Characterization of 5-HT$_{1A/1B}^-$ mice: An animal model sensitive to anxiolytic treatments

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**A B S T R A C T**

Selective serotonin (5-HT) re-uptake inhibitors (SSRIs) are commonly used in the treatment of generalized anxiety disorder in Humans. However, because only few animal models display overt anxious-like behavior, detailed preclinical studies of the anxiolytic properties of antidepressants are still lacking. Here, we studied the neurochemical and behavioral effects of a double 5-HT$_{1A/1B}$ receptor knockout in mice (5-HT$_{1A/1B}^-$) as compared to their wild-type littermates (5-HT$_{1A/1B}^+$/+). It is known that single deletion of either 5-HT$_{1A}$ or 5-HT$_{1B}$ receptor induces behavioral changes that are not correlated with differences in brain serotonergic tone. Deletion of both receptors resulted in (i) higher emotionality of animals, as observed in three unconditioned paradigms of anxiety (open field, elevated plus maze and novelty suppressed feeding tests); (ii) a $200\%$ increase in the mean spontaneous firing rate of 5-HT neurons in the dorsal raphe nucleus (DRN) compared to 5-HT$_{1A/1B}^+$/+ mice; (iii) elevated basal dialysate levels of 5-HT in the DRN and frontal cortex; (iv) an exaggerated response to acute paroxetine administration in microdialysis experiments, and (v) increased basal core body temperature. These findings suggest that the deletion of both autoreceptors induces a strong anxious-like behavioral state associated with increased 5-HT neurotransmission. Interestingly, 5-HT$_{1A/1B}^-$ mice are still sensitive to the acute administration of diazepam. Moreover, while deletion of both receptors impacted on the response to acute SSRI treatment in the forced swim test, anxiolytic-like effects of a chronic SSRI treatment were still observed in 5-HT$_{1A/1B}^-$ mice. Thus, the 5-HT$_{1A/1B}^-$ mouse model could be of great interest to unveil the mechanisms of action of the anxiolytic effects of SSRIs.

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1. Introduction

General anxiety disorders are best treated with benzodiazepines, but these drugs induce several side effects such as dependency after chronic administration (Lader, 1994). Serotonin-selective reuptake inhibitors (SSRIs) antidepressants, as well as the serotonin–noradrenaline reuptake inhibitor venlafaxine, are also effective in the treatment of general anxiety disorders (Ball et al., 2005; Bielski et al., 2005; Rickels et al., 2003; Stein et al., 2005). However, compared to benzodiazepines, the decrease in side effects of SSRI is counterbalanced by their slower onset of therapeutic action (Klein et al., 1995; Rickels et al., 2003).

The anxiolytic properties of SSRIs are difficult to demonstrate in preclinical studies, because current animal models of anxiety are poorly predictive (Borsini et al., 2002). The generation of knockout (KO) mice has however showed promise in this regard. For example, 5-HT$_{1A}$ receptor KO mice (5-HT$_{1A}^-$) display an anxious phenotype (Heisler et al., 1998; Parks et al., 1998; Ramboz et al., 1998), associated with decreased exploratory activity, increased fear of aversive environments (Klemenhagen et al., 2006) and other aspects of anxiety, such as autonomic activation, increased stress responsiveness, and neuroendocrine abnormalities (see Toth, 2003 for a review). Moreover, increased 5-HT turnover (Ase et al., 2000) and increased 5-HT neuron firing (Richer et al., 2002) have been reported in 5-HT$_{1A}^-$ mice, even though not associated with increased basal extracellular 5-HT levels (5-HTtext), as measured by microdialysis in the striatum (He et al., 2001; Knobelman et al., 2001b), dorsal raphe nucleus (DRN) and frontal cortex (Bortolozzi et al., 2004; Guilloux et al., 2006). Moreover, acute
treatment with an SSRI, which increases [5-HT]ext levels in the DRN and frontal cortex of both 5-HT1A+/− and 5 HT1A+/+ mice (Guilloux et al., 2006), has a greater effect on the forced swim test in the 5-HT1A KO mice, whereas chronic SSRI treatment does not seem to affect the anxiety phenotype of these mice (Santarelli et al., 2003).

5-HT1B−/− mice do not display overt anxiety (Sari, 2004), but enhanced aggressiveness (Bruner and Hen, 1997; Ramboz et al., 1996; Sari, 2004; Saudou et al., 1994), an abnormality which correlates with brain 5-HT dysfunction (Siever, 2008). Even though axon terminal release of 5-HT is under the control of 5-HT1B autoreceptors (Chaput et al., 1986), changes in 5-HT dynamics are not prominent in the brain of 5-HT1B−/− mice (Ase et al., 2000, 2001). Nor do these mice show any significant increase in 5-HT neuron firing (Evard et al., 1999). Accordingly, their basal [5-HT]ext levels appear to be unaffected in 5-HT terminal areas (De Groot et al., 2002b; Gardier et al., 2003; Knobelman et al., 2001b; Malagie et al., 2001; Trillat et al., 1997). However, by comparison to wild-type mice, acute SSRI treatment produces a greater [5-HT]ext increase in the hippocampus and frontal cortex of 5-HT1B−/− mice (De Groot et al., 2002a,b; Malagie et al., 2001, 2002), even if this treatment has no effect in the forced swim test (Gardier et al., 2001).

Very few studies have been aimed at characterizing the effects of a disruption of both 5-HT1A and 5-HT1B receptors. 5-HT1A/1B−/− mice have been generated previously to evaluate the binding ability of the 5-HT1A receptor agonist, 8-OH-DPAT, to 5-HT1 receptors (Bonaventure et al., 2002) and the impact of this double deletion on sleep-wakefulness rhythms (Alexandre et al., 2004). However, a behavioral, neurochemical and electrophysiological characterization of these mice is still lacking.

In this context, we hypothesized that a mouse lacking both 5-HT1A and 5-HT1B receptors might represent an interesting animal model to test for anxiolytic-like activity of SSRIs. We thus generated double KO mice (5-HT1A1B−/−) measured the effects of the double mutation on [5-HT]ext in the DRN and frontal cortex of these mice, and correlated such measurements with behavioral responsiveness to either acute or chronic SSRI antidepressant treatment. Our results provide the first demonstration that 5-HT1A1B−/− mice display an anxiety-like phenotype associated with an increased basal firing rate of DRN 5-HT neurons with increased basal [5-HT]ext in both the DRN and frontal cortex, as well as a reversion of this anxious phenotype by chronic SSRI treatment.

2. Methods

2.1. Animals

5-HT1A receptor KO mice (5-HT1A−/−) generated by Parks et al. (Parks et al., 1998) on the C57Bl/6 background were crossed with homozygous 5-HT1B receptor KO mice (5-HT1B−/−) generated by Saudou et al. (1994) on the 129Sv background. These F1 heterozygous 5-HT1A+/− and 5-HT1B+/− mice were then bred to generate double 5-HT1A1B−/− mice F2 and their wild-type littermates (5-HT1A1B+/+ control mice). All the animals were genotyped by PCR (Supplementary Materials and Supplementary Fig. 1). Male animals F3 (6–8 weeks old) weighing 25–30 g were used. Procedures involving animals and their care were conducted in conformity with the institutional guidelines, in compliance with international laws and policies (Council directive # 87-848, October 19, 1987, Ministère de l’Agriculture et de la Forêt, Service Vétérinaire de la Santé et de la Protection Animale, permissions # 92-196 to AM.G).

2.2. Chemicals and drugs

Paroxetine hydrochloride, a gift from GlaxoSmithKline laboratory (Harlow, United Kingdom), was dissolved in NaCl 0.9% for acute administration (4 or 8 mg/kg intraperitoneally, i.p.) or in tap water for chronic administration (16 mg/kg/day for 4 weeks). Despite its relative lower selectivity for serotonin reuptake transporter, paroxetine displays greater affinity (Owens et al., 2001) and potency for this transporter than other SSRIs (Mochizuki et al., 2002). Paroxetine doses were chosen according to the literature showing that low doses are sufficient to induce acute effects in the elevated plus maze or in the forced swim test (David et al., 2003b; Koks et al., 1999), whereas higher doses need to be used for chronic treatment (Elizalde et al., 2008). Diazepam (1 mg/kg, i.p.: Sigma–Aldrich, Saint Quentin Fallavier, France) was dissolved in TWEEN 20%. Control animals were injected with the corresponding vehicle.

2.3. Behavior of 5-HT1A/1B−/− mice

2.3.1. The open field paradigm

Anxiety and locomotor activity of mice were measured using the open field test (David et al., 2009). Each animal was placed in a 43 × 43 cm open field chamber, and tested for 30 min. Mice were monitored throughout each test session by an infrared tracking system (Med Associates, USA: Rocha et al., 1998). Dependent measures in the center were the total time and the number of entries over a 30-min period of test. The overall motor activity was quantified as the total distance travelled.

2.3.2. The elevated plus maze test

Each mouse was allowed to explore the apparatus for 5 min. Global activity was assessed by measuring the number of entries into the four arms (David et al., 2009). Anxiety was assessed by comparing the activity in the open versus enclosed arms.

2.3.3. The novelty suppressed feeding test

The novelty suppressed feeding paradigm (NSF) is a conflict test that elicits competing motivations between the drive to eat and the fear of venturing into the center of brightly lit arena. Latency to begin eating is used as an index of anxiety-like behavior, because classical anxiolytic drugs decrease this measure as well as chronic SSRI treatment (David et al., 2009). Mice were food-deprived for 24 h prior to the test. Testing was performed in a 50 × 50 cm box covered with bedding, and illuminated by a 70-Watt lamp. The latency to eat pellets of food placed in the center of the box, on the top of a piece of white filter paper, was timed. Each mouse was tested individually for 5 min.

2.3.4. The mouse forced swim test

In the forced swim test, mice were dropped individually into glass cylinders (height: 25 cm, diameter: 10 cm) containing 10 cm water height, maintained at 23–25 °C. Animals were tested for a total of 6 min. For acute administration of treatments, vehicle or paroxetine (8 mg/kg) were administered 30 min before the test. Two mice were tested simultaneously, and the time of mobility was recorded during the last 4 min of the 6-min testing period, after 2 min of habituation. The same well-trained experimenter, who was unaware of the treatment administered, performed the test.

2.4. Basal temperature measurement and stress induced hyperthermia (SIH)

Stress exposure induce several autonomic responses, including hyperthermia. The SIH test is based on this phenomenon and consists of a simple experimental procedure, in which the body temperature of mouse is measured twice via a rectal procedure, with an interval of 10 min. The procedure of the rectal temperature measurement (handling, insertion of the probe) is the actual stressor in this test (Olivier et al., 2003; Van der Heyden et al., 1997). After dipping in oil silicon, 2 cm of a thermistor probe was inserted in the rectum of mice to monitor body temperature using a BIO-T9882 thermometer (BIOSB, Vitrolles, France: accuracy of ±0.1 °C) (Trillat et al., 1998b). Experiments were performed at 10:00 AM. Digital recordings of basal rectal temperature (T0) were obtained, and then body temperature was again measured 10 min (T10) after the first measurement (that serves as a stressor). The stress-induced hyperthermia was calculated as the difference between these two temperatures (ΔT = T10 – T0).

2.5. Microdialysis procedure

Anaesthetized mice were implanted with probes located in the frontal cortex ( stereotaxic coordinates in mm from bregma: A: −1.6, L: −1.3, V: −1.6) or in the DRN (A: −4.5, L: 0, V: −4.0; A, anterior; L, lateral; and V, ventral). Animals were allowed to recover from the surgery overnight. The next day, 20 h after surgery, the probes were continuously perfused with an artificial cerebrospinal fluid (flow rate = 1.5 μl/min in cortex, 0.5 μl/min in DRN) while animals were awake, freely moving in their cage. Dialysate samples were analyzed for [5-HIt]ext by a HPLC apparatus (limit of sensitivity = 0.5 fmol/sample; signal-to-noise ratio −2). Four fractions were collected to obtain basal values (means ± S.E.M.) before drug administration (Guird et al., 2007; Guilloux et al., 2006). At the end of the experiments, localization of microdialysis probes was verified histologically (Bert et al., 2004).

2.6. In vivo electrophysiological recordings

Anaesthetized mice were placed into a stereotaxic frame. The extracellular recordings of the 5-HT neurons in the dorsal raphe were carried out as previously described (Guiard et al., 2008). The single-barreled glass micropipettes were positioned using the following coordinates (in mm from lambda): AP, −0.5 to 1; L, 0 ± 0.1; V, 2.5 to 3.5. Presumed 5-HT neurons were identified by their characteristic slow, regular firing rate (0.5–2.5 Hz) and long duration action potential (2–5 ms).
Electrophysiological data were expressed as mean ± S.E.M of the firing rate. The number of spontaneously active 5-HT neurons was also determined in each experimental group and data were expressed as mean ± S.E.M of neurons recorded per tract.

2.7. RNA isolation and SERT expression

Coronal brain slices were performed using a Brain matrix, and dorsal raphe nuclei were separated under the microscope from the rest of the tissue and directly put in TRIzol (Invitrogen<sup>e</sup>, USA). Total RNA from raphe nuclei was extracted from brain sections in TRIzol and assayed by chromatography (Agilent Bioanalyzer, Santa Clara, USA). Samples with an expert scoring number (RIN) above 7.2 were considered of good quality. 2 μg of RNA were then reverse-transcribed with Anchored Oligo(dT)<sub>b</sub> primers (Deltheil et al., 2008). Small PCR products (80–200 base-pairs) PCR products were amplified in triplicates using a LightCycler<sup>®</sup> Carousel-Based System (Roche, Mannheim, Germany) and SYBR Green PCR Master Mix Reagent (LightCycler<sup>®</sup> RNA Amplification Kit SYBR Green, Roche). Primer-dimers were assessed by amplifying primers without cDNA. Primers were retained if they produced no primer-dimers or non-specific signal after 35 cycles. Results were calculated as relative intensity compared to β-actin, and the 2<sup>-ΔΔCT</sup> method with the tested primers was used to calculate the relative expression levels of the transcripts (Schmittgen and Livak, 2008).

2.8. Data analysis and statistics

For all experiments, statistical data analysis (StatView 5.0 Abacus Concepts, Berkeley, CA, U.S.A) used means ± S.E.M. Student t-tests, one- or two-way ANOVA (on repeated measures for body temperature) were used followed by Fisher Protected Least Significance Difference post hoc test. Significant level was set at p < 0.05.

3. Results

3.1. Behavioral phenotyping of 5-HT<sub>1A/1B</sub>+/− and 5-HT<sub>1A/1B</sub>−/− mice and their response to chronic paroxetine treatment

3.1.1. Open field test

Two-way ANOVA (treatment × genotype) on the number of entries in the center zone and the time spent in the center revealed significant main effects of genotype factor [F(1,47) = 5.96; p < 0.05], treatment factor [F(1,47) = 9.38; p < 0.01] and treatment × genotype interaction between these two factors [F(1,47) = 5.38; p < 0.05] (Fig. 1A). Two-way ANOVA (treatment × genotype) on the time spent in the center revealed significant main effects of genotype factor [F(1,47) = 25.21; p < 0.001], no significant effect of treatment factor [F(1,47) = 0.064; p = 0.80] and no treatment × genotype interaction [F(1,47) = 0.65; p = 0.42] (Fig. 1B). Thus, 5-HT<sub>1A/1B</sub>−/− mice displayed a basal anxious-like phenotype characterized by a decrease in the number of entries and the time spent in the center compared to control mice (p < 0.001). Chronic treatment with paroxetine reversed this phenotype in part: it increased the number of entries in 5-HT<sub>1A/1B</sub>−/− mice (p < 0.01) and flattened the baseline difference observed between mutant and control mice (p < 0.05). This treatment induced a 4-fold increase in the time spent in the center in 5-HT<sub>1A/1B</sub>−/− mice but this effect failed to reach statistical significance.

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Fig. 1. Effects of chronic paroxetine on 5-HT<sub>1A/1B</sub>−/− and 5-HT<sub>1A/1B</sub>+/− mice behavior in the open field (A, B, C) and in the elevated plus maze (D, E, F). Results are expressed as means ± S.E.M of parameters measured in 5-HT<sub>1A/1B</sub>+/− (empty bars) and 5-HT<sub>1A/1B</sub>−/− (full bars): Left panel: Open field: number of entries in the center (Fig. 1A), time spent in the center (in seconds, Fig. 1B), and total locomotor activity (Fig. 1C); Right panel: Elevated plus maze: time spent in the open arms (in seconds, Fig. 1D), number of entries in the open arms (Fig. 1E), and time spent in the closed arms (in seconds, Fig. 1F). ** p < 0.01 significantly different from corresponding vehicle group; $$$ p < 0.001. $$$ p < 0.001 significantly different from 5-HT<sub>1A/1B</sub>+/− mice. Two-way ANOVA followed by a PLSD post hoc test, n = 11–14 animals per genotype and per treatment.
Two-way ANOVA (treatment × genotype) on the total ambulatory distance revealed significant main effects of genotype factor \(F(1,47) = 48.08; \ p < 0.001\), no significant main effect of treatment factor \(F(1,47) = 0.07; \ p = 0.79\) and no treatment × genotype interaction \(F(1,47) = 0.06; \ p = 0.80\) (Fig. 1C). Thus, \(5\text{-HT}_{1A/1B}^-/-\) mice displayed a 3-fold decrease in basal locomotor activity compared to controls \(p < 0.001\). This decrease in locomotor activity seemed to be driven by the anxious phenotype, since there was no effect of genotype on the home-cage activity during 24 h (Supplementary Fig. S2 and Supplementary Materials).

### 3.1.2. Elevated plus maze test (EPM)

Two-way ANOVA (treatment × genotype) on the number of entries in the open arms and the time spent in the open arms revealed significant main effects of genotype factor [entries: \(F(1,46) = 11.81; \ p < 0.01\) time: \(F(1,46) = 17.45; \ p < 0.001\)], not of treatment factor [entries: \(F(1,46) = 2.19; \ p = 0.15\) time: \(F(1,46) = 0.39; \ p = 0.53\), and no treatment × genotype interaction [entries: \(F(1,46) = 0.24; \ p = 0.63\) time: \(F(1,46) = 0.71; \ p = 0.40\)] (Fig. 1D and E). Thus, \(5\text{-HT}_{1A/1B}^-/-\) mice displayed an anxious-like phenotype manifested by a decrease in the open arms entries \((p < 0.01)\) and time spent in the open arms \((p < 0.01)\) as compared to controls. No statistical differences between \(5\text{-HT}_{1A/1B}^+/+\) and mutant mice were then observed after chronic paroxetine administration (entries: \(p = 0.05\) time: \(p = 0.08\)).

Two-way ANOVA (treatment × genotype) on the time spent in the closed arms revealed significant main effects of genotype factor \([F(1,46) = 21.74; \ p < 0.001]\), not of treatment factor \([F(1,46) = 1.47; \ p = 0.23]\), and no treatment × genotype interaction \([F(1,46) = 1.18; \ p = 0.28]\) (Fig. 1F). The increase in time spent in the closed arms by untreated \(5\text{-HT}_{1A/1B}^-/-\) mice compared to their controls \((p < 0.001)\) disappeared after chronic paroxetine treatment \((p = 0.05)\).

### 3.1.3. Novelty suppressed feeding test (NSF)

Two-way ANOVA (treatment × genotype) on latency values revealed significant main effects of genotype factor \([F(1,46) = 8.15; \ p < 0.01]\), treatment factor \([F(1,46) = 15.45; \ p < 0.001]\), but no treatment × genotype interaction \([F(1,46) = 0.12; \ p = 0.73]\) (Fig. 2A). Untreated \(5\text{-HT}_{1A/1B}^-/-\) mice displayed a majored increase in the latency to feed compared to \(5\text{-HT}_{1A/1B}^+/+\) mice \((p < 0.05)\). Thus, the anxious phenotype of \(5\text{-HT}_{1A/1B}^-/-\) mice observed in the open field experiment was confirmed in this paradigm. The chronic paroxetine treatment decreased the latency to feed in both \(5\text{-HT}_{1A/1B}^+/+\) and \(5\text{-HT}_{1A/1B}^-/-\) mice \((p < 0.05\) for both strains), but a difference between genotypes still occurred following chronic paroxetine treatment \((p < 0.05)\).

Neither treatment nor genotype had an effect on food intake measured after the test [genotype \([F(1,46) = 2.62; \ p = 0.12]\), treatment \([F(1,46) = 2.34; \ p = 0.13]\) treatment × genotype interaction \([F(1,46) = 0.07; \ p = 0.79]\)] (Fig. 2B).

### 3.2. \(5\text{-HT}_{1A/1B}^-/-\) mice did not respond to acute or chronic paroxetine administration in the mouse forced swim test (FST)

In wild type mice, both acute or chronic paroxetine administration increased mobility time in the FST, as already described (David et al., 2003b). The response to these treatments was different in \(5\text{-HT}_{1A/1B}^-/-\) mice.

### 3.2.1. Acute administration (Fig. 3A)

Two-way ANOVA (treatment × genotype) on the mobility time revealed significant effects of genotype factor \([F(1,61) = 0.22; \ p = 0.64]\) and treatment factor \([F(1,61) = 1.15; \ p = 0.29]\), but an interaction between these two factors \([F(1,61) = 27.14; \ p < 0.001]\), \(5\text{-HT}_{1A/1B}^-/-\) displayed a change in basal activity i.e., mobility time was increased compared to WT littermates \((p < 0.01)\). Acute paroxetine administration \((8 \text{ mg/kg})\) increased the mobility time of \(5\text{-HT}_{1A/1B}^+/+\) \((p < 0.05)\), while it decreased it in \(5\text{-HT}_{1A/1B}^-/-\) mice \((p < 0.001)\).

### 3.2.2. Chronic administration (Fig. 3B)

Two-way ANOVA (treatment × genotype) on the mobility time revealed significant main effects of genotype factor \([F(1,46) = 6.88; \ p < 0.05]\), not of treatment factor \([F(1,46) = 0.56; \ p = 0.45]\), and an interaction between these two factors \([F(1,46) = 12.28; \ p < 0.001]\). Chronic paroxetine administration increased the mobility time in \(5\text{-HT}_{1A/1B}^+/+\) \((p < 0.01)\) but not \(-/-\) mice \((p = 0.12)\). In this second experimental cohort, \(5\text{-HT}_{1A/1B}^-/-\) mice also displayed an increase in basal activity compared to WT littermates \((p < 0.05)\). After 4-weeks of paroxetine administration, there was no difference between genotypes as mobility duration was increased in the \(5\text{-HT}_{1A/1B}^+/+\) group, without any change in the treated mutants.

### 3.3. \(5\text{-HT}_{1A/1B}^-/-\) mice were sensitive to acute administration of diazepam or paroxetine in the elevated plus maze test

Two-way ANOVA (treatment × genotype) on open arm entries revealed significant main effects of genotype factor \([F(1,68) = 19.62; \ p < 0.001]\).
5-HT 

Effects in 5-HT 
basal behavior than their controls (Fig. 3A: 5-HT IA/1B --/+ (empty bars) and 5-HT IA/1B ---/-- mice (full bars) received either the vehicle or acute paroxetine (8 mg/kg, i.p.) 30 min before the test (n = 13–19 animals per genotype and per treatment). Fig. 3B: 5-HT IA/1B +/+ (empty bars) and 5-HT IA/1B ---/-- mice (full bars) received either the vehicle or chronic paroxetine (16 mg/kg/day during 28 days) (n = 12–13 animals per genotype and per treatment). * p < 0.05; ** p < 0.01; *** p < 0.001 significantly different from the genotype-matched vehicle group; \( \Delta \) p < 0.05; \( \Delta \) p < 0.01; \( \Delta \) p < 0.001 significantly different from 5-HT IA/1B +/+ mice. Two-way ANOVA followed by PLSD post hoc test.

Effects of the acute administration of diazepam or paroxetine on 5-HT IA/1B +/+ and 5-HT IA/1B ---/-- mice behavior in the elevated plus maze test. Results are expressed as means \( \pm \) SEM of number of entries in the open arms (Fig. 4A), time spent in the open arms (in seconds, Fig. 4B), and time spent in the closed arms (in seconds, Fig. 4C). 5-HT IA/1B +/+ (empty bars) and 5-HT IA/1B ---/-- mice (full bars) received either the vehicle or diazepam (1 mg/kg; i.p.) (n = 10–15 mice per group). * p < 0.05; ** p < 0.01; *** p < 0.001 significantly different from the corresponding vehicle group; \( \Delta \) p < 0.05; \( \Delta \) p < 0.01; \( \Delta \) p < 0.001 significantly different from 5-HT IA/1B +/+ mice. Two-way ANOVA followed by a PLSD post hoc test, n = 11–15 animals per genotype and per treatment.

Two-way ANOVA (treatment \( \times \) genotype) on time spent in the open arms revealed significant main effects of genotype \( F(1,67) = 6.56; p < 0.05 \) and treatment \( F(2,67) = 10.0; p < 0.001 \), but no treatment \( \times \) genotype interaction \( F(2,67) = 2.44; p = 0.09 \) (Fig. 4B). Thus, 5-HT IA/1B ---/-- mice displayed more anxious-like basal behavior than their controls (p < 0.01). Paroxetine had no effects in 5-HT IA/1B ---/-- mice (p = 0.56), but a significant anxiogenic-like effect in 5-HT IA/1B +/+ mice (p < 0.05). By contrast, diazepam increased the time spent in open arms in both 5-HT IA/1B +/+ (p < 0.01) and 5-HT IA/1B ---/-- mice (p < 0.05).

Two-way ANOVA (treatment \( \times \) genotype) on time spent in the closed arms revealed significant main effects of genotype factor \( F(1,68) = 14.61; p < 0.001 \), treatment factor \( F(2,68) = 11.77; p < 0.001 \) and no treatment \( \times \) genotype interaction \( F(2,68) = 1.88; p = 0.16 \) (Fig. 4C). 5-HT IA/1B ---/-- mice spent more time in the closed arms than 5-HT IA/1B +/+ mice (p < 0.001). Paroxetine had no effect in both 5-HT IA/1B +/+ and 5-HT IA/1B ---/-- mice (p = 0.10 and p = 0.56, respectively). Diazepam decreased the time spent in the closed arms in both 5-HT IA/1B +/+ (p < 0.05) and 5-HT IA/1B ---/-- mice (p < 0.001).

3.4. Behavioral consequences of stress induced hyperthermia in 5-HT IA/1B +/+ and ---/-- mice

Two-way ANOVA on repeated measurements showed no effects of genotype \( F(1,40) = 0.62, p = 0.44 \), but an effect of stress

Fig. 3. Effects of acute (A) and chronic paroxetine (B) on the behavior of 5-HT IA/1B --/+ and --/-- mice in the forced swimming test. Results are expressed as means \( \pm \) SEM of the mobility time (in seconds). Fig. 3A: 5-HT IA/1B +/+ (empty bars) and 5-HT IA/1B ---/-- mice (full bars) received either the vehicle or acute paroxetine (8 mg/kg, i.p.) 30 min before the test (n = 13–19 animals per genotype and per treatment). Fig. 3B: 5-HT IA/1B +/+ (empty bars) and 5-HT IA/1B ---/-- mice (full bars) received either the vehicle or chronic paroxetine (16 mg/kg/day during 28 days) (n = 12–13 animals per genotype and per treatment). * p < 0.05; ** p < 0.01; *** p < 0.001 significantly different from the genotype-matched vehicle group; \( \Delta \) p < 0.05; \( \Delta \) p < 0.01; \( \Delta \) p < 0.001 significantly different from 5-HT IA/1B +/+ mice. Two-way ANOVA followed by PLSD post hoc test.

Fig. 4. Effects of the acute administration of diazepam or paroxetine on 5-HT IA/1B +/+ and 5-HT IA/1B ---/-- mice behavior in the elevated plus maze test. Results are expressed as means \( \pm \) SEM of number of entries in the open arms (Fig. 4A), time spent in the open arms (in seconds, Fig. 4B), and time spent in the closed arms (in seconds, Fig. 4C). 5-HT IA/1B +/+ (empty bars) and 5-HT IA/1B ---/-- mice (full bars) received either the vehicle or diazepam (1 mg/kg; i.p.) (n = 10–15 mice per group). * p < 0.05; ** p < 0.01; *** p < 0.001 significantly different from the corresponding vehicle group; \( \Delta \) p < 0.05; \( \Delta \) p < 0.01; \( \Delta \) p < 0.001 significantly different from 5-HT IA/1B +/+ mice. Two-way ANOVA followed by a PLSD post hoc test, n = 11–15 animals per genotype and per treatment.
and DRN in 5-HT 3.5. Basal extracellular levels of 5-HT in the frontal cortex and DRN in 5-HT 1A/1B+/- mice displayed an elevated basal body temperature compared to 5-HT 1A/1B+/- mice (p < 0.01). This higher body temperature was confirmed in another set of experiments using another cohort of animals and performed at 2PM (data not shown). Ten minutes later (T10), analysis of ΔT demonstrated that 5-HT 1A/1B+/-, but not 5-HT 1A/1B-/- mice (p = 0.48), had an elevated body temperature (+1.1 °C), generated by the stress of inserting the rectal probe at T0 (p < 0.001).

3.5. Basal extracellular levels of 5-HT in the frontal cortex and DRN in 5-HT 1A/1B+/- and 5-HT 1A/1B-/- mice

Table 1 shows the means ± S.E.M. of basal [5-HT]ext levels in the frontal cortex (in fmol/20 μl) and DRN (in fmol/10 μl) of the various groups of mice studied. Basal [5-HT]ext were significantly increased in both the frontal cortex [F(1,27) = 14.46, p < 0.001] and DRN [F(1,40) = 8.54, p < 0.01] in 5-HT 1A/1B-/- mice compared to controls.

3.6. Deletion of 5-HT 1A and 5-HT 1B receptors potentiated the increase in 5-HT outflows induced by paroxetine administration

In the frontal cortex and DRN (Fig. 6A–D), the lack of both 5-HT 1A and 5-HT 1B autoreceptors potentiated the effects of paroxetine on 5-HT 1A/1B: two-way ANOVA (treatment × genotype) on AUC values revealed significant main effects of genotype factor [frontal cortex: F(1,25) = 78.84; p < 0.001. DRN: F(1,22) = 9.04; p < 0.01], treatment factor [frontal cortex: F(1,25) = 151.68; p < 0.001. DRN: F(1,22) = 22.53; p < 0.001] and treatment × genotype interaction [frontal cortex: F(1,25) = 76.60; p < 0.001. DRN: F(1,22) = 9.82; p < 0.01]. The acute administration of paroxetine (4 mg/kg, i.p.) increased [5-HT]ext in both regions in 5-HT 1A/1B+/- (frontal cortex: p < 0.05; DRN: p < 0.001) and 5-HT 1A/1B-/- mice (frontal cortex: p < 0.01; DRN: p < 0.001), but this effect was much stronger in 5-HT 1A/1B-/- than in 5-HT 1A/1B+/- mice (frontal cortex: p < 0.001. DRN: p < 0.01). After chronic paroxetine treatment, the paroxetine challenge produced a significant increase in cortical [5-HT]ext in 5-HT 1A/1B+/- mice on basal firing rate in the dorsal raphe nucleus (DRN)

A student t-test on the firing rate of DRN 5-HT neurons indicated that the mean spontaneous firing rate of these neurons was significantly higher in 5-HT 1A/1B-/- mice than in controls (2.4 ± 0.1 Hz vs. 1.2 ± 0.1 Hz, respectively; p < 0.001, Fig. 7A and B). Interestingly, the mean number of DRN 5-HT neurons recorded per track was also significantly higher in 5-HT 1A/1B-/- mice than in controls (Fig. 7C, n = 15 tracks per genotype; p < 0.001).

3.8. Influence of 5-HT 1A/1B deletion in mice on SERT expression in the raphe nuclei

qPCR analysis of SERT expression in the DRN shown a 28% decrease in SERT mRNA levels in 5-HT 1A/1B-/- mice when compared to 5-HT 1A/1B+/- mice (Fig. 8; [F(1,24) = 13.88, p < 0.001).

4. Discussion

This neurochemical and behavioral characterization of a mouse line knock-out for both 5-HT 1A and 5-HT 1B receptor genes complements the few studies performed in similar models (Alexandre et al., 2004; Bonaventure et al., 2002). 5-HT 1A/1B-/- mice display a robust, “hyper-anxious” phenotype associated with (i) increased basal firing rate of DRN 5-HT neurons; (ii) increased basal [5-HT]ext levels in both the DRN and frontal cortex; (iii) increased basal body temperature, and (iv) increased reactivity to stress induced by the FST. This “hyper-anxious” phenotype is corrected in part by either acute diazepam or chronic SSRI administration.

4.1. 5-HT 1A/1B-/- mice basal phenotype

The anxious-like basal phenotype of the double KO mice appears to be driven mainly by the lack of the 5-HT 1A rather than that of 5-HT 1B receptors. Indeed, there is convincing evidence that 5-HT 1A-/- mice may represent a “genetic model of anxiety” (Toth, 2003), while studies performed in 5-HT 1B-/- mice suggest that these exhibit similar or less anxious-like behavior than controls (Brunner et al., 1999; Crabbe et al., 1999; Lopez-Rubalcava et al., 2000; Malleret et al., 1999). Furthermore, 5-HT 1B receptor antagonists display anxiolytic-like properties (Dawson et al., 2006). The hypolocomotion of the double mutants in the open field may well be associated with their anxious phenotype as both anxious-like behavior and hypolocomotion share similar neural pathways or genes (Yilmazer-Hanke, 2008) and co-occur in this test (Holmes et al., 2002). Indeed, 24-hours home cage activity measurement indicated that 5-HT 1A/1B-/- mice display as much locomotion as their wild-type littermates. 5-HT 1A-/- mice also display similar activity compared to their littermates, while 5-HT 1B-/- mice display similar or increased total activity, as observed in the open

![Fig. 5. Behavioral consequences of stress induced hyperthermia in 5-HT 1A/1B+/- and -/- mice. Results are expressed as means ± SEM of rectal temperature in 5-HT 1A/1B+/- (○) and 5-HT 1A/1B-/- mice (●) measured at two different times (T = 0 and T = 10 min). *p < 0.01 significantly different from the TO; **p < 0.01 significantly different from 5-HT 1A/1B+/- mice. Two-way ANOVA followed by a PLSD post hoc test, n = 20–22 animals per genotype.](image-url)
field paradigm (Crabbe et al., 1999; Malleret et al., 1999; Saudou et al., 1994; Scearce-Levie et al., 1999; Zhuang et al., 1999).

This hyper-anxious state was also reflected in the FST, as 5-HT$_{1A/1B}$ mice displayed a robust basal increase in mobility, an effect also observed in single 5-HT$_{1A}$ mice (Guilloux et al., 2006; Parks et al., 1998; Ramboz et al., 1996), but not in 5-HT$_{1B}$ mice (Trillat et al., 1998a). This suggests that the genetic inactivation of 5-HT$_{1A}$ receptors enhanced the response of double mutant mice to stressful conditions, an effect that is not modified by inactivation of 5-HT$_{1B}$ receptors.

Using neurochemical techniques, we tried to link the anxious-like phenotype of 5-HT$_{1A/1B}^{-/-}$ mice to changes in serotonin neurotransmission. We found that the anxiogenic phenotype of 5-HT$_{1A/1B}^{-/-}$ mice correlated with a 40% increase in serotonergic tone in both the frontal cortex and DRN. Comparatively, a single mutation did not change basal frontocortical [5-HT]$_{ext}$, nor in single 5-HT$_{1B}^{-/-}$ mice (De Groote et al., 2002a; Gardier et al., 2003; Malagie et al., 2001).

These results suggest a lack of tonic control exerted by these autoreceptors on basal 5-HT release and that, in the single mutants, compensatory changes in the remaining autoreceptor subtype or other secondary developmental adaptations compensate for the absence of the missing autoreceptor.

Similarly, in pharmacological experiments, the co-perfusion of 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor antagonists in the raphe nuclei of rats has been shown to increase the local efflux of 5-HT, as measured by fast cyclic voltammetry, while no effects were observed when the antagonists were perfused separately (Roberts and Price, 2001), suggesting a synergistic effect of the blockade of both receptors on serotonergic tone. Conversely, it would seem that compensatory changes observed in terminal brain regions of 5-HT neurons in either 5-HT$_{1A}^{-/-}$ mice (Knobelman et al., 2001a; Ramboz et al., 1998) or 5-HT$_{1B}^{-/-}$ mice (Knobelman et al., 2001a; Shippenberg et al., 2000) may not be sufficient to dampen the increase in activity of these neurons.

Our electrophysiological results were consistent with the microdialysis data. Indeed, the mean spontaneous firing rate of

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**Fig. 6.** Effects of acute paroxetine administration on cortical (A, B) or DRN [5-HT]$_{ext}$ levels (C, D) in 5-HT$_{1A/1B}^{+/+}$ and 5-HT$_{1A/1B}^{-/-}$ mice. In A and C, results are expressed as means ± SEM of [5-HT]$_{ext}$ (in fmol per 20 µl) for the frontal cortex and DRN, respectively. 5-HT$_{1A/1B}^{+/+}$ (empty symbols) and 5-HT$_{1A/1B}^{-/-}$ mice (full symbols; n = 6–10 mice per group) were administered either the vehicle (○, ●) or paroxetine (4 mg/kg, i.p.) (■, ■) at arrow. In B and D, data are expressed as area under the curve values (AUC; mean ± SEM; n = 5–8 animals/genotype), i.e., relative amount of 5-HT efflux following the administration of either vehicle or paroxetine in percentage of baseline. The insert in 6A (dotted bars) shows the significant increase in [5-HT]$_{ext}$ measured in the frontal cortex after the administration of an acute dose of paroxetine in 5-HT$_{1A/1B}^{+/+}$ mice chronically treated with paroxetine. p < 0.05 and ***p < 0.001, significantly different from the corresponding vehicle-treated group; ▽▽ p < 0.01 and ▽▽▽ p < 0.001, significantly different from 5-HT$_{1A/1B}^{+/+}$ mice. One-way or two-way ANOVA followed by a PLSD post hoc test.
5-HT neurons was doubled in 5-HT1A/1B−/− mice, with 65% of 5-HT neurons still firing in the range measured in 5-HT1A/1B+/+ mice, as also observed in 5-HT1A−/− mice (Richer et al., 2002). Thus, the deletion of the 5-HT1B receptor did not amplify the consequences of the deletion of the 5-HT1A receptor on the firing rate of DRN 5-HT neurons. The alterations in 5-HT neurotransmission could also be related to changes in the activity or density of the 5-HT reuptake transporter. Indeed, a 28% decrease in SERT mRNA expression was measured in the DRN in 5-HT1A/1B−/− mice, which should decrease 5-HT clearance and might thus be responsible for increased basal dialysate 5-HT levels. By contrast, it is known that SERT distribution is unchanged in the frontal cortex or DRN of 5-HT1A−/− or 5-HT1B−/− mice (Ase et al., 2001). While a repressive effect of high serotonin levels on SERT expression cannot be ruled out, no study has yet observed such an effect. Indeed, chronic SSRIs treatments are known to reduce SERT binding and protein levels without affecting mRNA, but by stimulating microRNA-16 levels (Baudry et al., 2010). Furthermore, no studies have looked at the effects of chronic treatment that increase serotonin levels (without acting directly on the serotonin transporter like monoamine oxidase A inhibitors) on serotonin transporter expression.

These neurochemical variations may account for the elevated basal body temperature found in these 5-HT1A/1B−/− mutant mice. Basal body temperature was not changed in single 5-HT1A−/− mice (Gross et al., 2002), while stress-induced hyperthermia was described in single 5-HT1B−/− mice (Bouwknecht et al., 2001). Thus, surprisingly, the increased basal body temperature found in double mutant mice may result from the deletion of terminal 5-HT1B autoreceptors rather than of somatodendritic 5-HT1A autoreceptors.

The respective role of pre- versus postsynaptic 5-HT1A and 5-HT1B receptors is difficult to fully discriminate. Microdialysis, electrophysiology and body temperature measurements are techniques that mainly assess the presynaptic receptors function/activity, and thus we observed that deletion of both these receptors relieve the inhibition exerted by these autoreceptors on serotonergic neurons. Behavioral analyses reflect roles of both pre- and postsynaptic receptors, thus rendering difficult the discrimination of each. However, as we published previously using genetic and pharmacologic approaches in the FST (Guilloux et al., 2006), postsynaptic 5-HT1B receptors might be important for obtaining an antidepressant response to SSRI administration.

4.2. Acute SSRI treatment increases dialysate [5-HT] levels response, but has no antidepressant-like effect in the FST in 5-HT1A/1B−/− mice

Genetic blockade of 5-HT1A and 5-HT1B receptors resulted in greater SSRI increases of dialysate 5-HT levels in the FCX and the DRN of 5-HT1A/1B−/− compared to 5-HT1A/1B+/+ mice. Thus, in 5-HT1A/1B−/− mice, the 5-HT level response to acute SSRI treatment was similar to that of a chronic treatment in 5-HT1A/1B+/+ mice, which leads to desensitization of both 5-HT1A (Blier et al., 1987; Hervas et al., 2001) and 5-HT1B autoreceptors (Moret and Briley, 1996; Sayer et al., 1999), that is responsible for a greater SSRI-induced increase in dialysate 5-HT levels (insert in Fig. 6A). By
However, the increase in 5-HT dialysate after acute paroxetine dampens the effects of SSRI in the forced swim test (Malagie et al., 2001) or rats (Hervas et al., 2001). Interestingly, however, the increase in 5-HT dialysate after acute paroxetine in 5HT1A/1B−/− mice was not reflected in a greater antidepressant-like effect in the FST (Fig. 3).

Indeed, because 5-HT1A−/− mice display a much greater sensitivity to acute SSRI in the FST (Guilloux et al., 2006), we expected to observe similar effects in 5-HT1A/1B−/− mice. Here, we observed that a paroxetine dose that induced an antidepressant-like effect in 5-HT1A/1B−/−, decreased the mobility time in 5-HT1A/1B−/− mice. Such a phenomenon could be due to the very high elevation of dialysate 5-HT levels in both the FCX and DRN following paroxetine administration (observed in microdialysis experiments), which could be reflected in this behavioral test as a serotoninergic syndrome (i.e., mice are unable to swim during most of the test) (David et al., 2003b). However, at other low doses tested (1 and 4 mg/kg, i.p.), paroxetine had no antidepressant-like effect in 5-HT1A/1B−/− mice (data not shown). Interestingly, paroxetine did not induce a similar effect after chronic administration, thus suggesting that, in the FST, effects obtained after acute or chronic paroxetine administration rely on different mechanisms. However, in keeping with our previous work, and here in 5-HT1A/1B−/− mice after both acute and chronic paroxetine administration, we confirmed that activation of 5-HT1A receptors dampens the effects of SSRI in the forced swim test (Guilloux et al., 2006), while activation of 5-HT1B receptors is necessary to produce an antidepressant-like response in this test (Gardier et al., 2003).

### 4.3. Chronic SSRI treatment reverses the anxious-like phenotype of 5HT1A/1B−/− mice

To increase the validity of the present animal model of anxiety, we confirmed in the elevated plus maze test that 5-HT1A/1B−/− mice respond to the acute administration of diazepam, as reported in other mice with elevated anxiety (Griebel et al., 2000; Olivier et al., 2001). SSRIs can also be effective in relieving anxious states in patients, without inducing the dependence observed with benzodiazepines. Here, chronic (28-days) paroxetine treatment attenuated the basal anxious-like phenotype of 5-HT1A/1B−/− mice in all behavioral tests performed. In 5-HT1A/1B−/+ mice, it only had an effect in the NSF. These findings are consistent with clinical studies reporting beneficial effects of chronic SSRI treatment in anxiety disorders in humans, with only minor or no effects in control subjects (e.g., Stocchi et al., 2003). Similarly, SSRIs are effective in animal models of high anxious-like states, but not in controls or in non-anxious strains of mice (David et al., 2009; Dulawa et al., 2004). The possible mechanisms through which chronic paroxetine treatment has an anxiolytic-like effect could involve serotonin, but also other neurotransmitter systems. Indeed, paroxetine possesses moderate affinity (≤50 nmol/L) for the norepinephrine and dopamine transporters (Owens et al., 2001) and in vivo, paroxetine has been shown to increase cortical extracellular levels of norepinephrine in mice (David et al., 2003a). Thus, the behavioral effects measured in 5-HT1A/1B−/− mice after chronic SSRI treatment could reflect a combination of the action of antidepressants on both the serotoninergic and other monoaminergic systems. Furthermore, the present data confirm the hypothesis that some behavioral effects of chronic SSRI might not require the activation of 5-HT1A receptors (Holick et al., 2008), nor, as shown here, the activation of 5-HT1B receptors. Other molecular and cellular phenomena, such as hippocampal neurogenesis could contribute to the anxiolytic-like effects of SSRIs, even though no study has yet clearly demonstrated the 5-HT1A and/or 5-HT1B receptor dependency of this process.

### 4.4. Concluding remarks

Table 2 summarizes major similarities and differences reported between single (literature) and double KO (here) mice for 5-HT1A

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Comparison between single and double constitutive KO mice for 5-HT1A and/or 5-HT1B receptors.</th>
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<tr>
<td>Electrophysiology on 5-HT neurons in the DRN</td>
<td>5-HT1A/1B−/− (compared to respective controls)</td>
</tr>
<tr>
<td>Basal</td>
<td>70% (Malagie et al., 2002)</td>
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<tr>
<td>Acute SSRI</td>
<td>40% in FCX and DRN</td>
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<tr>
<td>Chronic SSRI</td>
<td>60% in FCX and DRN</td>
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<tr>
<td>Microdialysis</td>
<td>70% in FCX and DRN</td>
</tr>
<tr>
<td>Basal [5-HT]ext</td>
<td>Unchanged in the FCX and DRN (Guilloux et al., 2006)</td>
</tr>
<tr>
<td>Effects of Acute SSRI on [5-HT]ext</td>
<td>FCX and DRN: 70% (Guilloux et al., 2006)</td>
</tr>
<tr>
<td>Effects of Chronic SSRI on [5-HT]ext</td>
<td>Not tested</td>
</tr>
<tr>
<td>Behavior</td>
<td>Anxiety (Heisler et al., 1998; Parks et al., 1998; Rambez et al., 1998)</td>
</tr>
<tr>
<td>Body temperature</td>
<td>Basal</td>
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<td>Unchanged (Groenink et al., 2003)</td>
<td>Unchanged (Groenink et al., 2003)</td>
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and/or 5-HT₁B receptors. The phenotype of 5-HT₁A/₁B⁻/⁻ mice shows many similarities with that of 5-HT₁A⁻/⁻ mice. However, deletion of both receptors did not block the response to antidepressant treatment in several tests of anxious-like behavior. While the present study focused on the impact of the combined deletion of 5-HT₁A and 5-HT₁B receptors on the brain serotoninergic system, other neurotransmitter systems may be affected, especially since 5-HT₁B heteroreceptors are also known to control the release of GABA and glutamate (Maura et al., 1998). The 5-HT₁A/₁B⁻/⁻ mice model nevertheless emphasizes the role of both 5-HT₁A and 5-HT₁B receptors in the physiopathology of anxiety, and reveals that the deletion of these two receptors does not significantly impact on the anxious-like properties of a chronic SSRI treatment.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.neuropharm.2011.02.009.

References


