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Research Paper

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Background: Altered function of serotonin receptor 1A (5-HT_{1A}R) has been consistently implicated in anxiety, major depressive disorder and resistance to antidepressants. Mechanisms by which the function of 5-HT_{1A}R (expressed as an autoreceptor in serotonergic raphe neurons and as a heteroreceptor in serotonin [5-HT] projection areas) is altered include regulation of its expression, but 5-HT_{1A}R trafficking may also be involved. **Methods:** We investigated the consequences of the lack of Yif1B (the 5-HT_{1A}R trafficking protein) on 5-HT neurotransmission in mice, and whether Yif1B expression might be affected under conditions known to alter 5-HT neurotransmission, such as anxious or depressive states or following treatment with fluoxetine (a selective serotonin reuptake inhibitor) in humans, monkeys and mice. **Results:** Compared with wild-type mice, Yif1B-knockout mice showed a significant decrease in the forebrain density of 5-HT projection fibres and a hypofunctionality of 5-HT_{1A} autoreceptors expressed on raphe 5-HT neurons. In addition, social interaction was less in Yif1B-knockout mice, which did not respond to the antidepressant-like effect of acute fluoxetine injection. In wild-type mice, social defeat was associated with downregulated Yif1B mRNA in the prefrontal cortex, and chronic fluoxetine treatment increased Yif1B expression. The expression of Yif1B was also downregulated in the postmortem prefrontal cortex of people with major depressive disorder and upregulated after chronic treatment with a selective serotonin reuptake inhibitor in monkeys. **Limitations:** We found sex differences in Yif1B expression in humans and monkeys, but not in mice under the tested conditions. **Conclusion:** These data support the concept that Yif1B plays a critical role in 5-HT_{1A}R functioning and brain 5-HT homeostasis. The opposite changes in its expression observed in anxious or depressive states and after therapeutic fluoxetine treatment suggest that Yif1B might be involved in vulnerability to anxiety and depression, and fluoxetine efficacy.

Introduction

Serotonin (5-HT) system abnormalities have been associated with a number of psychiatric diseases, including depression and anxiety. The most commonly prescribed drugs for major depressive disorder (MDD), generalized anxiety disorder and social anxiety disorder are selective serotonin reuptake inhibitors (SSRIs). Thorough investigations of the mechanisms of action of SSRIs have shown that these drugs increase extracellular levels of 5-HT and promote activation of serotonergic receptors. Moreover, the 5-HT system plays a key role in antidepressant response, even after nonserotonergic treatments such as ketamine[1] or deep brain stimulation.[2] Activation of Gi/Go-protein-coupled 5-HT_{1A} receptors (5-HT_{1A}Rs) leads to the inhibition of neuronal firing and neurotransmitter release through modulations in the formation of cyclic adenosine monophosphate (cAMP) and in calcium and potassium channels. The 5-HT_{1A}Rs are expressed as heteroreceptors in neurons targeted by serotonergic neurotransmission, and as autoreceptors in serotonergic raphe neurons whose activation triggers negative feedback on their firing and 5-HT release.[3] Desensitization to 5-HT_{1A} autoreceptors is necessary for SSRI efficacy,[4] and changes in 5-HT_{1A}R levels have been associated with depression and social anxiety disorder in humans.[5–7] As well, in a genetic mouse model of depression in which SSRI treatment reversed spontaneous helplessness (as observed in the tail suspension test), marked changes in 5-HT_{1A}R expression and function were reported.[8]

Several models of genetic deletion of the 5-HT_{1A}R in mice have shown that such deletion leads to anxious and/or

depressive-like behaviour depending on which receptor (autoreceptor or heteroreceptor) was deleted and during which period of development (early postnatal period, juvenile or adulthood; see Albert and colleagues[9] for a review). Interestingly, a recent study showed that a change in only the targeting of the 5-HT_{1A}R, without any alteration in its level of expression, could also be implicated in depressive-like behaviour and SSRI effects.[10] This led us to address the question of how the mislocalization of 5-HT_{1A}R could affect its function and its implication in the development of anxious or depressive states, or in antidepressant response to SSRIs. We focused our investigations on Yif1B, the trafficking protein partner of the 5-HT_{1A}R, which we have previously shown to interact directly with the C-tail of the 5-HT_{1A}R and to play a key role in the targeting of this receptor to the distal part of dendrites.[11] In culture, knocking down Yif1B expression in hippocampal neurons leads to mislocalization of the 5-HT_{1A}R but not of other receptors, such as somatostatin 2A, purinergic P2X2 or 5-HT_{3A} receptors.[11,12] In the present study, our aim was to show that genetic deletion of Yif1B in mice (Yif1B-KO) affected serotonergic neurotransmission and social anxiety behaviour. We then looked for possible changes in Yif1B expression when central 5-HT tone was modified, such as in depression in humans or after SSRI treatment in monkeys and mice. Our investigations were focused on the prefrontal cortex (PFC), a brain structure known to be involved in depression and anxiety.[13,14]

Methods

Experimental design and statistical analysis

We performed experiments on both sexes in humans and monkeys. For mice, depending on the experiment, we used only males, or analyzed both sexes separately. All efforts were made to use the smallest number of animals possible but still obtain reliable data. Animals were allocated to experimental groups by matching in the monkey studies and in the social interaction test in mice. For the pharmacological studies in mice, animals were randomized in each genotype to be injected with the drug or saline. The numbers of animals or people per group are indicated in the figure captions. We used Prism GraphPad or Statistica software to perform statistical analyses; tests used are detailed in the figure captions. We considered p values to be significant at ≤ 0.05. We designed the experiments and edited the manuscript based on the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

Animals

Experiments on C57BL6/J and CD-1 mice were performed on 2- to 3-month-old animals from Janvier Breeding Centre and from a local animal facility (NAC) for homozygous Yif1B-KO and wild-type (WT) mouse littermates. The Yif1B mutant colony was kept on a mixed background 129SvEv/J-C57BL6/J by breeding littermates. The 3-primer polymerase chain reaction (PCR) strategy we used for genotyping has been described previously.[15] Experimental mice were housed with companions of the same sex and breeding mice in "trio" under standard conditions (type L2 cage with Celle Bel MAXI-25 cellulose bedding; 12 hour light/dark cycle; and constant temperature [23.0 ± 1.0°C] and humidity [60%]), with food and water ad libitum. All efforts were made to minimize animal suffering. For chronic SSRI treatment, mice received fluoxetine (10 mg/kg i.p. in 0.9% NaCl) or saline daily for 22 days at 1100 h. Mice were euthanized by cervical dislocation. Experiments strictly followed institutional guidelines for the care and use of animals, in compliance with national and international laws and policies (Council directives no. 87–848, October 19, 1987, French Ministère de l'Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions no. 75–805 to J.M.). All protocols were approved by the ethical committee (C2EA34) and were licensed by the Directorate General for Research and Innovation (French Ministère de l'Enseignement Supérieur et de la Recherche; protocol authorization 00717.06).

Tissues examined from rhesus macaque monkeys were from a previously published study.[16] Animals were housed in stainless steel cages with freely available water. They were fed monkey chow (Teklad 25% Monkey Diet; Harlan/Teklad) in amounts sufficient to maintain stable body weights and supplemented with a chewable vitamin tablet 3 days per week. Animals were healthy and were not exposed to stress. Environmental enrichment was provided daily. Monkeys were treated with fluoxetine (2 mg/kg/d for 8 weeks, then 3 mg/kg/d) or vehicle for 39 weeks by drinking. At 20–24 hours after their last drug or vehicle exposure, monkeys received initial sedation with a combination of midazolam and medetomidine (Versed 0.3 mg/kg and Domitor 0.06 mg/kg i.m.), followed by a lethal overdose of pentobarbital (75 mg/kg i.v.) via the saphenous vein. Following euthanasia, an autopsy saw (810 Autopsy Saw; Stryker, 810–2-11-REV) was used to remove the cranial dome. The brain was bisected into hemispheres, and each hemisphere dissected into coronal blocks that were immediately frozen in dry ice–chilled isopentane and stored at −80°C. All procedures were conducted in accordance with the guidelines established by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80–23)[17] and the Animal Care and Use Committee of the University of Mississippi Medical Center (Jackson, Mississippi).

Humans

The patients examined were a subset from a published study.[18] The paired controls had never met the criteria for an Axis I illness and had no history of a neurologic disorder (Appendix 1, Fig. S2A, available at jpn.ca/190134-a1). Recruitment, informed consent, postmortem brain tissue collection and retrospective psychiatric assessment were performed under a protocol approved by the institutional review boards at the University of Mississippi Medical Center and the University Hospitals Cleveland Medical Center (Cleveland, Ohio). All work was carried out in accordance with the Belmont Report.[19]

Extracellular recording of 5-HT cells in mouse dorsal raphe nucleus

We made extracellular recordings of dorsal raphe nucleus serotonergic neurons firing, as previously described.[20] Briefly, male mice were killed, their brains were rapidly removed, and a block of brainstem was cut into coronal sections (400 µm thick) using a vibratome and immersed in ice-cold artificial cerebrospinal fluid. Extracellular recordings of serotonergic neurons were obtained using glass microelectrodes filled with 2 *M* NaCl (10–15 MΩ). Firing was evoked in the otherwise silent neurons by adding the α1-adrenoceptor agonist phenylephrine (3 µ*M*) into the superfusing artificial cerebrospinal fluid.[21] Baseline activity was recorded for 5 to 10 minutes before application of the 5-HT_{1A}R agonist ipsapirone (60 n*M*, 3 minutes) via a 3-way tap system. We evaluated the effect of ipsapirone by comparing the mean discharge frequency during the 2 minutes before its addition to the superfusing artificial cerebrospinal fluid with the mean discharge frequency at the peak of drug action.

Immunofluorescence and quantitative analysis of labelling

Male mice were deeply anesthetized (pentobarbital, 60 mg/kg i.p.) and perfused with 4% paraformaldehyde in phosphate-buffered saline. After post-fixation, brains were sectioned with a vibratome (40 µm) at the level of the PFC and brainstem. Floating sections were incubated in antibody buffer (4% bovine serum albumin, 4% normal donkey serum, 0.1% Triton X-100, phosphate-buffered saline; 1 hour) with primary antibodies (4°C, overnight) and after phosphate-buffered saline washes, with secondary antibodies for 2 hours. Then, 5-HT and 5-HT transporter (SERT) were labelled with rabbit polyclonal anti-5-HT (1:500, 20079; Immunostar) and goat polyclonal anti-SERT (1:500, sc-1458; Santa Cruz) antibodies, respectively, followed by donkey Alexa555-conjugated anti-rabbit IgG (1:500, A-31572; ThermoFisher Scientific) or donkey biotinylated anti-goat IgG (1:500, D-20698; ThermoFisher Scientific) and Alexa488-conjugated streptavidin (1:500, S32354; ThermoFisher Scientific). After phosphate-buffered saline washes, sections were mounted in Fluoromount-G solution (CliniSciences). Immunofluorescence images were captured through the *z*-axis using a TCS SP5 microscope (PL-APO 63X, NA1.4 oil, imaging medium; Leica). For quantification, image stacks were acquired from a total of 80 sections for 5-HT labelling (11 to 18 sections from 3 animals per genotype) and 20 sections for SERT labelling (1 to 4 sections from 3 to 4 animals per genotype). The acquired images were analyzed on 3D stacks using Volocity (version 6.3.0; Perkin Elmer) or Neurolucida (version 11.08.2; MBF Bioscience).

DiI staining and quantification of dendritic spines

Male mice were deeply anesthetized (pentobarbital, 60 mg/kg i.p.) and perfused with 4% paraformaldehyde in phosphate-buffered saline. After post-fixation, 70 µm thick brain sections were prepared using a vibratome. Lipophilic carbocyanine DiI crystals (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; D-282; Molecular Probes, Invitrogen) were delivered to the hippocampus (CA1) sections using a commercially available biolistic device "gene gun" (Helios Gene Gun System, 165–2431; Bio-Rad). Sections were maintained in 50 m*M* phosphate-buffered saline, pH 7.4, at room temperature for 24 hours in the dark to allow the fluorescent dye crystals to diffuse fully along the membranes of the neuronal profiles. The DiI-labelled sections were post-fixed with 4% paraformaldehyde in phosphate-buffered saline for 30 minutes at room temperature; rinsed 3 times for 10 minutes each; and then mounted on Superfrost slides using the glycerol-based medium Mowiol (Calbiochem). Labelled dendrites were imaged using a TCS SP5 microscope (Leica). Serial stack images were scanned with a step size of 0.13 µm along the *z*-axis to allow for the detection of dendrites along 75 µm from the dendrite tip, and then projected to reconstruct a 3D image using Neurolucida. Dendritic spine density was evaluated on 3D stacks using the plug-in AutoSpine in Neurolucida.

Measurement of 5-HT and 5-hydroxy-indoleacetic acid

Immediately after dissection of the brainstem, hippocampus, frontal cortex and remaining cortex of male mice, tissues were homogenized in 10 volumes (vol/wt) of 0.1 N $HClO_4$ containing 0.5% $Na_2S_2O_6$ and 0.5% disodium EDTA, and centrifuged at 30 000 × *g* at 4°C. The supernatants were neutralized using 2 *M* KH_2PO_4/K_2HPO_4, pH 7.6, and centrifuged again. Aliquots (20 µL) of the final supernatants were injected into a high-performance liquid chromatography column (Ultrasphere IP; 25 cm, 0.46 cm outside diameter, 5 µm) protected with a Brownlee Newgard precolumn (3 cm, 5 µm). The mobile phase was as follows: 70 m*M* K_2HPO_4, 2 m*M* triethylamine, 1.5 m*M* octane sulfonic acid, 0.1 m*M* disodium EDTA and 11% methanol, adjusted to pH 3.10 with solid citric acid. The elution was performed at a flow rate of 1 mL/min, and the potential for electrochemical detection was settled at 0.65 V. Quantitative determinations were made by comparison with appropriate external standards using a CR 3A Shimadzu integrator.

Hypothermia induced by 8-OH-DPAT

The core body temperature of gently restrained male mice was measured at an ambient temperature of 23 ± 2° using a thermocouple probe (Betatherm) inserted into the rectum. The basal value was determined just before subcutaneous injection of the 5-HT_{1A}R agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT; 0.5 mg/kg s.c.) or vehicle, and body temperature was measured every 10 minutes for 2 hours.

Mouse behaviours

Behaviours were recorded during the light phase and automatically scored when experiments were performed in an apparatus equipped with an infrared floor or plate and a

camera connected to a computerized system (Viewpoint). All experiments were performed using 2- to 3-month-old animals. All experimental groups were different except for the social interaction test, which was performed with the same group of mice that underwent the open field test.

The open field test was performed in an open box of 50 × 50 cm. Male mice were placed in the arena for 20 minutes under normal light conditions (100 lux). The arena was subdivided into a central zone (20 × 20 cm) and a peripheral zone. The time spent in the central zone and the number of entries into the centre, as well as the distance travelled, were measured.

The social interaction test was conducted after 2 consecutive days of habituation in the open field box. On the third day, male mice who were of the same genotype but unfamiliar to each other were paired and placed in the arena under normal light conditions (100 lux). Animal behaviour was recorded with a camera, and active social interaction events (sniffing, fighting, chasing, grooming) were scored using ODlog (Macropod software) by an observer who was unaware of the genotype. Under our testing conditions, social interactions were not aggressive.

The elevated plus maze test consists of 2 open arms and 2 closed arms (40 × 5 cm) at 50 cm above the floor. Male mice were placed at the centre of the plus maze facing a closed arm and left free to explore for 10 minutes. The number of entries, time spent in the open arms and total distance covered in the entire maze were measured.

For the forced swim test, male and female mice were placed for 6 minutes in plastic buckets (19 cm in diameter, 23 cm deep) half-filled with 25°C water. Immobility (defined as the absence of movement and/or small movements necessary to keep the head above the water) was scored.

The repeated forced swim model of depression was adapted from the repeated open-space forced swim test.[22] Swimming was allowed for 6 minutes per day for 3 consecutive days in the forced swim test plastic buckets. On the fourth day, male mice were administered fluoxetine (20 mg/kg s.c.) or vehicle 30 minutes before the test, and immobility was quantified.

The chronic social defeat stress procedure was applied as described previously.[23] A male C57BL/6 mouse was introduced into the home cage of a male CD-1 mouse for 10 minutes. Then, these 2 mice were then physically separated with a perforated Plexiglas divider in the centre of the cage, allowing for 24-hour sensory interaction. To prevent any habituation to the same CD-1 mouse, each C57BL/6 intruder was exposed to a novel aggressor each day for 10 consecutive days. Control mice were housed in pairs in cages with a similar Plexiglas divider. Immediately after the last defeat session, socially stressed and control mice were housed singly in a new cage. Then, 24 hours after the last defeat session, the anxious/depressive phenotype was assessed and confirmed using the open field and social avoidance tests as previously reported.[23,24] Animals were killed 24 hours later to collect brain samples.

Western blots

We immunolabelled Yif1B in tissue punches of human PFC (Brodmann area 10) and monkey (frontal polar) cortical blocks. Equal volumes of protein samples (30 mg protein) were resolved on 12.5% sodium dodecyl sulfate–polyacrylamide gel and blotted on nitrocellulose membrane. Blots were incubated overnight at 4°C with rabbit anti-Yif1B polyclonal antibody (1:1000).[12] After washing, secondary polyclonal antibodies (rabbit HRP; 1:3000) were added. For normalization, anti-actin monoclonal antibody was used (primary 1:10000; secondary 1:5000; Chemicon). Each sample was immunoblotted in duplicate. The relative optical density of Yif1B bands was analyzed using imaging software (MCID Elite 7.0; Imaging Research) after normalization with the optical density of the corresponding actin band.

Real-time PCR

Total mRNA was extracted from male and female mouse PFC using the NucleoSpin RNA II extraction kit (Macherey-Nagel, GmbH & Co., KG). Reverse transcription was performed using the SuperScript III reverse transcriptase and random primers with the following cycling protocol: 25°C for 10 minutes; 37°C for 2 hours; and 85°C for 5 seconds. The cDNA samples were stored at −20°C. Amplification reactions were performed with ABsolute qPCR SYBR Green ROX Mix (Thermo Scientific) using a 7300 Real-Time PCR System (Applied Biosystems). The cycling protocol applied was as follows: 95°C for 3 minutes; 40 cycles of 95°C for 30 seconds; 54°C for 60 seconds; and 72°C for 30 seconds. Then, Yif1B cDNA amplification was performed (forward primer: ATGATTGGCGGCGTCCTCAC; reverse primer AGGTGGAAGGTGAGCCAGTA). We used *HPRT* as a housekeeping gene for normalizing gene expression results using GCAAACTTTGCTTTCCCTGG and TACTGGCAACATCAACAGGA as forward and reverse primers, respectively. We used the $2^{\Delta\Delta Fr}$ method to quantify the fold change in mRNA expression in the experimental groups.

Results

Yif1B deletion modifies 5-HT$_{1A}$R functionality

We evaluated 5-HT$_{1A}$ autoreceptor function by direct agonist activation using in vitro extracellular recordings from dorsal raphe nucleus slices of WT and Yif1B-KO mice. We observed no significant change in basal firing frequency in Yif1B-KO versus WT mice (Fig. 1A). The addition of the specific 5-HT$_{1A}$R agonist ipsapirone resulted in less inhibition of dorsal raphe nucleus 5-HT neuron firing in Yif1B-KO mice compared to WT mice, revealing a diminished efficacy of 5-HT$_{1A}$R stimulation in the Yif1B-KO mice. Because the 5-HT$_{1A}$ heteroreceptor expressed in hippocampal pyramidal neurons was shown to control their dendritic architecture,[25] we assessed the density of dendritic spines as a read-out of 5-HT$_{1A}$ heteroreceptor function in Yif1B-KO versus WT mice. Labelling with DiI revealed a significant decrease in the number of spines on CA1 pyramidal neurons in Yif1B-KO mice compared with WT mice (Fig. 1B), suggesting a decreased neurotrophic effect mediated by 5-HT$_{1A}$R.[26] Taken together, these results suggest that 5-HT$_{1A}$R functionality was decreased in Yif1B-KO mice. We then measured the ability

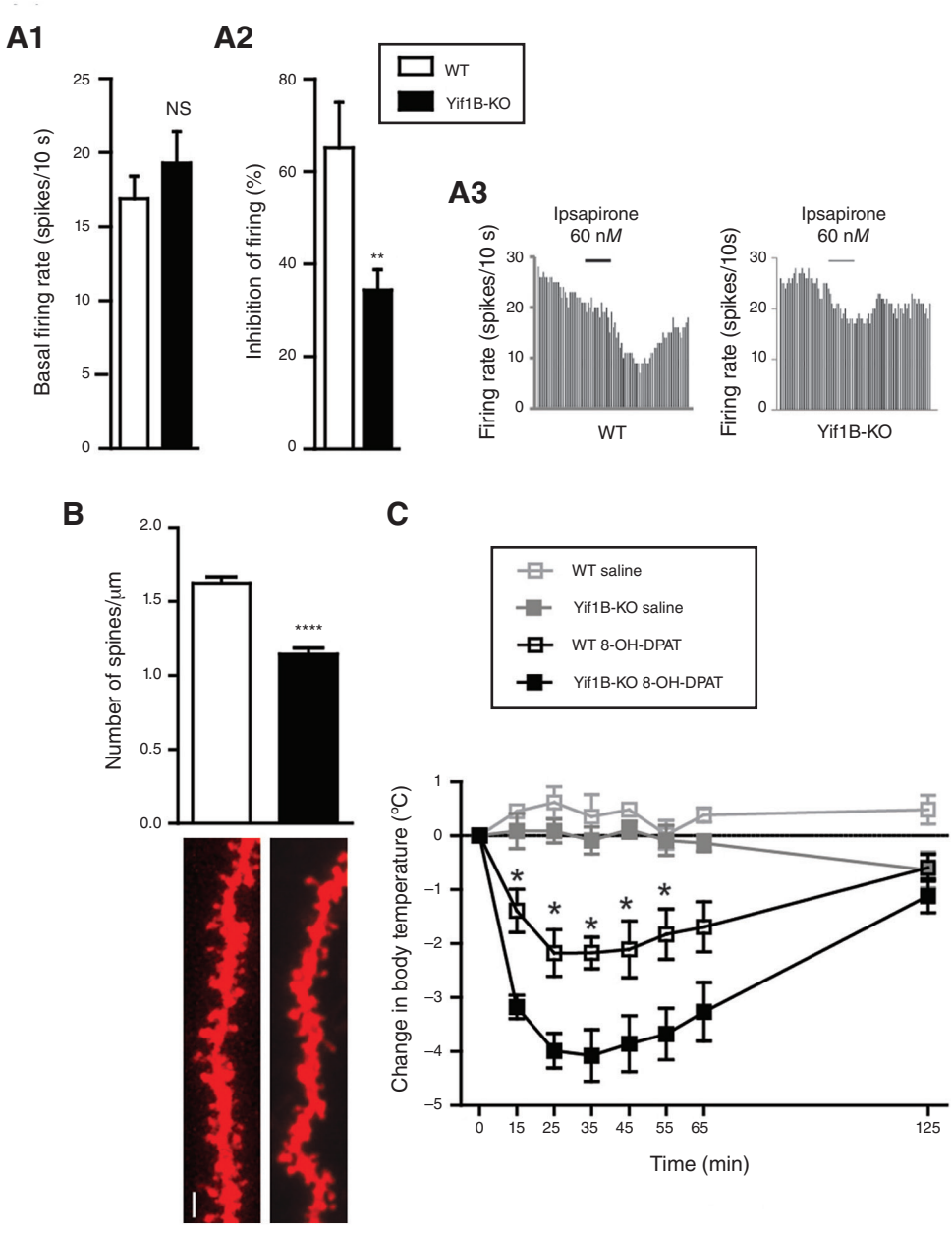

Fig. 1: 5-HT$_{1A}$R functionality in male Yif1B knockout (Yif1B-KO) versus wild-type (WT) mice. (A) Electrophysiological activity recording of serotonergic neurons in the dorsal raphe nucleus demonstrated no difference in basal firing frequency between genotypes (WT, n = 10; Yif1B-KO, n = 9), but the efficacy of the 5-HT$_{1A}$R agonist ipsapirone (60 n*M*) in inhibiting 5-HT cell activity was significantly lower in Yif1B-KO than in WT mice (WT, n = 7; Yif1B-KO, n = 13; t test, $p < 0.01$) as illustrated by representative integrated firing rate histograms (in spikes per 10 s) of neurons from WT and Yif1B-KO mice. (B) Quantification of the number of spines in terminal dendritic segment from pyramidal neurons of CA1 in WT and Yif1B-KO mice using DiI staining demonstrated a significant decrease in the Yif1B-KO mice (WT, n = 10; Yif1B-KO, n = 20; t test, $p < 0.0001$; see representative fluorescent dendrite images, scale bar 1 µm). (C) 8-OH-DPAT-induced hypothermia in Yif1B-KO versus WT mice. Mice were injected with saline (WT, n= 3; Yif1B-KO, n = 4) or 8-OH-DPAT (0.5 mg/kg subcutaneous; WT, n = 6; Yif1B-KO, n = 10), and rectal temperature was recorded for 2 hours. Data (means ± standard error of the mean [SEM]) represent changes in body temperature after injection. Repeated-measure 2-way analysis of variance for 8-OH-DPAT effects revealed an interaction effect for time × genotype effect ($F_{7,98}$ = 46.29, $p < 0.001$). Bonferroni post hoc tests revealed that the effect of 8-OH-DPAT was significantly different 15 to 55 minutes after injection ($p < 0.05$ for each time point) in Yif1B-KO compared to WT mice. Graphs represent means ± SEM; *$p < 0.05$; ****$p < 0.0001$. 5-HT$_{1A}$R = serotonin receptor 1A; 8-OH-DPAT = 8-hydroxy-2-di-n-propylamino) tetralin; CA1 = hippocampus section; DiI = 1,1-dioctadecyl-3,3,3',3'-tetramethylindocarbo-cyanine perchlorate; NS = nonsignificant.

of the 5-HT_{1A}R agonist 8-OH-DPAT to induce hypothermia in Yif1B-KO and WT littermates. Baseline temperature values were similar for both genotypes (WT, 36.90 ± 0.14°C; Yif1B-KO, 36.77 ± 0.12°C; mean ± standard error of the mean; *t* test, $p = 0.49$). Acute administration of 8-OH-DPAT induced a time-dependent decrease in body temperature in both genotypes compared with saline injection, but the effect was significantly enhanced in Yif1B-KO mice compared with WT mice (Fig. 1C).

Yif1B deletion induces changes in brain serotonergic innervation

Whereas 5-HT levels did not differ between Yif1B-KO and WT in the brainstem, they were significantly lower in serotonergic projection areas such as the hippocampus and cortex (including frontal cortex) in Yif1B-KO mice (Fig. 2A). Changes in 5-hydroxy-indoleacetic acid (5-HIAA) levels mirrored those observed for 5-HT (Fig. 2A), leading to similar [5-HIAA]/[5-HT] ratios as 5-HT turnover and metabolism indexes in Yif1B-KO mice compared with WT mice. We then compared the density of brainstem serotonergic soma and their neuronal projections in Yif1B-KO and WT mice using immunostaining for 5-HT and SERT as specific markers. We observed no significant differences in the number of serotonergic neurons in the dorsal raphe nucleus between the 2 genotypes (Fig. 2B). In contrast, SERT immunolabelling showed a lower mean total length of serotonergic fibres per section in the PFC of the Yif1B-KO mice (Fig. 2C). The decreased length of SERT immunopositive fibres (–21%) was in accordance with the decreased frontal cortex content of 5-HT in Yif1B-KO mice compared with WT mice.

Anxious/depressive behaviour in Yif1B-KO mice

Analysis of the locomotor activity in the open field and elevated plus maze tests and the duration and number of entries in the open field arena centre (Fig. 3A), as well as the percentage of entries in open arms in the elevated plus maze test (Fig. 3B) demonstrated that deletion of Yif1B did not affect locomotion and anxiety in these tests. Concerning the depressive-like behaviour assessed by the duration of immobility in the forced swim test, we also found no significant difference between Yif1B-KO and WT mice, regardless of sex (Fig. 3C and Appendix 1, Fig. S1). Finally, on the social interaction test, the Yif1B-KO mice spent significantly less time in active social interaction than the paired WT mice (Fig. 3D).

Together, these data showed that anxiety and depressive-like behaviours did not differ between Yif1B-KO and WT mice when animals were tested individually, but increased social anxiety appeared in the Yif1B-KO mice when the animals underwent the social interaction test as paired littermates.

Regulation of Yif1B expression in humans, monkeys and mice

Because deletion of Yif1B in mice affected social interactions that are known to be affected in MDD in humans, we then investigated the levels of Yif1B in samples from patients with MDD. The individual characteristics of the patients with MDD and controls (9 females and 10 males in each group) are detailed in Table 1. Compared with sex-matched controls, we found a significant decrease in Yif1B protein levels in the PFC of women with MDD but not in men with MDD (Fig. 4A). Interestingly, previous investigations showed that 5-HT_{1A}R protein levels in the PFC of the same human samples were also significantly lower only in women with MDD,[18] and comparison with our Yif1B data revealed the existence of a significant correlation between 5-HT_{1A}R and Yif1B levels in the human PFC (Appendix 1, Fig. S2).

Although the patients with MDD were on antidepressant medication, none tested positive for these drugs in post-mortem toxicology (Table 1). For this reason, we could not compare patients with MDD on or off antidepressants with healthy controls. To assess possible antidepressant treatment effects on Yif1B expression, we performed relevant investigations in macaques that had been treated for 9 months with fluoxetine at a clinically therapeutic dose. Clinically relevant blood levels of fluoxetine were associated with a marked increase in PFC levels of Yif1B protein in females, but not in males (Fig. 4B). Such sex-related differences in both humans and monkeys led us to explore whether a possible sex effect also occurred with respect to the effect of chronic fluoxetine treatment on Yif1B expression in WT mice. We noted a marked increase in PFC levels of Yif1B mRNA after a 22-day treatment with fluoxetine, but this effect was similar in both female and male mice (Fig. 4C). In striking contrast, we observed that chronic social defeat stress, previously shown to robustly induce a depressive-like state in male mice,[23] caused a significant decrease in PFC Yif1B mRNA levels (Fig. 4D), suggesting that fluoxetine-induced upregulation of Yif1B mRNA might be related to the SSRI antidepressant action.

Effect of fluoxetine on Yif1B-KO mice

To further assess the apparent discrepancy between the WT-like behaviour of Yif1B-KO mice in the forced swim test and Yif1B downregulation in the PFC in male mice subjected to chronic stress (Fig. 3D), we used a repeated forced swim test protocol that had been shown to induce stress-coping in male mice.[28] Comparison of Yif1B-KO and WT mice under these conditions allowed us to evaluate how deletion of Yif1B could affect the time course of resignation and the response to acute SSRI. We observed no significant difference in the duration of immobility between Yif1B-KO and WT mice on the 3 days of testing. In both groups, the time course of immobility was similar, up to a plateau reached as soon as the first minute of test days 2 and 3 (Fig. 5A), suggesting that Yif1B-KO mice responded similarly to their WT littermates in terms of stress coping. We then evaluated the response of Yif1B-KO and WT mice to an acute injection of fluoxetine administered 30 minutes before the forced swim test on day 4. The WT mice responded to the fluoxetine injection by decreasing their immobility time at the beginning of the test with a time course pattern

similar to the one observed on the first day of the forced swim test for WT and Yif1B-KO mice. In contrast, Yif1B-KO mice who received fluoxetine did not show any significant change in immobility compared with Yif1B-KO mice injected with saline (Fig. 5B).

Discussion

In the present study, we showed that deletion of the *YIF1B* gene, coding for a targeting partner of the 5-HT_{1A}R, led to decreases of both 5-HT_{1A}R functionality and 5-HT innervation,

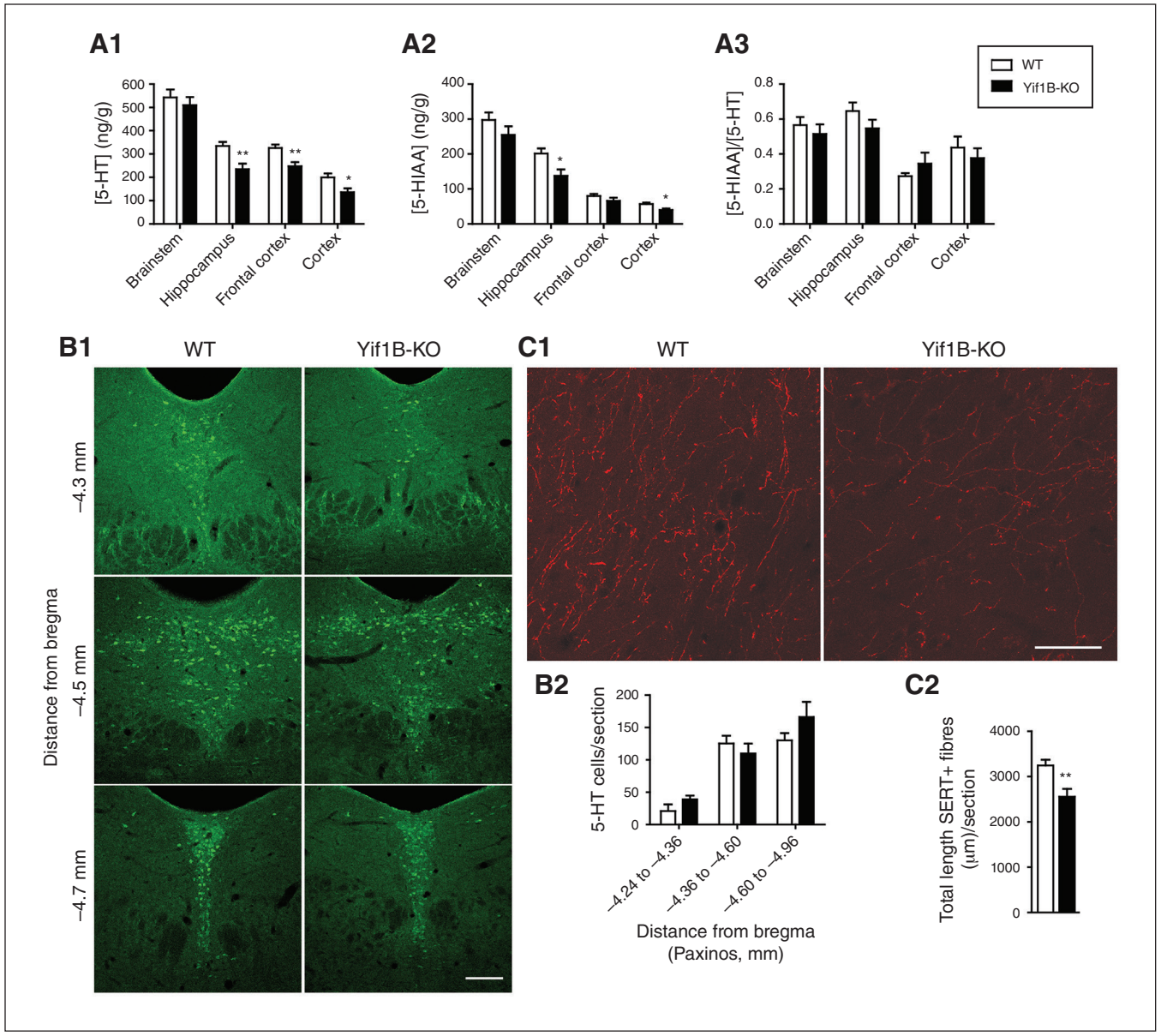

Fig. 2: Biomarkers of the brain serotonergic system in male Yif1B knockout (Yif1B-KO) and wild-type (WT) mice. (A1) 5-HT levels (ng/g of tissue) were significantly lower in the hippocampus (*t* test, $p = 0.003$), the frontal cortex (*t* test, $p = 0.003$) and the remaining cortex (*t* test, $p = 0.042$) in Yif1B-KO ($n = 10$) compared with WT ($n = 10$) mice. (A2) 5-hydroxy-indoleacetic acid (5-HIAA) levels (ng/g of tissue) in brain areas from Yif1B-KO ($n = 10$) versus WT ($n = 10$) mice followed the same trends; however, differences reached statistical significance only for the hippocampus and the cortex (*t* test, $p = 0.013$). (A3) The 5-HT turnover index (determined as the [5-HIAA]/[5-HT] ratio) in the same brain areas revealed no difference between genotypes. (B1) 5-HT immunoreactive neurons in the dorsal raphe nucleus showed similar antero-posterior distribution (–4.24 to –4.96 from the bregma; Franklin and Paxinos atlas[27]) in both genotypes ($n = 3$ per genotype). (B2) Two-way analysis of variance, antero-posterior level effect ($F_{2,11} = 31.52$, $p < 0.0001$), genotype effect ($F_{1,11} = 1.116$, $p = 0.31$) and no interaction of genotype × antero-posterior level ($F_{2,11} = 1.093$, $p = 0.36$). (C1) Serotonergic innervation in the prefrontal cortex of WT ($n = 4$) and Yif1B-KO ($n = 3$) mice revealed by SERT immunostaining. (C2) Quantification of total serotonin (5-HT) immunoreactive fibre length demonstrated a significant effect of genotype (*t* test, $p = 0.049$). Graphs represent means ± standard error of the mean (SEM); $^*p < 0.05$, $^{**}p < 0.01$. Scale bars, 250 µm (B1), 100 µm (C1).

and increased social anxiety. We also demonstrated that Yif1B expression was downregulated in the postmortem PFC of female patients with MDD and male mice subjected to chronic social defeat, but it was upregulated in female monkeys and in mice of both sexes after chronic SSRI treatment. Finally, we showed that the deletion of Yif1B prevented the antidepressant-like effects of acute SSRI treatment in male mice.

We found that Yif1B deletion affected 5-HT_{1A}R functionality, in line with previous data showing the essential role of this protein for targeting of the receptor.[11,12] Surprisingly, although hypothermia induced by 8-OH-DPAT is usually ascribed to 5-HT_{1A} autoreceptor activation,[29,30] we found that the decrease of 5-HT_{1A} autoreceptor functioning inferred from electrophysiological recordings was associated with greater hypothermia induced by 8-OH-DPAT in Yif1B-KO mice. Indeed, findings in the literature have indicated that postsynaptic 5-HT_{1A} heteroreceptors in the cortex and dentate gyrus also contribute to hypothermia induced by 8-OH-DPAT.[29] Although we indirectly observed decreased functionality of 5-HT_{1A}R in the hippocampus of Yif1B-KO mice, an upregulation of 5-HT_{1A} heteroreceptors in other brain areas might have occurred as an adaptive response to the decrease in central 5-HT tone found in these mice. Accordingly, further investigations of regional changes in brain 5-HT_{1A} heteroreceptor expression are needed to assess whether some upregulation accounted for the enhanced hypothermia induced by 8-OH-DPAT in Yif1B-KO mice.

Although a hyposerotonergic state and the deletion of 5-HT_{1A}R have been associated with anxiety and depression in mice,[5,7–14] Yif1B-KO mice in the present study did not display depressive-like or anxious phenotypes. However, they did spend less time in active social interaction than WT mice, indicating an increase of social avoidance. Social anxiety is a trait of several psychiatric diseases, and social interaction levels have been correlated with 5-HT levels and 5-HT_{1A}R activation. Impaired social communication is a core symptom of autism spectrum disorders and alterations in serotonergic tone have been observed in models of autism, including reduced density of serotonergic axons and altered 5-HT content.[30,31] On the other hand, lower 5-HT_{1A}R binding has been detected in the anterior cingulate cortex of patients with social anxiety disorder,[5] and pharmacological stimulation of 5-HT_{1A} autoreceptors increases social interaction in rats.[32] Social interaction is also impaired in patients with MDD, and convergent studies have documented the comorbidity of social anxiety disorder with MDD.[33,34] Interestingly, in our study, of the women with MDD who had lower levels of Yif1B in the PFC, only a small proportion had comorbid social anxiety disorder, but no men with MDD met the criteria for social anxiety disorder (Table 1) and displayed changes in Yif1B expression compared with controls. In mice experiencing chronic social defeat stress, Yif1B was downregulated in the PFC. Chronic social defeat stress is used to model a depressive-like state,[24] but it has also been proposed to induce anxiety-related disorders with social

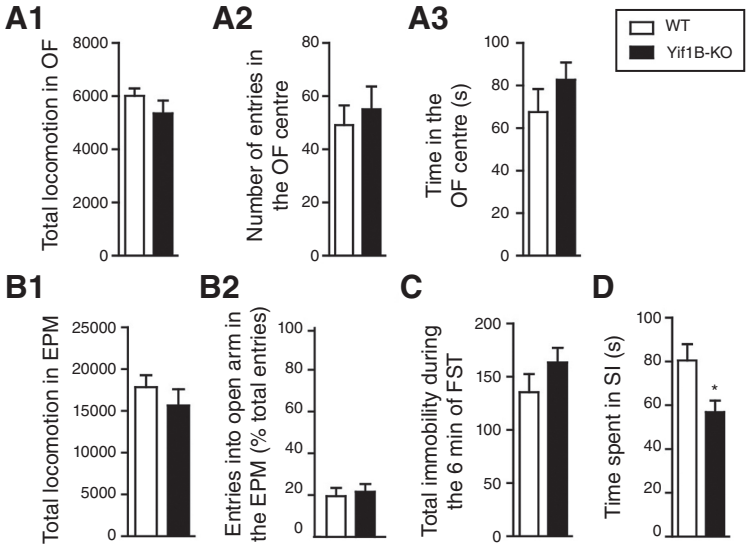

Fig. 3: Phenotypic characterization of male Yif1B knockout (Yif1B-KO) versus wild-type (WT) littermate mice with anxiety- and depressive-like tests. (A) Behavioural performance in the open field (OF) test during a 20-minute session (total locomotion, number of entries in the centre, time spent in the centre) was similar in WT (n = 14) and Yif1B-KO (n = 16) mice. (B) Behavioural performance in the elevated plus maze (EPM) test: total locomotion and percentage of entries in open arms were similar in Yif1B-KO (n = 6) and WT (n = 7) mice. (C) Total immobility recorded during the 6 minutes of the forced swim test (FST) was identical in WT (n = 8) and Yif1B-KO (n = 14) mice. (D) The social anxiety profile of the Yif1B-KO mice was revealed by a decrease in time spent in active social interaction (SI) (WT, n = 6; Yif1B-KO, n = 7; t test, p = 0.022). Graphs are means ± standard error of the mean (SEM); *$p < 0.05$.

avoidance.[35] Measurement of Yif1B levels in patients with social anxiety disorder but without MDD, and in mouse models presenting solely with a social interaction deficit or depressive behaviour, should be performed to determine whether changes in Yif1B expression are correlated specifically with 1 of these 2 pathological states.

We then addressed the potential link between Yif1B levels and SSRI treatment, considering its effect on serotonergic neurotransmission. Chronic SSRI treatment enhanced Yif1B expression in the PFC in monkeys and mice, but acute fluoxetine administration in Yif1B-KO mice had no effect on immobility in a repeated forced swim test protocol. This lack of response in Yif1B-KO mice might have been causally related to the relative deficit in serotonergic innervation of the PFC in these mice. This observation agrees with the findings of previous studies in mice with different polymorphisms of the

Table 1: Characteristics of patients with MDD and comorbidity with social anxiety disorder

Characteristic	Women	Men
Patients with MDD, *n**	9	10
Age at MDD onset, yr†	29.0 ± 2.7	44.0 ± 5.7
Duration of illness, yr†	22.0 ± 4.1	11.0 ± 3.5
Taking antidepressant medication, *n*	7	6
Toxicology-positive for drugs (postmortem), *n*	1 (temazepan)	1 (diazepam)
Comorbidity with social anxiety disorder, *n*	< 5‡	0

MDD = major depressive disorder.
*According to DSM-IV criteria.
†Mean ± standard error of the mean.
‡To protect participant privacy, findings with values of less than 5 have been rounded.

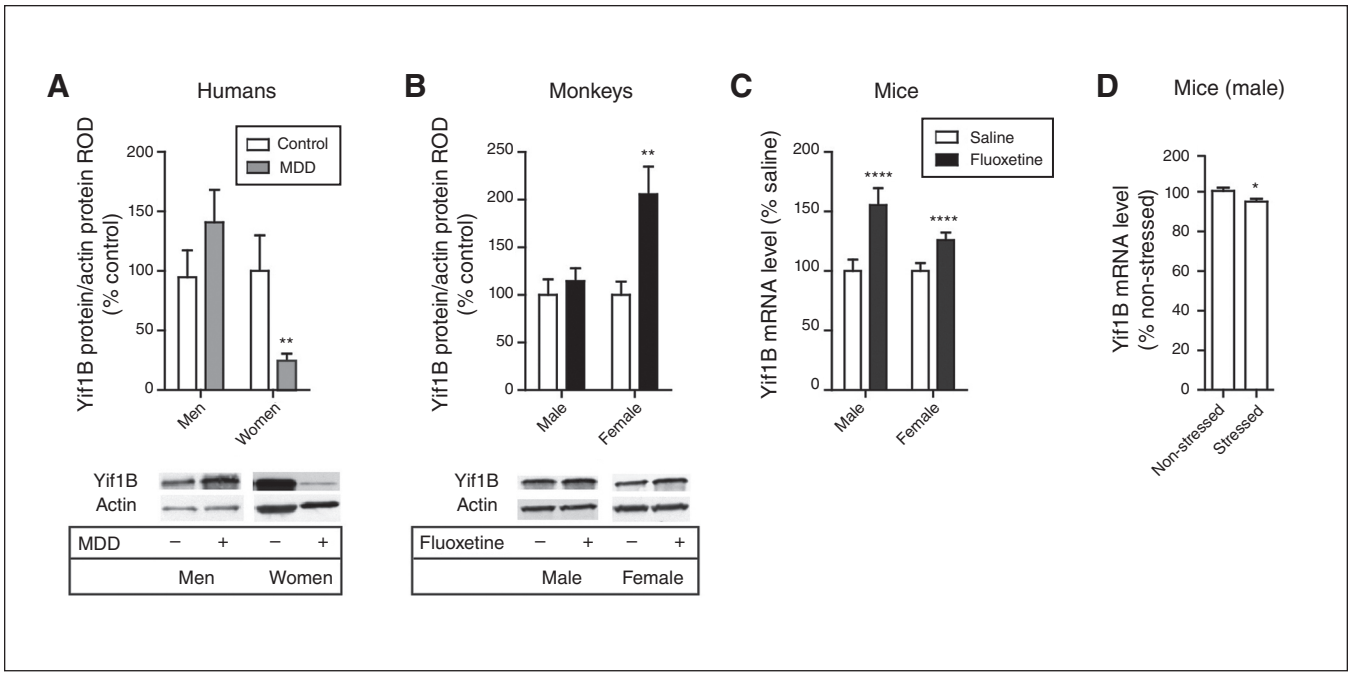

Fig. 4: Yif1B expression in depressive conditions and after chronic selective serotonin reuptake inhibitor (SSRI) treatment. (A, B) Quantification of Yif1B protein levels (normalized to actin relative optical density [ROD]) in the prefrontal cortex, targeting a specific region of serotonergic innervation, in humans and monkeys. (A) Quantification in healthy controls (men and women; n = 9–10) and in people with major depressive disorder (MDD) (n = 9–10) demonstrated an effect of MDD in women (paired t test, 2-tailed, p = 0.031 compared with sex-matched controls), but not in men (MDD v. controls, p = 0.05). (B) After 9 months of fluoxetine treatment in monkeys (n = 5–6), quantification of Yif1B protein levels revealed an effect of the drug in females (paired t test, 2-tailed, p = 0.011) but no change in males (p = 0.51). Representative Western blot bands are presented below each graph (Yif1B, 34 kDa; actin, 46 kDa). (C, D) Quantification of Yif1B mRNA in the prefrontal cortex of wild-type (WT) mice after SSRI treatment and in social-stressed mice. (C) A 22 day-treatment with fluoxetine (10 mg/kg daily) in male and female WT mice (n = 10–12) revealed an effect of the drug (2-way analysis of variance; drug effect, $F_{1,42}$ = 18.92, p < 0.0001; no sex effect and no interaction effect of genotype × gender, p = 0.12 for both). (D) After 10 days of social stress (1 session per day) in WT male mice (n = 13–18), we observed an effect of stress (t test, p = 0.020). Graphs represent means ± standard error of the mean (SEM). *p < 0.05; **p < 0.01; ****p < 0.0001.

TPH2 gene. In particular, mice carrying a 1473G allele of the *TPH2* gene (expressing low *TPH2* activity and showing a strong decrease in brain 5-HT content) failed to respond to SSRI in the forced swim test.[36,37]

Comparison of Yif1B expression levels under "low" or "high" serotonergic conditions in humans and monkeys on one hand, and in mice on the other hand, demonstrated a sex effect only in humans and monkeys. This sex-specific alteration could represent an underlying biological mechanism associated with the higher incidence of depression in women, because it has previously been proposed as an explanation for the significantly decreased levels of 5-HT_{1A}R and NUDR (a 5-HT_{1A}R transcriptional regulator) in the PFC of women with MDD.[38] Further studies are needed to investigate whether the sex effect in the Yif1B expression in humans and monkeys might be related to the presence of functional estrogen response element sites in the Yif1B promoter gene in these species.

As expected from the function of Yif1B as a targeting protein for the dendritic localization of the 5-HT_{1A}R, Yif1B deficiency changes the receptor activity. This could be directly demonstrated for the 5-HT_{1A} autoreceptor by extracellular recordings, but further experiments need to be performed in the CA1 region of the hippocampus[39] and in layers II/III or V of the PFC using whole-cell patch-clamp recordings.[40,41] Furthermore, to correlate 5-HT_{1A}R mislocalization and changes in its functionality, especially in the PFC where 5-HT_{1A}R has been reported to be expressed in the axon hillock and modulate neuronal activity,[42] immunostaining of the 5-HT_{1A}R will need to be performed.[43,44] In the present study, we showed that Yif1B exerts a regulatory control on serotonergic neurotransmission and is necessary for SSRI antidepressant action. It remains unknown whether Yif1B downregulation in pathological conditions known as the hyposerotonergic state results from the pathology in humans or is causative (Appendix 1, Fig. S3). Our studies clearly showed that constitutive deletion of Yif1B in mice led to social anxiety, but this state could be an indirect consequence of 5-HT_{1A}R hypofunction and/or 5-HT innervation alteration, because serotonergic neurotransmission during the critical period of development is involved in the anxiety feature in adults.[45,46] In Yif1B-KO mice, the decrease of serotonergic innervation could be a

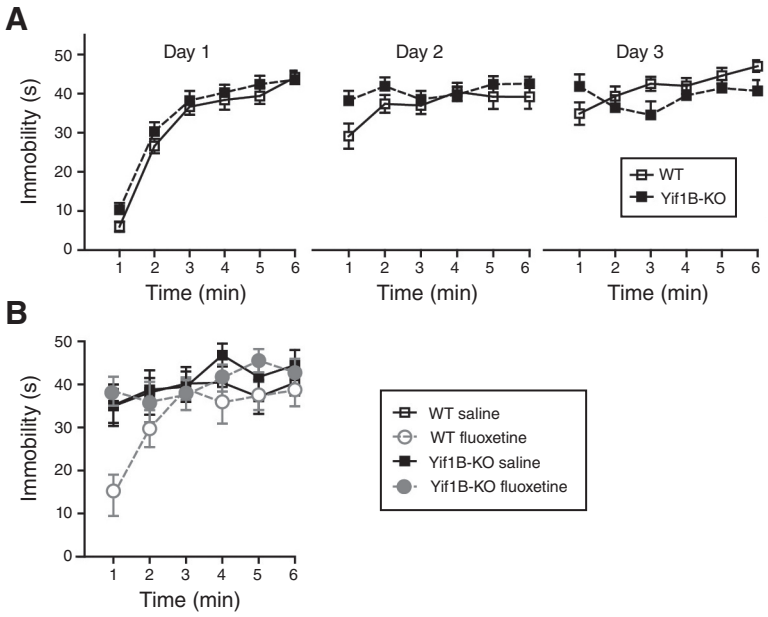

Fig. 5: Behavioural immobility of wild-type (WT) and Yif1B knockout (Yif1B-KO) mice in the repeated forced swim test. (A) Male mice (WT, $n = 17$; Yif1B KO, $n = 22$) were sensitized by immersion in water over 3 consecutive days during 6-minute sessions to induce an increase in resignation. Three-way analysis of variance revealed no effect of genotype ($F = 0.243$, $p = 0.62$) and no interaction of day × period × genotype ($F = 1.093$, $p = 0.36$). (B) Response to acute injection of fluoxetine (20 mg/kg intraperitoneally, 30 minutes before the test) on the fourth day differed markedly in Yif1B-KO mice compared with WT mice (WT saline, $n = 9$; Yif1B-KO saline, $n = 9$; WT fluoxetine, $n = 8$, Yif1B-KO fluoxetine, $n = 13$). Three-way analysis of variance revealed a significant effect on the interaction of period × genotype × treatment ($F = 2.8072$, $p = 0.042$). Two-way analysis of variance and post hoc tests on each period showed that response to fluoxetine was significantly different between genotypes during the first minute of the test (multiple comparisons with WT mice injected with saline, $p = 0.002$ for fluoxetine in WT mice, $p = 0.97$ for saline in Yif1B-KO mice and $p = 0.54$ for fluoxetine in Yif1B-KO mice). Graphs represent mean ± standard error of the mean (SEM); $^{**}p < 0.01$. MDD = major depressive disorder; ROD = relative optical density; SSRI = selective serotonin reuptake inhibitor.

consequence of axonal growth alteration according to a mechanism independent of the 5-HT_{1A}R. Indeed, Yif1B that belongs to the rabGTPases-interacting protein family[47] influences the anterograde traffic even in cells that do not express the 5-HT_{1A}R,[15] and rabGTPases have been proposed to play a key role in neurite outgrowth.[48]

Because chronic SSRI treatment is effective in patients with social anxiety disorder,[49] further studies are needed to investigate the effect of such treatment on the levels of Yif1B concomitantly with an anxious phenotype in WT mice subjected to social stress. This may provide essential data on the mechanisms that lead to the efficacy of fluoxetine in social anxiety disorder.

Finally, it is important to remember that transcriptional modulation of the 5-HT_{1A}R may be a novel therapeutic approach to treating depression.[50] Increasing the targeting of the 5-HT_{1A}R at its functional sites could be another way of increasing its functionality. In this context, the development of selective medications that could increase Yif1B levels might well be of therapeutic relevance.

Limitations

This study had several limitations: psychiatric diagnoses in the human participants were determined by a retrospective assessment of informant-based interviews; the treatment length and psychotropic medications administered to the human participants with depression varied; the number of human participants in each cohort was limited; fluoxetine was administered to monkeys and mice without the induction of stress as a model of human depression; the sex difference in Yif1B level observed in human tissue in depression and in monkey tissue after treatment with fluoxetine was not replicated in male versus female mice treated with fluoxetine.

Conclusion

Compared with WT mice, Yif1B-KO mice showed a significant decrease in the forebrain density of 5-HT projection fibres, a decrease in the number of spines in terminal dendritic segment from pyramidal neurons of CA1, and a hypofunctionality of 5-HT_{1A} autoreceptors expressed on raphe 5-HT neurons. Locomotion, assessment of anxiety-related behaviours and the duration of immobility in the forced swim test were not changed in Yif1B-KO mice, but the social interaction test was significantly affected in these animals. We found a significant effect of sex: women with depression had decreased levels of Yif1B protein compared to men with depression, and female monkeys treated with fluoxetine exhibited a significant increase in Yif1B protein levels compared to male monkeys. Despite these sex-related effects in human and monkey brain tissue, we found no sex-related difference in the Yif1B mRNA levels of mice treated with fluoxetine. Thus, Yif1B plays a critical role in 5-HT_{1A} receptor function, and brain 5-HT homeostasis and fluoxetine treatment may normalize the deficit in Yif1B protein levels in women with depression. Development of selective medications that could increase Yif1B levels might be of therapeutic relevance for treating mood disorders.

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