# Sleep deprivation induces dopamine system maladaptation and escalated corticotrophin-releasing factor signaling in adolescent mice

**Li-Heng Tuan**
National Tsing-Hua University: National Tsing Hua University
**Jin-Wei Yeh**
National Taiwan University College of Medicine
**Lukas Jyuhn-Hsiarn Lee**
National Health Research Institutes
**Li-Jen Lee** ( ljlee@ntu.edu.tw )
National Taiwan University College of Medicine https://orcid.org/0000-0001-9108-3654

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# Abstract

Sleep disruption is highly associated with the pathogenesis and progression of a wild range of psychiatric disorders. Furthermore, appreciable evidence shows that experimental sleep deprivation (SD) on humans and rodents evokes anomalies in the dopaminergic (DA) signaling, which are also implicated in the development of psychiatric illnesses such as schizophrenia or substance abuse. Since adolescence is a vital period for the maturation of the DA system as well as the occurrence of mental disorders, the present studies aimed to investigate the impacts of SD on the DA system of adolescent mice. We found that 72 h SD elicited a hyperdopaminergic status, with increased sensitivity to the novel environment and Amphetamine (Amph) challenge. Also, altered neuronal activity and expression of striatal DA receptors were noticed in the SD mice. Moreover, 72 h SD influenced the immune status in the striatum, with reduced microglial phagocytic capacity, primed microglial activation, and neuroinflammation. The abnormal neuronal and microglial activity were putatively provoked by the enhanced corticotrophin-releasing factor (CRF) signaling and sensitivity during the SD period. Together, our findings demonstrated the consequences of SD in adolescents including aberrant neuroendocrine, DA system, and inflammatory status. Sleep insufficiency is a risk factor for the aberration and neuropathology of psychiatric disorders.


# 1. Introduction

Nowadays adolescents at large are facing the conundrum of insufficient sleep. As suggested by the National Sleep Foundation, at least 8 h of sleep a day is adequate for adolescents [1]. However, a significant number of epidemiological studies revealed that adolescents generally slept less than the recommended [2, 3]. While sleep insufficiency is a pervasive phenomenon among adolescents, it is acknowledged as a serious health risk factor [4]. In addition to impaired cognitive, learning, and memory performance [5], sleep-insufficient adolescents also fail victim to poor mental health, such as depressive or anxiety symptoms, low self-esteem, increased health-risk behaviors, as well as suicidal ideation [6]. Notably, there is a growing recognition that the distortion of neurobiological remodeling during adolescence may underlie the pathogenesis of many psychiatric illnesses, such as schizophrenia, substance-use disorders, feeding/eating disorders, and affective/anxiety disorders [7]. Furthermore, sleep and circadian rhythm disruption are important aspects of various neuropsychiatric illnesses. It is observed that sleep dysfunctions are not only comorbid with many psychiatric diseases but also prevalently preceding the diseases or even relating to symptom severity [8, 9]. Raising evidence further suggests that the comorbidity between sleep disruption and psychiatric diseases might reflect their common affected mechanisms. Therefore, the neuropathological impacts of curtailed sleep on psychological health and their implication of disrupting the maturational trajectories in the adolescent brain should be deliberately investigated.

One of the salient developmental characterizations during adolescence is the maturation of the DA system [10]. During this distinct period, both cortical and subcortical DA circuitries undergo substantial changes in DA concentration, innervation pattern, receptor density, and response to environmental/pharmacological challenges. These changes may contribute to adolescents' behavioral

transition such as increased exploration, risk-taking, reward- and novelty-seeking behaviors [11]. DA plays a vital role in the fine-tuning of regulating affective, cognitive, motivational, and social processes, as well as arousal and sleep. Therefore, it is little surprise that the abnormalities in DA connectivity are well-recognized in the pathophysiology of various psychiatric disorders [12]. Of particular note, adolescents exhibited relatively heightened stress-coping responses, such as greater hypothalamic-pituitary-adrenal (HPA) axis activity and blunted habituation to stressors, which exert profound effects on the DA functions. These heightened activities may result in long-term molecular or behavioral maladaptation [13]. Accordingly, the extensive development of DA circuitry during adolescence might become a unique window for both positive and negative factors which affect the mental welfare of the individual for life.

Findings gleaned from both animal and human studies demonstrate that sleep loss distorts the DA system and its related functions. Experimental SD paradigms are well-acknowledged to induce psychosis-like phenotypes [14–16], in which the DA system serves an important role [17]. In particular, affected sensitivity and availability of DA receptors [18, 19], and increased DA turnover in the striatum [20] are also reported in SD paradigms. Together, the aforementioned literature posits DA neurotransmission as a vulnerable target of SD and subsequent neuropsychopathologies. However, while studies to date focused mainly on adult subjects, information is scarce regarding the consequence and mechanism of sleep insufficiency in the maturating DA system during adolescence.

An explicit role of microglia, the resident immunocompetent cells in the central nervous system (CNS), has been discovered in the scenario of insufficient sleep. For example, SD elicits microglia activation and neuroinflammation [21–23]. Notably, we have reported that during development, sleep loss halts essential neural maturing processes executed by microglia including compromised microglia-mediated synaptic pruning in sleep-deprived adolescent mice [22, 24]. Of particular concern, aberrant microglial functions are tightly intertwined with the etiology and progress of a wide range of neuropsychiatric disorders [25]. Given the pronounced reactivity of microglia faced by sleep loss, abnormal microglia functions are likely to underpin the susceptibility to neuropsychopathologies of sleep-disrupted individuals, especially the ones that are in the process of brain maturing.

Illuminated by the above evidence, the current study sought to investigate the impacts of 72 h SD on the neuronal and microglial properties in the subcortical regions involved in the DA system in adolescent mice. We provided evidence implicating that 72 h SD resulted in a hyperdopaminergic status and striatal microglia activation, which were likely both attributable to the escalated activity of the CRF system. These data provided an extensive understanding of the neuronal and molecular mechanisms associated with the DA system underlying the deleterious consequences of sleep loss on adolescents' mental health.

# 2. Methods

## 2.1. Animals

Adolescent male C57/BL6J mice of postnatal day (P) 21 were obtained from the National Laboratory Animal Center, Taiwan. All animal experiments were approved by the Institutional Animal Care and Use of National Taiwan University College of Medicine and were conducted in compliance with the ethical guideline. At P34, mice were randomly assigned to the normal sleep (NS) and the sleep deprivation (SD) group.

## 2.2. 72 h SD paradigm

Seventy-two hours of SD (72 h SD) was carried out using the modified multiple-platform method as previously described [22, 24]. The SD paradigm was launched at the beginning of a dark phase. Mice of the SD group were placed on small platforms (3 cm in diameter) surrounded by water with their cage mates to avoid social isolation and immobility stress. Mice of the NS group remained in their home cage. Both groups of mice were paired-housed under 12 h/12 h light/dark cycle and with *ad libitum* access to standard mouse chow and water.

## 2.3. Minocycline treatment

Minocycline hydrochloride (MINO, MilliporeSigma, St. Louis, MO, USA) was dissolved in drinking water containing glucose (2% w/w) to encourage the drinking will of mice. MINO was administrated at 40 mg/kg per day, provided three days before the beginning of the SD paradigm and throughout the experiment. Mice of the vehicle-taking groups were provided with glucose-containing water without MINO. Both MINO and vehicle were freshly prepared every day. The liquid consumption was monitored twice a day to ensure that mice acquired sufficient volumes of water (at least 3 mL for 20 g body weight).

## 2.4. Amphetamine challenge test

Right after of the SD paradigm, mice of the SD group were immediately returned to their home cage. Both NS and SD groups were then moved to the room for behavioral assessment and allowed for 30-min habituation. Subsequently, the individual mouse was placed in the center of a white acrylic square apparatus (45 cm in width, 50 cm in height) under light exposure approximately 100 lux for 60 min. D-amphetamine sulfate (Amph, MilliporeSigma), dissolved in 9% saline (2.5 mg/kg), was then administrated to the mouse through intraperitoneal (i.p.) injection. After injection, the activities of the mouse were recorded with a camera positioned above the apparatus and then analyzed using the Topscane software (CleverSys, Reston, VA, USA).

## 2.5. Histological examination

To evaluate the impact of SD, a cohort of mice was sacrificed 2 h after the completion of the SD paradigm. Another cohort was killed 2 h after the Amph challenge to determine the effect of Amph-induced neuronal activities in the brain. In general, mice were deeply anesthetized by sodium pentobarbital (i.p., 150 mg/kg) and perfused transcardially with 0.1 M phosphate buffer (PB) followed by 4% paraformaldehyde. Brains were removed and postfixed in 4% paraformaldehyde overnight and stored in phosphate buffer saline (PBS) containing 0.1% sodium azide.

# 2.5.1. Immunohistochemistry

Coronal sections of 30 μm were cut using a vibratome (VT 1000S, Leica, Wetzlar, Germany). Sections containing the striatum (from bregma 1.10 to 0.38 mm) and hypothalamus (from bregma − 0.58 to -1.22 mm) were acquired in the 1:6 series and 1:3 series, respectively. Free-floating immunohistochemical staining method was conducted as described [22]. The primary and secondary antibodies and dilution were listed in Table 1.

Table 1
Antibodies used in the current study

| Antibody | Dilution | Assay | Source | Identifier |
|---|---|---|---|---|
| Alexa-488 goat anti-mouse IgG | 1:500 | IF | Jackson Immuno Research Laboratories, West Grove, PA, USA | 115-545-205 |
| Alexa-594 goat anti-rabbit IgG | 1:500 | IF | Jackson Immuno Research Laboratories, West Grove, PA, USA | 111-585-144 |
| Alexa-647 goat anti-rat IgG | 1:500 | IF | BioLegend, San Diego, CA, USA | 405416 |
| Biotinylated goat anti-rabbit IgG | 1:500 | IHC | Jackson Immuno Research Laboratories, West Grove, PA, USA | 111-065-144 |
| Mouse anti-TH | 1:2000 | IF | Sigma-Aldrich, St. Louis, MO, USA | T1299 |
| Rabbit anti-c-fos | 1:1000 | IHC, IF | Cell Signaling, Danvers, MA, USA. | P01100 |
| Rabbit anti-Iba1 antibodies | 1:1000 | IHC, IF | GeneTex, Irvine, CA, USA. | GTX100042 |
| Rat anti-CD68 | 1:1000 | IF | Bio-Rad, Hercules, CA, USA | MCA1957 |
| Goat polyclonal anti-Dopamine D1 receptor (D1R) | 1:200 | WB | Santa Cruz Biotech, Dallas, Texas, USA | sc-31479 |
| Mouse monoclonal anti-dopamine D2 receptor (D2R) | 1:200 | WB | Abcam, Cambridge, UK | sc-5303 |
| Rabbit polyclonal anti-dopamine transporter (DAT) | 1:2000 | WB | Abcam, Cambridge, UK | ab111468 |
| Rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | 1:20000 | WB | GeneTex, Irvine, CA, USA. | GTX100118 |
| HRP goat anti-rabbit IgG | 1:2000 | WB | Vector Labs, Burlingame, CA, USA | BA-1000 |
| HRP Goat anti-mouse IgG | 1:2000 | WB | Vector Labs, Burlingame, CA, USA | BA-9200 |

IF: Immunofluorescence

IHC: Immunohistochemistry

WB: Western blot

| Antibody | Dilution | Assay | Source | Identifier |
|---|---|---|---|---|
| HRP rabbit anti-goat IgG | 1:2000 | WB | Vector Labs, Burlingame, CA, USA | BA-5000 |
| IF: Immunofluorescence | | | | |
| IHC: Immunohistochemistry | | | | |
| WB: Western blot | | | | |

For evaluating the density of c-fos- or Iba-1-positive cells, micrographs containing the caudate-putamen (CPu), nucleus accumbens (NAc), and paraventricular hypothalamic nucleus (PVN) were photographed under a 10X objective with a light microscope (DM750; Leica). The number of c-fos- or Iba-1-positive cells was measured within a 200 x 200 μm counting frame positioned in the regions of interest including the CPu, the core and shell of NAc (NAcC and NAcS), and PVN using the ImageJ software (NIH, Bethesda, MD, USA). Brain regions of interest were identified based on the anatomical landmarks (e.g. fiber tracts or ventricles) according to a mouse brain stereotaxic atlas [26]. The cell densities in 4–6 sections per mouse were averaged to represent the result of each mouse.

## 2.5.2. Immunofluorescence and cell counting

Brain sections containing the striatum, hypothalamus, and midbrain (from bregma − 2.92 to -3.80 mm) were acquired in the 1:6, 1:3, and 1:3 series and transferred to the blocking solution. Free-floating immunofluorescence method was conducted as described [22]. The primary and secondary antibodies and dilution were listed in Table 1.

In the assays considering the midbrain DA neurons, bilateral brain sections covering the ventral tegmental area (VTA) and substantia nigra (SN) were taken. DA neurons were identified by the tyrosine hydroxylase (TH) immunofluorescence. Image acquirement and cell counting were performed as described [24]. Multiple z-stacks were acquired under a 20x objective with a confocal microscope (LSM780; Carl Zeiss, Oberkochen, Germany) using a 2.69 μm z-interval combining the tiling function to encompass the bilateral midbrain dopaminergic nuclei, VTA and SN. The numbers of DA neurons in VTA and SN were obtained from 8 sections in each mouse and multiplied by the number of sections serial to represent the total estimates of each mouse. As for microglial density assessment, a 200 x 200 μm counting frame was positioned in the VTA, pars compacta, and reticulata of the SN (SNc and SNr), which were outlined by the TH-positive cells.

## 2.5.3. Morphometric analyses and CD68 expression of microglia

Confocal imaging was conducted using the LSM880 (Carl Zeiss) confocal microscope. Z-stacks of microglia within the CPu or NAcC were acquired under a 63x oil objective with a 0.7x digital zoom using a 0.6 μm z-interval. Reconstruction of microglia and analysis of CD68-positive lysosomal expression within microglia were performed as previously described [24] with minor modification. In brief, Z-stacks were

preprocessed by ImageJ and imported into Imaris (Version 9.8; Bitplane, Zurich, Switzerland). The Surface module was used to determine the volume of Iba1- and CD68-positive signals with a uniform analysis threshold across Z-stacks. The intracellular lysosomal content within microglia was presented as the percentage of CD68 volume within microglia; normalized to the average results of the NS group in the same imaging acquisition.

## 2.5.4. Golgi-Cox impregnation and dendritic spine counting

The FD Rapid Golgi Stain Kit (FD NeuroTechnologies, Columbia, MD, USA) was used to label the neurons as described previously [24]. Micrographs of the neurons in the CPu, NAcC, and NAcS were captured by a light microscope (Axio Imager M1, Carl Zeiss) under the 100x oil-objective with a 0.5 μm z-interval. The density of the dendritic spines was evaluated manually using the Neurolucida software (MicroBrightField Bioscience, Williston, VT, USA). The primary dendrites (the first protrusion from the soma) were not included in the analysis.

# 2.6. Biochemical analyses

Mice of NS and SD groups were decapitated 2 h after the 72-h SD paradigm and their brains were quickly taken. Bilateral striatum samples were quickly dissected on ice, immediately placed in dry ice, and stored at -80$^{o}$C until further analyses.

## 2.6.1. RT-PCR

TRIzol™ Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was used to isolate total RNA in the unilateral striatum of an individual mouse. Single strand cDNA was synthesized from 1 ng of total RNA using the Magic RT Mastermix cDNA Synthesis Kit according to the manufacturer's protocol (Bio-Genesis Technologies, Taipei, Taiwan). Quantitative PCR for target genes in each sample was performed with an SYBR Green Kit (Bio-Genesis Technologies) using 1 μl of cDNA per reaction and was conducted in duplicate for each sample on a PCR machine (QuantStudio3, Thermo Fisher Scientific). Relative gene expression was acquired by the dd$C_t$ method normalized endogenous control Gapdh and relative to the mean results of the NS group. Primer pairs for the genes of interest were listed in Table 2.

Table 2
qPCR primers used in the current study.

| Target gene | Primer sequence: |
|---|---|
| *C1q* | Forward: 5’- CAAGGACTGAAGGGCGTGAA-3’, Reverse: 5’-CAAGCGTCATTGGGTTCTGC-3’ |
| *C3* | Forward: 5’-TCAGATAAGGAGGGGCACAA-3’, Reverse: 5’-ATGAAGAGGTACCCACTCTGGA-3’ |
| *Caspase-1* | Forward: 5’-GGCACATTTCCAGGACTGACTG-3’, Reverse: 5’-GCAAGACGTGTACGAGTGGTTG-3’ |
| *CD11b* | Forward: 5’-TGGCCTATACAAGCTTGGCTTT-3’, Reverse: 5’-AAAGGCCGTTACTGA GGTGG-3’ |
| *CRH* | Forward: 5’-GTTGAATTTCTTGCAAC GGAG-3’, Reverse: 5’-GACTTCTGTTGAGGTTCCCCA-3’ |
| *CRF-R1* | Forward: 5’-TTCTACGGTGTCCGCTACAA-3’, Reverse: 5’-ATGACGGCAATGTGGTAGTG-3’ |
| *CRF-R2* | Forward: 5’-TACCGAATCGCCCTCATTGT-3’, Reverse: 5’-CCACGCGATGTTTCTCAGAAT-3’ |
| *CX3CR1* | Forward: 5’-ACCCCAGTTCATGTTCACAAA-3’, Reverse: 5’-GAAGAAGGCAAAGACCACCA-3’ |
| *D1R* | Forward: 5’-AAGATGCCGAGGATGACAAC-3’, Reverse: 5’-TCGACAGGGTTTCCATTACC-3’ |
| *D2R* | Forward: 5’-CCTGTCCTTCACCATCTCTTGC-3’, Reverse: 5’-TAGACCAGCAGGGTGACGATGA-3’ |
| *GAPDH* | Forward: 5'-ACGGGAAACCCATCACCAT-3', Reverse: 5'-CCAGCATCACCCCATTTGA-3 |
| *IL-1β* | Forward: 5'-CCAGGATGAGGACATGAGCACC, Reverse 5’-TTCTCTGCAGACTCAAACTC CAC-3’ |
| *IL-18* | Forward: 5'-GACAGCCTGTGTTCGAGGATATG-3’, Reverse: 5’-TGTTCTTACAGGAGAGGGTAGAC-3’ |

| Target gene | Primer sequence: |
|---|---|
| *IL-6* | Forward: 5'-GCTACCAAACTGGATATAATCAGGA-3', Reverse: 5'-CCAGGTAGCTATGGTACTCCAGAA-3' |
| *NLRP3* | Forward: 5'-TGCTCTTCACTGCTATCAAGCCCT-3', Reverse: 5'-ACAAGCCTTTGCTCCAGACCCTAT-3' |
| *TNF-a* | Forward: 5'-AGGCTGC CCCGACTACGT-3', Reverse 5'-GACTTTCTCCTG GTATGAGATAGCAAA-3' |

Table 3
Microglial morphometric analyses

| Parameters | CPu | | NAcC | |
|---|---|---|---|---|
| | NS (n = 24) | SD (n = 23) | NS (n = 23) | SD (n = 23) |
| Bifurcation nodes | 92.08 ± 3.18 | 102.83 ± 3.16 * | 140.26 ± 6.37 | 141.26 ± 5.75 |
| Terminal endings | 106.54 ± 3.40 | 117.17 ± 3.46 * | 158.57 ± 6.85 | 164.13 ± 6.49 |
| Total process length (μm) | 973.51 ± 29.92 | 979.31 ± 26.46 | 1119.93 ± 47.86 | 1053.25 ± 34.35 |
| Soma volume ($\mu m^2$) | 231.48 ± 6.38 | 248.52 ± 5.09 * | 250.49 ± 7.07 | 257.07 ± 7.05 |

## 2.6.2. Western blot

Frozen unilateral striatum samples were lysed homogenized in RIPA buffer (GenStar Biosolutions, Beijing, China) containing 1% of protease inhibitor mixture (Thermo Fisher Scientific) and centrifuged at 12,000 rpm for 30 min at 4°C for supernatants collection. The bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher Scientific) was performed to determine the protein concentration. Supernatants were denatured by Laemmli sample buffer for 30 min at 65$^{o}$C. 40 μg protein sample was loaded per lane, separated by 1 % SDS-PAGE gel, and transferred to PVDF membranes (Immobilon-P, Millipore, Burlington, MA, USA). The membranes were blocked in a solution of 5% low-fat milk in Tris-buffered saline with Tween-20 (TBST) and then incubated with diluted primary antibodies in TBST overnight at 4$^{o}$C, followed by incubating with peroxidase-conjugated secondary antibodies for 1 h at room temperature. The immunoreactive bands were scanned by chemiluminescent HRP substrate (Immobilon™ Western, Millipore) with a UVP AutoChemi™ System (UVP Inc, Upland, CA, USA). The intensity of the immunoreactive bands was evaluated by ImageJ (NIH) and normalized to GAPDH in a semiquantitative manner.

# 2.7. Statistical analysis

Data are represented as mean ± SEM. Significance was assessed with Student's t-test. $p < 0.05$ was considered to indicate a significant difference.

# 3. Results

## 3.1. Amph induced hyperlocomotion and greater neuronal activity in adolescent SD mice

In concern of the strong association between sleep disruption and aberrant DA activity, we first examined the effect of 72 h SD on the response to a single dose of Amph, which could elevate the extrasynaptic DA level. We observed greater locomotor activity in mice of the SD group compared to those in the NS group, both before and after Amph treatment (Fig. 1a **& b**). The elevated basal locomotor activity (before Amph administration) in the SD group implicated the failure of habituation in a novel environment. Such impaired habituation has also been observed in DA transporter (DAT)-knockdown mice, a model of hyperdopaminergic tone [27]. Following the treatment of a low dose Amph (2.5 mg/kg), the change of activity in the NS group was minimal, whereas the locomotion of SD mice was largely enhanced (Fig. 1a **& c**). This result implicated that SD increases the response to psychostimulants targeting the DA system. Amph treatment elevates the extrasynaptic DA level, which may further aggravate the hyperdopaminergic status in SD mice resulting in their greater locomotor activity. These findings suggested that 72 h SD inferences the DA system and biases the novel environment- and psychostimulant-induced responses toward a heightened status.

Next, we examined the Amph-induced neuronal activity by measuring the c-fos protein expression in the striatum because it receives the DA inputs from the midbrain DA neurons. In consistence with the behavioral phenotypes, increased density of c-fos-positive cells was observed in three subregions of the striatum, namely the CPu, NAcC, and NAcS, in mice of the SD paradigm (Fig. 2a-c). We further examined the c-fos expression in midbrain DA neurons (Fig. 3a-c). A significant increase of c-fos-positive labeling in DA neurons was noticed in the SNc and VTA whereas the total numbers of DA neurons in both midbrain regions were unaffected (Fig. 3d **& e**). The basal levels of c-fos-positive cells in the aforementioned regions of the striatum and midbrain were comparable between the two groups (**Suppl. Figure 1a & b**), implicating the changes in the SD mice are stimulation-dependent. Our findings suggested that sleep loss augments the sensitivity to psychostimulants targeting the DA circuitry in a manner similar to repetitive psychostimulant reinforcement [28, 29], which is also associated with the neuropathology of schizophrenia [30].

## 3.2. 72 h SD altered the expression of striatal DA receptors

A collection of studies revealed the alleviated effects of antipsychotic drugs that act as blockers of DA receptors on the SD-induced hyperdopaminergic status [31, 20, 32]. It is therefore tenable to suspect alternations in DA receptors being accountable for the SD-induced reactions. We then examined the expression of striatal DA receptors in adolescent SD mice **(**Fig. 4a). An increase in dopamine D1 receptor (D1R) protein expression was noticed in SD mice (Fig. 4b); however, the D1R mRNA level was comparable between groups (Fig. 4e). Since the density of striatal D1R declines remarkably during adolescence, until it reaches a constant level in adulthood [33, 34], our data implied an SD-mediated disruption of

developmental D1R elimination. Reduced dopamine D2 receptor (D2R) expression at both protein and mRNA levels was observed in the SD compared with the NS group (Fig. 4c **& f**). These findings indicated that SD downregulates the expression of striatal D2R, paralleling the previous studies of the SD paradigm in humans and rats [18, 19]. The protein expression of DAT which reuptakes extrasynaptic DA was similar between groups (Fig. 4d).

In adolescent mice, the SD paradigm may impair the elimination of dendritic spines, which are mainly glutamatergic synapses, in the cortex and hippocampus [35, 22, 24]. Accordingly, the spine density of striatal medium spiny neurons (MSNs) was measured in Golgi-stained samples (**Suppl. Figure 2a**). We found comparable spine densities between the NS and SD groups in all three striatal subregions (**Suppl. Figure 2b**). These results suggest that the striatal excitatory synapses might not be the major target of SD.

## 3.3. 72 h SD decreased microglial phagocytic capacity in the striatum

In addition to the role as the passive responders to disrupted homeostasis in the CNS, microglia also mediate neuronal plasticity which has been highlighted in the neuro-maladaptation in the context of erratic DA functions, such as stress and drug abuse [36]. Moreover, microglia participate in the shaping of adolescent neural circuitries by phagocytic elimination of synaptic components [37] or D1R [38], which presumably takes place during sleep [39–41]. To determine the impact of SD on microglia-mediated neuroimmune signaling for the refinement of synapses or receptors in the striatum, we quantified the transcriptional level of microglia-specific receptors and their neuronal ligands. Decreased mRNA level of CX3CR1, a pivotal mediator for the neuron-microglia communication in synaptic refinement [42], was found in SD mice in comparison with NS mice (Fig. 5a). Also, the level of CD11b, a subunit of complement receptor 3 (CR3) in the classical complement system, was decreased significantly in the SD group compared with the NS group (Fig. 5b). The expression of C1q and C3 was comparable between groups (Fig. 5c **& d**).

Seeing the downregulation of microglial receptors related to phagocytic elimination of neuronal components, we next evaluated the phagocytic capacity of microglia by double-labeling of Iba1 and CD68 (Fig. 5e), a microglial lysosomal membrane protein [43]. In consensus with the downregulated phagocytic-related receptors, there was a significant decrease in the occupancy of CD68 within microglia in the NAcC but not the CPu in SD mice compared to NS controls (Fig. 5f-h). Taken together, these findings implicated an SD-induced reduction of microglial phagocytosis in the striatum, which is important for the developmental remodeling of the DA system, by downregulating vital microglial receptors and lysosomal abundance.

## 3.4. 72 h SD primed microglia activation and neuroinflammation in the striatum

A growing body of evidence strongly links microglial activation with SD-induced cognitive impairments [21–23] as well as the pathophysiology of various psychiatric illnesses [25]. Microglia activation and subsequent neuroinflammatory events cause substantial influences on neuronal activity and behavioral deficits related to affected regions [44]. In the light of the above evidence, we first explored the densities of Iba1-positive microglia in the striatum (Fig. 6a-b). There was a significant increase in striatal microglia in SD mice compared with NS mice (Fig. 6c). Secondly, we examined the density of microglia within the midbrain VTA, SNc, and SNr (Fig. 7a-b). No difference in microglial density in these midbrain regions was observed between SD and NS groups (Fig. 7c). Interestingly, our results showed elevated microglial density in the striatum which receives the DA projection but not in the midbrain nuclei where DA neurons reside.

Microglial processes respond dynamically to the changes in the surrounding microenvironment and their morphological shift often reflects altered physiological conditions [45]. We next three-dimensionally reconstructed Iba1-positive individual microglia and examined their morphological features (Fig. 8a **& d**). Significant increases in the node, endings, and soma volume were noted in microglia in the CPu of the SD group (Table 2). Furthermore, the microglia of SD mice exhibited more intersections in Sholl analysis and greater numbers of segments, showing increased ramification (Fig. 8b **& c**). In the NAcC (Fig. 8d-f), a subtle reduction of intersections in the distal part of the microglial process was noted in the SD group (Fig. 8e), while the number of segments was comparable between groups **(**Fig. 8f). Together, morphometric findings suggested that 72 h SD increases striatal microglial population and shifts microglial morphology toward hyper-ramification.

Considering that the pro-inflammatory cytokines released by microglia under the circumstance of neuroinflammation may perturb neuronal activity, we measured the level of pro-inflammatory factors. The transcriptional levels of pro-inflammatory cytokines, namely the tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-1 beta (IL-1β), and IL-18, were significantly elevated in SD mice compared to NS controls (Fig. 9a-d). Furthermore, the mRNA levels of NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) and Caspase-1, the upstream activators of IL-1β and IL-18 [46], were also up-regulated in SD mice (Fig. 9e **& f**). Collectively, these findings indicated SD-mediated priming of microglia activation and neuroinflammatory status in the striatum.

We next investigated whether microglia-mediated neuroinflammation is responsible for the striatal phenotypes of sleep-deprived adolescent mice. Minocycline (MINO) is an antibiotic that has also been recognized as an inhibitor of microglial activation [47]. MINO has been demonstrated to inhibit microglial activation, ameliorate Amph-induced hyperlocomotion, prepulse inhibition (PPI) deficits, and reduce drug-seeking behaviors in rodent models associated with evoked immune responses [48–50]. In light of this, we administered MINO via drinking water three days before SD and throughout the SD period. There was a significant reduction of microglial density in the CPu and NAcS of MINO-treated SD (MSD) mice compared with the vehicle-taking SD (VSD) mice (**Suppl. Figure 3a**). However, MINO failed to prevent Amph-induced hyperlocomotion in SD mice. There was no difference in the traveled distance between MSD and VSD mice in the Amph challenge test (**Suppl. Figure 3b**). These results indicated that while

MINO treatment prohibits the SD-induced striatal microglial activation, the hyperdopaminergic performances in SD mice might be attributed to other mechanisms.

## 3.5. 72 h SD augmented CRF signaling in the hypothalamus and midbrain

Sleep loss shared a wide spectrum of endocrine and neurochemical imbalances with stressful encounters. Stressful encounters are postulated to produce alternations in the neurological and behavioral dysfunctions of the mesocorticolimbic DA circuitries, such as psychotic symptoms, reinforcement, and behavioral sensitization to psychostimulants [51, 13]. In particular, a plethora of studies has identified the CRF system as a prominent character regulating the DA neuron-involved maladaptation to stress [52]. CRF is mainly produced by the hypothalamic neurons in the PVN and governs the activity of the HPA axis [53]. Notably, sleep loss induces activation of the HPA axis in the effort to maintain wakefulness and arousal [54, 55]. In our previous study, we observed elevated plasma corticosterone levels in mice after 72 h SD [24], implying a sign of activated HPA axis in our SD paradigm. Based on the above evidence, we aimed to determine whether SD enhances CRF signaling in the adolescent brain. Firstly, the level of c-fos expression in the PVN, where the CRF-secreting neurons are located, was evaluated (Fig. 10a **& b**). Mice subjected to SD showed a significant increase of c-fos-positive cells in the PVN compared with that in the NS group (Fig. 10c). Furthermore, the transcriptional level of CRF was also upregulated in the hypothalamic tissue (Fig. 10d), suggesting that SD elicited hypothalamic CRF signaling.

The VTA DA neurons are innervated by CRF-containing glutamatergic afferent terminals and express CRF receptors 1 and 2 (CRF-R1 and CRF-R2) [53], we then examined the expression of CRF receptors in the midbrain tissue at mRNA level. A significant upregulation of the CRF-R1 but not CRF-R2 expression was observed in the SD mice compared to the NS mice (Fig. 10e), indicating an SD-induced enhancement of CRF-R1 mediated signaling upstream of the DA system. Taken together, our results demonstrated an augmented CRF signaling and sensitivity following SD, both of which might contribute to the sensitization and hyperactivity of the DA system.

# 4. Discussion

The current study showed immense impacts of 72 h SD on the neurons and microglia in adolescent brains. SD produced a hyperdopaminergic status, with escalated locomotor activity, increased neuronal activity in the striatum and midbrain DA neurons under Amph stimulation, as well as altered expression of striatal DA receptors. We also discovered distinct features of microglial activation in the striatum of SD mice. However, whereas treatment with an anti-neuroinflammation agent MINO normalized the increment of the striatal microglial density in SD mice, it did not rescue the behavioral changes in the Amph challenge test, implying that microglia reactivity might not be the major stem of the SD-induced hyperdopaminergia. In contrast, SD mice showed increased activity and sensitivity of the CRF signaling, which potently primes both DA system and microglia activity. Collectively, these findings support a

fueling role of stress responses evoked by SD to the DA circuitry and the striatal microglia in adolescents. Our results also unraveled a potential mechanism of how sleep deprivation navigates adolescent individuals to the susceptibility to psychiatric illnesses.

# 4.1. 72 h SD induces a CRF signaling-mediated hyperdopaminergic status in adolescent mice

In good agreement with previous studies [14, 20], we found that SD elicited exaggerated responses to a novel environment and acute Amph stimulation in adolescent mice. Whereas most studies focused on changes in DA afferent regions such as the striatum and prefrontal cortex [14, 31], we herein demonstrated the significance of functional alternations in the midbrain DA neurons in the SD-induced hyperdopaminergic status. Using c-fos labeling as the indicator of neuronal activity, we found that aside from increased striatal c-fos density, SD also evoked robust c-fos expression in the VTA. It is to note that acute psychostimulant administration generally exerts inhibition effects through the somatodendritic-released DA [56] or forebrain innervation to the VTA DA neurons [57]. The increased VTA DA neuronal activity following the Amph challenge in the SD animals was akin to the pattern of repetitive drug administration [28, 29] as well as repeated stress exposure [58]. Notably, appreciable evidence demonstrated that drugs of abuse and stressful experience trigger common pathways to generate behavioral and neuronal sensitization by evoking neuroplastic changes in the VTA DA neurons [13]. Taken together, our findings postulate that the hyperdopaminergic status might attribute to the SD-induced sensitization in the mesolimbic DA system.

One of the prominent findings in the current study was that 72 h SD enhanced hypothalamic CRF signaling and increased midbrain CRF-R1 level. These results suggested that SD arouses CRF-mediated stress response and sensitivity. It is well known that the activation of the CRF system is a crucial aspect of the stress-induced sensitization of DA circuitries [52, 53, 13]. The CRF-containing neurons in the PVN, central amygdala, and bed nucleus of the stria terminalis innervate the VTA DA neurons and form excitatory synapses [59]. In the VTA, locally released CRF acts in concert with glutamate to modulate the activity of DA neurons mainly through CRF-R1 [53]. The regulatory effects of CRF on the VTA DA neurons are predominantly excitatory, which encompasses increasing neuronal firing [60], potentiating N-methyl-D-aspartate (NMDA) receptors [61], and evoking DA release in the target brain areas [62]. Systemic or intra-VTA administration of CRF receptor antagonists has demonstrated a vital role of CRF signaling in stress-induced drug-seeking behaviors [63, 64]. Besides, CRF may influence the DA circuitries indirectly by activating the HPA axis and the subsequent release of glucocorticoids. In our previous study, we found that 72 h SD elevated plasma corticosterone [24], which is highly lipophilic and able to diffuse into the CNS. Glucocorticoid receptors are widely expressed in neurons of the DA system [13]. Animal studies showed that corticosterone augments DA release into the NAc [65], and increases Amph or cocaine self-administration, and locomotor sensitization [66, 67]. Together, 72 h SD could trigger CRF-mediated stress responses, probably potentiates by both intra- and extra-hypothalamic CRF signaling pathways, leading to neuronal and behavioral sensitization of the DA system.

CRF signaling also mediates microglial functions directly under stressful conditions since microglia express both CRF-1R and CRF-2R [68]. CRF stimulation was found to enhance microglial production of proinflammatory cytokines TNF-α or IL-18 [69, 70], which is proposed to modulate the stress responses [71]. Taken together, enhanced CRF signaling upon SD might act as a potent trigger to both neuronal and microglial reactivity of the DA system.

Mounting evidence suggested a reciprocal relationship between sleep loss and stress responses [54, 55]. On one hand, the facilitated HPA activity is essential for maintaining wakefulness during the SD regimen, as a positive correlation was found between plasma cortisol level and arousal state [72]. On the other hand, exogenous CRF and glucocorticoid treatments could increase wakefulness, suppress sleep, and alter sleep architecture through interacting with sleep-wake regulatory neural circuitries, including the wake-promoting locus coeruleus and sleep-activating nuclei in the preoptic hypothalamus [73, 74]. Notably, the activity of VTA DA neurons and their projections to the NAc is necessary for arousal [75]. Correspondingly, the physiological arousing effects of the DA system were supported by pharmacological or genetic manipulations targeting DA receptors [76, 77]. The above pieces of literature, accompanied by our observations on elevated CRF and DA signaling after SD, have raised that the SD-dependent heightened CRF signaling might increase the excitability of the DA system, to fulfill the demand for inducing or sustaining arousal.

## 4.2. 72 h SD alters striatal DA receptor expression in adolescent mice

Besides exacerbated behavioral and neuronal responses to the Amph challenge, the current study also identified an SD-induced downregulation of striatal D2R at both mRNA and protein levels. Similarly, downregulated D2R had been recognized in the ventral striatum after chronic treatments of psychostimulants or agonists [78, 79]. One potential mechanism for SD-induced D2R reduction is the prolonged DA stimulation, which may lead to the surface internalization, endocytosis, and degradation of D2R [80, 81]. Previous studies demonstrated that SD elevates DA concentration or its metabolites in the striatum [82, 20]. Although D2R plays a crucial role in the maintenance of wakefulness [75, 76], sustained DA stimulation might result in the degradation of striatal D2R. Another tenable cause might be the activation of the adenosine receptor [83], which is co-expressed with D2R on striatal MSNs [84]. As a derivative of energy metabolism, extracellular adenosine accumulates during the waking state and employs modulatory effects through the activation of $A_1$ and $A_2$ receptors [85], which might decrease D2R binding affinity [86] or drive D2R surface internalization [87]. In addition, chronic stress exposure has been shown to reduce striatal D2R mRNA expression, emphasizing that the DA system is the affected pathway between sleep loss and stress responses [88, 89]. Collectively, the heightened DA signaling or energy expenditure evoked by SD or SD-related stress might be responsible for the reduction of D2R biosynthesis and expression.

In contrast to the downregulated striatal D2R expression, an increase in striatal D1R protein was observed in the SD mice. Specifically, the significance has occurred in the protein but not the mRNA level, raising

the assumption of decreased D1R elimination rather than upregulated biosynthesis in the SD paradigm. In rodents, functional availability of D1R and D2R in the CPu and NAc peaks approximately between P28 to P40 and declines thereafter to adult levels [33, 34]. A similar developmental pattern of DA receptors was also reported in the human postmortem striatum, implicating a highly-conserved pruning process of DA receptors during adolescence [90]. Remarkably, it has been shown that microglia and complement-mediated immune signaling might contribute to the elimination of D1R in the adolescent NAc [38]. This developmental D1R pruning is associated with the successful transition of social behaviors from adolescent to adult, emphasizing the importance of proper neuron-microglia interactions in the maturating DA system [38].

Hypersensitivity of D1R and reduction of D2R, which result in the imbalance between the signaling of these two receptors, have been proposed to predispose the individual to addiction [91, 92]. It is supported by a correlation between sleep disturbance and susceptibility to cocaine addiction [93]. Therefore, alternations of DA receptors, as maladaptive responses engaging neuronal and microglial changes, elicited by sleep loss are likely to bring detrimental consequences such as vulnerability to substance-use disorders or extended neuropathology in the DA system. The intricate mechanisms underpin should be carefully elucidated in the future.

## 4.3. 72 h SD activates striatal microglia in adolescent mice

Microglia activation is well recognized in various SD regimens [21–23]. Likewise, our data of increased microglia density, altered microglial morphology, and upregulated transcription of a plethora of pro-inflammatory components suggest that 72 h SD primes microglia activation and neuroinflammation in the adolescent striatum. In our current model, SD effectively evokes stress responses and activates the neuroendocrine system which has a direct impact on microglia. Stress-coping hormones such as glucocorticoids, CRF, and norepinephrine could elicit microglia activation and subsequent cytokine productions [68]. Besides, the increase of danger-associated molecular patterns (DAMPs) is also a potent activator of microglia and neuroinflammation in the context of SD [94]. Endogenous DAMPs bind to their associated pattern recognition receptors on microglia and activate the downstream inflammatory pathways, such as increasing the transcription of NLRP3 or pro-inflammatory cytokines [95]. We, therefore, proposed that DAMPs generated by increased synaptic activity or accumulated cellular stress during SD contribute to the priming of inflammatory pathways in the striatum.

In addition to the profiles of neuroinflammation-related cytokines, we observed an increase in the complexity of microglial processes in the CPu of the SD mice. Hyper-ramified microglia have been identified in various chronic stress models [96–98]. Microglia hyper-ramification might be a transition phase from the resting to the activated state in response to injury [99]. Emerging evidence further suggests that microglia, equipped with a wild array of receptors, are activated by neurotransmitters and purinergic signaling [68], and elaborate their processes to intensify the surveillance, contacts, and modulation of the surrounding neuronal structures [100]. In the SD paradigm, aberrant neuronal activity might stimulate microglial morphological changes and interactions with the surrounding microenvironment.

Microglial-mediated neuronal activity and structural remodeling might take place during the sleep state [39–41]. In particular, our previous studies demonstrated that the lack of sleep prevents microglial engulfment and phagocytosis of synaptic materials in adolescent mice [22, 24]. Here, we provided evidence of downregulated CX3CR1 and CD11b, in the striatal homogenate of the SD mice. These molecules are microglia-specific components that participate in the developmental synaptic refinement [101] and synaptic remodeling of the DA system [38, 48]. Resonating with the mRNA assessment, the lysosomal marker CD68 was also found to decrease in the NAcC microglia of SD mice, implying a reduction in the microglial phagocytic capacity. Since microglia and complement-mediated immune signaling play role in the elimination of D1R in the adolescent NAc [38], our results suggested that the deficiency in microglia phagocytic capacity is a potential culprit for the increased remnant of striatal D1R after SD.

Microglia activation and neuroinflammation have been acknowledged by converging clinical and animal studies as risk factors for developing substance use disorders or other psychiatric illnesses associated with abnormal DA functions [25]. MINO administration has been shown to inhibit the microglial activation and neuroinflammatory events associated with the DA system, including drug-seeking behaviors, behavioral sensitization to psychostimulants, and reduced PPI [48–50]. In the present study, six days of MINO treatment reduced the SD-induced microglia increase in the striatum; however, it did not prevent the hyperactivity after Amph stimulation. These results implicated that microglia activation is not the only source of SD-induced hyperdopaminergic status. On a cautionary note, the abnormal DA signaling might be a convergence of different etiologies or multiple factors. Besides the activation of stress response systems, attenuated pruning processes, and priming of pro-inflammatory cascades occurred in our SD paradigm, which might not be prevented by a single MINO treatment. The consequences of various harmful factors brought by SD are highly intertwined with each other; we might develop therapeutic strategies using combined pathways.

# 4.4. Conclusion

Our current findings present an interplay between neuroendocrine, DA, and immune systems in sleep-deprived adolescent mice. We proposed that prolonged sleep loss stimulates the DA system to achieve the demand of arousal maintenance by activating the stress response systems. Furthermore, the elevated stress responses result in various neuronal and microglial adaptions in the DA systems, including altered striatal DA receptors, decreased microglial pruning capacity, and primed neuroinflammatory signaling, which contributes to enhanced sensitivity to psychostimulants in sleep-deprived adolescents. While a wealth of studies showed that SD models have prominent face validity to neuropsychiatric disorders such as schizophrenia, herein we provide evidence indicating similar underlying mechanisms covering the three major aspects, namely the aberrant neuroendocrine, DA, and immune signaling, between SD and a wild range of neuropsychiatric illnesses. The detrimental effects of sleep insufficiency on the DA system are potent risk factors for the development of mental disorders and should be considered deliberately in the prevention or therapeutic strategy for neuropsychiatric disorders.

# Abbreviations

Amph: Amphetamine

CNS: central nervous system

CR3: complement receptor 3

CRF: corticotrophin-releasing factor

CRF-R1: corticotrophin-releasing factor-receptor 1

CRF-R2: corticotrophin-releasing factor-receptor 2

CPu: caudate-putamen

CX3CR1: CX3C chemokine receptor 1

DA: dopamine/dopaminergic

D1R: dopamine D1 receptor

D2R: dopamine D2 receptor

DAT: dopamine transporter

GAPDH: glyceraldehyde 3-phosphate dehydrogenase

HPA: hypothalamic-pituitary-adrenal

Iba1: ionized calcium-binding adaptor molecule 1

IL-18: interleukin-18

IL-1b: interleukin-1 beta

IL-6: interleukin-6

MINO: minocycline

MSD: minocycline-treated SD

MSN: medium spiny neuron

NAcC: core of nucleus accumbens

NAcS: shell of nucleus accumbens

NLRP3: NOD-, LRR- and pyrin domain-containing protein 3

NS: normal sleep

PSD95: postsynaptic density protein 95

PPI: prepulse inhibition

PVN: paraventricular hypothalamic nucleus

TH: tyrosine hydroxylase

TNF-a: tumor necrosis factor-alpha

SD: sleep deprivation

SNc: substantia nigra pars compacta

SNr: substantia nigra pars reticulata

VSD: vehicle-taking SD

VTA: ventral tegmental area

# Declarations

### Compliance with Ethical Standards

### Disclosure of potential conflicts of interest

None.

### Research involving Human Participants and/or Animals

Animals.

### Informed consent

Not applicable.

### Ethics approval

All animal experiments were approved by the Institutional Animal Care and Use of National Taiwan University College of Medicine and were conducted in compliance with the ethical guideline.

### Consent to participate

Not applicable.

### Consent for publication

All authors have reviewed and approve the contents of the manuscription and consent for publication.

### Availability of data and materials

Not applicable.

### Competing interests

All authors declared no competing interests, neither financially nor non-financially.


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### Authors' contributions

LHT and LJL designed all the experiments and wrote the manuscript. LHT and JWY performed experiments and analyzed the data. JHL analyzed the data and proof-read the manuscript. All authors read and approved the final manuscript.


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# Figures

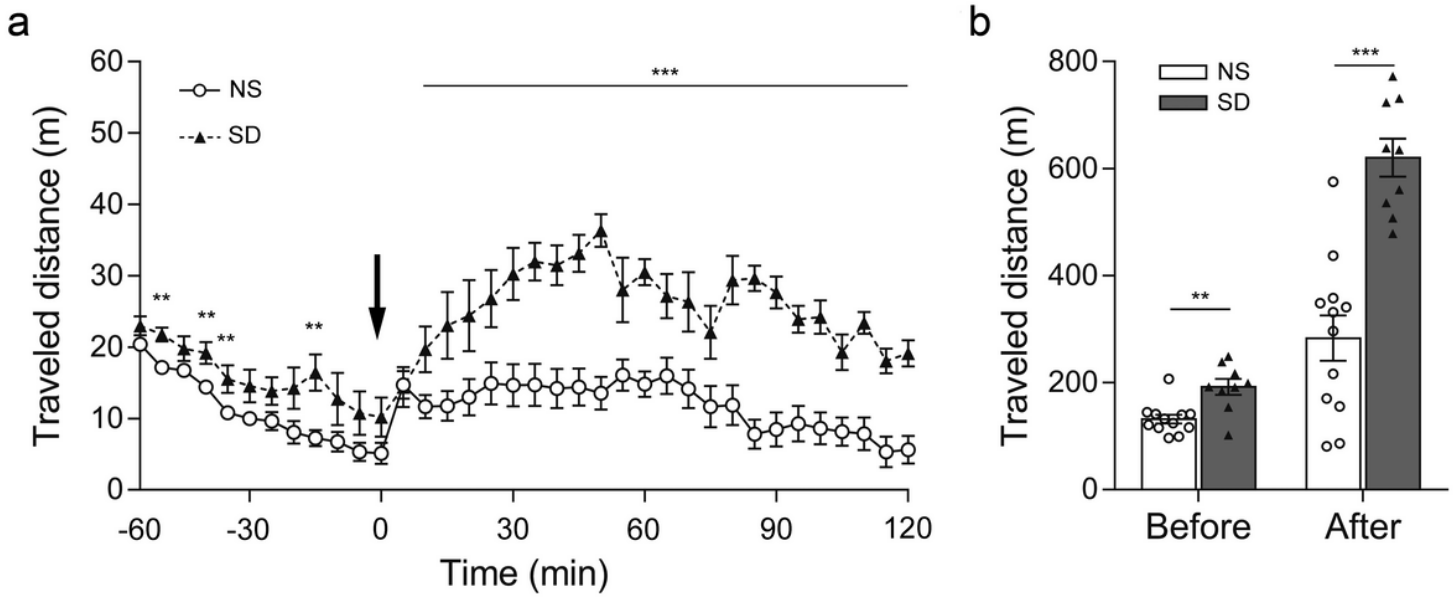
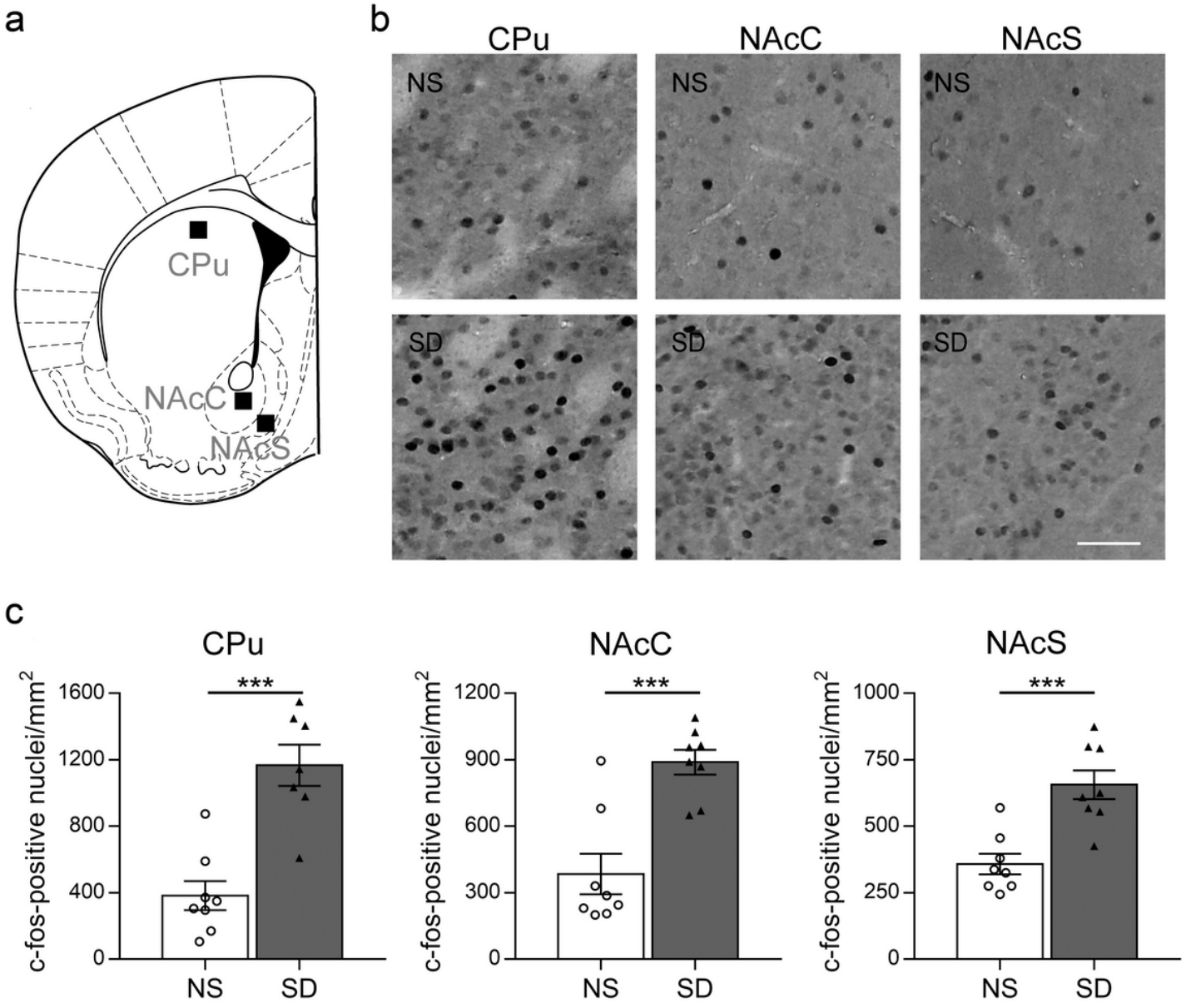



**Figure 1**

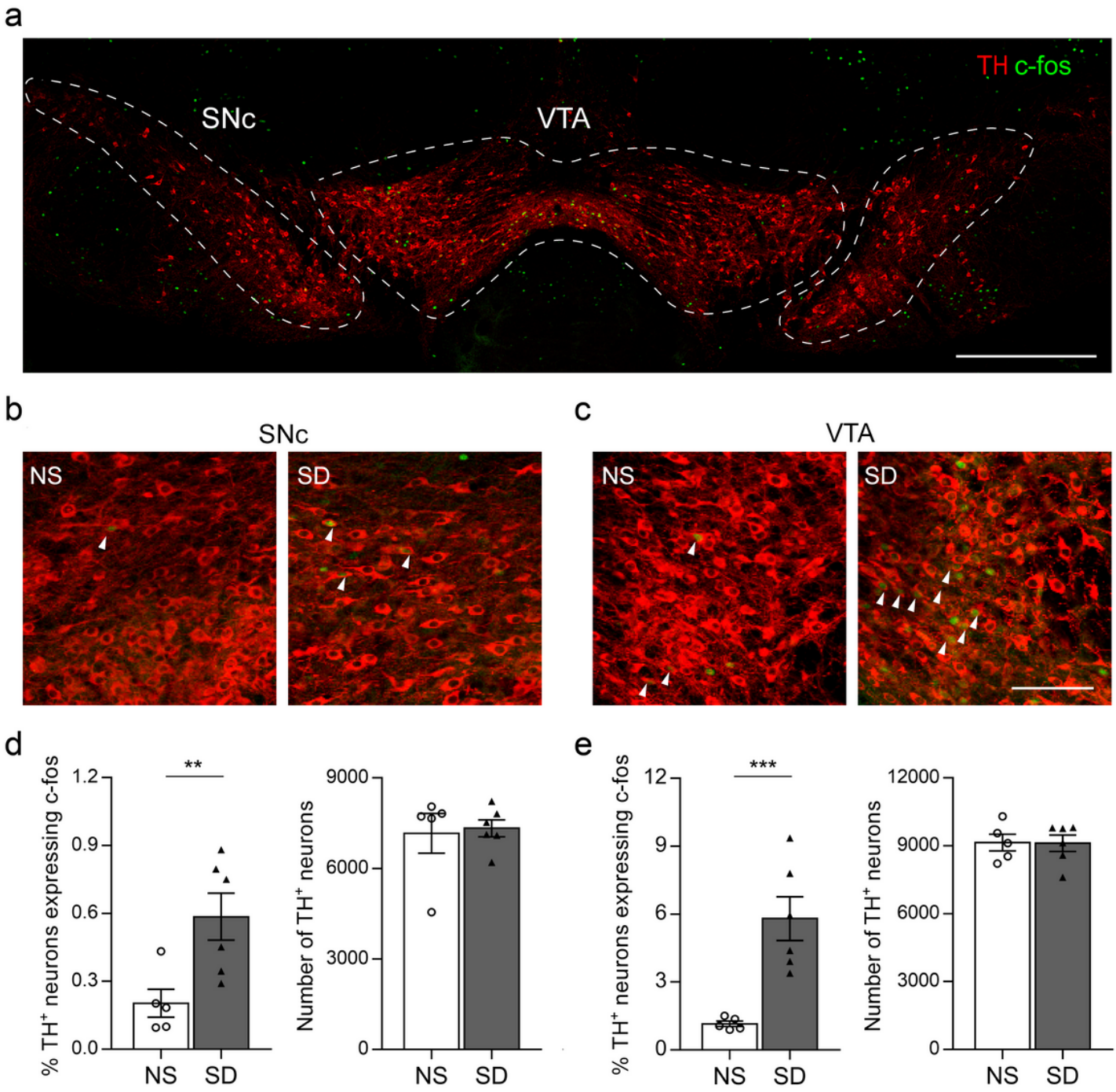
Locomotor activity in the Amph challenge test. Mice of NS and SD groups were brought to the novel environment and the locomotor activity was recorded. Amph (the arrow in a) was given 60 min later. The locomotor activity of mice is represented as the traveled distance of every 5 min (a) and the total traveled

distance before and after the Amph injection (b). Data collected from NS (N = 12) and SD (N = 9) groups are presented as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.




## Figure 2

Striatal neuronal activity following the Amph challenge test. The subregions of the striatum including the CPu, NAcA, and NAcS were selected (a). The densities of c-fos-positive nuclei in the three subregions of the striatum were evaluated (b). Amph induced higher c-fos expression in mice of the SD group in all subregions (c). Scale bar: 50 mm in (b). Data collected from NS (N = 8) and SD (N = 7) groups are presented as means ± SEM. $^{***}p < 0.001$.




## Figure 3

Neuronal activity of the midbrain DA neurons following the Amph challenge test. DA neurons in the SNc and VTA were labeled by TH immunofluorescence (a). The number of total TH-positive DA neurons and the percentage of c-fos-positive DA neurons was assessed (b & c). Amph induced higher c-fos expression in SNc and VTA DA neurons of the SD group, whereas no change was observed in the total number of TH-positive DA neurons (D & E). Scale bar: 500 mm in (a), 100 mm in (c). Data collected from NS (N = 5) and SD (N = 6) groups are presented as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.

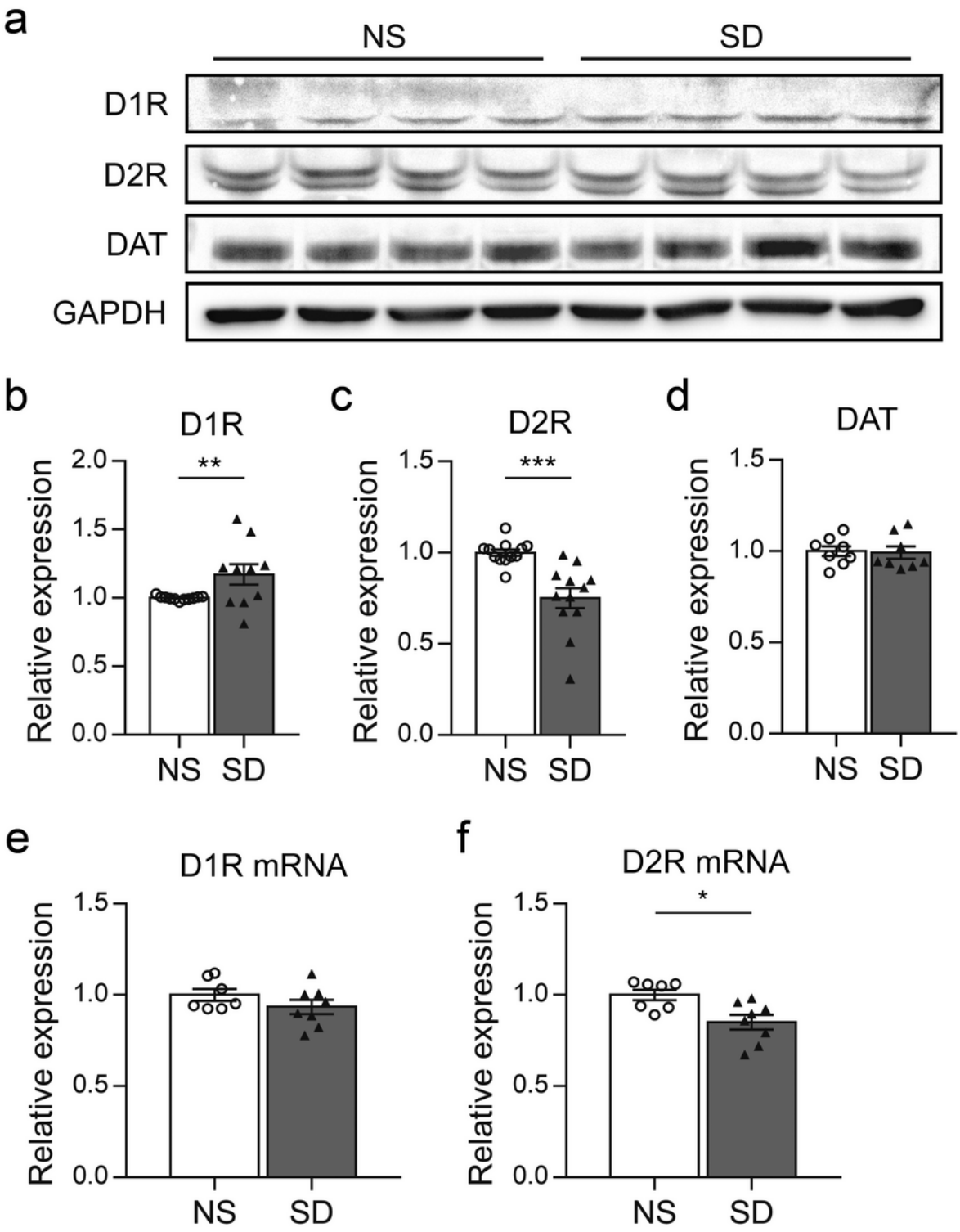
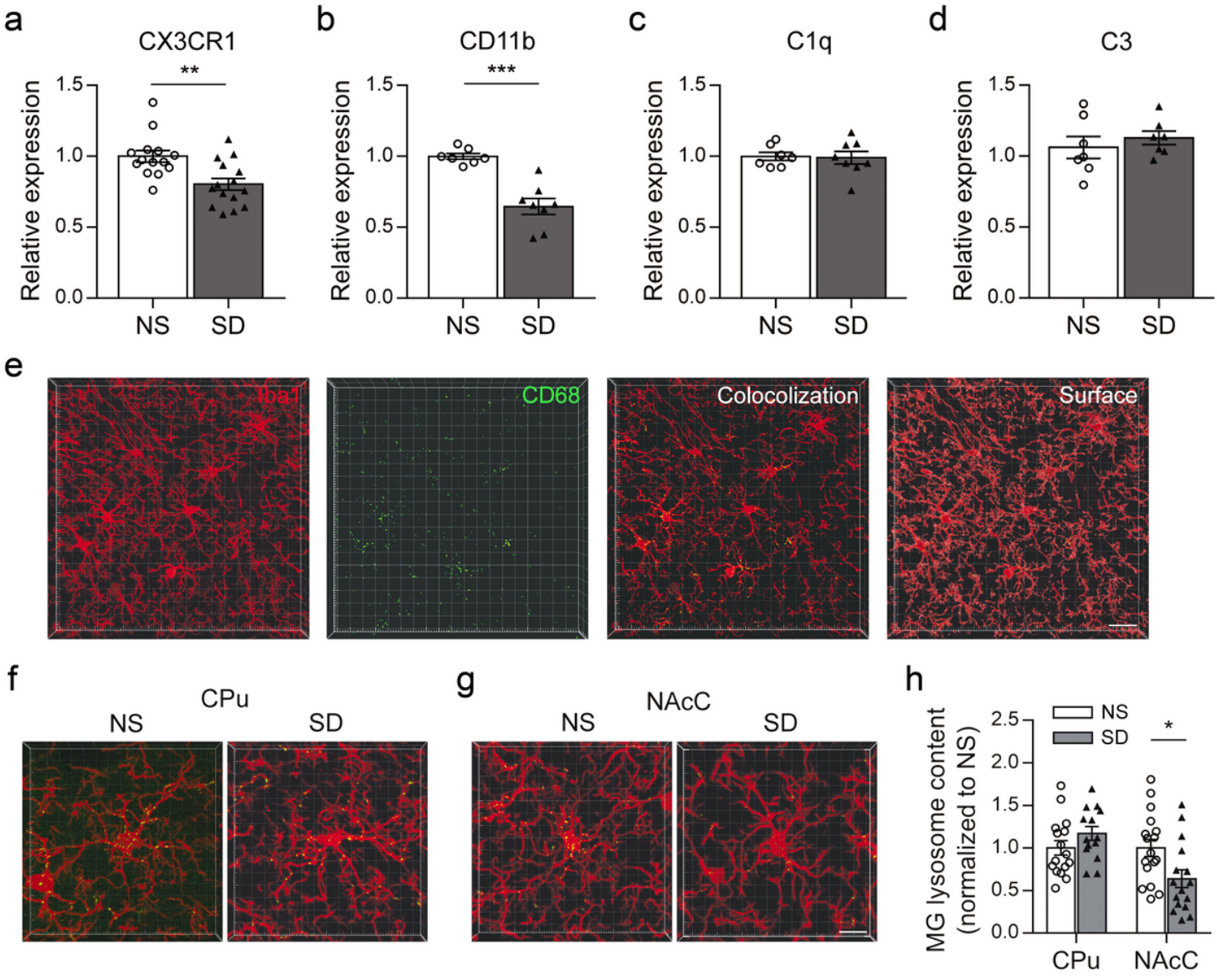


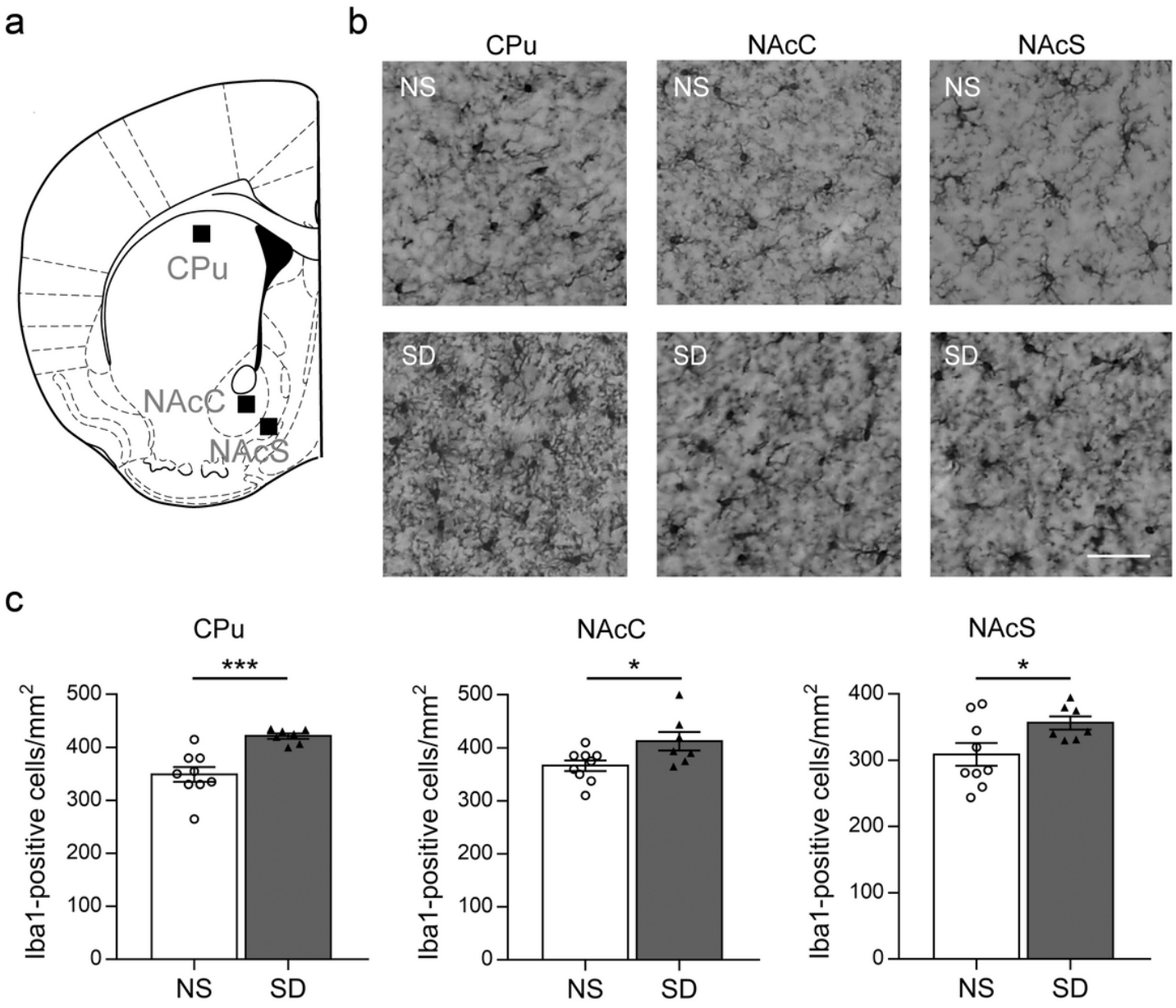

**Figure 4**

Expression of striatal DA receptors. The protein levels of D1R, D2R, and DAT in the striatal homogenate were revealed by western blot (a) and quantified (b). GAPDH was used as an internal control. The mRNA level of D1R was unchanged (e), while D2R mRNA was reduced significantly (f). N = 7-12 mice in each group. Data are means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.



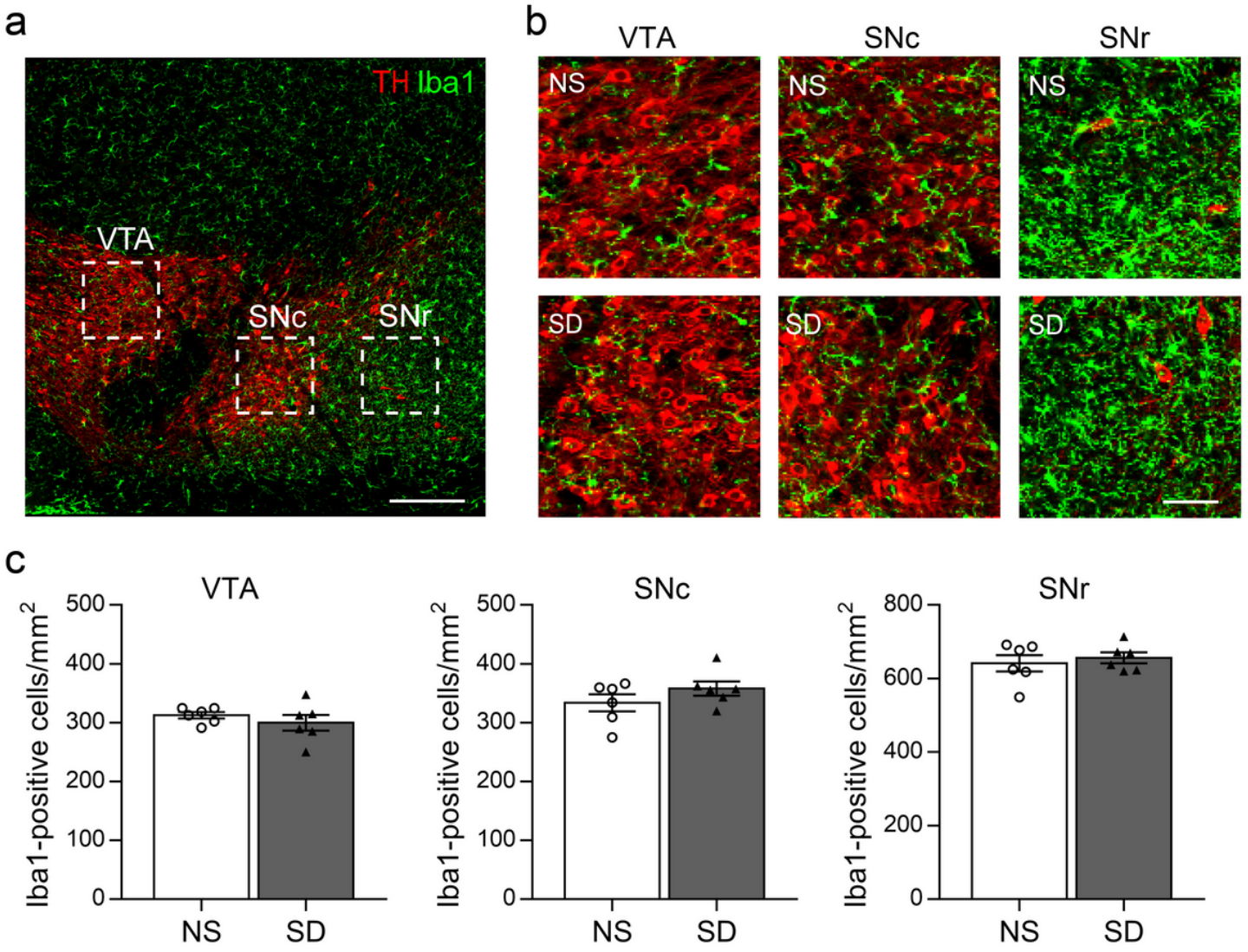

## Figure 5

Expression of critical components for neuronal-microglial interaction and microglial phagocytic capacity in the striatum. The mRNA expression of microglial-specific receptors CX3CR1 (a), and CD11b (b) were downregulated in the SD mice, while C1q (c) and C3 (d), ligands for the complement pathway, remained unaltered. N = 7-15 mice in each group. Microglial phagocytic capacity was further characterized by assessing the volume of CD68 within microglia (e). Microglia in the CPu and NAcC were captured by a confocal microscope and reconstructed (f & g). A reduction of CD68 volume within the microglia in the NAcC was found in the SD mice (h). Scale bar: 20 mm in (e), 5 mm in (g). n = 14-17 z-stacks from 5 mice in each group. Data are means ± SEM. *$p < .05$, **$p < 0.01$, ***$p < 0.001$.



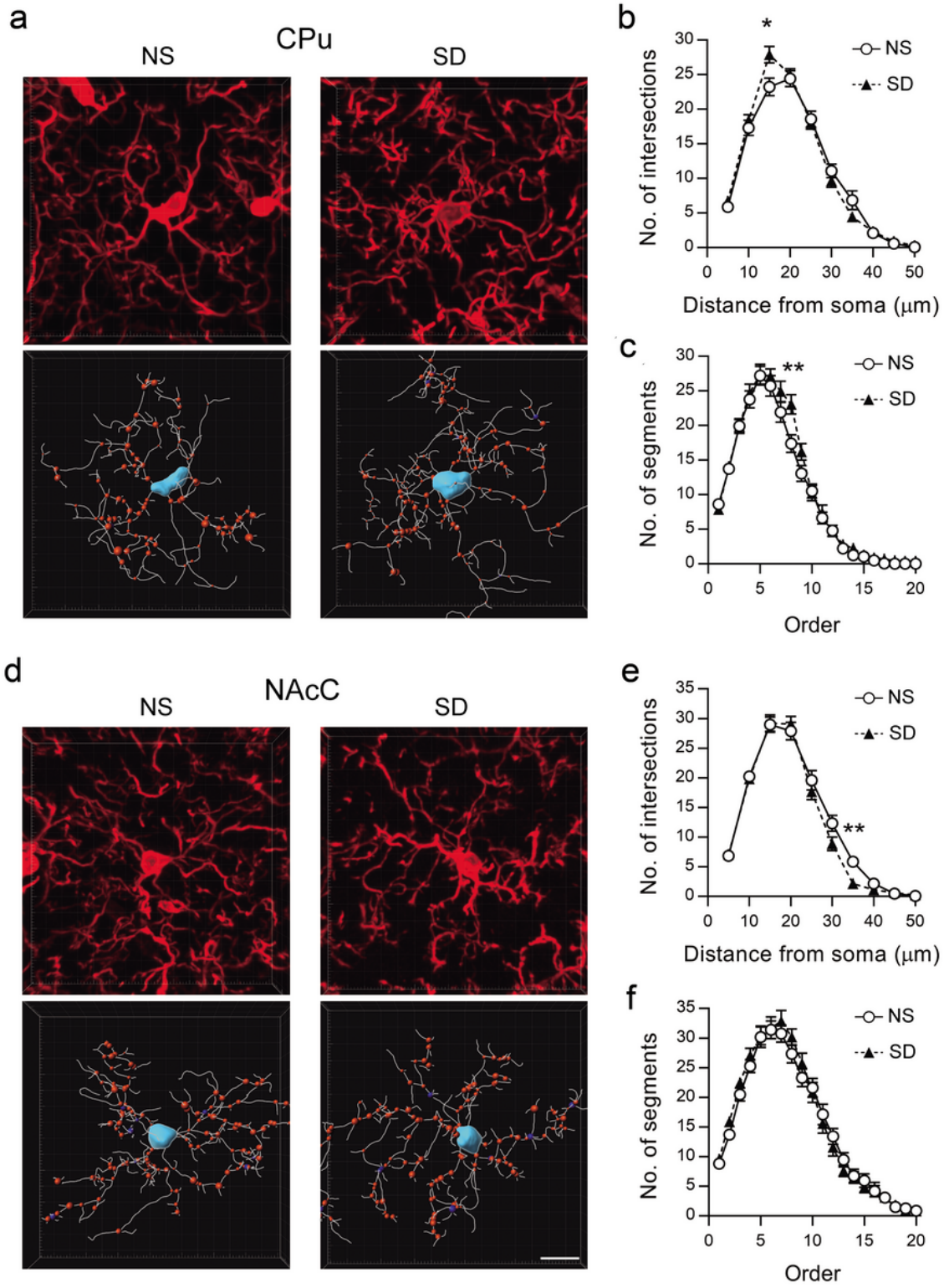

## Figure 6

Microglia density in the striatum. The subregions of the striatum including the CPu, NAcA, and NAcS were selected (A). The densities of Iba1-positive microglia in the three subregions were evaluated (B). Higher microglia densities in the three subregions of the striatum were noted (C). Scale bar: 50 mm. Data collected from NS (N = 9) and SD (N = 7) groups are presented as means ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$.




## Figure 7

Microglia density in the midbrain. Microglia and DA neurons were labeled by Iba1 and TH double immunofluorescence (a). The density of microglia within the VTA, SNc, and SNr, were measured (b). Microglia density was comparable between the NS and SD groups in all three regions. Scale bar: 200 mm in (a), 50 mm in (b). Data are means ± SEM. N = 6 mice in each group.




## Figure 8

Morphometric analyses of microglia. The morphology of individual microglia within the CPu and NAcC were reconstructed (a & d). In the CPu, microglia of the SD group exhibited increased intersections in the Sholl analysis (b) and greater number of segments (c). In the NAcC, a subtle reduction of intersections was found in microglia of the SD group (e), while the number of segments was comparable between the

two groups (f). Scale bar: 10 mm. n = 23-24 microglia from 4 mice in each group. Data are presented as means ± SEM. *$p < 0.05$, **$p < 0.01$.

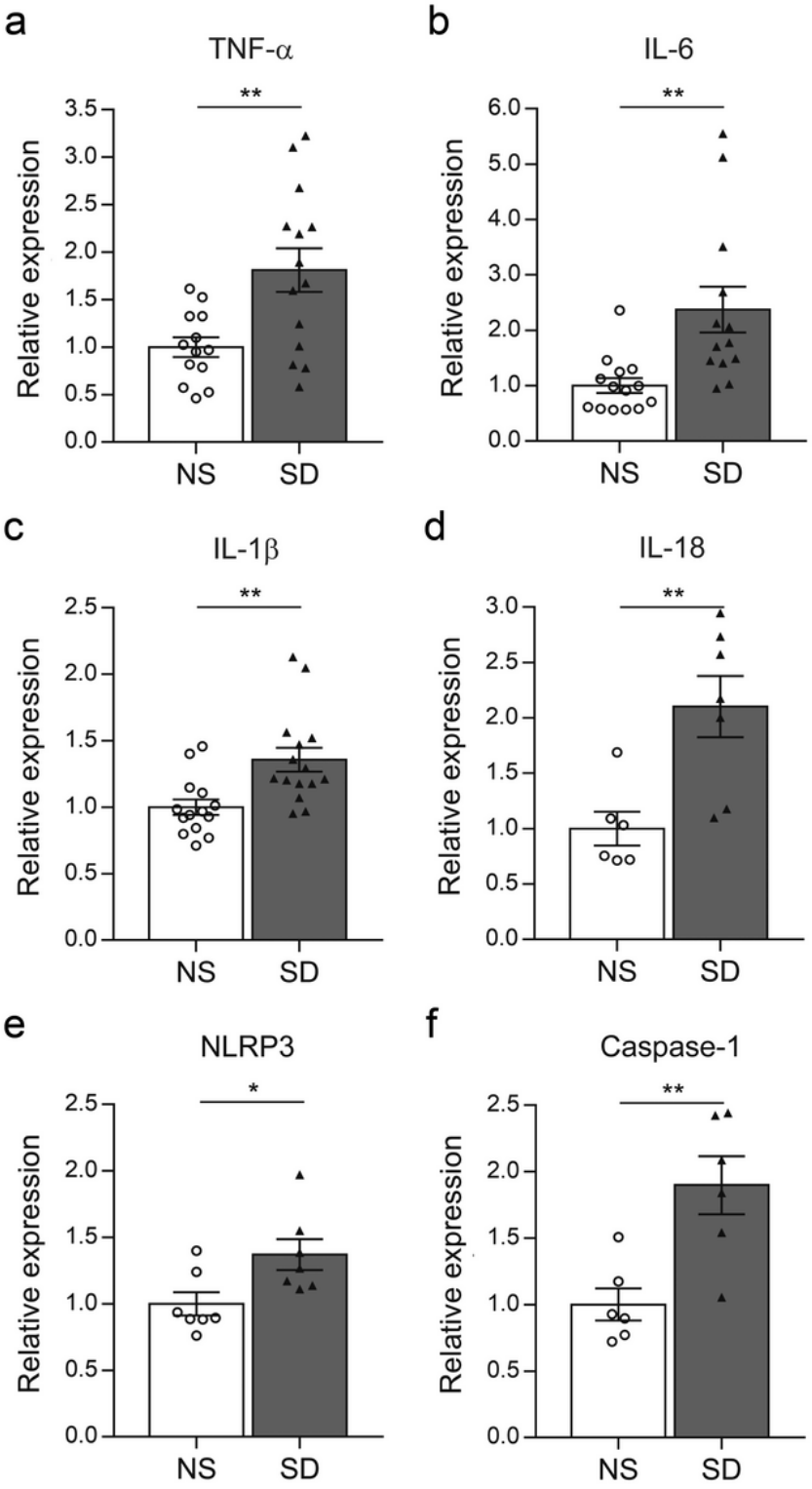



**Figure 9**

mRNA level of TNF-a (a), IL-6 (b), IL-1b (c), IL-18 (d), NLRP3 (e), and Caspase-1 (f) in the striatal homogenate were evaluated through the qPCR assay. n=7-15 mice in each group. Data are means ± SEM. *$p < 0.05$, **$p < 0.01$.

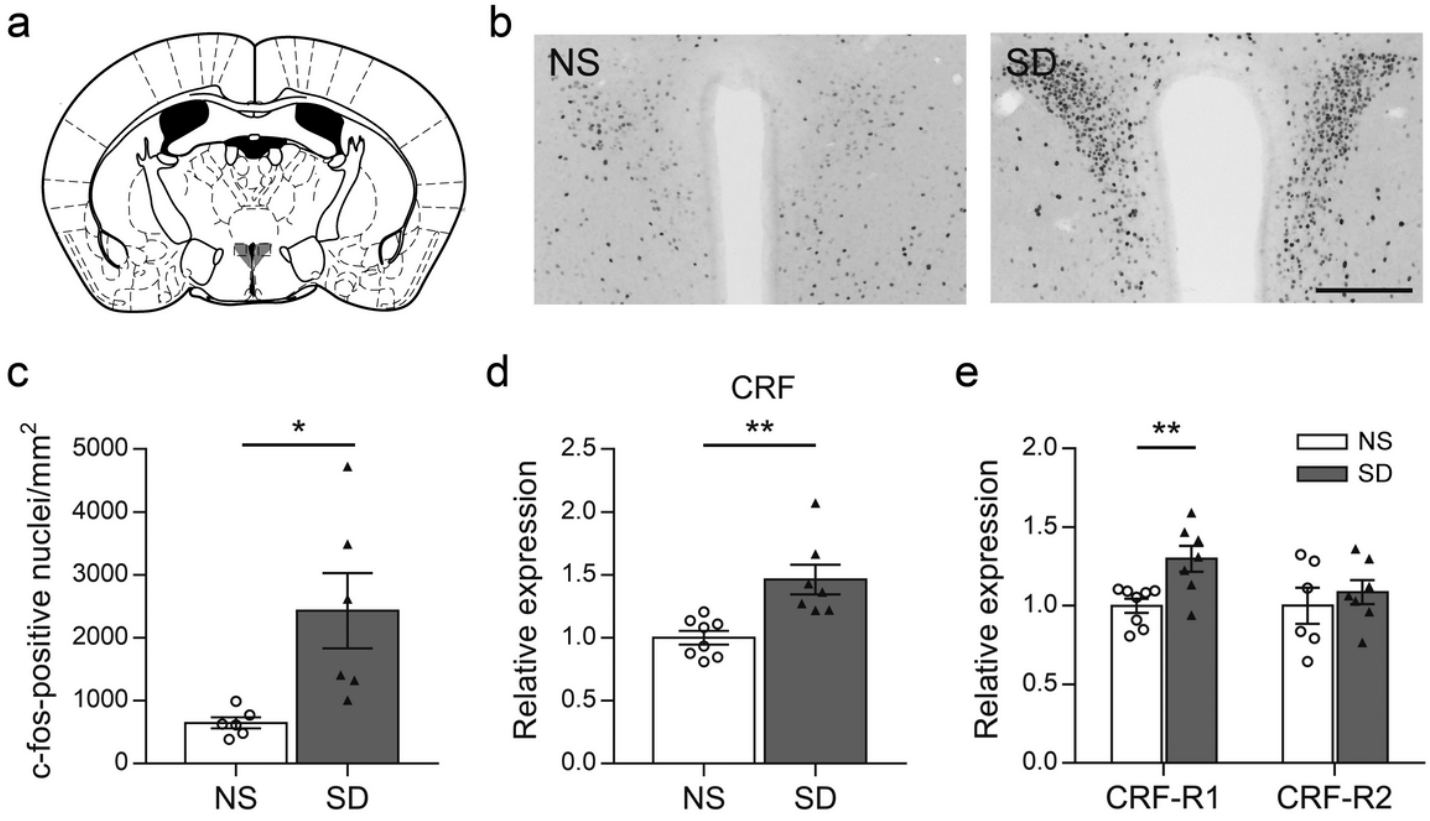



**Figure 10**

The activity of CRF system. The neuronal activity in the PVN was evaluated by measuring the density of c-fos-positive nuclei (a & b). The density of c-fos-positive nuclei was significantly increased in the SD group (c). N = 6 mice in each group. Scale bar: 200 mm. The mRNA expression of CRF and CRF-R1 were upregulated in the hypothalamus and midbrain of SD mice (d & e). N = 6-8 mice in each group. Data are means ± SEM. *$p < 0.05$, **$p < 0.01$.

# Supplementary Files

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