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FoxO1 regulates leptin-induced mood behavior by targeting tyrosine hydroxylase

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ABSTRACT

Purpose: While leptin has been associated with various psycho-physiological functions, the molecular network in leptin-mediated mood regulation remains elusive.
Methods: Anxiolytic behaviors and tyrosine hydroxylase (TH) levels were examined after leptin administration. Functional roles of STAT3 and FoxO1 in regulation of TH expression were investigated using in vivo and in vitro systems. A series of animal behavioral tests using dopaminergic neuron-specific FoxO1 KO (FoxO1 KO^{DAT}) were performed and investigated the roles of FoxO1 in regulation of mood behaviors.
Results: Here, we show that administration of leptin induces anxiolytic-like phenotype through the activation of signal transducer and activator of transcription 3 (STAT3) and the inhibition of forkhead box protein O1 (FoxO1) in dopaminergic (DA) neurons of the midbrain. Specifically, STAT3 and FoxO1 directly bind to and exert opposing effects on tyrosine hydroxylase (TH) expression, where STAT3 acts as an enhancer and FoxO1 acts as a prominent repressor. Accordingly, suppression of the prominent suppressor FoxO1 by leptin strongly increased TH expression. Furthermore, our previous results showed that specific deletion of FoxO1 in DA neurons (FoxO1 KO^{DAT}) led to a profound elevation of TH activity and dopamine contents. Finally, FoxO1 KO^{DAT} mice exhibited enhanced leptin sensitivity as well as displayed reduced anxiety- and depression-like behaviors.
Conclusions: This work establishes a novel molecular mechanism of mood behavior regulation by leptin and suggests FoxO1 suppression by leptin might be a key for leptin-induced behavioral manifestation in DA neurons.

1. Introduction

Obesity has been shown to contribute to numerous pathophysiological processes of both physical and mental illnesses. Importantly, obese individuals have been reported to be more vulnerable to the development of mood disorders [1–4], though the molecular network in obesity-mediated mood regulation is poorly understood. In addition, obesity is closely associated with the development of resistance to leptin [5–7], a 16-kDa peptide secreted by the adipose tissue. Compelling evidence has demonstrated that leptin acts mainly on the central nervous system (CNS) to regulate nutrient intake and energy homeostasis [8]. More interestingly, it has been further suggested that leptin signaling has been linked to mood regulation [4,9,10].

Specifically, leptin has been shown to play a role in the regulation of reward and anxiety-like behaviors [10–20]. For instance, repeated administration of leptin into leptin-deficient *ob*/*ob* mice showed anxiolytic-like behaviors indicated by increased time spent in the open arms in the elevated plus maze [21]. Moreover, specific deletion or mutation of leptin receptors in the dopaminergic (DA) neurons induced anxiogenic-like behavior in mice due to an alteration in the dopamine signaling [20,22]. Taken together, these studies raise the possibility that signaling pathways activated by leptin in the dopaminergic neurons might be essential for the mood regulation. However, underlying molecular network on how leptin is contributed to the regulation of mood behavior is not fully understood. Therefore, identification of the molecular pathways mediating leptin's action on mood regulation would be critical to understand leptin's diverse physiological function.

In the arcuate nucleus of the hypothalamus (ARC), activation of signal transducer and activator of transcription 3 (STAT3) and inhibition

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of forkhead box protein O1 (FoxO1) were shown in response to leptin and these transcription factors directly involved in control of food consumption through direct actions on *Pomc* and *Agrp* genes [23,24]. Although leptin receptors are widely expressed and leptin acts in extra-hypothalamic regions [25], no distinct targets in other brain regions regulated by leptin are revealed despite of diverse physiological roles of leptin.

In the present study, we show that systemic leptin administration induced anxiolytic-like behaviors together with a significant increase of TH expression in the mouse midbrain. Leptin-mediated TH expression was associated with a markedly increased phosphorylation of signal transducer and activator of transcription 3 (STAT3) and forkhead box protein O1 (FoxO1). Molecular experiments showed that both STAT3 and FoxO1 directly bind to the promoter region of TH, where these two transcriptional factors exhibited opposing effects on the TH expression. Specifically, TH activity was enhanced upon STAT3 activation, whereas was suppressed by FoxO1 activation, in which the latter demonstrated a greater potency in regulation of TH expression. Therefore, activation of STAT3 and inactivation of FoxO1 simultaneously by leptin signaling strongly increased TH expression. Correspondingly, DA neuron-specific FoxO1 knock-out mice (FoxO1 KO^{DAT}) showed enhanced leptin sensitivity and markedly increased TH expression [26], suggesting that leptin-mediated TH expression is highly potentiated in the absence of FoxO1 in the DA neurons. Consequently, deletion of FoxO1 in DA neurons (FoxO1 KO^{DAT}) was sufficient to induce anxiolytic as well as anti-depression-like behavioral phenotypes, confirming our finding that leptin-mediated mood regulation might be prominently through the inactivation of FoxO1 followed by TH expression increment.

2. Materials and Methods

2.1. Reagents

Dulbecco's modified Eagle's medium (DMEM) was obtained from HyClone Laboratories Inc. (Thermo Scientific, UT, USA). Fetal bovine serum (FBS) and penicillin-streptomycin (P/S) were purchased from Gibco (Life Technologies, NY, USA). Recombinant mouse leptin was purchased from Sigma (Sigma Aldrich, MO, USA, Cat#. L3772) or National Hormone and Peptide Program - A.F. Parlow (Torrance, CA, USA). Wortmannin (Cat#. 9951), LY294002 (Cat#. 9901) and PD98059 (Cat#. 9900) were obtained from Cell Signaling Technology (MA, USA). Niclosamide (Cat#. sc-250564) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Akt (Cat#. 2920, 1:10,000), phosphor-Akt (Ser473) (Cat#. 4060, 1:10,000), Erk1/2 (Cat#. 9107, 1:5000), phosphor-Erk1/2 (Thr22/Thr24) (Cat#. 9101, 1:5000), FoxO1 (Cat#. 2880, 1:2000), phosphor-FoxO1 (Thr24) (Cat#. 9464, 1:2000), STAT3 (Cat#. 9139, 1:5000), phosphor-STAT3 (Tyr705) (Cat#. 9131, 1:2000) were purchased from Cell Signaling Technology (MA, USA). Anti-Tyrosine hydroxylase (TH) (Cat#. AB152, 1:1000) was purchased from Millipore (EMD Millipore, MA, USA). Anti-myc (Cat#. 11667149001, 1:1000) was purchased from Roche (Roche, Basel, Switzerland) and anti-flag (Cat#. F3165, 1:5000) was obtained from Sigma (Sigma-Aldrich, MO, USA). Antibody against β-actin (Cat#. ab6276, 1:10,000) was obtained from Abcam (Cambridge, UK). GAPDH (Cat#. sc-25778, 1:5000) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents were purchased from Sigma-Aldrich unless otherwise stated.

2.2. Cell Culture and Plasmid Vector Transfection

HEK293T, mouse neuroblastoma Neuro2A (described in Khanh et al., main reference 7), and hypothalamic N1 [27] cells were maintained in DMEM media containing 10% FBS and 1% P/S and cultured in a humidified atmosphere of 5% CO_2 at 37°°C. Cells were transfected with Lipofectamine 2000 (Invitrogen, CA, USA) according to the manufacturer's instructions. For luciferase assays, cells were lysed 24 h after transfection. For mRNA and protein levels, cells were harvested 48–96 h after transfection. All transfections were performed in triplicate. Vectors encoding myc-tagged FoxO1-WT, FoxO1-ADA (constitutively nuclear form with the mutations T24A, S253D and T316A), FoxO1-DN (dominant negative form lacking trans-activation domain with a deletion truncating after amino acid 265) and flag-tagged STAT3-WT, STAT3-CA (constitutively nuclear form with the mutation at Tyr705), STAT3-DN (dominant negative form with a mutation) and control vector, pCMV5, were used for FoxO1 or STAT3 overexpression experiments. All the STAT3-related vectors (STAT3-WT, STAT3-CA, and STAT3-DN) are gifts from Dr. Yong Xu in Baylor College of Medicine.

For experiments of activation of leptin receptor signaling, cells were transfected with low dose (100 ng) of plasmid vector encoding the long-form leptin receptor (LepRb) or control pcDNA 3.1 vector (100 ng). After 24 h transfection, cells were treated with leptin at a final concentration of 100 nM for an additional time from 2 to 24 h depending on particular experiments.

2.3. Extraction of Nuclear and Cytoplasmic Fractions

Cells were washed with 1× PBS and harvested. The cell membranes were lysed in ice-cold buffer A (10 mM HEPES pH 8.0, 10 mM KCl, 0.1 mM EDTA pH 8.0, 0.1 mM EGTA pH 8.0, 1 mM DTT, 100 mM NaF) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). The cytoplasmic supernatant fraction was collected after centrifugation. The nuclear pellet was then lysed in ice-cold buffer C (20 mM HEPES pH 8.0, 400 mM NaCl, 1 mM EDTA pH 8.0, 1 mM EGTA pH 8.0, 1 mM DTT, 100 mM NaF) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland) and was centrifuged to collect the supernatant nuclear fraction. The nuclear and cytosolic protein was then analyzed by Western blotting.

2.4. Animals

All animal experiments in this study were approved by the Institutional Animal Care and Use Committee (IACUC) of the Yonsei University Wonju College of Medicine. C57BL/6 and *db*/*db* mice were purchased from RaonBio (Yong In, South Korea). FoxO1 KO^{DAT} (KO, knock-out) mice were generated by crossing $DAT^{IREScre}$ with $FoxO1^{loxP/loxP}$ ($FoxO1^{F/F}$) mice [28–30]. Littermate mice homozygous for the floxed FoxO1 allele ($FoxO1^{F/F}$) without $DAT^{IREScre}$ allele served as controls (WT, wild-type). All mice were maintained in controlled room temperature (22–24°°C) with a 12-hour-light/dark cycle (light on/off at 6 *a.m./p.m.*). Mice were fed with normal chow (Zeigler, Gardners, PA, USA, Cat. No. Rodent NIH-31 Modified Auto, 4.02 kcal/g) or high-fat diet (HFD; Research Diets, Cat#. D12492, 60% fat, 5.24 kcal/g).

2.5. Measurement of Behavioral Phenotype

10 to 14-week-old male C57BL/6, FoxO1 WT, and FoxO1 KO^{DAT} mice were subjected to the behavior tests. Mice were maintained in their home cages with food and water ad libitum. Testing mice were transferred to behavioral room (temperature was maintained at 22–24 °C and the light intensity was 280 lx) and acclimated for 3 h. Either leptin (5 mg/kg) or saline was intraperitoneally administered into C57BL/6 mice 1 h prior to conducting behavior tests. All behavioral tests were conducted between 10:00 and 16:00 and data obtained were blindly analyzed by two third-party investigators.

2.5.1. Open-Field Test (OFT) Physiology605!

The open field arena (600(d) × 600(w) × 200(h) mm) was used to measure the locomotor function and anxiety-like behavior of mice [31]. The center and peripheral zone were designed using SMART v3.0 video tracking system (Panlab Harvard Apparatus, MA, USA) and were 150 mm^2 and 600 mm^2, respectively, where the former was located exactly at the center of the latter. Each mouse was placed individually at the south-east corner of the arena and left freely to explore for 10 min

in a lit room (280 lx). The distance travelled, mean speed, time spent in the center and peripheral zone were monitored and analyzed using SMART v3.0 video tracking system (Panlab Harvard Apparatus, MA, USA).

2.5.2. Elevated Plus-Maze (EPM)

The elevated plus-maze (660(d) × 660(w) × 370(h) mm) consisted of four arms arranged in a cross shape, of which two arms had no side and end walls, and the other two had side and end walls and elevated to a height of 370 mm [32]. The length of each arm was 300 mm with the arm width of 60 mm. The center area, in which the four arms were intersected was 60 mm^2. Mice were placed in the center zone facing the north closed arm and allowed to freely explore the maze for 10 min in a lit room (280 lx), where each entry was defined as all four paws were entering each arm. The percentage of time spent in the open and closed arms, number of transition, and distance travelled were monitored and analyzed using SMART v3.0 video tracking system (Panlab Harvard Apparatus, MA, USA).

2.5.3. Light-Dark Box (LDB)

Light-dark box was used to measure the time mice spent in either the brightly illuminated (620 lx, 250(d) × 250(w) × 240(h) mm) or dark compartment (7 lx, 250(d) × 250(w) × 240(h) mm) [33]. The compartments were divided by a plastic wall, where an opening (70 mm^2) between the two compartments was located at the bottom center of the dividing wall with a manually controlled door (80 mm^2). Each mouse was placed in the middle of the dark compartment facing away from the opening and the door was manually opened after 5 s. Mice were allowed to freely explore both compartments for 10 min in a dark room (7 lx) and were video-recorded with Canon EOS 700D (Canon Inc., Tokyo, Japan). The latency to the lit side, time spent in each compartment, and number of transitions were analyzed.

2.5.4. Forced Swimming Test (FST)

Forced swimming test was used to measure depression-like phenotype of mice. The test was conducted by placing a mouse in a semi-transparent 5 L cylinder (188.5(d) × 229(h) mm, Kartell, Milan, Italy, Cat#. KA.819) containing 3 L of water of the temperature of 22–24°°C for 7 min in a lit room (270 lx) [34]. The water was discarded and refilled after each trial. Behavioral phenotypes were recorded with Canon EOS 700D (Canon Inc., Tokyo, Japan). As each mouse was placed in the water-filled cylinders, the movements of a mouse during the first 2 min were not measured as it tended to exhibit hyper-activity. A mouse was considered to be immobile when it exhibited the floating or balancing behavior. The latency to immobilization and immobilized time were analyzed.

2.5.5. Tail Suspension Test (TST)

Tail suspension test was used to measure depression-like phenotype of mice. Each mouse was hung 250–270 mm above the bottom surface by taping their tails, at approximately 10 mm from the tip of the tail, to a non-transparent container (250(w) × 400(h) mm) [35]. Each mouse was recorded with Canon EOS 700D (Canon Inc., Tokyo, Japan) for 10 min in a lit room (270 lx). A mouse was considered immobile if it hung down without apparent movements. The latency to immobilization and immobilized time were analyzed.

2.6. Leptin Administration and Brain Dissection

C57BL/6 mice were fed normal chow and water ad libitum. Mice were daily administered (intraperitoneally, *i.p.*) with normal saline or 3 mg/kg body weight of leptin (National Hormone and Peptide Program, Torrance, CA, USA) prepared in saline in an experimental period of 3 days. Body weight was monitored daily before administration. After 2 h of the last dose of leptin, the mice were killed. Serum and organs were collected for further analyses. After removing the brain from the skull, the hypothalamus, cortex, cerebellum, and midbrain were dissected and harvested under the low power dissecting microscope. All brain samples were snap-frozen on dry ice and stored at −80°°C for further analysis. For immunohistochemistry for p-STAT3, mice were fasted for 18 h and administered saline or leptin (5 mg/kg). After 1 h, mice were perfused and tissues were obtained for further analysis.

2.7. Leptin Measurement

Leptin levels were measured using ELISA kits (Morinaga Institute of Biological Science, Yokohama, Japan) in accordance with manufacturer's instructions.

2.8. SDS-PAGE and Western Blotting

The cells were washed with 1× PBS and lysed by RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 1% Triton-X-100, 0.5% sodium deoxycholate and 0.1% SDS) containing protease and phosphatase inhibitors (Roche, Basel, Switzerland). For in vivo samples, the tissues were homogenized and lysed by RIPA buffer with protease and phosphatase inhibitors. The protein concentration was measured using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, CA, USA). Standard Western blotting was performed and the blots were visualized using the chemiluminescence UVP BioSpectrum® 600 Imaging System (UVP, CA, USA).

2.9. Reverse Transcription PCR (RT-PCR) and Quantitative Real-Time PCR

Total RNA was isolated using Ambion® Trizol reagent (Life Technologies, CA, USA) and 1 μg of total RNA was used to synthesize cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystem, CA, USA) in accordance with the manufacturer's instructions. For reverse transcription PCR (RT-PCR), cDNAs were amplified using TaKaRa® ExTaq (TaKaRa Bio Inc., Shiga, Japan) and the PCR products were analyzed on 1.5% agarose gel. The primers for LepRb (product size, 191 bp) were following: forward, 5′-GCTCTTCTGATGTATTTGGAAATC-3′, reverse, 5′-ACCTGATATTGAAGCGGAAATGG-3′ and for β-actin (product size 202 bp) were following: forward, 5′-AAATCGTGCGTGAC ATCAAA-3′, reverse, 5′-ATGCCACAGGATTCCATACC-3′.

For quantitative real-time PCR (qPCR), cDNA and primers were prepared with a *Power* SYBR® Green PCR Master Mix (Applied Biosystem, Warrington, UK) according to the manufacturer's instructions. The expression of TH gene was normalized to β-actin. The primers were used including TH, forward, 5′-TTGGCTGACCGCACATTT-3′, reverse, 5′-GCCCCCAGAGATGCAAGT-3′ and β-actin, forward 5-TTCTACAATGAGCT GCGTGTG-3′, reverse, 5′-GGGGTGTTGAAGGTCTCAAA-3′.

2.10. Luciferase Reporter Assays

The construct of 5′ proximal promoter region of the mouse TH gene with potential binding sites of FoxO1 and STAT3 (TH_luc, from −696 to −1) was cloned into pGL3-basic vector using *Xho*I and *Kpn*I sites. HEK293T cells were transfected with the following plasmids: FoxO1-WT (500 ng), FoxO1-ADA (mutated in T24 to A, S256 to D, and S316 to A), FoxO1-DN (500 ng), STAT3-WT (500 ng), STAT3-CA (500 ng), STAT3-DN (500 ng) or pCMV5 (500 ng) and pGL3B (250 ng) or TH_luc (250 ng) and renilla (100 ng). After 24 h transfection, the activities of renilla and luciferase were determined using the Dual Luciferase Reporter Assay System (Promega, WI, USA). The luciferase activity was normalized to renilla activity.

2.11. Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay were performed in isolated chromatin from cultured Neuro2A cells transfected with flag-tagged STAT3-CA using the following primers: target site, forward, 5′-CCTGCAAAAGTGGGC-3′, reverse, 5′-GCTCTCTAATCAAACC-3′; non-target site, forward, 5′-GGTCAGGTC

AGCAGGG-3′, reverse, 5′-CCCTCTAGACAGGGC-3′. The antibodies of anti-STAT3 (Cell Signaling, Cat#. 9139 and EMD Millipore, Cat#. 06-596), anti-flag (Sigma Aldrich, MO, USA, Cat#. F3165) and protein A/G-coupled agarose beads (Thermo Scientific, CA, USA, Cat#. 20422) were used to pull down the protein-chromatin complex. The chromatin fragments were purified by Phenol:Chloroform:Isoamyl alcohol (Sigma Aldrich, MO, USA, Cat#. P-2069) in accordance to the manufacturer's extraction protocol. The purified chromatin samples were then amplified by PCR using TaKaRa® ExTaq (TaKaRa Bio Inc., Shiga, Japan) and the PCR products were analyzed on 2% agarose gel. The PCR conditions for all primers were 95°°C for 3 min and 39 cycles of denaturation at 95°°C for 30 s, annealing at 58°°C for 60 s and extension at 72°°C for 60 s.

2.12. Statistical Analysis

The Prism 5.0 software was utilized for all statistical analyses. ANOVA or Student's *t*-test was used to assess the difference between groups and $P < 0.05$ was regarded as a statistical significance difference.

3. Results

3.1. Anxiolytic-Like Effect of Leptin is Associated With Elevated TH Expression in the Midbrain

To address molecular mechanisms underlying leptin-mediated mood behaviors, we first systemically administered leptin into the mice and subjected to a series of behavioral tests. Leptin-administered mice exhibited anxiolytic-like phenotypes indicated by increased time spent in the light side in the light-dark box (Fig. 1A) without change in latency to the lit side, and increased time spent in the open arms in the elevated plus-maze (EPM) (Fig. 1B and C) with no distinct changes in the locomotor function (Supplementary Fig. 1A). Unlike chronic stressed condition [36], leptin administration into normal mice seems not affect to depression-like behaviors (Supplementary Fig. 1B and C).

Considering previous reports that leptin administration in *ob*/*ob* mice induced anxiolytic phenotype [21], together with increased dopamine contents in the midbrain [18,37], we speculated that anxiolytic effect of leptin might be mediated via alterations in TH activity, a rate-limiting enzyme for dopamine synthesis. Examination of the expression of TH and long-form leptin receptor (LepRb), a major isoform mediating leptin signaling in the brain [38,39] revealed an abundant expression in the midbrain and hypothalamus (Fig. 1D and E). Next, we induced hyperleptinemic condition without alteration of body weight by leptin administration and monitored TH levels (Fig. 1F and Supplementary Fig. 1D). TH expression was significantly enhanced only in the midbrain and hypothalamus where LepRb were highly expressed, but not in the adrenal gland (Fig. 1G and H). In addition to leptin administration, chronic hyperleptinemic condition was generated by feeding mice with HFD (Fig. 1I). TH expression was markedly increased in the brain of HFD-induced mice (Fig. 1J and K). In sharp contrast, *db*/*db* mice, a mouse model with no functional leptin receptors, exhibited decreased TH expression in the midbrain and hypothalamus suggesting a significant role of leptin in the regulation of TH expression (Fig. 1L and M).

3.2. Reciprocal Role of STAT3 and FoxO1 in TH Regulation

It has been suggested that JAK/STAT3 and IRS2/PI3K/Akt/FoxO1 are two major downstream signaling pathways of leptin signaling, leading to activation/inactivation of the two transcriptional factors, STAT3 and FoxO1, on target genes [8]. Intriguingly, we noticed that the increased TH expression by leptin in the midbrain and hypothalamus was concomitant with the activation of leptin downstream factors including STAT3, Akt, and FoxO1 (Fig. 2A–D and Supplementary Fig. 2A) while no distinct changes in PI3K/Akt/FoxO1 and STAT3 pathways were seen in the adrenal glands (Supplementary Fig. 2B and C). Based on the previous finding and leptin's action [23,24], we thought there might be a reciprocal action of FoxO1 and STAT3 on TH expression. Sequence analysis revealed two putative STAT3 binding sites close to the identified FoxO1 binding site (Fig. 2E). Chromatin immunoprecipitation (ChIP) assays established that STAT3 directly binds to the target TH promoter region containing the potential binding site, but not to the non-target sequences (Fig. 2F). Reporter assays using a luciferase construct containing the promoter region of TH demonstrated that over-expression of wild-type (WT) STAT3 enhanced the TH activity which was further increased by the constitutively active form of STAT3 (STAT3-CA) (Fig. 2G and H). In contrast, over-expression of a dominant negative mutant form of STAT3 (STAT3-DN) had no effect on reporter activity (Fig. 2G and H). Consistent with the reporter assay, overexpression of STAT3 increased endogenous TH mRNA and protein levels (Fig. 2I). Notably, the stimulatory effect of STAT3 on the TH transcription was strongly suppressed by FoxO1 (Fig. 2J and K, and Supplementary Fig. 2D and E). These results highly indicate that STAT3 and FoxO1 directly bind to the promoter and play opposing roles in regulation of TH expression.

3.3. Identification of FoxO1 as a Prominent Inhibitor in Leptin-Mediated TH Expression

To determine whether leptin regulates TH expression by bidirectional shuttling of FoxO1 and STAT3 between the nucleus and cytoplasm, Neuro2A cells in which functional leptin receptor and TH are expressed [26] were treated with leptin and monitored shuttling of FoxO1 and STAT3. Upon leptin treatment, phosphorylated FoxO1 (p-FoxO1) is exported from the nucleus, whereas phosphorylated STAT3 (p-STAT3) is imported into the nucleus (Fig. 3A and B).

Considering STAT3 and FoxO1 exhibit opposing effects on TH expression, we hypothesized that leptin enlists STAT3 and FoxO1 to regulate TH gene expression. To address this possibility, LepRb was transiently transfected into HEK293T cells and confirmed the activity by monitoring STAT3 and FoxO1 phosphorylation after leptin treatment (Supplementary Fig. 3A). In this condition, leptin significantly increased TH gene promoter activity only in the presence of functional LepRb (Fig. 3C). In addition, endogenous TH expression was enhanced by leptin treatment in Neuro2A cells (Fig. 3D). To further characterize regulatory roles of FoxO1 and STAT3 on TH expression, the effect of leptin on TH expression was examined in the presence or absence of a PI3K inhibitor (wortmannin), STAT3 inhibitor (niclosamide) or both (Fig. 3E and Supplementary Fig. 3B and C). Treatment of wortmannin (2 μM) and niclosamide (0.1 μM) completely blocked PI3K and p-STAT3 activities, respectively (Supplementary Fig. 3B and C). The effect of leptin on the TH promoter activity and mRNA expression was completely blocked by 2 μM wortmannin (Fig. 3E and F). However, blockade of p-STAT3 using niclosamide was failed to suppress leptin-induced TH activation (Supplementary Fig. 3D). In addition, combined treatment of low dose wortmannin (1 μM) in which PI3K activity is partially inhibited (Supplementary Fig. 3B) and niclosamide (0.1 μM) showed synergistic reduction of TH promoter activity (Fig. 3E). Furthermore, pretreatment with another PI3K inhibitor, LY294002, also significantly blocked the effect of leptin on the TH transcription (Fig. 3G and Supplementary Fig. 3E). In contrast, there was no change in leptin-induced TH reporter activity upon treatment with the ERK inhibitor, PD98059 (Supplementary Fig. 3F). These results strongly indicate that STAT3 and PI3K pathways, but not the ERK pathway, play critical roles in leptin-mediated TH expression with a prominent role of PI3K/Akt/FoxO1 pathway.

3.4. FoxO1 Inhibition is Essential for Leptin-Mediated TH Expression

To further investigate the potential contributions of FoxO1 and STAT3 on the TH transcription upon leptin treatment, the effect of leptin on TH expression was examined in the context of FoxO1 and STAT3 overexpression. Different from the effect of STAT3 on pro-

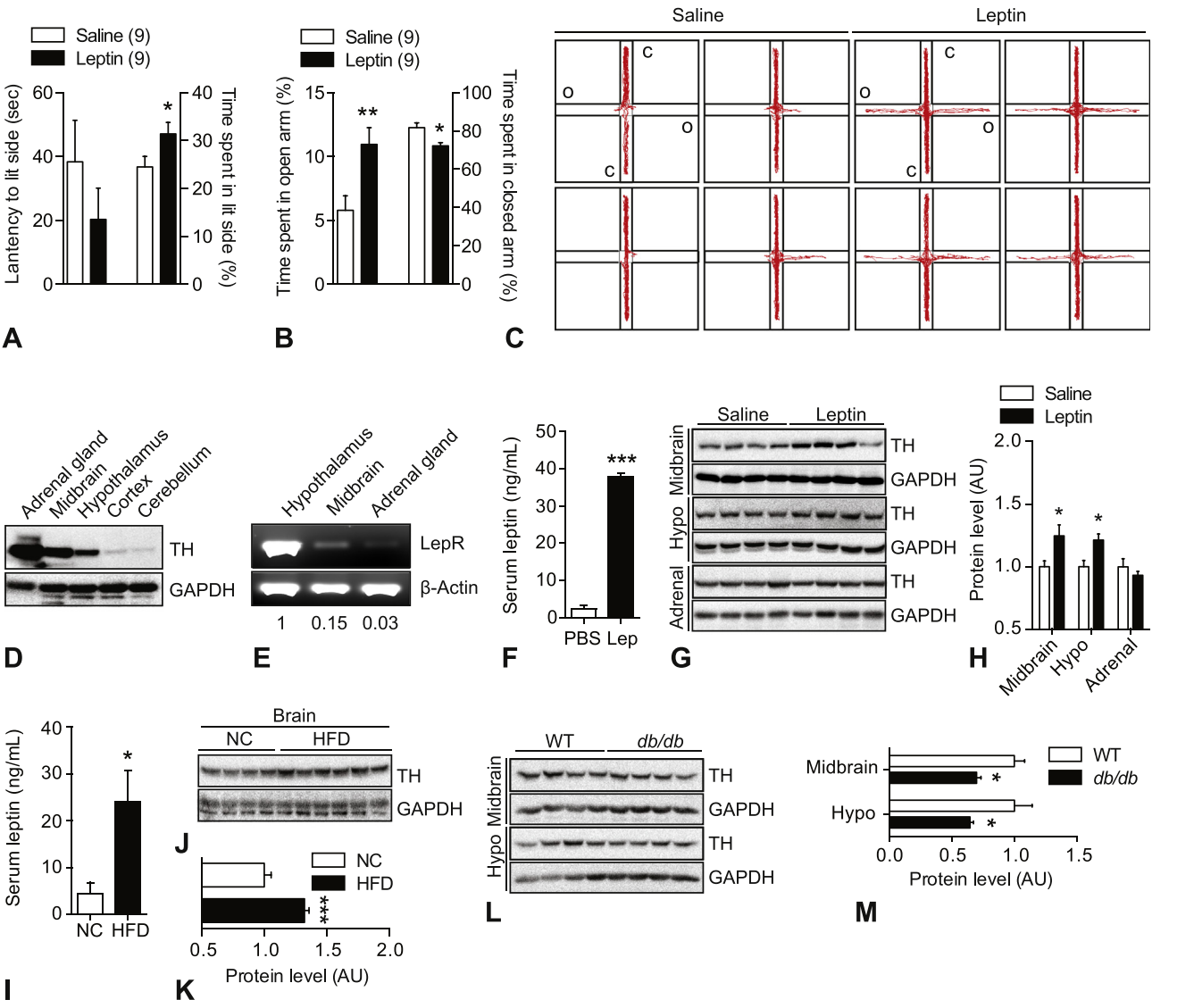

Fig. 1. Leptin-mediated anxiolytic-like behaviors is accompanied with TH increment. A. Latency to the lit side (sec) and time spent in the lit side (%) of saline or leptin administered C57BL/6 mice were measured using the light-dark box. Sec, second. B. Time spent in the open or closed arms (%) of saline or leptin administered mice were measured using the elevated plus-maze. C. Travel pathway in the elevated plus-maze. c, closed arm. o, open arm. D. Western blot for TH in indicated tissues. E. Expression of LepR in indicated tissues. The digits below each lane indicate relative levels of LepR expression. F. Serum leptin levels after leptin administration for 3 days. G, H. Immunoblots (G) and relative levels (H) of TH in the indicated regions of mice administered saline or leptin for 3 consecutive days. Hypo, hypothalamus. I. Serum leptin levels after 6 weeks of HFD. J, K. Immunoblots (J) and relative levels (K) of TH in the brain of C57BL/6 mice fed with either normal chow (NC) or high-fat diet (HFD) for 6 weeks. L, M. Immunoblots (L) and relative levels (M) of TH in the indicated regions of *db/db* mice. All mice were fed with NC unless stated otherwise. The results are expressed as mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-tests).

opiomelanocortin (POMC) gene regulation [24], stimulatory effect of STAT3 alone on the TH expression by leptin was not strong as its effect on the POMC expression (Fig. 4A and Supplementary Fig. 4A and B). However, STAT3 seems necessary for leptin-mediated transcriptional regulation of TH, as the gene was failed to be increased when STAT3 activity was inhibited (Fig. 4A). The FoxO1 suppression by leptin might play prominent role in TH expression as the overexpression of FoxO1 completely blunted the effect of leptin and inhibition of FoxO1 using FoxO1-DN strikingly potentiated the effect of leptin on TH expression (Fig. 4B and C, Supplementary Fig. 4C and D). Taken together, our results suggest that suppression of the inhibitory effect of FoxO1 is imperative for full activation of TH.

3.5. Deletion of FoxO1 in DA Neurons Shows Improved Mood Behaviors

In parallel with our finding of the prominent inhibitory role of FoxO1 on the TH expression, mice with permanent deletion of FoxO1 specifically in DA neurons (FoxO1 KO^{DAT}) showed markedly enhanced leptin sensitivity (Supplementary Fig. 5A–C) and displayed a robust increase in TH activity and dopamine contents in the midbrain [26]. These findings led us to postulate that the anxiolytic effects of leptin might be mediated through the suppression of FoxO1 activity in DA neurons. Therefore, we employed dopamine neuron-specific FoxO1 KO (FoxO1 KO^{DAT}) mice to suppress FoxO1 activity specifically in the DA neurons. The FoxO1 KO^{DAT} mice displayed anxiolytic-like behaviors in a series of behavioral tests, such as the open field (Fig. 5A–B), elevated plus-maze test (Fig. 5C–D), and light-dark box (Fig. 5E), indicated by increased time spent in the center, open arms, and light compartment, respectively (Fig. 5A–E), without an apparent difference in locomotor function (Fig. 5F). In addition, FoxO1 KO^{DAT} mice demonstrated antidepression-like behavior, illustrated by reduced immobilized time in the forced-swim (Fig. 5G) and tail hanging tests (Fig. 5H). Combined with previous findings [26], these results indicate that suppression of FoxO1 by leptin in DA neurons might lead to an increase in TH expression and dopamine levels, which could be essential for behavioral manifestations induced by leptin signaling activation.

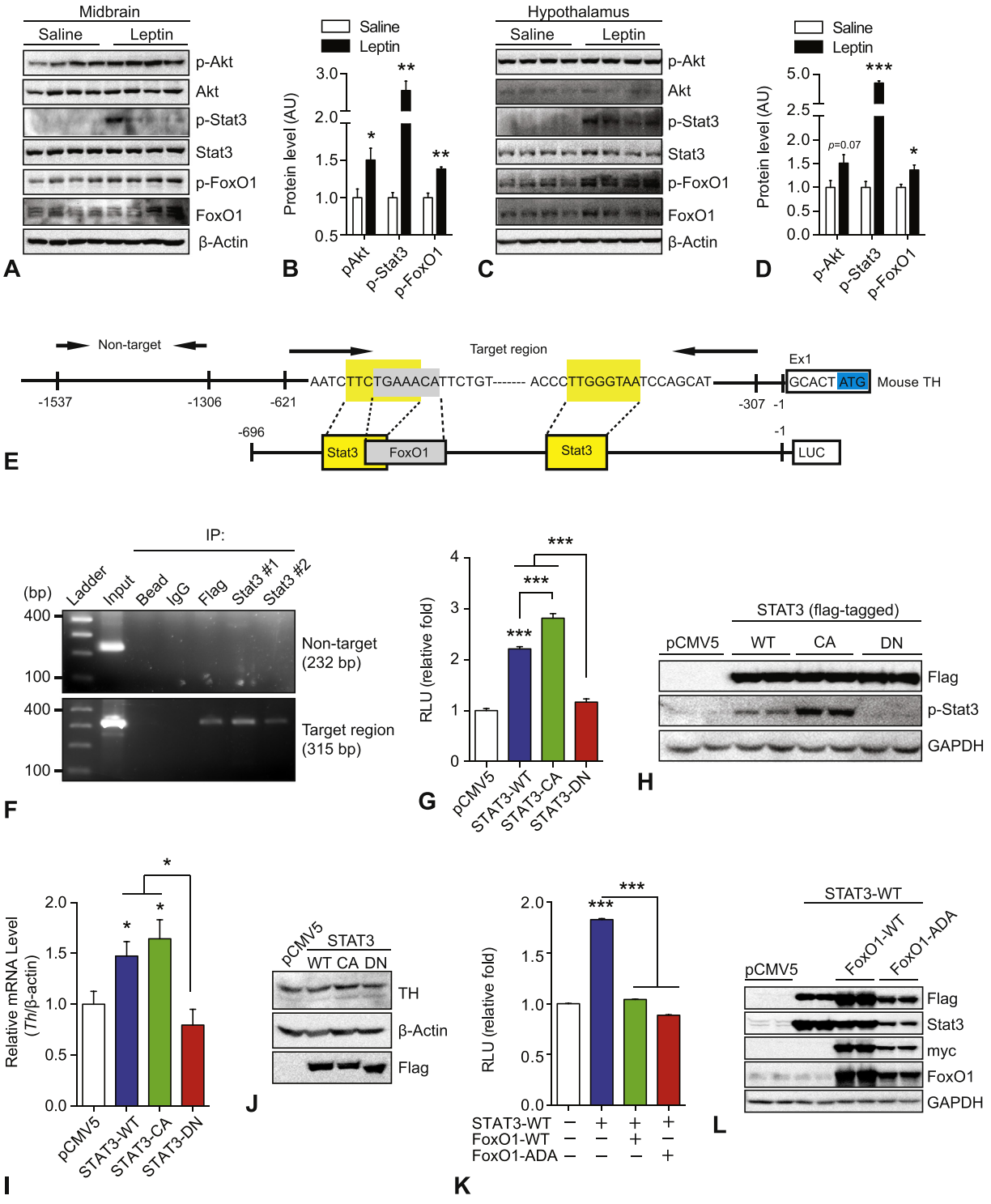

Fig. 2. Direct transcriptional regulation of TH by STAT3 and FoxO1. A, B. Immunoblots (A) and relative levels (B) of indicated proteins in the midbrain of C57BL/6 mice administered saline or leptin for 3 consecutive days. C, D. Immunoblots (C) and relative levels (D) of indicated proteins in the hypothalamus of mice administered saline or leptin for 3 consecutive days. E. Schematics for the mouse TH promoter (top) and luciferase construct containing potential binding sites for FoxO1 and STAT3 (bottom). F. ChIP assays using Neuro2A cells transfected with flag-tagged STAT3-CA showing a specific binding of STAT3 on the TH promoter. G. Relative luciferase activity after expression of STAT3 (WT), constitutive active STAT3 (CA) and dominant negative STAT3 (DN). H. Immunoblots confirming the expression of STAT3-WT, -CA, and -DN. I. TH mRNA levels after overexpression of STAT3-WT, -CA and -DN in Neuro2A cells. J. TH levels after overexpression of STAT3-WT, -CA and -DN in Neuro2A cells. K. Opposing effects of STAT3 and FoxO1 on the regulation of TH promoter activity. L. Immunoblots confirming expression of STAT3-WT and FoxO1-WT and -ADA. The results are expressed as mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-tests in A and D, one-way ANOVA, Turkey's post-tests in other experiments).

4. Discussion

Altered mood regulation has been recognized in obese population. DeJesus et al. reported that the prevalence of anxiety and body mass index form a U-shaped association, where both the underweight and overweight population show a tendency toward development of anxiety [1]. Moreover, mice fed with high fat diet developed anxiogenic-like behavior, as well as memory deficit [2]. Considering that leptin

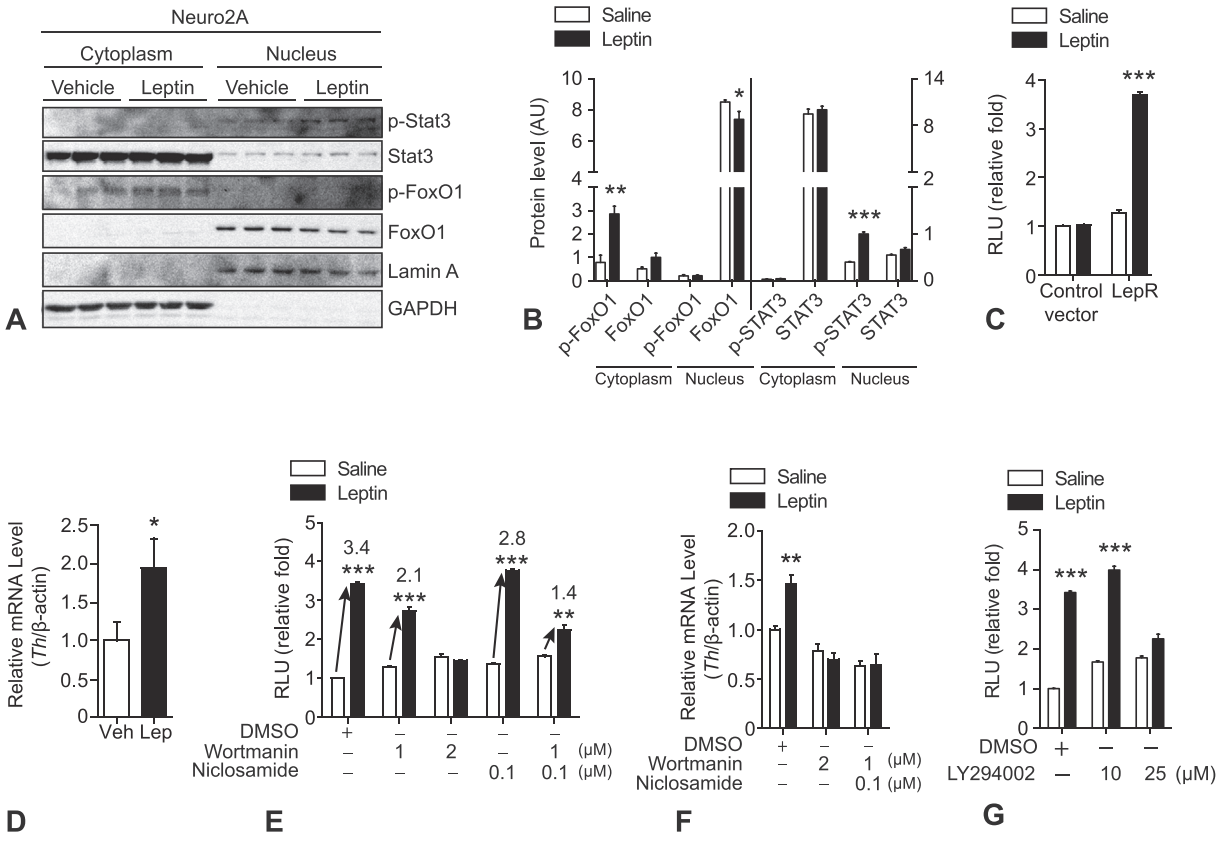

Fig. 3. Leptin-mediated TH expression is via reciprocal actions of STAT3 and FoxO1. A, B. Immunoblots (A) and relative levels (B) showing translocation of STAT3 and FoxO1 between the cytoplasm and nucleus after leptin treatment in Neuro2A cells. C. TH promoter activity after leptin treatment. D. Effect of leptin on TH mRNA levels in Neuro2A cells. E. Effects of leptin on TH promoter activity with or without wortmannin and niclosamide. The digits above each dark bar indicate the corresponding fold changes. F. Effect of leptin on TH mRNA expression with or without wortmannin and niclosamide in Neuro2A cells. G. Effects of leptin signaling activation on TH promoter activity with or without dose-dependent treatment of PI3K inhibitor (LY294002). The results are expressed as mean ± SEM. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test in D, two-way ANOVA, Bonferroni's post-tests in other experiments).

resistance develops during obesity progression, and previous studies which suggested a possible contribution of leptin in mood regulation [10,20–22,36], we postulated that obesity-mediated altered mood regulation could be contributable to defective leptin sensitivity. Previous findings in which leptin receptor-deficient *db/db* mice exhibited anxiogenic-like phenotype and administration of leptin in *ob/ob* mice manifested anxiolytic-like behavior support our notion that leptin administration might contribute to regulation of mood behavior [21,22]. However, the molecular mechanism by which leptin controls mood behaviors is not known. Our study demonstrates that leptin administration induces anxiolytic-like behavior through the activation of STAT3 and suppression of FoxO1. STAT3 and FoxO1 exert opposing effects on the tyrosine hydroxylase (TH) expression, where STAT3 acts as an enhancer and FoxO1 acts as a prominent repressor, indicating that leptin-mediated anxiolytic-like behavioral regulation is majorly through TH modulation. Identification of FoxO1 as a prominent suppressor for TH expression led us to establish a dopamine-neuron specific FoxO1 KO mouse model (FoxO1 KO^{DAT}), which demonstrated

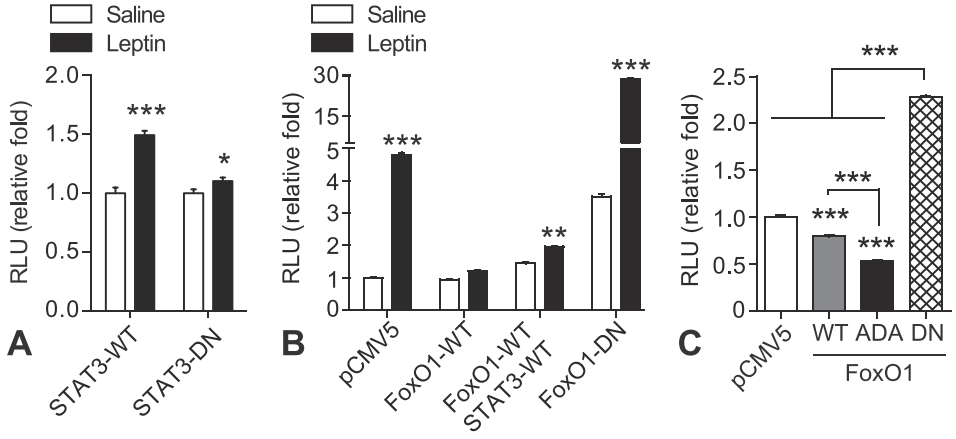

Fig. 4. FoxO1 plays a prominent inhibitory role on TH expression. A. Effects of leptin signaling activation with or without STAT3-WT or -DN overexpression on the TH promoter activity. B. TH promoter activity upon leptin signaling activation with or without overexpression of FoxO1-WT, -DN and STAT3-WT. C. TH promoter activity after FoxO1 (WT), constitutive active FoxO1 (ADA) and dominant negative FoxO1 (DN) overexpression. The results are expressed as mean ± SEM (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test in A, two-way ANOVA with Bonferroni's post-test in B, one-way ANOVA with Turkey's post-test in C).

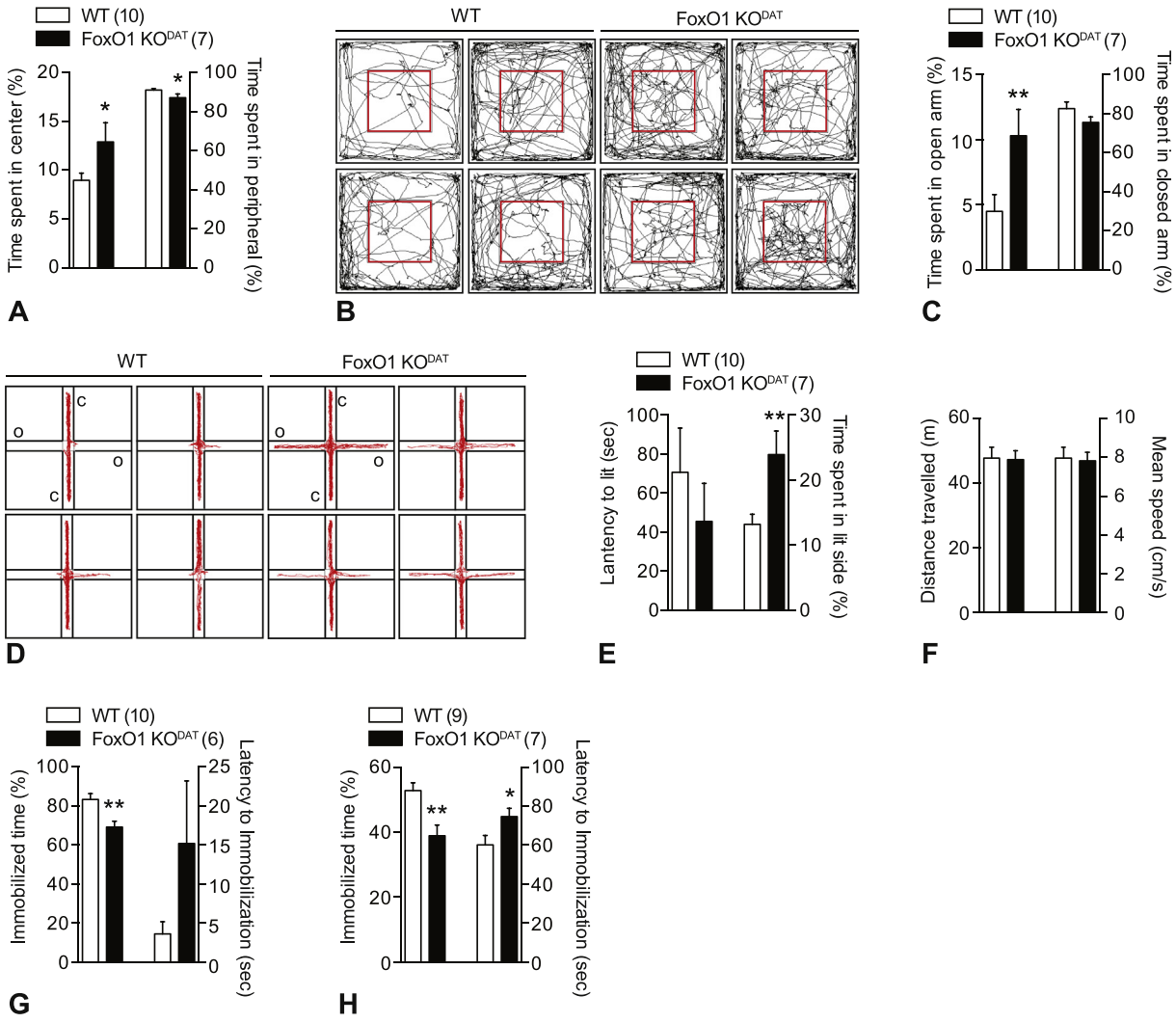

Fig. 5. Anti-anxiety- and anti-depression-like behaviors in FoxO1 KO^{DAT} mice. A. Time spent (%) in the center, and periphery of WT and FoxO1 KO^{DAT} mice were measured using the open field test. B. Travel pathway in the open field test. C. Time spent (%) in the open and closed arms of WT and FoxO1 KO^{DAT} mice were measured using the elevated plus maze. D. Travel pathway in the elevated plus maze. C, closed arm. O, open arm. E. Latency to lit side (sec) and time spent in the lit side (%) of WT and FoxO1 KO^{DAT} mice were measured using the light-dark box. F. Distance travelled (left), and mean speed (right) of WT and FoxO1 KO^{DAT} mice were measured using the open field test. G. Time of immobility (left) and latency to immobilization (right) of WT and FoxO1 KO^{DAT} mice were monitored using the forced swim test. H. Time of immobility (left) and latency to immobilization (right) of WT and FoxO1 KO^{DAT} mice were measured using the tail-suspension test. All mice used for behavioral tests were fed with NC. The results are expressed as mean ± SEM (*$P < 0.05$, **$P < 0.01$, Student's *t*-tests).

enhanced leptin sensitivity with increased TH and dopamine levels in the midbrain. Altogether, having monitored FoxO1 KO^{DAT} mice exhibiting anxiolytic behaviors, we suggest that repression of TH-inhibiting FoxO1 activity by leptin might play a pivotal role in leptin-mediated behavioral manifestations.

Systemic administration of leptin was accompanied with the significant increase in TH levels specifically in hypothalamus and midbrain where leptin receptor is densely expressed (Fig. 1) and had led us to postulated that leptin might regulate mood behaviors via alterations in the transcription and expression of TH, the rate-limiting enzyme for dopamine synthesis. In fact, we found that downstream transcription factors of leptin-signaling, STAT3 and FoxO1 directly bind to the TH promoter region and modulate the TH expression, eventually leading to increasing of dopamine contents [26].

Although several studies focused on serotonergic, GABAergic, and adrenergic systems, DA also has been suggested as a neurotransmitter in anxiety regulation [40,41]. Increased dopamine level in synaptic cleft has been known to enhance anxiety but deletion of DA receptors also induced anxiety delineating dual roles of dopamine signaling in anxiety regulation [20,41]. As we observed increased DA levels in the midbrain and SN in FoxO1 KO^{DAT} mice, the increased DA levels might contribute at least in part to the less anxious phenotype in the FoxO1 KO^{DAT} mice. Changes in downstream targets induced by FoxO1 deletion in the DA neurons might be an additional reason for the presentation of the anxiolytic phenotype.

It has been suggested that leptin triggers three major downstream signaling pathways: 1) JAK2 (Tyr1138)/STAT3, resulting in phosphorylation and nuclear translocation of STAT3; 2) JAK2 (Tyr985)/SHP2, leading to the activation of the ERK pathway; and 3) IRS2/PI3K, which causes nuclear exclusion of FoxO1 through phosphorylation, and thereby inhibiting FoxO1 transcriptional activity on target genes [8]. Intriguingly, our data showed that leptin-mediated TH expression is via activation of JAK2/STAT3 and IRS2/PI3K/pAkt/pFoxO1 pathways, leading to activation of STAT3 and inactivation of FoxO1 simultaneously, but not the ERK pathway as inhibition of ERK pathway did not affect leptin-mediated TH expression (Supplementary Fig. 3F). Our results further demonstrated that STAT3 and FoxO1 directly target TH gene to regulate its transcription in an opposing manner, in which the former acts as an amplifier, whereas the latter acts as a repressor. Intriguingly, pretreatment with PI3K inhibitor alone or overexpression of FoxO1 was

sufficient to block the effect of leptin on TH expression. In addition, the stimulatory effect of STAT3 on the TH expression was completely abolished upon introduction of FoxO1. These findings highlight that leptin effect on the TH activation might be primarily through the inhibition of FoxO1 rather than the activation of JAK2/STAT3. In support of our findings, FoxO1 KO^{DAT} mice in which FoxO1 was specifically deleted in DA neurons exhibited a marked increase in TH and dopamine levels, emphasizing the prominent suppressive role of FoxO1 in TH expression [26]. Increased TH and dopamine levels were accompanied with enhanced leptin sensitivity in the FoxO1 KO^{DAT} mice and had led us to examine whether FoxO1 KO^{DAT} mice might recapitulate anxiolytic-like behavioral phenotype observed in the leptin-administered mice. A battery of behavioral tests including open field, elevated-plus, and dark-light tests indeed demonstrated that FoxO1 KO^{DAT} mice manifest anxiolytic-like behaviors similar to that of the leptin-administered mice, delineating present finding that FoxO1 might play the prominent inhibitory role in leptin-mediated TH expression and mood behaviors. Although we have suggested the FoxO1 and STAT3 as the potential main mediators of leptin action in DA neurons, other factors regulated by leptin might also play a role for the TH expression and mood behaviors. In this regard, anti-depression-like phenotype monitored in FoxO1 KO^{DAT} mice is an intriguing finding because mice did not show changes in depression-like behaviors upon leptin administration. A future study identifying the functional roles of FoxO1 in the regulation of depression-like behavior might suggest a specific FoxO1 function independent of leptin actions in DA neurons.

Reciprocal action of STAT3 and FoxO1 on the regulation of TH is another intriguing finding. The TH regulatory mechanism by leptin was similar to that of POMC and AgRP in which STAT3 acts as an enhancer and FoxO1 acts as a repressor [23]. However, in contrast to the previous finding on POMC and AgRP expression by leptin, our data demonstrates that the suppressive effect of FoxO1 was more dominate than the stimulatory effect of STAT3 on the TH expression. It has been suggested that leptin modulates various physiological functions within the body, including appetite, reproduction, and energy expenditure via regulation of specific target genes in varying physiological circumstances [8]. Therefore, unveiling of gene regulation in specific regions under varying circumstances by leptin would be supportive to expand the knowledge pool of the physiological role of leptin. In conclusion, our results establish a novel molecular mechanism by which TH regulation by leptin is via STAT3 and FoxO1, resulting in regulation of mood behaviors in dopaminergic neurons. Particularly, FoxO1 suppression by leptin is a critical step for leptin-mediated behavioral manifestation.

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Author Contribution

DHS and KVD conceptualized the research, performed experiments, analyzed data and wrote the manuscript. DJY, JSS, SKK, NK, and JYK performed experiments and analyzed data. JHP and RAD established $FoxO1^{loxP/loxP}$ animal and edited the manuscript. YHC, DMS, and KWK conceptualized the research, analyzed data, wrote, edited and finalized the manuscript.

Additional Information

The authors declare no competing interests.

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Disclosure

The authors have nothing to disclose.

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