JPET Fast Forward. Published on August 29, 2017 as DOI: 10.1124/jpet.117.242925
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Title: FTBMT, a novel and selective GPR52 agonist, demonstrates antipsychotic-like and procognitive effects in rodents revealing a potential therapeutic agent for schizophrenia

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Running title: FTBMT, a novel GPR52 agonist

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Number of text pages: 39

Number of tables: 4

Number of figures: 7

Number of references: 54

Number of words in the Abstract: 217

Number of words in the Introduction: 715

Number of words in the Discussion: 1133

Nonstandard abbreviations used: FTBMT:

4-(3-(3-fluoro-5-(trifluoromethyl)benzyl)-5-methyl-1*H*-1,2,4-triazol-1-yl)-2-methylbenzamide

Recommended section assignment: Neuropharmacology

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Abstract

GPR52 is a Gs-coupled G protein-coupled receptor (GPCR) predominantly expressed in the striatum and nucleus accumbens (NAc), and was recently proposed as a potential therapeutic target in schizophrenia. In the current study, we investigated the in vitro and in vivo pharmacological activities of a novel GPR52 agonist, 4-(3-(3-fluoro-5-(trifluoromethyl)benzyl)-5-methyl-1*H*-1,2,4-triazol-1-yl)-2-methylbenzamide (FTBMT). FTBMT functioned as a selective GPR52 agonist in vitro and in vivo, as demonstrated by the activation of cyclic adenosine monophosphate (cAMP) signaling in striatal neurons. FTBMT inhibited MK-801-induced hyperactivity, an animal model for acute psychosis, without causing catalepsy in mice. The c-fos expression also revealed that FTBMT preferentially induced neuronal activation in the shell of the NAc, compared with the striatum, thereby supporting its antipsychotic-like activity with less catalepsy. Furthermore, FTBMT improved recognition memory in a novel object recognition test and attenuated MK-801-induced working memory deficits in a radial arm maze test in rats. These procognitive effects were supported by the results of FTBMT-induced c-fos expression in brain regions related to cognition, including the medial prefrontal cortex, entorhinal cortex, and hippocampus. Taken together, these findings suggest that FTBMT shows antipsychotic and procognitive properties without causing catalepsy in rodents. Given its unique pharmacological profile, which differs from that of current antipsychotics, FTBMT may provide a new therapeutic option for the treatment of positive and cognitive symptoms of schizophrenia.

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Introduction

Schizophrenia is a severe, chronic, and disabling mental disorder associated with a lifetime risk of approximately 1% (Saha et al., 2005). The core clinical features of schizophrenia include positive, negative, and cognitive symptoms. Typical antipsychotics for the treatment of schizophrenia can reduce the severity of positive symptoms via dopamine D_2 receptor (D2R) antagonism; however, these treatments induce extrapyramidal symptoms (EPS). To overcome this side effect, another category of drugs, atypical antipsychotics, have been developed, which decreased the incidence of EPS. However, concerns with tolerability, in relation to weight gain and endocrinopathies, still remain (Krebs et al., 2006). More importantly, these therapeutic agents do not demonstrate satisfactory efficacy for negative and cognitive symptoms, which essentially determine the quality of life and associated costs of care (Miyamoto et al, 2012). Thus, new classes of drugs for negative and cognitive symptoms have been developed based on several hypotheses; however, no compounds achieved solid efficacy in those symptoms although some compounds showed modest activity (Kingwell, 2014; Li et al, 2015; Nakazawa et al, 2012).

GPR52 was first reported as a novel human gene identified by performing homology searches, using basic local alignment search tool with amino acid sequences of known G protein-coupled receptors (GPCRs), of publicly available databases such as high throughput genome or expressed sequence tags databases (Sawzdargo et al., 1999). Following its identification from human genomic DNA, the precise distribution of GPR52 mRNA in human tissues was determined (Komatsu et al, 2014). It is predominantly found in the brain, with the highest expression levels in the striatum and nucleus accumbens (NAc), which have been implicated in psychosis (Epstein et al, 1999; Sarpal et al, 2015). Interestingly, GPR52 shows near complete co-localization with D2R, but not dopamine D_1 receptor (D1R), in these regions (Komatsu et al., 2014). GPR52 is also clearly detected in critical

brain regions that are involved in cognitive functions, including the prefrontal cortex, entorhinal cortex, cingulate cortex, and mammillary nucleus (Komatsu et al., 2014). In contrast to the striatum, GPR52 is partially co-localized with D1R, but not D2R, in the prefrontal cortex (Komatsu et al., 2014).

The fact that intrinsic ligands for GPR52 are not identified and GPR52 shares the limited homology with other GPCRs such as GPR21 (71%), histamine H2 (27%) and 5-HT4 (26%) human receptors (Sawzdargo et al., 1999) makes it difficult to analogize its function and actually its function was not elucidated so far. In such situation we have been trying to reveal its biological function. Reserpine, an antihypertensive agent (Fraser, 1996) and a series of small compounds raise intracellular cyclic adenosine monophosphate (cAMP) levels in recombinant cells expressing GPR52, which indicated that GPR52 is coupled to a Gs protein (Komatsu et al., 2014; Setoh et al., 2014). Given that D2R is a Gi-coupled receptor, which decreases intracellular cAMP levels when activated, the stimulation of GPR52 may counteract D2R signaling of the medium spiny neurons in the striatum and NAc. Indeed, the systemic administration of a GPR52 agonist, 3-(2-(3-chloro-5-fluorobenzyl)-1-benzothiophen-7-yl)-*N*-(2-methoxyethyl)benzamide, (compound 7m), and overexpression of GPR52 attenuate methamphetamine-induced hyperactivity in mice (Komatsu et al., 2014; Setoh et al., 2014). Moreover, compound 7m did not produce a cataleptic response. However, the underlying mechanism of action for the antipsychotic-like activity of GPR52 agonists, which do not induce catalepsy, and their effects on cognitive function, remain unknown.

We recently developed a novel GPR52 agonist, 4-(3-(3-fluoro-5-(trifluoromethyl)benzyl)-5-methyl-1*H*-1,2,4-triazol-1-yl)-2-methylbenzamide (FTBMT), which had favorable physicochemical and pharmacokinetic properties (Tokumaru et al., 2017). The aim of the current study was to elucidate in vitro and in vivo

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pharmacological activities of FTBMT as a potential therapeutic agent in schizophrenia. In order to determine if FTBMT functioned as a selective GPR52 agonist in vitro and in vivo, we first investigated the effect of FTBMT on cAMP signaling in Chinese hamster ovary (CHO) cells expressing GPR52, primary cultured striatal neurons, and striatal tissues. We next evaluated the antipsychotic-like effect of FTBMT on MK-801-induced hyperactivity and its cataleptogenic activity in mice, and then assessed FTBMT-induced neural activation in the striatum and NAc using c-fos immunohistochemistry (Pinna et al., 1997; Satow et al., 2009). Furthermore, we tested whether FTBMT improved cognitive functions in a novel object recognition task (NORT) and a radial arm maze task in rats (Young et al., 2009) and, finally, investigated neural activation in brain regions involved in cognitive functions (Preston et al., 2013).

Materials and Methods

Animals

Male C57BL/6J, C57BL/6N, and imprinting control region (ICR) mice, and Wistar rats were obtained from CLEA Japan, Inc. (Tokyo, Japan). Male Long-Evans rats were obtained from Japan SLC Inc. (Hamamatsu, Japan). Pregnant Sprague-Dawley rats were supplied by Charles River Laboratories Japan, Inc. (Yokohama, Japan). Homozygous GPR52 knockout (KO) mice were generated on 129SvEv background (Original 129SvEv mice were derived from the University of Cambridge (Cambridge, UK) and bred at the research animal facilities at Takeda) and these GPR52 KO mice were also backcrossed once or three times with C57BL/6J strains as previously described (Komatsu et al., 2014). Their correspondent wild-type (WT) littermate mice on each background were generated and used as control animals. The animals were maintained in a light-controlled room on a 12-h light/dark cycle (lights on at 0700 h) and were acclimated for approximately 1 week prior to the experiment. The care and use of the animals and the experimental protocols were approved by the Experimental Animal Care and Use Committee of Takeda Pharmaceutical Company Limited adhered to Act on Welfare and Management of Animals, a Japanese law (Amendment Act No. 46 of 2014).

Drugs

FTBMT (Tokumaru et al, 2017) (Figure 1a), was synthesized at Takeda Pharmaceutical Company Limited (Osaka, Japan). Olanzapine was extracted from Zyprexa (Eli Lilly and Company, Indianapolis, IN) at KNC Laboratories Co. Ltd. (Kobe, Japan). FTBMT and olanzapine were suspended in 0.5% (w/v) methylcellulose in distilled water. Aripiprazole was obtained from AK Scientific Inc. (Union City, CA) and suspended in a 1% (v/v) solution of Tween 80 in distilled water. Methamphetamine hydrochloride (Dainippon

Sumitomo Pharma, Osaka, Japan) and (+)-MK-801 hydrogen maleate (MK-801; Wako, Osaka, Japan, and Sigma-Aldrich, St Louis, MO) were dissolved in saline. All compounds were administered at a dose of 10 or 20 mL/kg body weight in mice and 2 mL/kg body weight in rats.

Tissue sampling

For quantitative polymerase chain reaction (qPCR) analyses, male ICR mice (8–10 weeks old), GPR52 KO mice and their WT littermates (2–10 months old) on C57BL/6J background were sacrificed 1–2 h after the oral administration of FTBMT at a dose of 3–100 mg/kg. After whole brains were collected, the entire striatum and hippocampus were immediately isolated with fine forceps on ice. However, in the comparison study between the striatum and NAc, each tissue was separated from coronal slices (1 mm) and was prepared using a Precision Brain Slicer (Braintree Scientific, Braintree, MA) as previously described (Sakuma et al, 2015). All tissues were frozen on dry ice and stored at -80 ºC until analysis. For the phospho-cAMP response element binding protein (CREB) analyses, the mice were sacrificed 1 h following the oral administration of FTBMT (3–30 mg/kg) using a focused microwave irradiation system (MMW-05; Muromachi Kikai, Tokyo, Japan). Striatum and hippocampal tissues were then isolated using fine forceps on ice and lysed with denaturing cell extraction buffer (#FNN0011; Thermo Fisher Scientific, Waltham, MA) containing a protease inhibitor cocktail and p-APMSF (Sigma-Aldrich, St. Louis, MO). The lysates were stored at -80 ºC until analysis.

Cell culture

CHO cells (ATCC, No. CRL-9096, Manassas, VA) stably expressing GPR52 receptors (human, mouse, and rat genes) and naïve CHO cells were cultured in Ham’s F-12 (Thermo

Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Moregate Biotech, Brisbane, Australia) and 100 U/mL penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA). For the preparation of rat primary striatal neurons, striatal tissue, which was dissected from rat embryos on embryonic day 18–19, was dissociated using Nerve-Cells Dispersion Solutions (Sumitomo Bakelite, Tokyo, Japan). Cells were plated on a 96-well poly-D-lysine-coated plates (BD Biosciences, San Jose, CA) at a density of 3×10^4 cells/well, for the [^{3}H]-gamma-aminobutyric acid (GABA) release assay, or a 24-well poly-D-lysine-coated plates (BD Biosciences, San Jose, CA) at a density of 1.86×10^5 for other assays. Cells were then cultured with Neurobasal medium (Thermo Fisher Scientific, Waltham, MA), containing B27 supplement (Thermo Fisher Scientific, Waltham, MA), 2 mM L-glutamine (Lonza, Basel, Switzerland), 20 μg/mL gentamicin (Lonza, Basel, Switzerland), 100 U/mL penicillin (Lonza, Basel, Switzerland), and 100 μg/mL streptomycin (Lonza, Basel, Switzerland) at 37 °C in a humidified atmosphere of 95% air and 5% CO2. On 13–16 days in vitro (DIV), cells were treated with FTBMT in Hanks' balanced salt solution (Thermo Fisher Scientific Inc., Waltham, MA), containing 5 mM HEPES (DOJINDO Laboratories, Kumamoto, Japan), pH 7.5 and 0.5% fatty acid free bovine serum albumin (Wako, Osaka, Japan) for 15 or 60 min at 37 °C, and then homogenized in RLT buffer (Qiagen, Germantown, MD) or lysed with denaturing cell extraction buffer (Thermo Fisher Scientific, Waltham, MA) containing a protease inhibitor cocktail and p-APMSF (Sigma-Aldrich, St. Louis, MO). Lysates were stored at -80 ºC until analysis.

cAMP assay

CHO cells stably expressing GPR52 receptors and naïve CHO cells (10,000 cells/well) were stimulated with FTBMT in cAMP assay buffer for 30 min at 37 °C. Intracellular

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cAMP levels were determined using an Alphascreen cAMP detection kit (PerkinElmer, Waltham, MA), according to the manufacturer's instructions. EC_{50} values were analyzed using nonlinear regression analyses with GraphPad PRISM software (GraphPad Software Inc., San Diego, CA, USA). In the cAMP assay using recombinant CHO cells, the response at 1 μM of *N*-(2-hydroxyethyl)-3-(2-(3-(trifluoromethyl)benzyl)-1-benzofuran-4-yl)benzamide (compound 7a) was used as the 100% control, as described previously (Setoh et al, 2014).

Off-target profiling

Selectivity against other receptors, ion channels and enzymes was determined in Ricerca Biosciences which is now eurofins, according to the experimental conditions specified at company website:
http://www.eurofins.com/biopharma-services/discovery/services/in-vitro-pharmacology/

Preparation and processing of acute NAc slices

NAc slices were prepared as described in a previous paper with minor modifications (Nishi et al., 2008). NAc slices were prepared from male ICR mice (8–10 weeks old). Coronal slices (500 μm) were prepared using a vibrating blade microtome, DTK-1000 (D.S.K, Kyoto, Japan). Each NAc slice was placed in a polypropylene incubation tube (SARSTEDT, North Rhine-Westphalia, Nümbrecht, Germany) with 2 mL of fresh Krebs-HCO_3-buffer containing 124 mM NaCl, 4 mM KCl, 26 mM $NaHCO_3$, 1.5 mM $CaCl_2$, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$ and 10 mM D-glucose (all reagents were supplied by Wako, Osaka, Japan), pH 7.4 with 10 μg/mL of adenosine deaminase (Roche Life Science, Indianapolis, IN). The slices were allowed to recover at 30 ºC, under constant oxygenation with 95% O2/5% CO2 for 60 min. After the first 30 min of incubation, the buffer was replaced with fresh

Krebs-HCO_3-buffer without adenosine deaminase, followed by an additional 30 min of incubation. Subsequently, slices were treated with FTBMT for 5 min, transferred to tubes, and frozen on dry ice. Frozen tissue samples were denatured using sonication and boiling in 1% (w/v) sodium dodecyl sulfate (Wako, Osaka, Japan), containing 50 mM sodium fluoride (Wako, Osaka, Japan) and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO).

[^{3}H]-GABA release assay

The culture medium for primary striatal neurons (96 well plate) was replaced with assay buffer, which had the following composition: 10 mM HEPES, 135 mM NaCl, 5 mM KCl, 0.6 mM $MgSO_4$, 1 mM $CaCl_2$, 6 mM D-glucose, and 100 µM aminooxyacetic acid (all reagents were supplied by Wako, Osaka, Japan), pH 7.4. Cells were incubated with 0.3 µM [^{3}H]-GABA (NET191X; PerkinElmer, Waltham, MA) in assay buffer for 60 min at 37 °C, and then washed three times with assay buffer. To measure the basal release of [^{3}H]-GABA, cells were incubated with FTBMT in assay buffer for 30 min at 37 °C, following which the assay buffer was collected. To measure the *N*-Methyl-D-aspartate (NMDA, Wako, Osaka, Japan)-evoked release of [^{3}H]-GABA, cells were pre-incubated with FTBMT in assay buffer for 10 min at 37 °C, following which the cells were stimulated with 20 µM NMDA for 20 min at 37 °C. Thereafter, the assay buffer was collected, and radioactivity was measured using a microplate scintillation counter, TopCount NXT (PerkinElmer, Waltham, MA).

Western blotting and enzyme-linked immunosorbent assay (ELISA)

The protein concentrations of lysates from striatal neurons, NAc slices and microwaved brain tissues were determined using a Bicinchoninic acid assay (BCA) kit (Thermo Fisher Scientific, Waltham, MA). For Western blotting, the lysates (10 µg of protein/lane) were

subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 4-12% NuPAGE Bis-Tris gel (Thermo Fisher Scientific, Waltham, MA) and then transferred to a nitrocellulose membrane (Thermo Fisher Scientific, Waltham, MA). The membranes were incubated in blocking buffer (Phosphate-buffered saline (Thermo Fisher Scientific, Waltham, MA) containing 5% (w/v) nonfat dry milk (Wako, Osaka, Japan) and 0.1 % (w/v) Tween 20 (Sigma-Aldrich, St. Louis, MO)) for 60 min at room temperature. The membranes were probed with antibodies to dopamine and cAMP-related phosphoprotein 32 kDa(DARPP32) (#2302, 1:500; Cell Signaling Technology, Danvers, MA), and phospho-DARPP32 (Thr34) (#P1025-34, 1:300; Phosphosolutions, Aurora, CO). Immunoreactive bands were visualized using horseradish peroxidase (HRP)-linked anti-rabbit IgG antibodies (#7074, 1:5000; GE Healthcare, Buckinghamshire, UK) and an ECL plus system (GE Healthcare, Buckinghamshire, UK). Protein levels were quantified by densitometry using Quantity One (Bio-Rad, Pleasanton, CA). Amounts of phospho-CREB and total CREB in striatal neurons and microwaved tissues were determined using the CREB [pS133] Phospho-ELISA and CREB (total) ELISA Kits (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions.

Quantitative gene expression analysis

Total RNA purification and qPCR, which have been previously outlined in detail (Sakuma et al., 2015), were applied to the striatum and NAc samples isolated from coronal slices. Other samples were processed and analyzed using the previous method cited above, with modifications. Briefly, total RNA was purified using QIAzol Lysis Reagent (Qiagen, Hilden, Germany), followed by use of the RNeasy kit (Qiagen, Hilden, Germany) and DNase I (Qiagen, Hilden, Germany). The RNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kits (Thermo Fisher Scientific, Waltham, MA).

Real-time qPCR was performed using a 7900HT Sequence Detection System (Thermo Fisher Scientific, Waltham, MA), with TaqMan Universal PCR Master Mix, and Gene Expression Assay primer and probe sets (Assay ID for c-fos; Mm00487425_m1 and Rn02396759_m1, Egr-2; Mm00456650_m1 and Rn00586224_m1, Arc; Mm00479619_g1, GAPDH; 4352338E and 4352339E, Thermo Fisher Scientific, Waltham, MA). The quantities of target genes were determined using absolute quantification or relative quantification according to the ΔΔCt method.

Immunohistochemical staining

Vehicle or FTBMT (30 mg/kg) were intraperitoneally administered to male C57BL/6N mice (9 weeks old). After 2 h, the animals were anesthetized and perfused with saline (Otsuka, Tokushima, Japan), followed by saline with 4% paraformaldehyde (Wako, Osaka, Japan), via the left cardiac ventricle prior to brain sampling. The sampled brains were cryoprotected with 30% sucrose (Wako, Osaka, Japan), and frozen coronal sections (40 μm) were prepared using a freezing cryostat CM1850 (Leica, Nussloch, Germany) and used for staining. The sections were incubated with anti-c-Fos antibody (sc-52, 1:4000; Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using a VECTASTAIN Elite ABC Kit (PK-6101; Vector Laboratories, Burlingame, CA) and diaminobenzidine (DOJINDO Laboratories, Kumamoto, Japan). All of the procedures were performed using the free-floating method. After processing, the sections were mounted, and images, including the unilateral nucleus, were acquired using light microscopy ECLIPSE E800 (Nikon, Tokyo, Japan). The number of c-Fos-positive cells was automatically measured from the obtained images using Image-Pro Plus (MediaCybernetics, Rockville, MD). The counts from 2 or 4 sections from each animal were averaged; the mean value was used for subsequent statistical analyses.

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Locomotor activity

The locomotor activity in male ICR mice (7–8 weeks old), homozygous GPR52-KO mice and their WT littermates (2–10 months old) on 129SvEv background was measured using locomotor activity monitors, MDC-LT (Brain Science Idea Co., Ltd, Osaka, Japan). Mice were individually placed in transparent polycarbonate cages (30 x 40 x 20 cm) and acclimated for more than 60 min. FTBMT (3–30 mg/kg) or vehicle was orally administered 60 min prior to the subcutaneous administration of MK-801 (0.2 mg/kg) or vehicle. Activity counts were measured for 60 min after administration of FTBMT or vehicle until administration of MK-801 and for 90 min after the administration of MK-801 or vehicle.

Bar test

Male ICR mice (6–7 weeks old) were treated orally with vehicle, FTBMT, olanzapine, or aripiprazole, 60 min prior to the bar test. The mice were lifted by the tail and placed with their front paws on a steel bar (diameter, 2 mm), which was elevated 6.5 cm above the table. Their hind legs were on the plane surface. The time during which both forelimbs remained on the bar (cataleptic response) was measured, with a maximum time limit of 30 sec. The procedure was repeated three times and the cataleptic response time was averaged for each mouse.

Plasma prolactin concentrations

Male Wistar rats (7 weeks old) received an oral dose of FTBMT (1–100 mg/kg), haloperidol (1 mg/kg), or vehicle. Trunk blood was collected in EDTA-coated tubes, 1 h later, and centrifuged at 1600 g for 20 min at 4 ºC. Plasma was stored at -20 ºC until analysis. Plasma prolactin concentrations were measured using a Rat Prolactin Enzyme

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Immunoassay Kit (SPI-bio, Montigny-le-Bretonneux, France).

NORT

The NORT was slightly modified from the previously described method (Bevins and Besheer, 2006; Shiraishi et al., 2016). On the first day, male Long-Evans rats (7 weeks old) were acclimated to the behavioral test room for over 1 h, and then individually familiarized with the empty test box (a gray-colored polyvinyl chloride box measuring 40 × 40 × 50 cm) for 10 min. Testing comprised of two 3-min trials, which were called the acquisition and retention trials. These trials were separated by a 48-h inter-trial interval. On the second day, in the acquisition trial, rats were allowed to explore two identical objects (A1 and A2) for 3 min. On the fourth day, in the retention trial, rats were again allowed to explore a familiar object (A3) and a novel object (B) for 3 min. Object exploration was defined as licking, sniffing, or touching the object with their forelimbs while sniffing. Leaning against the object to look upward, standing, or sitting on the object was excluded. The exploration time for each object (A1, A2, A3, and B) in each trial was scored manually. FTBMT, at a dose of 3 or 10 mg/kg, or vehicle, was orally administered immediately after the acquisition trial. The novelty discrimination index (NDI) was calculated using the following equation: novel object interaction / total interaction × 100 (%). The person who performed the measurement was blinded to if FTBMT or vehicle had been administered to each rat.

Radial arm maze test

The experiment was performed using male Long-Evans rats (9 weeks old), as previously described (Shiraishi et al., 2016; Zou et al., 1998), with modifications. An eight-arm radial maze (each arm was 50 cm long, 10 cm wide, and 40 cm high; the maze was elevated 50 cm above the floor) was used to evaluate the working memory of Long-Evans rats (9 weeks

old). After fasting for 24 h, all rats were food-restricted to 85% of free-feeding body weight on the first day of exposure to the maze. Reinforcement consisted of three food pellets (45 mg dustless precision pellets, Bio-Serv Inc., Frenchtown, NJ), which were placed in the food cup at the end of each arm. On the first day of habituation to the maze, reinforcement pellets were placed near the entrance and at the midpoints of each arm. Three rats were placed on the maze and allowed to explore and consume the pellets for 8 min. On the second day of habituation, each rat was allowed to explore independently and consume the pellets placed at the midpoints and in the food cups at the end of each arm for 5 min. Training sessions started on the third day, and pellets were placed in the food cups at the end of each arm. Rats were placed on the maze facing away from the person performing the experiment and facing the same arm at the start of each trial. The entry of rats into each arm was recorded in sequence. Rats were allowed to explore until they completed one entry into each arm, or 5 min had elapsed. Entries into previously chosen arms and failure to consume the pellets were counted as an error. The learning criterion was defined as fewer than two errors in two consecutive days. FTBMT at 10 mg/kg or vehicle was orally administered 1 h before the test. MK-801 (0.08 mg/kg, s.c., as a salt) or saline was administered 30 min before the test. The person who performed the measurement blinded to if the rat had been administered FTBMT or vehicle.

Statistical analysis

All statistical analyses were performed using the EXSUS statistical software package (CAC Croit Corporation, Tokyo, Japan). Aspin Welch test (Algina, 2005) was used to evaluate significant differences between the two groups. In dose-response experiments, the homogeneity of variances was assessed using a Bartlett's test (Snedecor and Cochran, 1989), after which a two-tailed Williams test (Williams, 1971) or two-tailed Shirley-Williams test

(Shirley, 1977) was performed for parametric and non-parametric data, respectively. A two-way analysis of variance (ANOVA), followed by an Aspin Welch test or two-tailed William tests as a post-hoc test, was used for the experiments evaluating the GPR52-mediated effects of FTBMT in GPR52 KO mice. In the NORT experiments, the differences between exploration times spent with familiar and novel objects for each group were analyzed using paired t-tests. Statistical significance was set at $P < 0.05$.

Results

FTBMT-activated cAMP signaling in vitro

FTBMT increased intracellular cAMP levels in CHO cells expressing human, mouse, or rat GPR52, with pEC_{50} values of 7.03 ± 0.04, 6.85 ± 0.02, and 6.87 ± 0.02 M, respectively (Figure 1b). However, FTBMT, at doses up to 10 μM, did not demonstrate an effect on naïve CHO cells (data not shown). The selectivity of FTBMT was further tested against 98 other targets, including receptors, ion channels, and enzymes (Ricerca Biosciences, Concord, OH). FTBMT (10 μM) did not show significant inhibition or stimulation (50%) in these biochemical assays (Table 1, 2, 3 and 4). In primary rat striatal neurons that highly express endogenous GPR52, FTBMT elevated the levels of cAMP (Figure 1c; pEC_{50} = 6.82 ± 0.05 M) and phosho-CREB (Figure 1d), which increased the expression of downstream CREB target genes, c-fos and Egr-2 (Figure 1e). The stimulation of Gs-coupled adenosine A_{2A} receptor (A2A-R) expressed on GABAergic neurons in the striatum increased GABA release via the cAMP signaling pathway (Ferre et al., 2008; Shindou et al., 2002). Similarly, FTBMT increased the basal and NMDA-stimulated release of [^{3}H]-GABA in primary striatal neurons (Figure 1f). In NAc slices highly expressing GPR52, as well as in the striatum, FTBMT also activated cAMP signaling, which was demonstrated by the increased levels of phospho-DARPP-32 at Thr34 catalyzed by protein kinase A (Figure 2).

FTBMT exhibited antipsychotic-like activity without causing catalepsy in mice

Consistent with in vitro results, the oral administration of FTBMT (30 mg/kg) to WT mice increased phospho-CREB levels in the striatum. The effect was diminished in GPR52 KO mice (Figure 3a, $P < 0.001$; significant main effect of the strain: $F_{1, 29} = 15.95$, $P < 0.05$; significant strain × dose interaction: $F_{2, 29} = 3.38$, $P < 0.05$; post hoc two-tailed Williams test).

GPR52 agonists, including FTBMT, have previously been shown to decrease methamphetamine-induced hyperactivity (Tokumaru et al., 2017), which is a model that is widely used to assess antipsychotic-like effects on the basis of the dopamine hyperfunction hypothesis of schizophrenia (Jones et al., 2011). The antipsychotic-like activity of FTBMT was further evaluated in a MK-801-induced hyperactivity model, which is based on the glutamate hypofunction hypothesis of schizophrenia (Goff and Coyle, 2001; O'Neill and Shaw, 1999). In mice, FTBMT demonstrated a dose-dependent inhibition of MK-801-induced hyperactivity, at doses from 3–30 mg/kg (Figure 3b). This effect was absent in GPR52 KO mice, even at the highest dose of 30 mg/kg of FTBMT (Figure 3d, $P < 0.01$; significant main effect of the strain: $F_{1, 68} = 9.13$, $P < 0.01$; significant main effect of the drug: $F_{1, 68} = 27.43$, $P < 0.001$; significant strain × drug interaction: $F_{1, 68} = 12.62$, $P < 0.001$; post-hoc Aspin-Welch test). On the other hand, FTBMT also decreased basal locomotor activity (Figure 3c).

A potential side effect of FTBMT on motor function was evaluated using bar tests in mice. The oral administration of FTBMT at doses up to 100 mg/kg did not cause any cataleptic responses in the bar test (Figure 3e), while olanzapine- and aripiprazole-induced catalepsy at their respective minimum effective dose (MED) for methamphetamine-induced hyperactivity (the MEDs for hyperactivity of olanzapine, aripiprazole, and FTBMT were 1 mg/kg, 0.3 mg/kg, and 10 mg/kg, respectively). Moreover, hyperprolactinemia caused by D2R blockade was not observed in rats treated with FTBMT (3–100 mg/kg, p.o.) whereas haloperidol (1 mg/kg) showed significant elevation of plasma prolactin level (Figure 4).

FTBMT displayed preferential activity for neurons in the NAc shell compared with the striatum in vivo

FTBMT exhibited a larger separation between doses for antipsychotic-like effects and those

for catalepsy, compared with olanzapine and aripiprazole. The MEDs for hyperactivity vs. catalepsy were 10 mg/kg vs. > 100 mg/kg, 1 mg/kg vs. < 1 mg/kg, and 0.3 mg/kg vs. < 0.3 mg/kg, for FTBMT, olanzapine, and aripiprazole, respectively. This may be explained by the activation of different subregions of the striatum and NAc. For example, the activation of medium spiny neurons in the dorsolateral striatum (DLS) has been implicated in antipsychotic-induced catalepsy (Ohno et al., 2008; Wan et al., 1995). Thus, FTBMT-induced neural activation in the DLS, dorsomedial striatum (DMS) and NAc was assessed using c-fos immunohistochemistry. When compared with the vehicle injection, the intraperitoneal injection of FTBMT (30 mg/kg) significantly increased the number of c-fos positive cells in the DMS ($P < 0.05$) and NAc shell ($P < 0.01$), but not in DLS (Figure 5a and b).

The brain region-specific activation patterns following treatment with antipsychotics have been quantitatively evaluated using transcriptional changes of several IEGs (Nguyen et al., 1992; Robbins et al., 2008; Sakuma et al., 2015). Our previous study (Sakuma et al., 2015) demonstrated that the induction of three IEGs, namely, c-fos, Egr2, and Arc, in the NAc was common following treatment with four antipsychotic drugs, i.e., haloperidol, aripiprazole, olanzapine, and clozapine. Using the same method, the region-specific activation due to FTBMT application was evaluated and compared with data from out previous study using olanzapine and aripiprazole (Sakuma et al., 2015). Although FTBMT increased the expression of c-fos, Egr-2, and Arc in the NAc to the same levels as olanzapine and aripiprazole, it had a smaller effect in the striatum, compared with the other antipsychotic drugs (Figure 5c).

FTBMT improved recognition and spatial working memory in rats

The effects of FTBMT on cognitive function were assessed using a NORT and radial arm

maze test in rats. FTBMT was orally administered to rats, at a dose of 3 or 10 mg/kg, immediately after the acquisition trial for NORT. In the retention trial, which was performed 48 h after the acquisition trial, FTBMT administration significantly increased the exploration time for novel objects compared with familiar ones (Figure 6a; $P < 0.01$) and the novel discrimination index (Figure 6b; $P < 0.01$) at both doses, suggesting an improvement in recognition memory. In a radial arm maze test, MK-801 was used to induce impairment of spatial working memory as reflected by the increase in the number of errors (Enomoto et al., 2008). A 1-h pretreatment with FTBMT (10 mg/kg, p.o.) significantly decreased the number of errors induced by MK-801 (Figure 6c; $P < 0.05$), which suggested an improvement in spatial working memory.

FTBMT stimulated neuronal activity in brain regions related to cognition

GPR52 mRNA was expressed in brain regions related to cognition, including the entorhinal cortex, frontal cortex, and medial prefrontal cortex. As described in Figure 5a, c-fos immunohistochemistry was used to investigate FTBMT-induced neuronal activation in these brain regions. FTBMT (30 mg/kg, i.p.) significantly increased the number of c-fos positive neurons in the entorhinal cortex ($P < 0.05$), frontal cortex ($P < 0.01$), and medial prefrontal cortex ($P < 0.01$) (Figure 7a and b). The entorhinal cortex is commonly perceived as a major input and output structure for hippocampal formation, and is considered the hub of cortico-hippocampal circuits (Preston and Eichenbaum, 2013). Thus, the activation of the hippocampus, a key region for memory storage, was further assessed. In the hippocampus, the oral administration of FTBMT at doses of 3 mg/kg, 10 mg/kg, and 30 mg/kg, significantly induced gene expression of Egr-2 ($P < 0.05$), c-fos ($P < 0.01$), and Arc ($P < 0.001$), respectively (Figure 7c). The induction of c-fos gene expression in the hippocampus due to FTBMT (30 mg/kg, p.o.) diminished in GPR52 KO mice (Figure 7d,

JPET Fast Forward. Published on August 29, 2017 as DOI: 10.1124/jpet.117.242925
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$P < 0.001$; significant main effect of the strain: $F_{1, 15} = 19.82$, $P < 0.01$; significant main effect of the drug: $F_{1, 15} = 8.52$, $P < 0.01$; significant strain × drug interaction: $F_{1, 15} = 10.99$, $P < 0.01$; post-hoc Aspin-Welch test). This activation of hippocampal neurons was confirmed by the increased phosphorylation of CREB, which regulates the synthesis of proteins that are important for the formation of long-term memory in the hippocampus (Barco et al., 2003).

DISCUSSION

Our study demonstrated that FTBMT functions as a selective GPR52 agonist in vitro and in vivo, and has antipsychotic-like and procognitive properties, but no cataleptogenic activity, in rodents. Its behavioral effects were supported by neurochemical findings such as FTBMT-induced c-fos expression in the NAc shell related to antipsychotic-like effects (Jennings et al., 2006), and in the medial prefrontal cortex, entorhinal cortex, and hippocampus, which are related to cognitive function (Matsuo et al., 2009). These results indicate that FTBMT could be a new therapeutic agent for the treatment of positive and cognitive symptoms of schizophrenia.

On antipsychotic-like activity, FTBMT suppressed MK-801-induced hyperactivity whereas FTBMT also suppressed basal locomotor activity (Figure 3b, c). That may suggest the former effect is not specific for stimulant-evoked portion of locomotor activities. However similar manner of actions was seen in an A2A-R agonist that showed suppression of MK-801-induced hyperlcomotion at the doses where it decreased basal locomotor activity (Malec et al., 2006). In the case of atypical anti-psychotics such as olanzapine, although some preferentiality for stimulant-evoked hyperlocomotion was seen, seperation from the effect on basal locomotor activity was not neccesarily wide (Ninan and Kulkarni, 1999). Nevetherless it is nortworthy that FTBMT didn’t show cataleptic responses up to 30-fold the minimam effective dose for antipsychotic-like effects (Figure 3e).

The data presented in the current study provide new insights into the potential for GPR52 agonists to become a new class of drugs for schizophrenia. Previous studies have highlighted the potential role of GPR52 in targeting the positive symptoms of schizophrenic. Evidence for this hypothesis comes from behavioral studies of methamphetamine responsiveness using GPR52 transgenic mice and the GPR52 agonist compound 7m (Komatsu et al., 2014; Setoh et al., 2014). However, there is a lack of biochemical evidence

to support and understand these behavioral data. In the current study, we used biochemical analyses to demonstrate that FTBMT activated cAMP signaling in vitro and in vivo. GPR52-mediated cAMP signaling was evaluated in the neurons of the striatum and NAc. FTBMT increased intracellular cAMP, phospho-CREB, and IEG expression in primary striatal neurons (Figure 1c, d, and e). Moreover, FTBMT increased the basal and NMDA-stimulated release of GABA in primary striatal neurons (Figure 1f). In line with these findings, FTBMT increased phospho-CREB levels via GPR52 in the striatum in vivo (Figure 3a). These pharmacological effects are quite similar to those of A2A-R agonists. The activation of A2A-R in striatopallidal GABAergic neurons counteracts changes in the cAMP level following Gi-coupled D2R signaling, thus increasing GABA release and leading to the inhibition of amphetamine-induced hyperactivity (Khisti et al., 2000; Kull et al., 1999; Rimondini et al., 1997). CREB signaling, which occurs downstream of cAMP signaling, also impacts both the dopamine and glutamate systems in the striatum (Greengard et al., 1999). Some dissociation was seen between the effective doses in behavioral tests and those in biochemical assays. It may be due to the limitation of tissue sampling that does not necessarily collect responsible neurons for some specific behavioral effect resulting in the lower sensitivity. Nevertheless the present biochemical findings qualitatively support the antipsychotic-like activity of FTBMT.

We also evaluated the effects of FTBMT in pharmacological models based on the glutamate hypothesis. Since protein kinase A directly regulates NMDA receptor-mediated Ca^{2+} increases, GPR52 activation was expected to potentiate NMDA receptor signaling via cAMP signaling (Skeberdis et al., 2006). FTBMT reversed NMDA receptor blocker MK-801-induced hyperactivity and cognitive impairment (Figure 3b and 6c). In addition, FTBMT enhanced NMDA-evoked GABA release in primary striatal neurons (Figure 1f). These results suggest that FTBMT may enhance NMDA receptor functions.

Next, we demonstrated a potential mechanism by which FTBMT had no cataleptogenic activity. Atypical antipsychotics reduce EPS liability, probably due to their weak D2R and potent 5-HT_{2A} receptor antagonism (Meltzer, 1999). However, FTBMT had a wider safety margin compared with atypical antipsychotics, such as olanzapine and aripiprazole (Figure 3d). This profile is similar to some potential antipsychotic agents, such as the A2A-R agonist CGS 21680, the metabotropic glutamate receptor 1 allosteric antagonist CFMTI, and the phosphodiesterase 10A inhibitor TAK-063 (Rimondini et al., 1997; Satow et al., 2009; Suzuki et al., 2015). Among these, CGS 21680 and CFMTI increased c-fos positive neurons in the NAc shell, but not in the DLS, which was similar to the results observed with FTBMT (Figure 5a and b) (Pinna et al., 1997; Satow et al., 2009). The findings from c-fos immunohistochemical staining were validated with the comparison studies of region-specific IEG expression using FTBMT, olanzapine, and aripiprazole (Figure 5c). Given that the NAc has been proposed as the central therapeutic site of activity for antipsychotics, the preferential neuronal activation in the NAc may reflect the antipsychotic-like activity of FTBMT, without catalepsy induction (Epstein et al., 1999; Jones et al., 2011; Raij et al., 2009). However, the mechanism by which FTBMT preferentially induces neuronal activation in the NAc remains to be elucidated.

Finally, we demonstrated that FTBMT improved cognitive functions associated with the medial prefrontal cortex, frontal cortex, and hippocampus. Seven cognitive domains that are commonly disrupted in schizophrenia, which were identified by the Measurement and Treatment Research to Improve Cognition in Schizophrenia initiative, were as follows: attention/vigilance, working memory, reasoning and problem solving, processing speed, visual learning and memory, verbal learning and memory, and social cognition (Nieuwenstein et al., 2001). Among these cognitive domains, the NORT and radial arm maze test could be used to probe visual learning and memory, and working memory in

rodents (Young et al., 2009). In these tests, FTBMT improved both cognitive functions in rats. Previous behavioral studies have demonstrated that the medial prefrontal cortex is important for working memory in the radial arm maze test, while the frontal cortex and hippocampus are involved in visual learning and memory in the NORT (Clark et al., 2000; Nagai et al., 2007; Taylor et al., 2003). Thus, the increased neuronal activity in the frontal cortex, medial prefrontal cortex, entorhinal cortex, and hippocampus with FTBMT corroborated with these previous results. The changes in these brain regions may represent the activation of the prefrontal-entorhinal-hippocampus circuit, which plays a crucial role in the encoding and retrieval of memories (Preston et al., 2013).

The results from the current study provided some evidence that GPR52 could be a potential therapeutic target in schizophrenia; however, the elucidation of its etiological role in the condition is still required validate this. To the extent of our knowledge, there is no evidence demonstrating the association between GPR52 and schizophrenia in human genetic and pathological studies. Since GPR52 is still an orphan receptor, the identification of its corresponding endogenous ligand may lead to new findings about its etiological link with schizophrenia.

In conclusion, given its antipsychotic and procognitive properties without causing catalepsy in rodent models, FTBMT, a selective and potent GPR52 agonist, may provide a new therapeutic option for the treatment of positive and cognitive symptoms of schizophrenia.

JPET Fast Forward. Published on August 29, 2017 as DOI: 10.1124/jpet.117.242925
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Acknowledgment

We thank Hiroyuki Ota for providing technical advice on behavioral assays. We also thank Yoshiteru Itoh for synthesis and supply of FTBMT.

Authorship contributions

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Wrote or contributed to the writing of the manuscript: Nishiyama wrote the first draft with contribution of all authors. Shimojo revised the draft and prepared the final version.

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Footnotes

This work was supported by Takeda Pharmaceutical Company Limited.

Figure legends

Figure 1

FTBMT-induced activation of cAMP signaling in vitro. (a) Chemical structure of FTBMT (b) FTBMT-stimulated cAMP response in CHO cells expressing human, mouse, or rat g-protein coupled receptor 52 (GPR52; n = 4). Dose-dependent effects of FTBMT on (c) cAMP production (n = 3), (d) phosphorylated CREB (n = 3), (e) IEG (c-fos and Egr-2) expression (n = 3), and (f) basal and 20 μM NMDA-stimulated release of [^{3}H]-GABA (n = 4–8) in primary striatal neurons. The cultured neurons (13–16 DIV) were stimulated with FTBMT for 15 (c, d and e), 30 (g), or 60 (f) min. All data are presented as means ± standard error of mean *P <0.05, ***P <0.001, $^{\#\#\#}$P <0.001, vs. respective controls.

Figure 2

FTBMT increased phospho-DARPP32 levels in the NAc slices. Acute NAc slices from mouse brains were treated with FTBMT (0.01, 0.1, and 1 μM) for 5 min. Phospho-DARPP32 and total DARPP32 in the NAc slices were detected by Western blot analysis (upper panel; representative images), and the corresponding bands were subsequently quantified using densitometry (lower panel; n = 9, *P <0.05 vs. vehicle control).

Figure 3

GPR52-mediated antipsychotic-like activity and low cataleptogenic activity of FTBMT in mice. (a) GPR52-mediated phosphorylation of CREB in the mouse striatum. WT and GPR52 KO mice were dosed with FTBMT (3 and 30 mg/kg, p.o.) or vehicle. Levels of p-CREB in the stratum 60 min post-dose were detected using an ELISA (*n* = 5–6, *P < 0.05

vs. vehicle (Veh) control group in WT mice). (b) Dose-dependent effects of FTBMT on MK-801-induced hyperactivity in mice. One hour after pretreatment with FTBMT (3–30 mg/kg, p.o.) or vehicle, mice were injected with MK-801 (0.2 mg/kg, s.c.), after which locomotor activities were monitored for 90 min (n = 9–10, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. MK-801-treated control group). (c) Effects of FTBMT on basal locomotor activity in mice. Locomotor activities were measured during one hour pretreatment prior to MK-801 administration (n = 9–10, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. vehicle-treated control group). (d) GPR52-mediated inhibitory activity of FTBMT on MK-801-induced hyperactivity. In accordance with method established in (b), WT and GPR52 KO mice were pretreated with FTBMT (30 mg/kg, p.o.) or vehicle prior to MK-801 injection (n = 18, $^{***}P < 0.001$ vs. MK-801-treated control group). (e) Effects of FTBMT, olanzapine and aripiprazole on cataleptic responses in the bar test in mice. Mice were treated orally with FTBMT (3–100 mg/kg), olanzapine (1–10 mg/kg), aripiprazole (0.3–10 mg/kg), or vehicle, 60 min prior to the bar test (n = 5–6, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle-treated control group). All data are presented as mean ± standard error of mean.

Figure 4

Effects of FTBMT and haloperidol on plasma prolactin concentrations in rats. FTBMT (3–100 mg/kg), haloperidol (1 mg/kg), or vehicle was orally administered to rats. One hour later, plasma was collected and prolactin concentration in plasma was measured by ELISA (n = 4, $^{***}P <0.001$ vs. vehicle control).

Figure 5

Preferential activity of FTBMT on neurons in the NAc shell compared with the striatum. (a) Representative photomicrographs of c-fos immunostaining in the DLS, DMS, and NAc shell. The brains of the animals were fixed by perfusion and processed for c-fos immunohistochemical analyses, 2 h after the injection of FTBMT (30 mg/kg, i.p.) or vehicle. Schematic drawings of coronal sections, which were adapted from the mouse brain atlas (Paxinos and Franklin, 2004), were used to count c-fos positive neurons. Scale bars = 50 μm. (b) Number of c-fos positive cells in the DLS, DMS, and NAc (n = 5, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. respective vehicle control). (c) Comparison study between the NAc shell and striatum, organized by IEG expression. IEG (i.e., c-fos, Egr2, and Arc) expression in the NAc shell and striatum taken from mice, 1 h after oral administration of FTBMT (3–100 mg/kg) or vehicle, was quantified using qPCR as previously described (n = 5–6, $^{*}P <0.05$, $^{**}P <0.01$, $^{***}P <0.001$ vs. vehicle control) (Sakuma et al, 2015). The data on olanzapine (0.3–10 mg/kg, p.o.) and aripiprazole (0.1–3 mg/kg, p.o.) were cited from our previous report (Sakuma et al, 2015). Data are presented as mean ± standard error of mean.

Figure 6

Procognitive activity of FTBMT in a NORT and radial arm maze test. (a, b) Effects of FTBMT, which was orally administered at doses of 3 and 10 mg/kg immediately after the acquisition trial, on recognition memory in a NORT. (a) The exploration times of familiar and novel objects 48 h after FTBMT administration (n = 10, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. respective exploration time for familiar objects), after which (b) the NDI was calculated using the equation described in the Materials and Methods section (n = 10, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. vehicle (Veh) control). (c) Effects of FTBMT on MK-801-induced working

memory deficits in a radial arm maze task in rats. FTBMT (10 mg/kg) or vehicle was orally administered to rats 30 min prior to the subcutaneous injection of MK-801 (0.08 mg/kg) or vehicle. The test was started 30 min after the MK-801 injection (n = 8, 13, and 14 for vehicle alone, MK-801 plus vehicle and MK-801 plus FTBMT, respectively, $^{###}P < 0.001$ vs. vehicle-treated control, $^{*}P < 0.05$ vs. MK-801-treated control). All data are presented as mean ± standard error of mean.

Figure 7

FTBMT-induced neuronal activation in brain regions related to cognition. (a) Photomicrographs showing c-fos-positive neurons in the frontal cortex (FC), medial prefrontal cortex (mPFC), and entorhinal cortex (EC). Immunohistochemical analyses of c-fos were performed as described in Figure 5a. Scale bars = 50 μm. (b) Number of c-fos positive cells in the FC, mPFC, and EC (n = 5). Induction of (c, d) IEG expression and (e) p-CREB by FTBMT in the hippocampus. Two hours after the administration of FTBMT (3–30 mg/kg, p.o.) to (c, e) naïve mice, and (d) GPR52 KO mice and WT littermates, the hippocampal tissues were collected and analyzed using gene expression assays (c; n = 6–7, d; n = 4–5) and an ELISA for p-CREB (e; n = 4–5). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$; $^{\&\&\&}P < 0.001$ vs. respective vehicle (Veh)-treated controls. All data are presented as mean± standard error of mean.

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Table 1. Effects of FTBMT on various enzyme activities

Enzyme	% inhibition
ATPase, Ca„ $^{2+,}$ Skeletal Muscle	-1
ATPase, Na+/K+, Heart	-2
Carbonic anhydrase II	4
Choline esterase, acetyl, ACES	18
Cyclooxygenase-1 (COX-1)	-1
Cyclooxygenase-2 (COX-2)	-20
HMG-CoA reductase	-13
Lipoxygenase (5-LO)	-5
Monoamine oxidase A (MAO-A)	6
Monoamine oxidase B (MAO-B)	26
Nitric oxide synthase, inducible (iNOS)	7
Nitric oxide synthase, neuronal (nNOS)	11
Peptidase, factor Xa	-4
Matrix metalloproteinase-1 (MMP-1)	-3
Matrix metalloproteinase-7 (MMP-7)	0
Matrix metalloproteinase-13 (MMP-13)	1
Phosphodiesterase PDE3	28
Phosphodiesterase PDE4	6
Phosphodiesterase PDE5	24
Phosphodiesterase PDE6	17
Phosphodiesterase PDE10A1	0
Protein kinase A (PKA), non-selective	17
Protein kinase C (PKC), non-selective	-5
ROCK1	9
Protein tyrosine kinase, EGF receptor	-1
Steroid 5α-reductase	0
Xanthine oxidase	10

FTBMT was tested at 10 μM in the all assays.

EGF, epidermal growth factor; HMG CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A.

ROCK1, Rho Associated Coiled-Coil Containing Protein Kinase 1

Negative value of percent inhibition indicate activation of enzyme activity.

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Table 2. Effects of FTBMT in various receptor binding assays (1)

Receptor	% inhibition
Adenosine A1	33
Adenosine A2A	0
Adenosine A2B	-12
Adrenergic α1, non-selective	3
Adrenergic α2, non-selective	5
Adrenergic β1	-1
Adrenergic β2	-5
Adrenergic β3	0
Androgen (testosterone)	5
Angiotensin AT1	9
Angiotensin AT2	1
Bradykinin B1	-5
Bradykinin B2	16
Calcium channel L-type, benzothiazepine	19
Calcium channel $_{\text{L-type}}$, dihydropyridine	-2
Calcium channel L-type, phenylalkylamine	-6
Calcium channel N-type	-9
Cannabinoid CB • 1	-4
Cholecystokinin CCK1 (CCKA)	6
Cholecystokinin CCK2 (CCK_B)	0
Dopamine D1	7
Dopamine D2L	5
Dopamine D3	8
Dopamine D4.2	3
Endothelin ETA	-13
Estrogen ERα	-9
GABAA, chloride channel	2
GABAA, flunitrazepam, central	6
GABAA, muscimol, central	-1
GABAB, non-selective	0
GABAB1A	5
GABAB1B	2
Glucocorticoid	12

FTBMT was tested at 10 μM in the all assays.

Negative value of percent inhibition indicates stimulation of receptor activity.

Table 3. Effects of FTBMT in various receptor binding assays (2)

Receptor	% inhibition
Glutamate, AMPA	2
Glutamate, kainate	2
Glutamate, NMDA, agonism	-7
Glutamate, NMDA, glycine	3
Glutamate, NMDA, phencyclidine	4
Glycine, strychnine	-4
Growth hormone secretagogue (ghrelin)	0
Histamine H1	-11
Histamine H2	2
Imidazoline I2, central	6
Insulin	-9
Muscarinic M1	4
Muscarinic M2	-6
Muscarinic M3	-6
Nicotinic acetylcholine	-2
Opiate δ (OP1, DOP)	4
Opiate κ (OP2, KOP)	-9
Opiate μ (OP3, MOP)	-10
Potassium channel (KATP)	-1
Potassium channel (SKCA)	-1
Progesterone PR-B	4
Prostanoid, Thromboxane A2 (TP)	-6
Serotonin 5-HT1, non-selective	-11
Serotonin 5-HT2, non-selective	14
Serotonin 5-HT2B	6
Serotonin 5-HT3	10
Serotonin 5-HT4	4
Sigma, non-selective	28

FTBMT was tested at 10 μM in the all assays.

AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ; NMDA, N-methyl-D-aspartic acid.

Negative value of percent inhibition indicates stimulation of receptor activity.

Table 4. Effects of FTBMT in various receptor binding assays (3)

Receptor	% inhibition
Sodium channel, Site 2	1
Serotonin transporter (SERT)	8
Tachykinin NK1	4
Tachykinin NK2	-12
Tachykinin NK3	20
Transporter, dopamine (DAT)	-1
Transporter, GABA	2
Transporter, norepinephrine (NET)	10
Vasopressin V1A	14
Vasopressin V2	-1

FTBMT was tested at 10 μM in the all assays.

Negative value of percent inhibition indicates stimulation of receptor activity.

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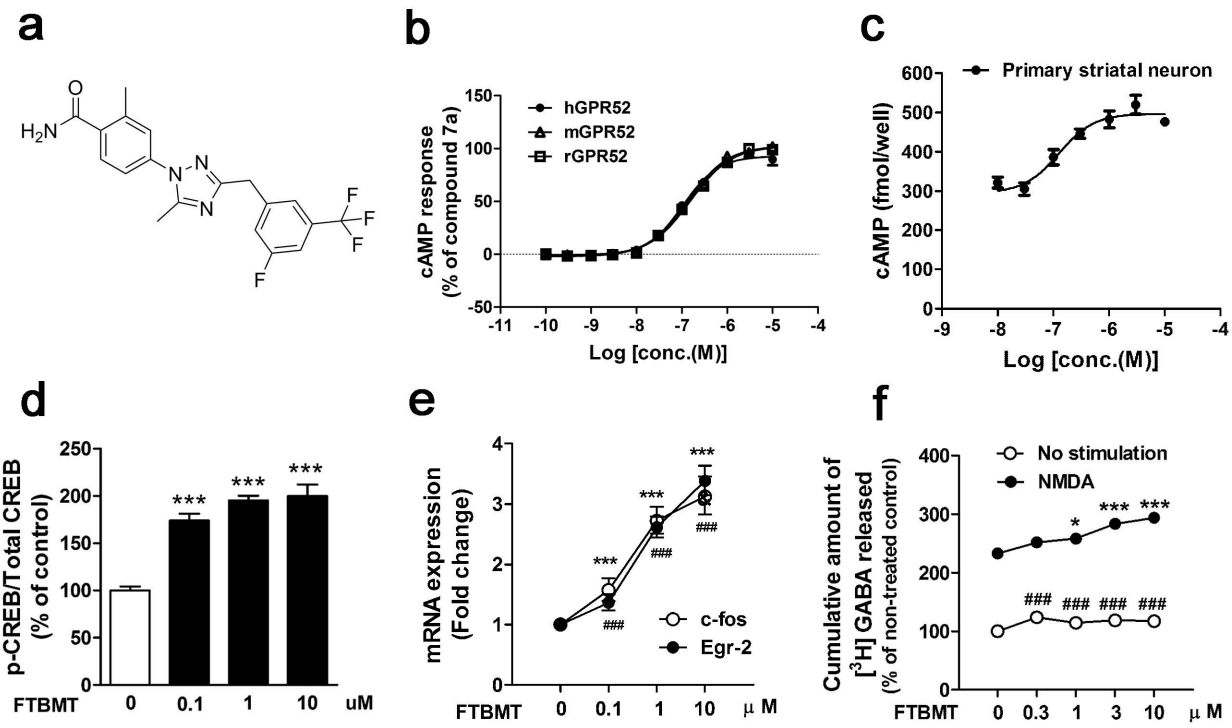
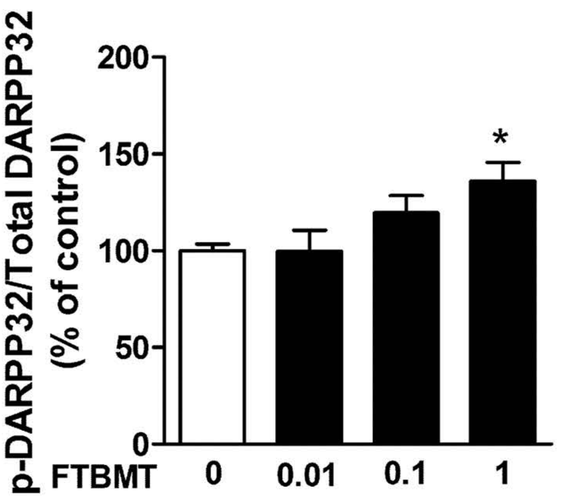


Figure 1

a



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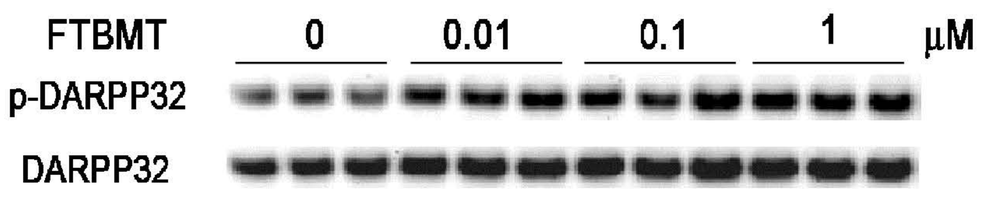

Figure 2

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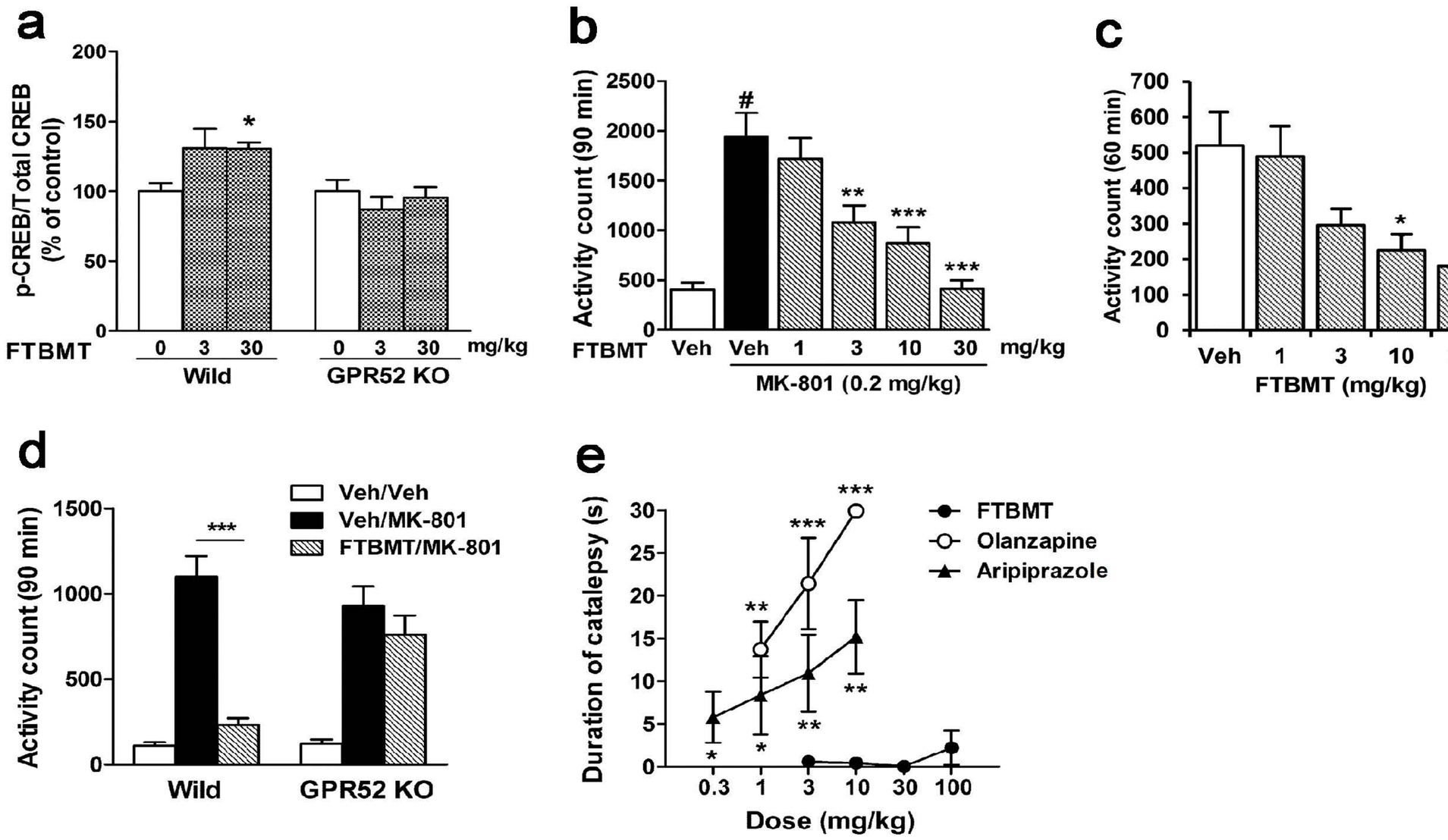

Figure 3

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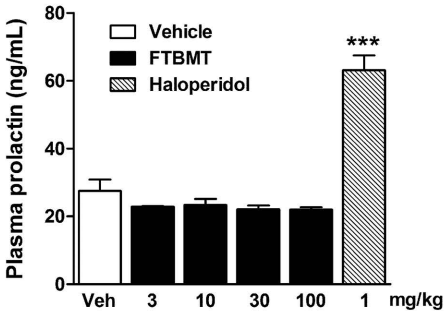

Figure 4

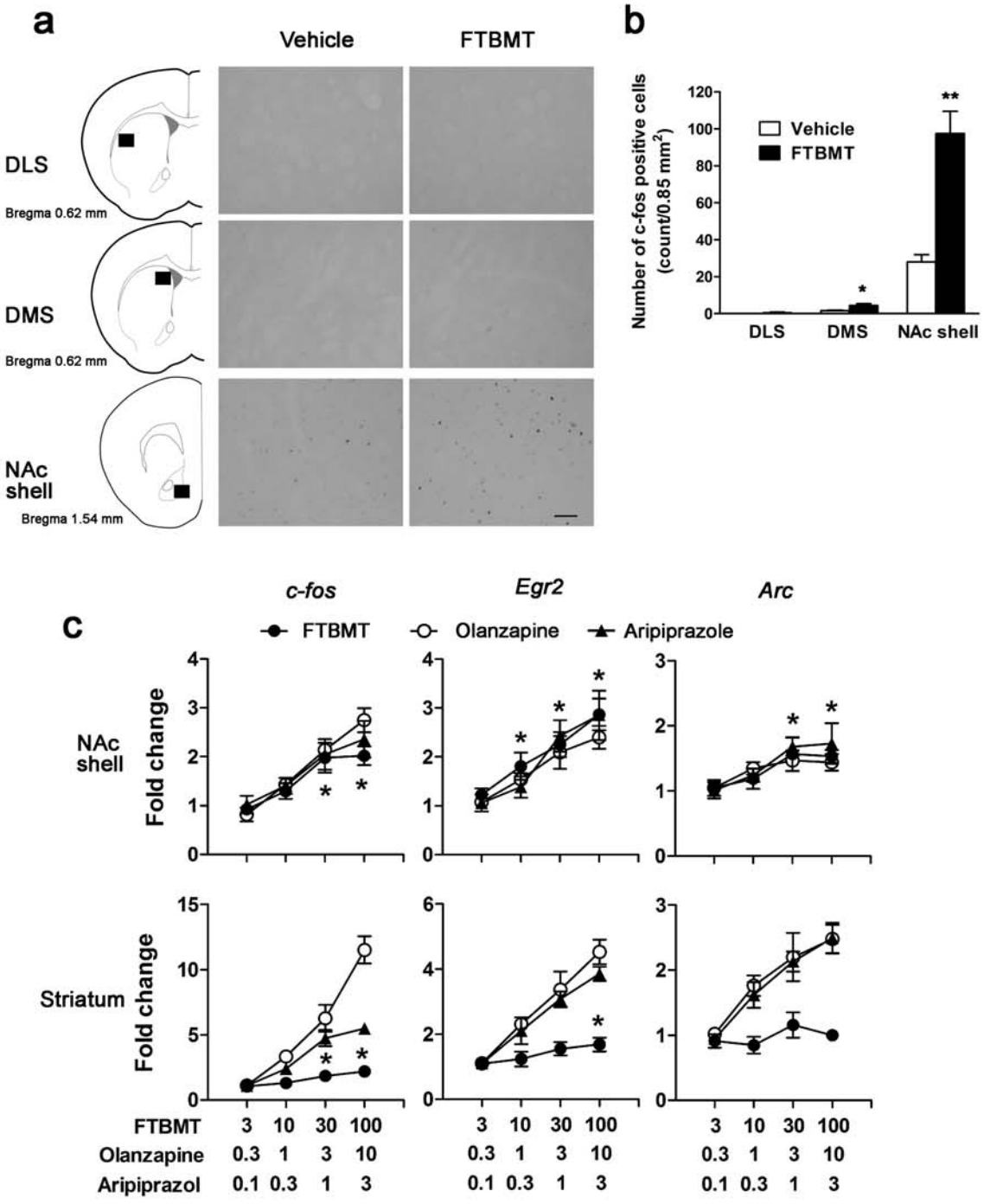

Figure 5

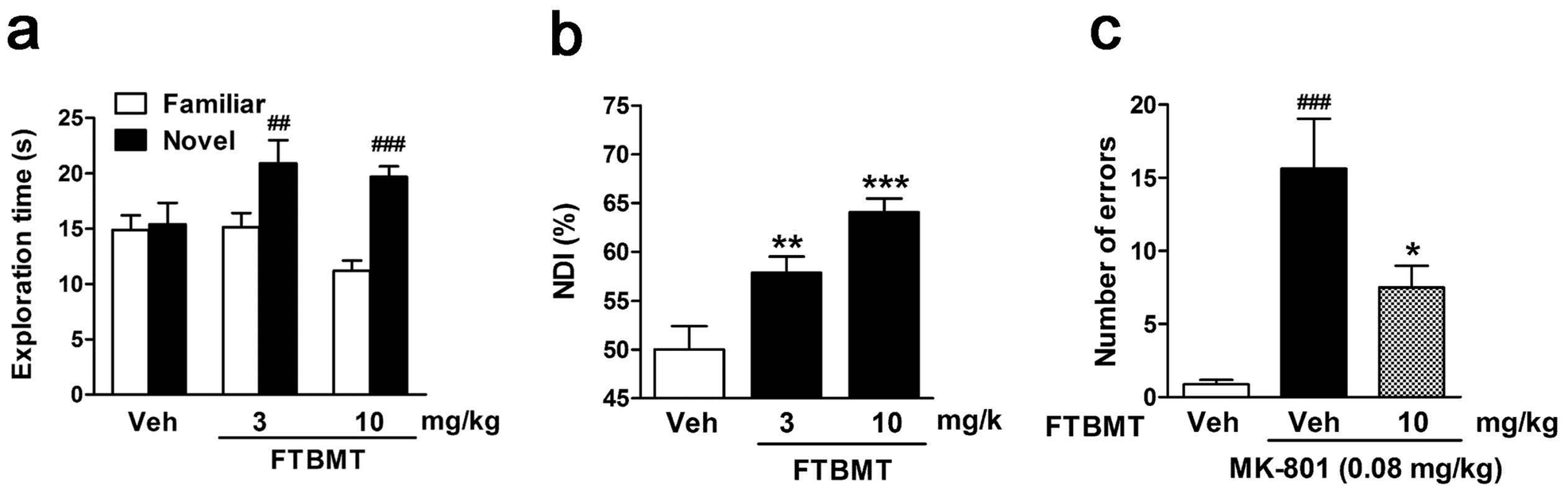

Figure 6

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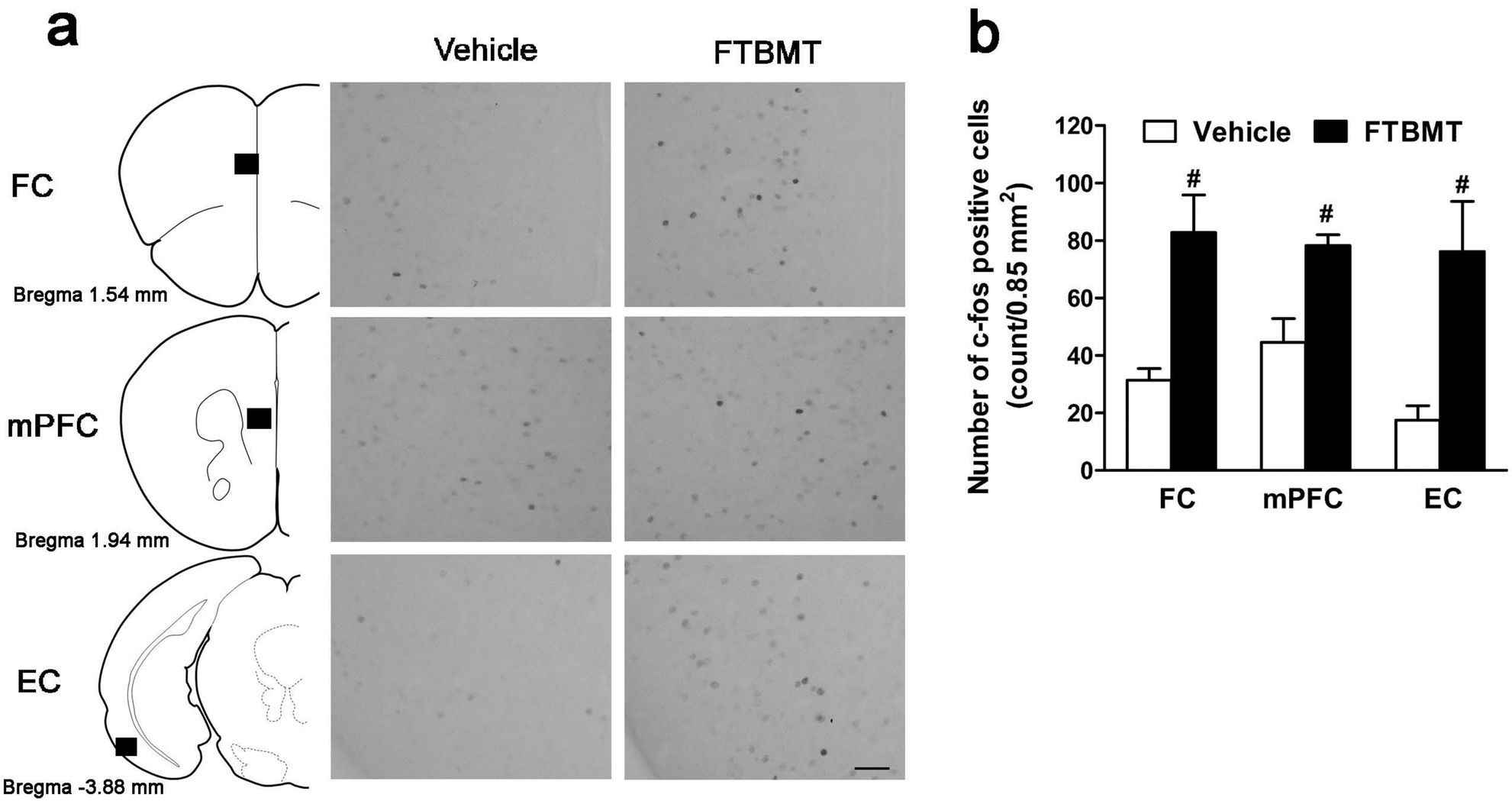

Figure 7

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