n=11, females n=11), $NOCT^{ctrl}$: control group (males n=10, females n=10)).

Y-Maze: The self-domestication hypothesis suggests that scavenging wolves with better learning and memory abilities would have more probability to be domesticated (Li et al., 2014). We then assessed whether there were changes on contextual memory ability via Y-Maze.

The Y-Maze is a symmetrical closed 3-arm maze, shaped like a capital letter "Y", with each arm forming a 120 ° angle and each arm having a movable gate valve to restrict animal activity (21cm long × 7cm wide × 15.5cm high, Shanghai Xinruan Informatlon Technology Co. Ltd, shanghai, China). Three arms can be randomly set as: novel arm, start arm, and other arms. The activity data of experimental animals were recorded using overhead cameras. The experiment was conducted at room temperature during the daytime (9:00-12:00) ($NOCT^{flox/flox}$: experimental group (males n=6, females n=6), $NOCT^{wt}$: control group (males n=8, females n=8)).

Brain tissue section staining: The fresh brain tissue was removed and placed in 4% paraformaldehyde fixative solution at room temperature for 24h. After the formaldehyde fixative completely penetrated into the tissue, the tissue was transferred to 10%-20%-30% sucrose for gradient dehydration. After dehydration, the fixed dehydrated tissue was infiltrated with OTC (Sakura, Japan) embedding agent for 30min and then put into -80°C refrigerator for quick freezing to complete embedding. The tissue was cut into 10 microns slices using a frozen microtome and stored in a -80°C refrigerator.

Myelin staining: The tissue sections were removed from the -80 refrigerator, dried at 37℃ for 20 min, fixed with 4% paraformaldehyde for 20 min, wash 3 times for 5 minutes each time with 1×PBS buffer, dyed with FluoroMyelin™ Red dye for 20 min, cleaned wash 3 times for 5 minutes each time with 1×PBS buffer. Finally, anti-fluorescence quenching tablets containing DAPI were used to seal the tablets. The experimental and control groups each included three mice for sectioning and staining, with sections taken from the cerebral cortex region (Figure 5).

Mitochondrial determination:

Mitochondrial extraction kit (Beyotime, China) was used to extract mitochondria from brain tissue, and then mitochondria were broken to extract mitochondrial protein (Acín-Pérez et al., 2023). Finally, mitochondrial protein content was quantified, and mitochondrial protein content represented mitochondrial content.

Transcriptome analysis

Raw RNA-seq reads were subjected to quality assessment using FastQC (v.0.11.9) with aggregated reports generated by MultiQC (v.1.9). Adapters and low-quality bases were trimmed using Trimmomatic (v.0.39). Processed reads were aligned to the reference genome (GRCm37) using STAR (v.2.7.10b) in two-pass mode with default splicing parameters. Transcript abundance estimation was performed by RSEM (v.1.3.3) with expectation-maximization algorithm, generating TPM and FPKM values. Differentially expressed genes (DEGs) were identified using DESeq2 (v.1.30.1) with |log2FC|>1 and FDR-adjusted *P*-value<0.05. Gene Ontology (GO) enrichment was analyzed KBOAS/DAVID. Weighted Gene Co-expression Network Analysis (WGCNA) was performed using the WGCNA package with soft-thresholding power determined by scale-free topology criterion.

Statistical analysis

GraphPad Prism (version 8.4.0, GraphPad Software, San Diego, CA) and R (4.2.3) was used for all statistical analyses and to create all plots. Differences between the control group and experimental group were analyzed using Student t-test. Statistical significance was indicated as follows: *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$.

RESULTS

Adaptive evolution of indels at the *NOCT* locus and their potential impact on expression reduction

A domestication-related trait should be seen in non-breed domesticated populations, and if there are no non-breed populations, traits seen across all breeds may potentially be domestication related (Lord et al., 2020). Accordingly, we retrieved previously published resequencing genomes from 365 canids containing 112 non-breed dogs, 213 dogs representing 110 breeds, 35 Eurasian grey wolves, and 3 coyotes/1 golden jackal/1 Andean fox as outgroups (Supplementary Table S1).

Based on a total of 7,530,193 indels detected, dogs were clearly distinguished from their closest wild relative (grey wolves) by both phylogenetic reconstruction through neighbor-joining method (Figure. 1A) and population structure assessment using non-parameter principal component analysis (PCA) (Figure. 1B) and Admixture (Figure. 1C). Population comparison identified 1642 genes with high fixation index (F_{ST}) (Top-5%) and 2103 genes with low nucleotide diversity (π) (Top-5%), whereby 754 genes were shared. Among the 754 selective candidate genes, eight belongs to circadian rhythm category (GO: 0007623), including *KCNH7*, *LEP*, *MYCBP2*, *NCOR1*, *NOCT*, *RAI1*, *SREBF1*, and *TOP1* (Figure. 1D, E). We then assessed their expression pattern across 10 different dog tissues and brain frontal cortex tissue from 5 dogs and 6 wolves. Only *NOCT* exhibited: 1) highest expression in the dog brain cortex compared to 11 other tissues; 2) significantly lower expression in dog brain versus wolf brain (student's T test, $P=0.0017$; Figure. 1F, G). The remaining eight genes showed neither brain-biased expression nor differential expression between dogs and wolves (Supplementary Figure. S1). Moreover, *NOCT* directly regulates post-transcription of circadian clock components and circadian control of many metabolic processes (Stubblefield et al., 2012), whereas the other genes primarily function downstream (e.g., in metabolism, neuronal activity) rather than

in rhythm generation (Supplementary Table S2). Additionally, cell-specific knockdown has suggested that *NOCT* was required presynaptically to mediate motor axon pathfinding (Lobb-Rabe et al., 2022) and impact behavior (Chen et al., 2018). Accordingly, we prioritized *NOCT* to investigated how indels might influence its expression and consequently behavioral determination.

Three indels surrounding *NOCT* exhibited significant allele frequency shifts from the wolves to the dogs, whereby the derived alleles were completely absent in the outgroups (Figure. 1H). Two located in the first intron (chr19:3593363 with TCC insertion and chr19:3602081 with AACT deletion) showing near-complete linkage disequilibrium from each other (r^2=0.90). The rest one is a TG deletion ~6 kb downstream (chr19:3586513). Regions harboring these indels overlapped with several predicted transcription factor binding sites (Supplementary Table S3), implying potential regulatory roles. The brain-biased expression of *NOCT* is probably controlled by multiple enhancer elements, a phenomenon termed enhancer redundancy (Spielmann et al., 2018), which may obstacle the consequence of the two intron indels. For simplicity, we explored the regulatory effect of the downstream deletion through dual fluorescence assay in MDCK (dog kidney) cells. Constructs carrying the downstream TG deletion drove significantly lower reporter expression compared to controls (either upstream-deletion or intact sequences) (Figure. 1I). This reduction aligned with the observed down-regulation of *NOCT* in dog brains relative to wolves, supporting a potential causal role of this indel in modulating *NOCT* expression.

NOCT knockdown mice exhibiting increased daytime wakefulness

Multiple assays on *NOCT* knockout mice have indicated a robust role in energy metabolism and circadian outputs (Green et al., 2007; Le et al., 2019; Stubblefield et al., 2012). To explore whether reduced *NOCT* expression in nocturnal animal could change behaviors and facilitate adaptation to anthropogenic environments, we employed the Cre-dependent CRISPR/CAS9 system to generate *NOCT* conditional knockout mice ($NOCT^{flox/flox:Cre+}$). We chose C57BL/6J mice strain as nocturnal model, which was justified by the 24-hour change pattern of total aqueous humor protein (Zhou & Liu, 2006). LoxP sites were inserted flanking the third exon, creating the $NOCT^{flox/flox}$ allele. The Cre recombinase was expressed specifically in the central nervous system using the nestin-Cre driver.

Prior to maturation (from birth to 100 days), no phenotypic differences were apparently observed among $NOCT^{wt}$, $NOCT^{flox/flox}$, and $NOCT^{flox/flox:Cre+}$ mice. However, adult $NOCT^{flox/flox:Cre+}$ mice (~120 days) frequently exhibited abnormal postures and locomotor behaviors, such as slowed movement (Supplementary Video 1). The condition deteriorated with age (>280 days), performing Parkinson-like syndrome in 13.89% of the subjects without sexual bias (Supplementary Video 2). This was obviously a disadvantage of fitness in the wild nature. Conversely, $NOCT^{flox/flox}$ mice were less susceptible to such abnormalities, with only 2.78% of senecence mice displaying head-tilt syndrome. Moreover, $NOCT^{flox/flox}$ mice consistently exhibited stronger locomotory activities compared to $NOCT^{wt}$ (Supplementary Video 3 as an example of 60 days). We further

investigated whether there were alterations of the sleep-wake cycle between *NOCT*$^{flox/flox}$ and *NOCT*^{wt} in sexually mature (6-week) and senescent age (18-month), respectively. While no differences in the average sleep duration per hour over the 24-hour period were observed in both ages (Student t test, P=0.0571 and 0.6783 for 6-week and 18-month; Figure. 2A, B), *NOCT*$^{flox/flox}$ mice kept wakeful significantly more during the 8:00-20:00 period compared to *NOCT*^{wt} mice (Student t test, P<0.0001 and 0.028; Figure. 2C, D), particularly around 14:00 for young age (Student t test, P=0.0003; Figure. 2E) and around 15:00 for senescent age (Student t test, P<0.0001; Figure. 2F). In concordant with these changes of sleep duration, *NOCT*$^{flox/flox}$ mice exhibited decreased nocturnal activity (walking around) and increased diurnal activity compared to *NOCT*^{wt} mice (Supplementary Figure. S2A-B).

This paralleled the behavioral changes observed in laboratory rats that transformed from nocturnal to diurnal activity patterns (Stryjek et al., 2013). Real-time PCR (Figure 2G) and Western blot experiments (Figure. 2H, I) consistently demonstrated decreased *NOCT* expression caused by the integration of loxP sites flanking the third exon in *NOCT*$^{flox/flox}$, which was further validated by the transcriptome of brain cortex (Figure. 2J). This reduction was comparable to the expression changes between dogs and wolves (Figure. 1G), Subsequently, we conducted multiple behavioral assays to examine the behavioral implications of *NOCT* reduction by comparing *NOCT*$^{flox/flox}$ mice and *NOCT*^{wt}.

NOCT reduction increasing diurnal exploratory locomotion

Increased diurnally exploratory locomotion likely enhanced the primary interactions between ancient humans and scavenging wolves under the self-domestication hypothesis (Hewson, 2003; Li et al., 2014; Tancredi & Cardinali, 2023). Accordingly, we investigated whether there were changes of diurnally exploratory activities between *NOCT*$^{flox/flox}$ and *NOCT*^{wt} in adolescent age (4-8 weeks after birth, i.e., 28-56 days). In the open field test (OFT) conducted during the daytime (9:00-18:00 with matched comparison), *NOCT*$^{flox/flox}$ mice exhibited more locomotion that covered a greater distance (Figure. 3A, t=3.631, df=50, P=0.006) and spent more resident time in the central area (Figure. 3B, t=2.226, df=50, P=0.0337), compared to the *NOCT*^{wt} mice. This elevation consistently appeared in *NOCT*$^{flox/flox}$ mice without sexual bias (Supplementary Figure. S3A, B). Furthermore, the exploratory locomotion of *NOCT*^{wt} mice significantly decreased in adulthood (60-90 days), whereas *NOCT*$^{flox/flox}$ mice maintained or even increased their exploratory behavior in the same age (Figure. 3C).

Considering the potential importance of locomotion in the initial stage of dog domestication, we further employed AAV interference to assess the impact of *NOCT* reduction. An engineered adeno-associated virus (AAV) combined with a green fluorescent protein (GFP) tag was used to decrease *NOCT* in the central nervous systems of 4-week-old (28-day) mice by caudal intravenous injection(Challis et al., 2019; Li et al., 2019), referred to here as *NOCT*^{AAV} mice. As a control group, the same AAV vector containing a blank tag was injected into the control mice (*NOCT*^{ctrl}). Although *NOCT*^{AAV} mice showed no differences from *NOCT*^{ctrl} mice

one week after injection (Supplementary Table S4; Figure. 3D, t=0.1009, df=42, *P*=0.9201), *NOCT*^{AAV} mice exhibited significantly more locomotion and spent more time in the central area compared to *NOCT*^{ctrl} mice four weeks after injection (Supplementary Table S4; Figure. 3E, t=3.293, df=42, *P*=0.0021). Brain cortex staining showed that *NOCT* in the brain was mainly expressed in the cerebral cortex (Supplementary Figure. S4A). Examination of mRNA and protein revealed a significant reduction of *NOCT* four weeks after injection (Supplementary Figure S4B, C, D). As the effect of the virus mitigated six weeks post-injection, illustrated by the virus activity (Supplementary Figure. S4E), this difference gradually weakened (Supplementary Table S4; Figure. 3F, t=0.7209, df=42, *P*=0.4765).

NOCT reduction enhancing fear/anxiety-related sensitive behavior

Weakened fear response could shorten the distance from human presence and was assumed to be the prerequisite of dog domestication under the tame-selection hypothesis (Belyaev, 1969; Trut et al., 2009). We assessed the fear-related behaviors between *NOCT*$^{flox/flox}$ and *NOCT*^{wt} mice (6-week) via the elevated plus maze that relied upon the unconditioned fear of heights/open spaces (File et al., 1993; Handley & Mithani, 1984; Hilton et al., 2023; Mendes-Gomes et al., 2011; Walf & Frye, 2007), despite mixed anxiety-like behavior. We considered the performance of the first trial only, due to the commonly observed decrease of exploration in the open arms during re-exposure to the maze (Shoji & Miyakawa, 2021). *NOCT*$^{flox/flox}$ mice spent significantly more resident time in the enclosed arm (Figure. 3G; t=3.3, df=38, *P*=0.0021) and less resident time in the open arm (Figure. 3H; t=3.248, df=38, p=0.0024) than *NOCT*^{wt} mice, indicating stronger fear behavior. In addition, the equal, even less time spent in the central platform in *NOCT*$^{flox/flox}$ mice (19.54% for *NOCT*$^{flox/flox}$ and 16.19% for *NOCT*^{wt}) roughly precluded reduced exploration and indicated equal, even less anxiety level in *NOCT*$^{flox/flox}$ mice, as illustrated in rats (Casarrubea et al., 2016).

Parallelly, we observed this enhanced fear-related sensitive behavior in *NOCT*^{AAV} mice (7-week; 3 weeks post-injection) compared to *NOCT*^{ctrl} mice (Figure. 3I, t=3.935, df=42, *P*=0.0003). Meanwhile, *NOCT*^{AAV} mice spent less time in the central platform (8.32% for *NOCT*^{AAV} and 15.66% for *NOCT*^{ctrl}), further confirming the influence of *NOCT* reduction in fear response. Again, this difference of fear response between *NOCT*^{AAV} and *NOCT*^{ctrl} mice alleviated six weeks after virus injection (Supplementary Figure. S3E, t=1.572, df=42, *P*=0.9025).

NOCT reduction improving short-term memory

The enhanced fear response appeared in *NOCT* knockdown mice opposed to the tame-selection hypothesis, whereas the increased exploratory locomotion supported the self-domestication hypothesis. Following the self-domestication hypothesis, scavenging wolves with better learning and memory abilities would come close to human settlements more frequently, acquire greater food resources, and thus had greater opportunities to survive (Li et al., 2014). Besides, circadian rhythm is strongly implicated in plasticity, cognition, learning, and memory. Accordingly, we assessed the performance of short-term memory between *NOCT*$^{flox/flox}$ and *NOCT*^{wt} mice (6-week)

through Y maze experiments. *NOCT*$^{flox/flox}$ mice showed more residence time in the food arm compared to *NOCT*^{wt} mice (Figure. 3J, t=2.781, df=28, *P*=0.0099), indicating better short-term memory (Motion trajectory, Figure. 3K). Since starvation-induced 20% weight loss pre-exposure to Y maze experiment would largely obstacle the health of the subjects, we didn't repeat the Y maze experiment in *NOCT*^{AAV} and *NOCT*^{ctrl} mice.

Transcriptome of mice with *NOCT* reduction indicating expression abnormality in myelination

We reviewed genes interacting with *NOCT*, which play roles in circadian rhythm regulation, energy metabolism, and nervous diseases (Figure. 4A). Considering that *NOCT* in the brain was mainly expressed in the cerebral cortex (Supplementary Figure. S4A), we sequenced the transcriptome from brain cortex of *NOCT*$^{flox/flox}$ and *NOCT*^{wt} with 60 days to unravel the genetic basis behind these locomotory changes in mice with *NOCT* reduction, using muscle and bone marrow that associated with movement as controls. Differentially expressed genes (DEGs) detected in muscle and bone marrow showed most overrepresentations in muscle-related and immunity-related pathways, respectively (Supplementary Table S5), whereas those in brain cortex indicated significant association with nervous system, especially myelination (Figure. 4B). Myelin regulation has been reported to sculpt circuits in learning and memory throughout the lifespan (Xin & Chan, 2020). We therefore focused on the expression changes in brain cortex for further investigation.

There were 397 DEGs detected in brain cortex, including 167 and 230 up- or down-regulated in *NOCT*$^{flox/flox}$ mice (Supplementary Figure S4F). The up-regulated DEGs showed the most overrepresentation in biological process of "myelination" (adjP=2.6e-8; Figure. 4C), whereas the down-regulated DEGs showed the most overrepresentation in "fatty acid metabolic process" (adjP=1.6e-8; Figure. 4D). In the AAV model, up-regulated DEGs of prefrontal cortex four weeks post-injection were also enriched in "positive regulation of myelination" (*P*=0.0041; Supplementary Table S6). The less significance of myelination in the AAV model might due to the differed efficiency of *NOCT* reduction, as the AAV model worked on RNA with partial interference, while the transgenic model worked on DNA with completed modification.

Considering the weakened reduction in the AAV model due to the partial interference, we further applied weighted correlation network analysis (WGCNA) to identify modules of highly correlated genes that synchronically changed expression level post-injection and assess their potential functions with myelination. Specifically, Module-greenyellow was mostly anti-associated with prefrontal cortex of *NOCT*^{AAV} (*P*=9E-6, Supplementary Figure. S5). Genes within this module were mostly enriched in "myelination" (adjP=1.7E-7) (Supplementary Table S7), consistently indicating alteration of myelination in *NOCT*^{AAV} cortex. Module-magenta was mostly associated with prefrontal cortex of *NOCT*^{AAV} (*P*=2E-5, Supplementary Figure. S5), with overrepresentation of "glutamatergic synapse" (adjP=4.9356E-8) (Supplementary Table S8). The dramatic changes of myelination and synapse in *NOCT*^{AAV} mice consistently supported the currently recognition that myelin can

constrain where axons sprout and form synapses with dendrites or with other axons, and stabilize the pattern of connectivity in neural circuits (Fields, 2014).

Brain cortex staining validating morphological changes of myelin in mice with *NOCT* reduction

As expression alternation in mice with *NOCT* reduction indicated enhanced myelination, we sequentially stained the myelin sheath of brain cortex and examined potential morphological changes in the transgenic model and the AAV model. In the transgenic model, the thickness and quantity of myelin sheaths in the brain of adolescent $NOCT^{flox/flox}$ mice (6-week) were significantly greater than those of adolescent $NOCT^{wt}$ mice (Figure. 5A). As age increased (18-month), the myelin sheath gradually disappeared in senescent $NOCT^{wt}$ mice, while it maintained more stably in senescent $NOCT^{flox/flox}$ mice (Figure. 5B). In the AAV model, we also observed greater density of myelin sheath in $NOCT^{AAV}$ mice compared to $NOCT^{ctrl}$ mice, four weeks after virus injection (with apparent viral effect) (Figure. 5C). Conversely, the difference of myelin sheath density became negligible between $NOCT^{AAV}$ and $NOCT^{ctrl}$ mice nine weeks after virus injection (with mitigated viral effect) (Figure. 5D).

No difference of mitochondrial content in brain cortex with or without *NOCT* reduction

Strengthened myelination should require more energy supply. As the main energy producer, mitochondrion was also the terminal that *NOCT* targeted to (Estrella et al., 2019). Therefore, we measured the mitochondrial content of the brain cortex with and without *NOCT* reduction through the transgenic model. In the comparison between $NOCT^{flox/flox}$ and $NOCT^{wt}$ mice with 6-week, the time when the two groups exhibited apparent behavioral changes and transcriptome alterations, we hardly found any significant difference on mitochondrial content in the brain cortex (Supplementary S6A), indicating potentially alternative energy supply. It has been proposed that myelin acted as a proton capacitor, wherein the two most abundant embedded proteins, *PLP* and *MBP*, formed a coupled system that facilitated energy storage by protons during sleep and provided a reserve during wakefulness, a way carried out by extra-mitochondrial oxidative phosphorylation (Morelli et al., 2025). Therefore, we compared the expression of myelin protein genes between $NOCT^{flox/flox}$ and $NOCT^{wt}$ mice with 42 days. Eleven genes that encode proteins participating in the myelin sheath were highly increased in $NOCT^{flox/flox}$, including *BCAS1*, *GAL3ST1*, *GJC3*, *MAG*, *MAL*, *MBP*, *MPZ*, *PLLP*, *PLP*, *PMP22*, and *UGT8A* (Supplementary Figure. S6B). Specifically, *MBP* and *PLP*, the two major proteins embedded in myelin sheath and convert protons to ATP via oxidative phosphorylation during wakefulness, were significantly up-regulated 4.38-fold (adjP=0.0060) and 3.12-fold (adjP=0.0099) in $NOCT^{flox/flox}$ mice, respectively (Supplementary Figure. S6B).

DISCUSSION

This study integrated genomic, transcriptomic, and functional analyses to explore circadian links to dog domestication. We identified a highly diverged indel that reduced the expression of a clock-controlled gene *NOCT*; this reduction was specifically observed in the frontal cortex of dogs compared to wolves. *NOCT*-reduction mice showed disrupted circadian sleep-wake

outputs (prolonged daytime wakefulness) and enhanced frontal cortex myelination, accompanied with prolonged exploratory locomotion, heightened fear/anxiety response, and improved short-term memory. These behavioral shifts were hypothesized to facilitate dog domestication (Li et al., 2014). Below, we discuss these findings, limitations, and future directions.

The domestication traits that preceding the conscious selection

The core of animal domestication relied on the adaptation to man-driving environment. Dogs are the sole carnivore species that have gone domestication. Their adaptations to human society were accordingly concentrated into aggressive-tame behavior transformation. Two main opinions on how this transformation occurred have been debated for decades; one persisted in human-driving artificial selection on communication skills (Hare et al., 2002; Topál et al., 2009) or tameness (Belyaev, 1969; Trut et al., 2009) during the initial stage, while the other proposed self-domestication hypothesis that scavenging wolves with better learning and memory abilities would prolong exposure to human settlement and finally domesticate themselves into human culture (Li et al., 2014). Given that unconscious selection often precedes directional selection (Gittleman, 1986), investigating the unconscious changes that occurred at the onset of domestication might give valuable insight into this mystery.

Animal domestication concentrated on the adaptation to anthropogenic environments (Kruska, 2005). Therefore, the diurnal pattern is reasonably assumed to be a distinct sign of dog adaptation to human-driving environments, regarding to the crepuscular pattern in grey wolves. Our scanning on highly diverged indels stick out clock gene *NOCT* as the candidate, which confirmed our previous study based on SNPs. The two comparisons consistently identified allele polymorphism in the wolves combined with frequency shifts from wolves to dogs, rather than novel mutations originated during domestication, wherein the derived alleles were absent in more distant outgroups. This pattern was believed to be the most important way that underpinned phenotypic changes throughout the domestication (Lord et al., 2020).

***NOCT* as a candidate bridging circadian rhythm and behavior in dog domestication**

NOCT was first discovered as a clock-regulated gene in the *Xenopus laevis* retina (Green & Besharse, 1996), possessing circadian rhythmic expression with highest peak level in mice liver during the early dark phase (Wang et al., 2001). It belongs to the CCR4 family of deadenylase, which remove poly(A) tails from mRNA (Baggs & Green, 2003).

NOCT is not critical for rhythm generation by the core clock, as mice deficient for Nocturnin ($Noc^{-/-}$) do not have overt circadian phenotypes (Green et al., 2007). However, as a circadian deadenylase, it is poised to play an important role in post-transcriptional regulation of metabolic genes under circadian control (Stubblefield et al., 2012), therefore was involved in a wide range of biological functions, including lipid metabolism, adipogenesis, glucose balance, inflammatory response, bone formation, oxidative stress response, etc (Estrella et al., 2019; Green & Besharse, 1996; Kawai et al., 2010; Kojima et al., 2015; Laothamatas et al., 2020).

Since it was highly diverged in dogs during domestication, which exhibited noticeable behavioral transformation, considering its potential impact on motor neuron (Lobb-Rabe et al., 2022) and behavior (Chen et al., 2018), we therefore focused on its role in mice brain.

Here we showed that the reduction of *NOCT* expression prolonged the individual's wakefulness in daytime, which was consistent with the reduced siesta sleep (ZT3-ZT9) observed in nocte (*NOCT* homologue) mutant flies (From the American Association of Neurological Surgeons (AANS) et al., 2018). This indeed indicated a changed amplitude of sleep-wake cycle (Figure 2E, F) instead of a reversal of the phase of core clock, which was consistent with previous studies that downregulation of *NOCT* led to amplitude disruption of circadian rhythm, while the expression of canonical clock genes (e.g., *Per* and *Cry*) remains largely unaffected—a mechanism corroborated by Green CB et al.'s research (Green et al., 2007). This sensitiveness of *NOCT* expression on lights and subsequent impacts on sleep-wake cycle and behavior might consequently compromise human-driving diurnal environments.

The behavioral variation due to circadian change supporting self-domestication of primary dogs

This circadian change of prolonged wakefulness was accompanied with increased and prolonged exploratory behavior, enhanced fear/anxious behavior, and improved short-term memory; all these behavioral changes were reproducible and heritable. Strong exploratory locomotion was found in dogs with an extended juvenile period (~120 days). This is a trait related to neoteny, a common domestication syndrome, since wolves of 45-60 days showed reduced exploratory activities (Šimić et al., 2020). We observed that mice with *NOCT* reduction indeed increased exploratory locomotion and desires in adolescent age, which was continuously found in the age beyond sexual maturation. These changes mimic the differences between dogs and wolves and shall enhance the opportunity to learn and became familiar with human society, which was assumed to be a domestication trait.

The prolonged exploratory behaviors exhibited in dogs and tame foxes were explained by the reduced fear response under the tame-selection hypothesis (Belyaev, 1969; Trut et al., 2009). However, in a wild nature, the reduced fear response may be hard for these dog progenitors to survive. This inference was in concordance with our previous finding that the expression changes of fear-related genes with extreme divergence between dogs and wolves tend to enhance synapse plasticity and benefit memory ability rather than to reduce fear response (Li et al., 2014). Wolves with better learning and memory abilities would perform nonaggressive response because they would understand that the presence of humans was harmless and come close to human settlements more frequently to acquire food, which shall facilitate self-domestication. In this study, mice with prolonged exploratory behaviors did not exhibit reduced fear response (Figure. 3A, G) but improved short term memory (Figure. 3K), which highly supported our assumption that affected learning and memory abilities would facilitate the behavioral shift, prolonged exposure to humans, and helped the

individuals to be self-domesticated.

It should be cautioned that the behavior transformation of dogs at the initial stage of domestication was a very complex process. Actually, we found a paucity of genes involved in nervous system were highly diverged between dogs and wolves (Li et al., 2013; Pendleton et al., 2018; Sutter & Ostrander, 2004; Zhang et al., 2020). However, it was unreasonable that multiple genes were selected simultaneously in the very beginning of the dog domestication. Few genes with important contribution to dog behavior transformation should be selected in the initial stage, and then many genes involved in that aspect were likely to have accumulated variants by genome hitchhiking, a way observed in the domesticated gayal (Li et al., 2023). This assumption has been evidenced in human self-domestication that *BAZ1B* was a major human gene patterning the modern human face and cognitive behavior (Zanella et al., 2019). Parallely, we found that *NOCT* reduction can alter the circadian rhythm to adapt to anthropogenic environment and cause behavioral changes that facilitated the first interaction with humans. These results made it to be a potential domestication gene in the initial stage of dog domestication.

NOCT as a potential entry point into the maintaining of myelination and improving of neurodegenerative diseases

Sleep rhythm disturbance (e.g., micro-architectural sleep alterations, nocturnal sleep fragmentation, decrease in nocturnal sleep duration, diurnal napping and even inversion of the sleep-wake cycle) were commonly observed in neurodegenerative diseases such as Alzheimer's disease (Peter-Derex et al., 2015) and Parkinson's disease (Stefani & Högl, 2020), where synchronized myelin changes happened and myelin protection and renewal can improve dysfunctions in cognition and behaviors (Altinoz et al., 2021; Chen et al., 2021). Compared to poor sleeper, good sleeper also exhibited higher myelin volume fraction (Andica et al., 2024), consistently indicating associations between sleep and myelination.

In mice, sleep is polyphasic, and around two thirds of these short sleep periods occur during the light/rest phase (Vyazovskiy & Delogu, 2014). Conditional knockout of clock gene Bmal1 in mice oligodendrocyte precursor cells induced more fragmented sleep time during dark/active phase, accompanied with deficit myelination and remyelination (Rojo et al., 2023). Various studies have shown that the circadian rhythm regulates various cellular molecular mechanisms and signaling pathways involved in remyelination (Tang et al., 2025).

Here we showed that *NOCT* reduction led to less sleep time in light/rest phase and thus more sleep time in dark/active phase (Figure. 1E, F), accompanied with enhanced myelination in the prefrontal cortex from adolescence to senescence (Figure. 5A). While the lack of myelin quantitative statistics and measurement of myelin rhythmic changes proposed cautions to interpret the results, these findings predicted a potential role of *NOCT* in the development of neurodegenerative diseases via regulating myelination in a way of circadian disruption. Indeed, variations within or surrounding *NOCT* have recently been associated with Alzheimer's disease (Andrews et al., 2019; Khani et al., 2024; Scheltens et al., 2021).

While *NOCT* primarily localizes to mitochondrion (Estrella et al., 2019), which

affected neurodegenerative diseases with dysfunction of energy production (Klemmensen et al., 2024), our result found no association between mitochondrial content and the observed myelination enhancement following *NOCT* reduction (Supplementary Figure. S6A). Alternatively, the up-regulation of *MBP* and *PLP*, down-regulation of lipid metabolic process, and dense myelin sheath indicated potentially additional energy-generating mechanisms that bypass mitochondrion, like proton transmission through myelin membrane architecture (Morelli et al., 2025) and iron-mediated extracellular Fenton chemistry (Dai et al., 2025; Li et al., 2024; Wu et al., 2025).

Cautions and Perspectives

There are several limitations that warrant consideration in the present study. Firstly, functional assays targeting the intronic indels (to assess their impact on expression) would strengthen the causal link between *NOCT* and the observed phenotypic changes, providing deeper insights into the underlying regulatory mechanisms. Additionally, potential species-specific differences may exist between the canine genetic findings and the murine transgenic models, particularly in terms of evolutionary adaptations of circadian regulatory pathways and neural circuit organization responding to *NOCT* modulation. For example, brain is the tissue with highest *NOCT* expression dogs (Figure 1F), whereas it is the liver in the case of mice (Kulshrestha & Devkar, 2023). Therefore, extrapolating findings from mouse models to canine biology requires caution, and future studies incorporating canine in vivo models would help bridging this gap.

Secondly, sleep architecture is a critical readout for evaluating circadian rhythm shifts, yet the current study lacks objective assessments via polysomnographic tools such as electroencephalography (EEG). Without direct quantification of sleep-wake cycles, sleep stages, and their temporal dynamics, our understanding of how *NOCT* modulation influences rhythmic behaviors remains indirect. Complementary analyses of diurnal expression profiles of *NOCT* and core clock genes, aligned with sleep-wake cycles, would further clarify these relationships.

Thirdly, the scope of our transcriptomic analyses is constrained by the availability of canine brain region-specific data, limiting our investigations primarily to the cerebral cortex. As the suprachiasmatic nucleus (SCN) within the hypothalamus serves as the master circadian pacemaker, and the hypothalamus integrates rhythmic signals across physiological systems, the absence of transcriptomic data for these key regions hinders a comprehensive understanding of NOCT's role in canine circadian regulation. Detailed diurnal expression profiles of *NOCT* and other rhythm-related genes in these regions would provide critical context for interpreting interspecific differences in basal expression levels between dogs and wolves.

CONCLUSION

By intersecting experimental data with genome population comparison between dogs and wolves, we found significant frequency shifts of three indels within a circadian gene *NOCT* during domestication, whose expression reduction altered circadian rhythm and caused behavioral changes that shall facilitate the initial self-domestication of the primary dogs. Our results also provided a promising

therapeutic approach of maintaining myelin via the regulation of circadian rhythm to alleviate impairments of neurodegenerative diseases. Future research should focus on expanding analyses to key circadian brain regions (e.g., SCN, hypothalamus), incorporating EEG-based sleep assessments, and validating findings in canine models. Such efforts will deepen our understanding of how circadian rhythm changes contributed to dog domestication.

DATA AVAILABILITY

The transcriptome data generated in this study have been deposited in the Genome Sequence Archive (GSA) at China National Center for Bioinformation database (https://ngdc.cncb.ac.cn/gsa; accession number GSA: CRA028553), Science Data Bank (https://www.scidb.cn; DOI: 10.57760/sciencedb.j00139.00265), and NCBI Sequence Read Archive (https://submit.ncbi.nlm.nih.gov/subs/sra; BioProjectID: PRJNA1298411).

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

J.L., Y.P.Z., and Y.L. designed research; J.X., Y.S. and Y.L. performed research; J.X., X.L.T., W.H.Y., Z.G., J.L., and Y.L. analyzed data; J.X., and Y.L. wrote the paper; J.L., and Y.P.Z. revised the paper. All authors read and approved the final version of the manuscript.

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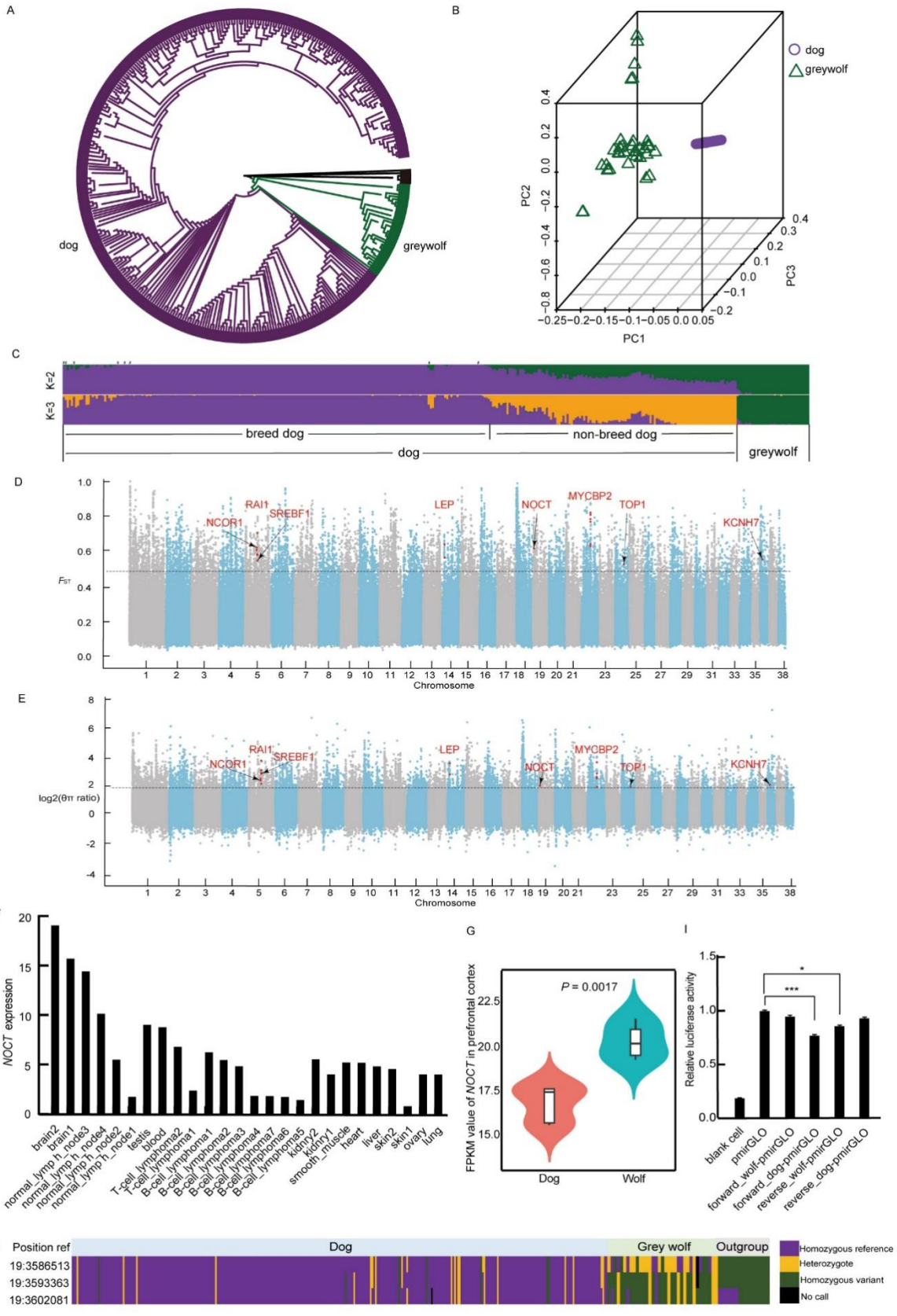

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Figure 1 *NOCT* as a candidate revealed by canid population analysis

A: Phylogenetic relationship among dogs, gray wolves, and distant outgroups, constructed by neighbor-joining method. Purple: dogs; green: grey wolves; black: coyotes, golden jackal, and Andean fox. B: Principal component analysis (PCA) between dogs and grey wolves. C: Model-based clustering of dogs/wolves using ADMIXTURE with K=2 and K=3. The pound denoted non-breed dogs that clustered with breed dogs. D,E: Genomic landscape of fixation index (F_{ST}) (D) and wolf/dog ratio of nucleotide diversity (π) (E) consistently identifying eight genes (denoted in red) associated with circadian rhythm. F: Expression patterns of *NOCT* among different tissues of dogs. G: Expression differentiation of *NOCT* in dog brain versus wolf brain. H: Dual luciferase reporter test results. The ordinate is the expression of four plasmid vectors. pmirGLO is the blank control, forward_wolf-pmirGLO is the expression vector of a 500 bp TGTG type positive strand fragment was inserted downstream of the pmirGLO vector, forward_dog-pmirGLO is a 500 bp size TG was inserted downstream of the pmirGLO vector, reverse_wolf-pmirGLO is an expression vector of a 500 bp CACA type anti-strand fragment was inserted downstream of pmirGLO, reverse_dog-pmirGLO is a 500 bp CA type anti-strand fragment expression vector was inserted downstream of the pmirGLO vector; The vertical coordinate Relative luciferase activity represents the relative expression level of luciferase; *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$; ****: $P<0.0001$. I: Three indels surrounding *NOCT* exhibiting significant allele frequency shifts from the wolves to the dogs.

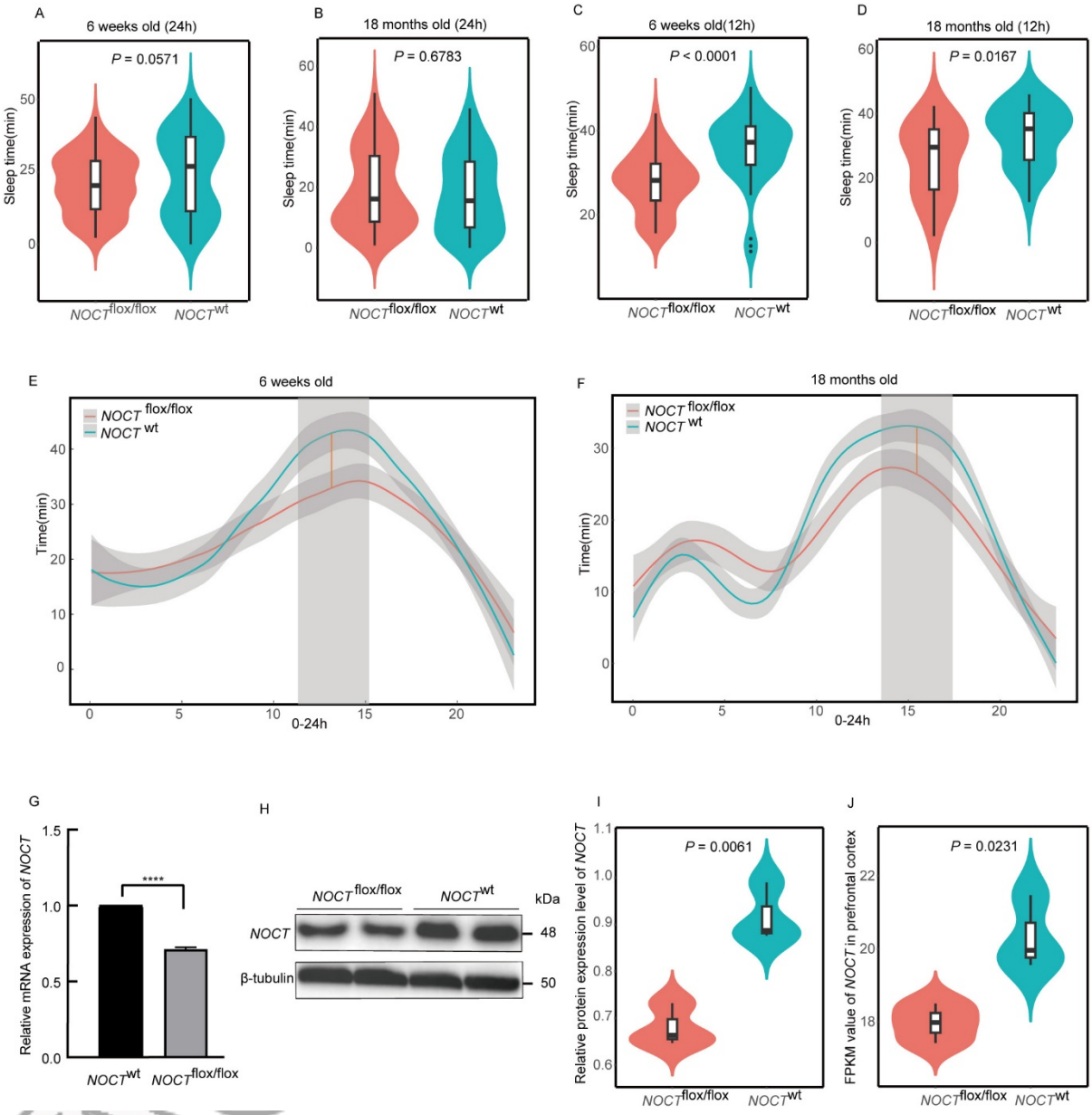

Figure 2 Alterations of the sleep-wake cycle in the transgenic mice with *NOCT* reduction

A: 24-hour sleep time of 6-week of *NOCT*$^{flox/flox}$ and *NOCT*^{wt} mice. B: 24-hour sleep time of 18-months of *NOCT*$^{flox/flox}$ and *NOCT*^{wt} mice. C: 12-hour (8:00-20:00) sleep time of 6-week of *NOCT*$^{flox/flox}$ and *NOCT*^{wt} mice. D: 12-hour (8:00-20:00) sleep time of 18-months of *NOCT*$^{flox/flox}$ and *NOCT*^{wt} mice. E: Change curve of 6-week transgenic mice sleep time in 24 hours. F: Change curve of 18-months transgenic mice sleep time in 24 hours. G: Expression of *NOCT* mRNA in the brain of *NOCT*$^{flox/flox}$ and *NOCT*^{wt} mice (n=3). Mean ± SEM. ****: P<0.0001. H: Westernblotting of *NOCT* protein in prefrontal cortex extracted from *NOCT*$^{flox/flox}$ and *NOCT*^{wt}. I: Quantitative analysis of *NOCT* protein in *NOCT*$^{flox/flox}$ and *NOCT*^{wt} (n=3), normalized by β-tublin. J: Quantitative analysis of *NOCT* protein levels (n=3), normalized to β-tublin.

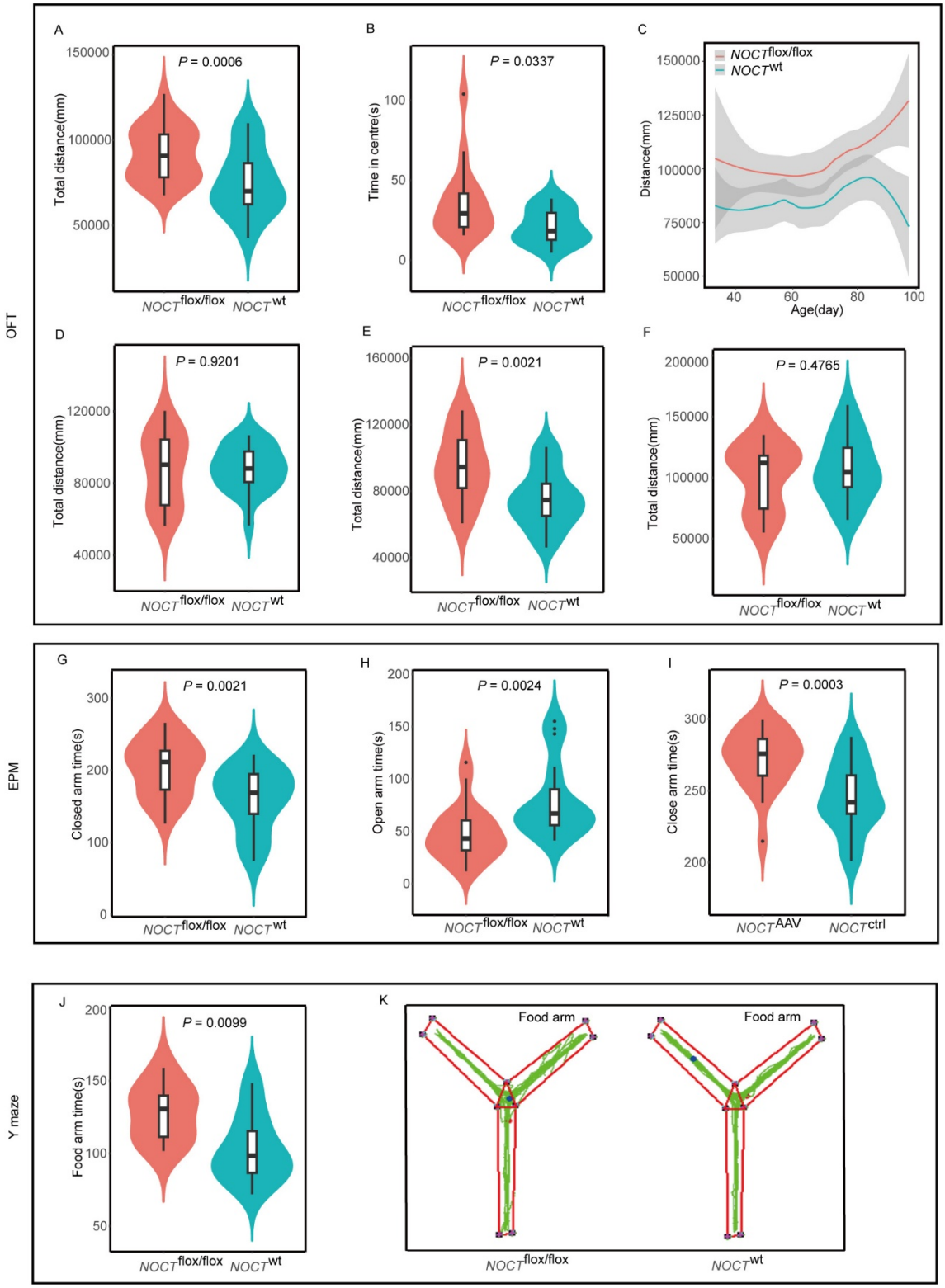

Figure 3 Altered behavioral trajectories of mice with *NOCT* reduction

A,B: The locomotion trait of *NOCT*$^{flox/flox}$ and *NOCT*^{wt} mice in OFT for 30 minutes evaluated by the total movement distance (A) and the residence time in central area (B). C: The curve of the movement distance of mice with age. D–F: The movement distance in 30-minute OFT

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between *NOCT*^{ctrl} and *NOCT*^{AAV} mice with virus post-injection for one week (*D*), four weeks (E), and eight weeks (F). G: The close arm duration time of *NOCT*$^{flox/flox}$ mice within 5 minutes (EPM). H: The Open arm duration time of *NOCT*$^{flox/flox}$ mice within 5 minutes (EPM). I: Four weeks after injection of the virus, the closed arm duration time of *NOCT*^{AAV} mice within 5 minutes (EPM). J: The food arm duration time of *NOCT*$^{flox/flox}$ mice within 5 minutes (Y-maze). K; The movement trajectory of mice in the Y-maze experiment; *NOCT*$^{flox/flox}$ trajectory was significantly stronger than *NOCT*^{wt}.

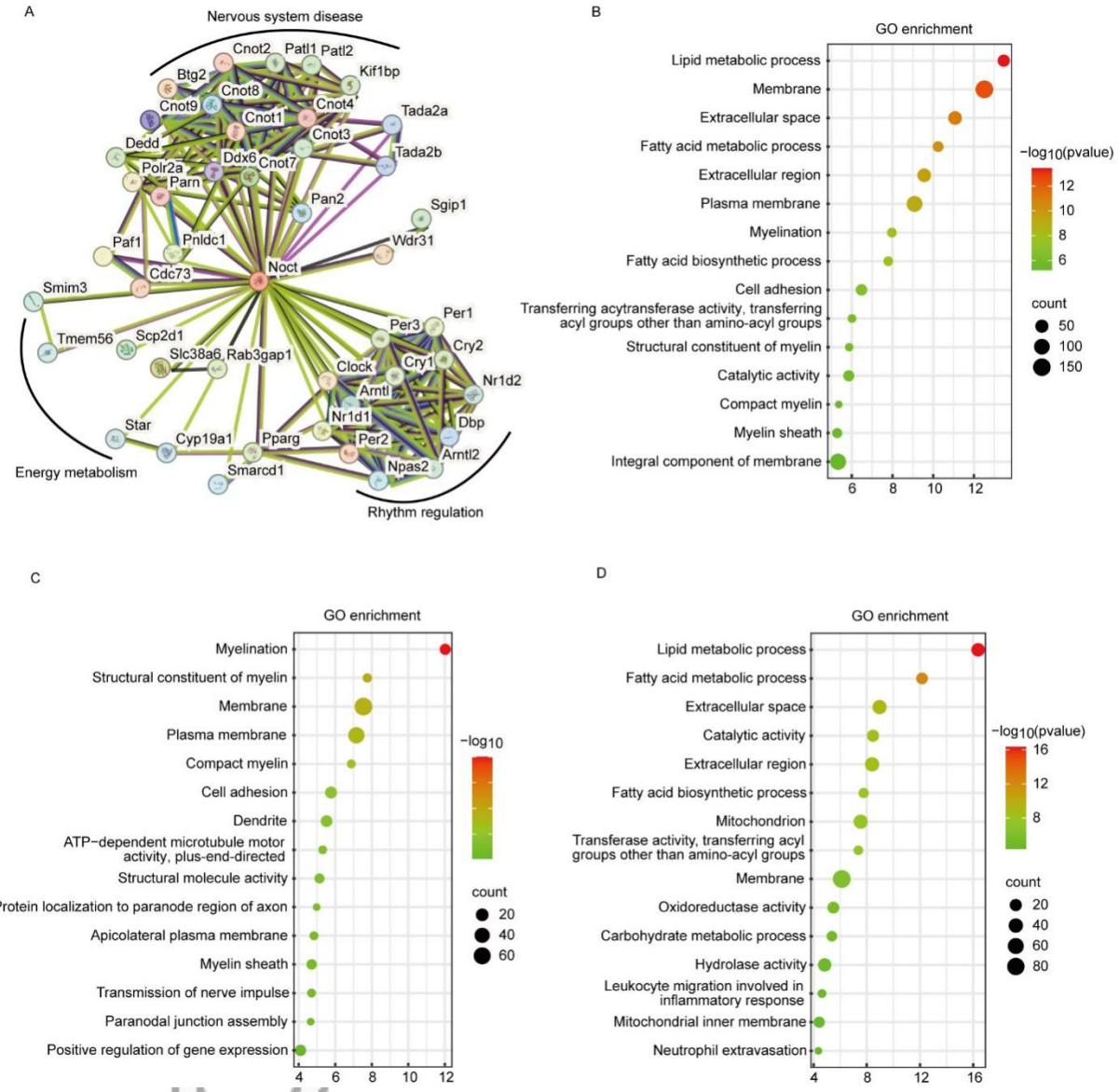

Figure 4 Transcriptome of mice with *NOCT* reduction regulating the expression of myelin sheath and energy metabolism pathways

A: *NOCT* gene interaction network. B: GO pathways enriched in differentially expressed genes in $NOCT^{flox/flox}$ and $NOCT^{wt}$. C: GO pathways enriched in up-regulated genes in $NOCT^{flox/flox}$ and $NOCT^{wt}$. D: GO pathways enriched in down-regulated genes in $NOCT^{flox/flox}$ and $NOCT^{wt}$.

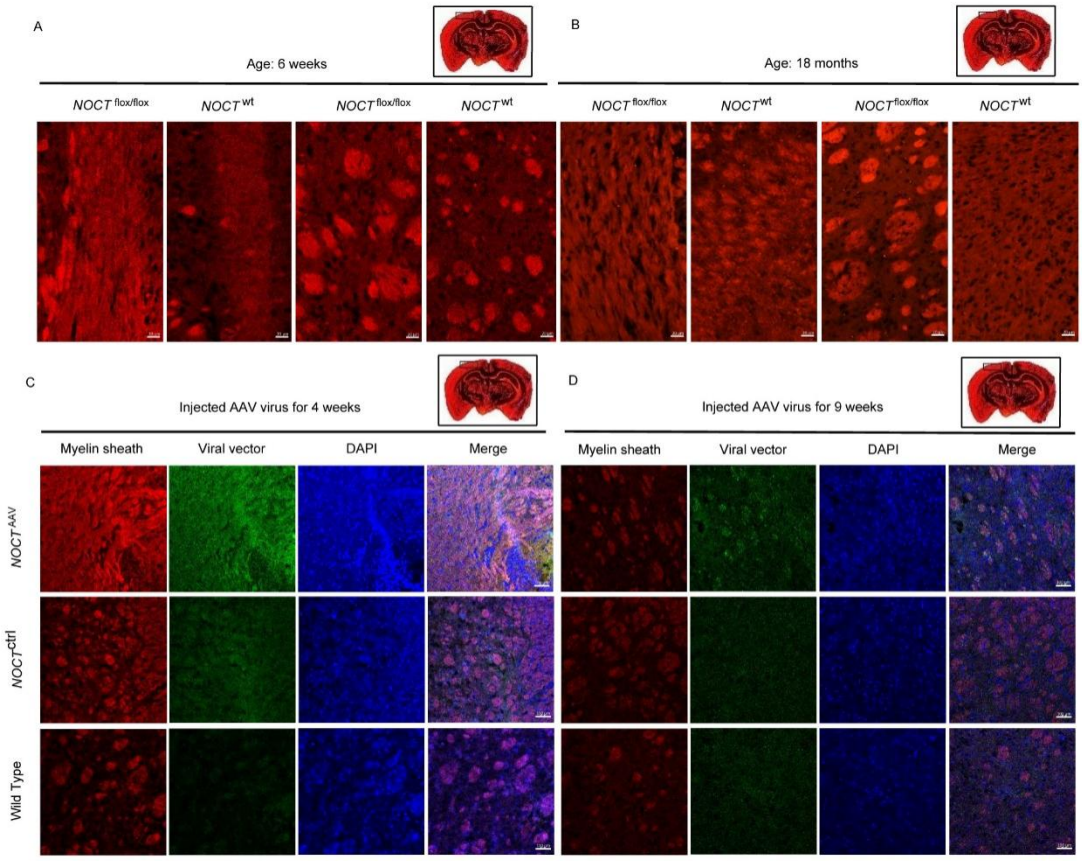

Figure 5 Changes in the myelin sheath of the mouse brain

A: Myelin staining in cortical regions of 6-week-old *NOCT*$^{flox/flox}$ mice. B: Myelin staining in cortical regions of 18-month-old *NOCT*$^{flox/flox}$ mice. C: Myelin staining in cortical regions of *NOCT*^{AAV} mice after 4 weeks of virus injection; the virus has the best inhibitory effect on *NOCT* gene expression after 4 weeks of injection, and its inhibitory effect gradually decreases thereafter. D: Myelin staining in cortical regions of *NOCT*^{AAV} mice after 9 weeks of virus injection; the virus has a weak inhibitory effect on the expression of *NOCT* gene (green fluorescence becomes weak).