SUPPLEMENT 1

Forkhead Box, Class O Transcription Factors in Brain: Regulation and Behavioral Manifestation

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Materials and Methods

Animal treatment After a one-week accommodation in the university animal facility, adult male C57BL/6 mice were treated with d-fenfluramine or imipramine dissolved in saline and injected intraperitoneally (IP). For intra-cerebroventricular (ICV) injection, mice were anesthetized with ketamine and xylazine (100 mg/kg and 10 mg/kg, respectively) to place a guide cannula (2.2 mm) stereotaxically (posterior 0.8 mm and left 1.6 mm to Bregma). Five days later, vehicle (10% dimethyl sulfoxide [DMSO]) or the PI3K inhibitor LY294002 (5 nmol in 1 μl of 10% DMSO, ICV) was infused into the left ventricle via an internal cannula. Animals were given d-fenfluramine (IP) 90 min after LY294002. At the end of treatment, mice were rapidly decapitated and brain regions (cerebral cortex, hippocampus, and striatum) were immediately dissected in ice-cold saline and used for immunoassays.

FoxO1 and FoxO3a mouse strains Homozygous FoxO1/3/4-flox/flox mice on a FVB background (1, 2) were cross-bred with Nestin-Cre mice on a C57BL/6 background (Jackson Laboratory, Bar Harbor, Maine) to generate homozygous Nestin-Cre:FoxO1-flox/flox (Brain-targeted FoxO1 knockout) mice. Genotype was confirmed by Polymerase Chain Reaction (PCR) using primers 5'-GCTTAGAGCAGAGATGTTCTCACATT, 5'-CCAGAGTCTTTGTATCAGGCAAATAA, and 5'-CAAGTCCATTAATTCAGCACATTGA for FoxO1 (2), and 5'-CGAGTGATGAGGTTCGCAAGAACC and 5'-TCCATGAGTGAACGAACCTGGTCG for Cre. Littermates with Nestin-Cre negative or FoxO1 wild type genotypes were used as FoxO1 controls. Homozygous FoxO3a-deficient and littermate wild-type mice were generated from the heterozygous FoxO3a-trap mice

on a C57BL/6 background (3). Genotyping of each mouse was confirmed at 30 days of age as previously described (3).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Brain RNA was extracted with Trizol Reagent (Invitrogen Life Technologies, Carlsbad, CA) and chloroform, precipitated with isopropanol, washed with 75% ethanol, and stored at -80ºC in DEPC-treated water. RT-PCR was performed using the SuperScript III One-step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA) with the forward and reverse primers of FoxO1 (5'-CCTGTCGTACGCCGACCTCATCAC-3' and 5'-GTCCATGGACGCAGCTCTTCTCCG-3'). PCR products (15 μl) were separated on a 2% agarose gel, visualized by ethidium bromide staining, and photographed with Fluor-S MultiImager (Bio-Rad, Hercules, CA).

Protein preparation and immunoblotting Proteins from brain homogenate or nuclear/cytosolic extracts were resolved in 7.5-10% SDS-polyacrylamide gels, and immunoblotted with antibodies to phospho-Ser^{256}-FoxO1, FoxO1, phospho-Ser^{253}-FoxO3a, phospho-Thr^{308}-Akt, phospho-Ser^{473}-Akt, Akt (Cell Signaling Technologies, Danvers, MA), phospho-Thr^{32}-FoxO3a, FoxO3a, CREB (Upstate Biotech, Lake Placid, NY), and β-tubulin (Sigma-Aldrich, St. Louis, MS). Following a reaction with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, the immunoreactions were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ), and protein bands were quantified with a densitometer.

Immunohistochemistry The immunohistochemistry method was as described previously (4). Briefly, brains were immersion-fixed in Bouin's fixative overnight at 4°C, processed in paraffin, and 4 μm brain sections were prepared on a microtome. Deparaffinized sections

were incubated with anti-FoxO1 or anti-FoxO3a, labeled with horseradish peroxidase-conjugated anti-rabbit IgG, and counter-stained with Hoechst 33,258 (Invitrogen Life Technologies, Carlsbad, CA). Immuno-fluorescence in brain sections was viewed with a digital confocal microscope and photographed using a 100x objective. Negative controls of FoxO1 and FoxO3a immunostains were obtained from brain specimens of FoxO1 knockout mouse and FoxO3a-deficient mouse, respectively.

Behavior Tests

Forced Swim Test (FST) (5) was conducted using an automated apparatus (Kinder Scientific, Poway, CA). Movements were continuously monitored by computer for 6 min with data recorded using the Motor Monitor Software. The resting time (representing immobility) during the last 4 min of test was analyzed (6).

Tail Suspension Test (TST) (7) was conducted using an automated TST system (Med Associates Inc, St. Albans, VT). Mouse tail was attached to a strain gauge that detects any movements of a mouse and movement was tested for 6 min. The duration of immobility during the last 4 min of the test was recorded by the computer software and calculated as the time the movement force was below a preset threshold (8).

For the Elevated Plus Maze Test (EPMT) (9), each mouse was placed on the central platform of the maze (San Diego Instruments, San Diego, CA), and movements were observed for 4 min with an Ethovision camera driven tracker system (Noldus, The Netherlands). Entries into (number of entries) and time spent (seconds) in each arm were recorded via computer during the 4-min testing time and data were analyzed using the Ethovision software.

For the Open Field Test (OFT) (10), each mouse was placed into an arena of 42 cm^2 with 20 cm (Coulburn Instruments, Whitehall, PA), and activity was monitored with the Ethovision system for 4 min. The locomotor activity was recorded via computer as total travel distance (cm) and travel velocity (cm/sec), and data were analyzed using the Ethovision software. Some mice received d-amphetamine (4 mg/kg, IP) treatment for 30 min before testing. The d-amphetamine-induced locomotor activity in each mouse was recorded and calculated as the % of its own baseline activity.

Results

A line of nestin-directed FoxO1 knockout mice was generated by cross-breeding FoxO1/3/4-flox/flox mice (2, 11) with Nestin-Cre mice carrying Cre recombinase under the control of a nestin promoter to preferentially knockout FoxO1 from brain (12) (Figure S2A). The PCR products from tail and brain DNAs in the Cre+ FoxO1-flox/flox mice confirmed a specific brain FoxO1 knockout pattern represented by a 149bp fragment in the tail DNA and a 190bp fragment in the brain DNA (Figure S2B). RT-PCR detection of FoxO1 mRNA in the cerebral cortex was lower in the Cre+ FoxO1-flox/flox mice than in littermate controls (which contain intact FoxO1) (Figure S2C). Immunoblots of FoxO1 in the cerebral cortex, hippocampus, and striatum revealed almost complete deletion of FoxO1 protein in the brain FoxO1 knockout mice, whereas FoxO1 is detectable in the lungs (Figure S2D).

References

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Table S1. Acute Imipramine Treatment

Brain Regions	Phopho-Ser^{256}-FoxO1			Phopho-Ser^{253}-FoxO3a		
	Mean ± SEM (% saline)		p-value	Mean ± SEM (% saline)		p-value
	Saline	Imipramine	(n=9)	Saline	Imipramine	(n=9)
Cerebral Cortex	100.00 ± 10.92	98.68 ± 10.92	0.96	100.00 ± 9.42	120.48 ± 18.39	0.38
Hippocampus	100.00 ± 17.32	148.13 ± 39.04	0.21	100.00 ± 9.24	111.96 ± 17.95	0.60
Striatum	100.00 ± 10.89	113.17 ± 32.67	0.37	100.00 ± 16.65	118.36 ± 16.72	0.46

FoxO, forkhead box, class O

Figure Legend

Figure S1. Dose-dependent response of FoxO1 and FoxO3a to d-fenfluramine in the cerebral cortex and hippocampus. C57BL/6 mice were treated with indicated doses of d-fenfluramine (IP, 2 hours) before FoxO proteins from homogenates of the cerebral cortex (CTX), and hippocampus (HIP), were examined by immunoblots. Values are expressed as % saline treatment (control or 0 mg/kg d-fenfluramine).

Figure S2. Generation of brain FoxO1 knockout mice. FoxO1/3/4-flox/flox mice were crossbred with Nestin-Cre mice. (A) Representative PCR genotyping products from tail DNA show different genotype combinations of Cre, FoxO1, FoxO3a, and FoxO4. The maps of the floxed FoxO constructs, the relative primer positions, and size of DNA fragments are as previously reported (1, 2). (B) PCR products of tail and brain DNA from wild type (Cre-), heterozygous (Cre+:FoxO1-fl/+), and homozygous (Cre+:FoxO1-fl/fl) FoxO1 knockout mice. (C) RT-PCR of brain FoxO1 mRNA from homozygous and heterozygous FoxO1 knockout and matching wild type mice. (D) Representative immunoblots of the levels of FoxO1 protein in the cerebral cortex, hippocampus, striatum, and lung in homozygous FoxO1 knockout and matching wild-type mice.

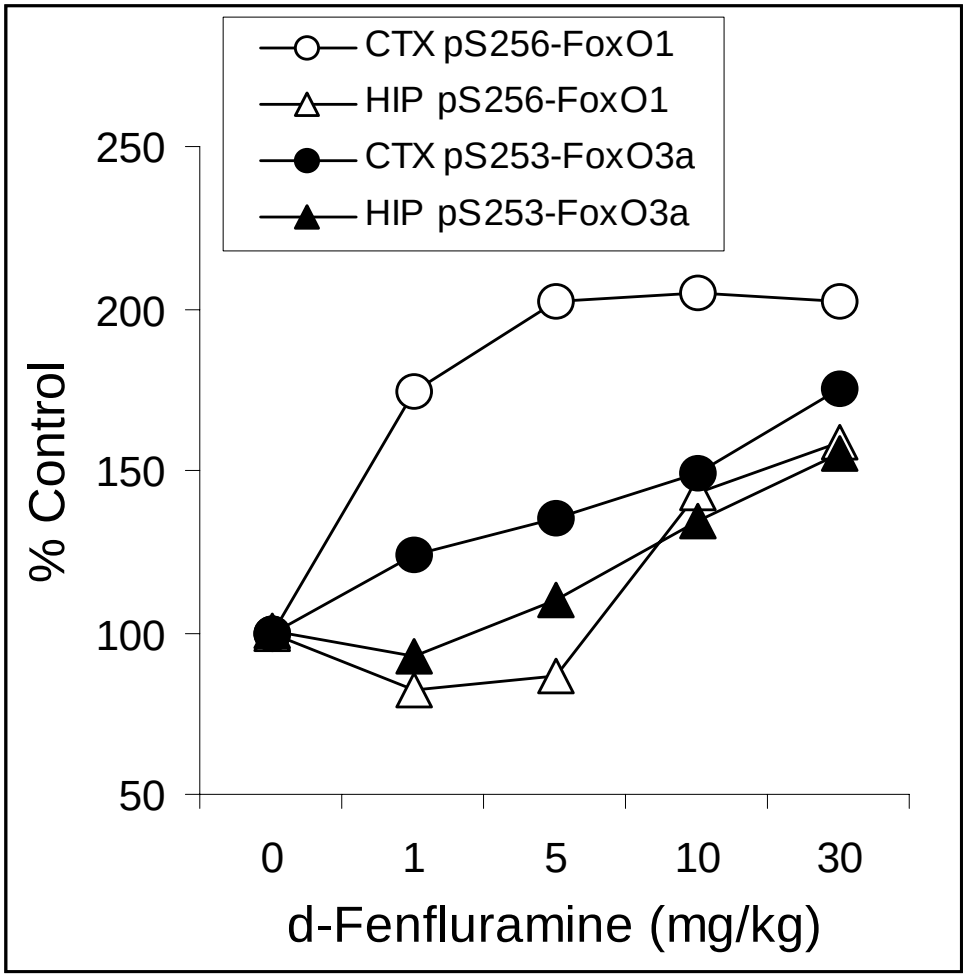
CTX pS256-FoxO1
HIP pS256-FoxO1
CTX pS253-FoxO3a
HIP pS253-FoxO3a
250
200
150
100
50
% Control
0
1
5
10
30
d-Fenfluramine (mg/kg)

Figure S1

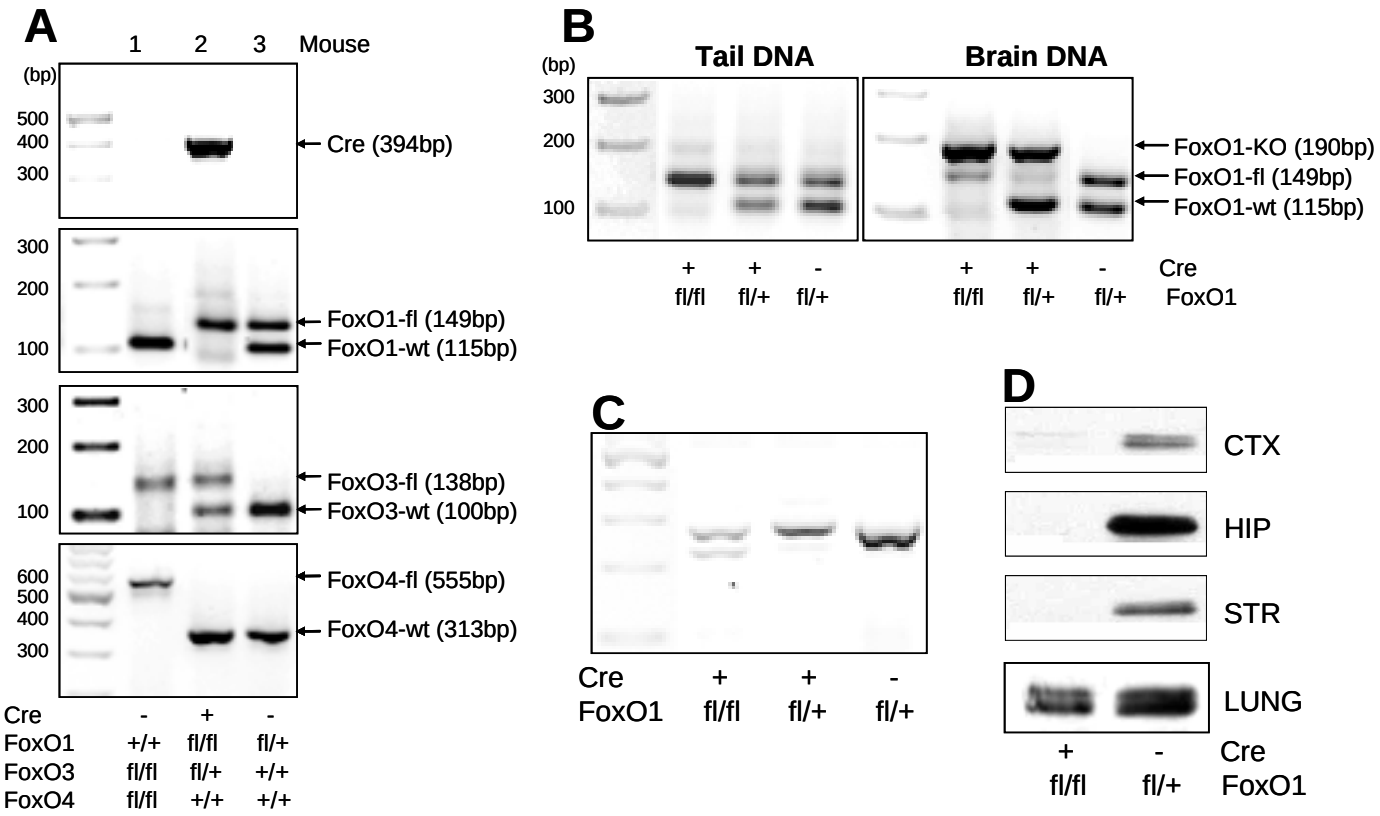
A
1
2
3
Mouse
(bp)
500
400
300
Cre (394bp)
300
200
100
FoxO1-fl (149bp)
FoxO1-wt (115bp)
300
200
100
FoxO3-fl (138bp)
FoxO3-wt (100bp)
600
500
400
300
FoxO4-fl (555bp)
FoxO4-wt (313bp)
Cre - + -
FoxO1 +/+ fl/fl fl/+
FoxO3 fl/fl fl/+ +/+
FoxO4 fl/fl +/+ +/+
B
(bp)
Tail DNA
Brain DNA
300
200
100
FoxO1-KO (190bp)
FoxO1-fl (149bp)
FoxO1-wt (115bp)
+ + - + + - Cre
fl/fl fl/+ fl/+ fl/fl fl/+ fl/+ FoxO1
C
Cre + + -
FoxO1 fl/fl fl/+ fl/+
D
CTX
HIP
STR
LUNG
+ - Cre
fl/fl fl/+ FoxO1

Figure S2