Implication of 5-HT(2B) receptors in the serotonin syndrome.

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Abstract: The serotonin (5-HT) syndrome occurs in humans after antidepressant overdose or a combination of drugs inducing a massive increase of extracellular 5-HT. Several 5-HT receptors participate in the expression of this syndrome in humans and animal models. Likewise, the 5-HT2B receptor has been proposed as a positive modulator of serotonergic activity, but whether it is involved in the expression of the 5-HT syndrome has not yet been studied. We analyzed thus, the putative role of the 5-HT2B receptor in the development of this disorder by the forced swimming test (FST) and behavioural assessment in the open field. In the FST, the genetic or pharmacological ablation of the 5-HT2B receptor facilitated the selective serotonin reuptake inhibitors (SSRI)-induced expression of symptoms related to the 5-HT syndrome like increase of immobility time, hind limb abduction and Straub tail. Similar responses were developed in the FST by both wild type (WT) and 5-HT2B-/- mice after the administration of 5-HT1A, 5-HT2A and 5-HT2C receptors agonists. In contrast, the 5-HT2B and 5-HT1B receptors agonists decreased immobility time. The SSRIs- induced 5-HT syndrome was blocked in both WT and 5-HT2B-/- mice by administration of 5-HT1A and 5-HT2C receptors antagonists. We then performed a behavioral characterization and confirmed that 5-HT2B-/- mice were more prone to develop the 5-HT syndrome symptoms after administration of high doses of SSRIs or the 5-HT precursor. These evidences suggest that the presence of 5-HT2B receptor hinders acute 5-HT toxicity once high levels of 5-HT are attained. Therefore, differential agonism/antagonism of these receptors should be considered in the search of therapeutic targets for treating this serious disorder.

*Cover letter

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Editor

Dear Sir,

Please find our manuscript entitled "**Implication of 5-HT$_{2B}$ receptors in the serotonin syndrome**" which we submit for consideration as a research article to Neuropharmacology.

The serotonin syndrome occurs in humans after antidepressant overdose or a combination of drugs inducing a massive increase of serotonin levels. It has been extensively analyzed in humans, but data on animal models are less consistent. Several serotonin receptors participate in the expression of the syndrome in humans as well as in animal models, but there is no definite consensus about which receptors mediate this syndrome. So far, 5-HT$_{1A}$, 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors have been shown to participate in the development of this acute serotonin toxicity. Our manuscript shows that in addition to these three receptors, the 5-HT$_{2B}$ receptor has a hindering role in the manifestations of this syndrome. We employed parallel pharmacological and genetic approaches, both of which led to the same conclusion: The different subtypes of 5-HT receptors are positively (5-HT$_{1B}$ and 5-HT$_{2B}$) or negatively (5-HT$_{2C}$, 5-HT$_{1A}$ and 5-HT$_{2A}$) involved in the behavioral responses, respectively impeding or facilitating the serotonin syndrome. Future studies should further dissect the specific mechanisms by which the lack of the 5-HT$_{2B}$ receptor facilitates the triggering of a serotonin syndrome. These informations are of prime relevance for developing more efficacious pharmacological treatments for this toxicological disorder.

Thus, this work summarizes the respective contributions of serotonin receptors in the appearance of the syndrome. We believe our findings to be both novel and of significant interest to merit dissemination in Neuropharmacology.

I hope that you will evaluate the importance of this work, and that you will find it in accordance with the aims of the Journal,

Sincerely,

Luc Maroteaux PhD

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***Title page**

Implication of 5-HT_{2B} receptors in the serotonin syndrome

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ABSTRACT

The serotonin (5-HT) syndrome occurs in humans after antidepressant overdose or a combination of drugs inducing a massive increase of extracellular 5-HT. Several 5-HT receptors participate in the expression of this syndrome in humans and animal models. Likewise, the 5-HT_{2B} receptor has been proposed as a positive modulator of serotonergic activity, but whether it is involved in the expression of the 5-HT syndrome has not yet been studied. We analyzed thus, the putative role of the 5-HT_{2B} receptor in the development of this disorder by the forced swimming test (FST) and behavioural assessment in the open field. In the FST, the genetic or pharmacological ablation of the 5-HT_{2B} receptor facilitated the selective serotonin reuptake inhibitors (SSRI)-induced expression of symptoms related to the 5-HT syndrome like increase of immobility time, hind limb abduction and Straub tail. Similar responses were developed in the FST by both wild type (WT) and 5-$HT_{2B}^{-/-}$ mice after the administration of 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors agonists. In contrast, the 5-HT_{2B} and 5-HT_{1B} receptors agonists decreased immobility time. The SSRIs- induced 5-HT syndrome was blocked in both WT and 5-$HT_{2B}^{-/-}$ mice by administration of 5-HT_{1A} and 5-HT_{2C} receptors antagonists. We then performed a behavioral characterization and confirmed that 5-$HT_{2B}^{-/-}$ mice were more prone to develop the 5-HT syndrome symptoms after administration of high doses of SSRIs or the 5-HT precursor. These evidences suggest that the presence of 5-HT_{2B} receptor hinders acute 5-HT toxicity once high levels of 5-HT are attained. Therefore, differential agonism/antagonism of these receptors should be considered in the search of therapeutic targets for treating this serious disorder.

Keywords: 5-HT_{2B} receptor; serotonin syndrome; SSRI antidepressants; forced swimming test; serotonin syndrome; mice

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ABSTRACT

The serotonin (5-HT) syndrome occurs in humans after antidepressant overdose or a combination of drugs inducing a massive increase of extracellular 5-HT. Several 5-HT receptors participate in the expression of this syndrome in humans and animal models. Likewise, the 5-HT_{2B} receptor has been proposed as a positive modulator of serotonergic activity, but whether it is involved in the expression of the 5-HT syndrome has not yet been studied. We analyzed thus, the putative role of the 5-HT_{2B} receptor in the development of this disorder by the forced swimming test (FST) and behavioural assessment in the open field. In the FST, the genetic or pharmacological ablation of the 5-HT_{2B} receptor facilitated the selective serotonin reuptake inhibitors (SSRI)-induced expression of symptoms related to the 5-HT syndrome like increase of immobility time, hind limb abduction and Straub tail. Similar responses were developed in the FST by both wild type (WT) and 5-$HT_{2B}^{-/-}$ mice after the administration of 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors agonists. In contrast, the 5-HT_{2B} and 5-HT_{1B} receptors agonists decreased immobility time. The SSRIs- induced 5-HT syndrome was blocked in both WT and 5-$HT_{2B}^{-/-}$ mice by administration of 5-HT_{1A} and 5-HT_{2C} receptors antagonists. We then performed a behavioral characterization and confirmed that 5-$HT_{2B}^{-/-}$ mice were more prone to develop the 5-HT syndrome symptoms after administration of high doses of SSRIs or the 5-HT precursor. These evidences suggest that the presence of 5-HT_{2B} receptor hinders acute 5-HT toxicity once high levels of 5-HT are attained. Therefore, differential agonism/antagonism of these receptors should be considered in the search of therapeutic targets for treating this serious disorder.

Keywords: 5-HT_{2B} receptor; serotonin syndrome; SSRI antidepressants; forced swimming test; serotonin syndrome; mice

1. **INTRODUCTION**

The serotonin (5-HT) syndrome is a serious disorder reported in humans that most commonly appears after antidepressant overdose or after combining several psychotropic medications (Kalueff et al., 2008). The exaggerated serotonergic function induced by these conditions can be simulated in experimental animals by the administration of serotonin-enhanced drugs like 5-HT precursors or 5-HT receptors agonists (Kalueff et al., 2008; Van Oekelen et al., 2002). Thus, signs of the 5-HT syndrome are possibly due to a huge increase of extracellular 5-HT activating 5-HT_{1A}, 5-HT_{2C}, 5-HT_{2A}, and 5-HT_{7} receptors among others (Cryan & Lucki, 2000; Van Oekelen et al., 2002; Landry et al. 2006; Fox et al., 2008). This acute 5-HT toxicity has been characterized in mice by the expression of certain behavioral and physiological responses as hind limb abduction, forepaw treading, backward movement, Straub tail, head weaving, tremor and low flat posture (Izumi et al., 2006; Kennett et al., 1985; Sternbach et al, 1991; Fox et al., 2007).

The forced swimming test (FST) is the behavioural paradigm most employed for screening new molecules potentially efficacious as antidepressants. A highly heterogeneous sensitivity to antidepressants effects and particularly to selective 5-HT reuptake inhibitors (SSRI), among several strains of mice was clearly demonstrated in the FST (Lucki et al. 2001; David et al., 2003). This fact suggests a fine genetic-dependent regulation in the components of the 5-HT neurotransmission systems involved in antidepressants effects. In particular, one of the signs of the 5-HT syndrome, the hind limb abduction was shown specifically by 129/Sv mice in the FST at high dose of SSRIs (Lucki et al., 2001). As for the 5-HT syndrome, many subtypes of 5-HT receptors have been proposed as regulating the response in the FST either acting at the presynaptic membrane (Bortolozzi et al., 2004; Jones and Lucki, 2005) or at a postsynaptical level (Cryan and Lucki, 2000; Boothman et al., 2003; Tatarczyńska et al., 2005; Cremers et al., 2007). For instance, the 5-$HT_{2B/2C}$ receptor agonists WAY 161503, Ro 60-0175 and Ro 60-0332 all decrease immobility time in rats in the FST, whereas the non-selective 5-HT_{2}

receptors agonist m-CPP increases immobility scores (Cryan et al., 2000). These results implicate 5-HT_{2B} and/or 5-HT_{2C} receptors in the behavioural effects of antidepressants, but it has been difficult to dissect the role of each receptor subtype given that non-selective drugs were employed in most of these studies. We have recently observed that stimulation of 5-HT_{2B} receptors in mice induced an antidepressant-like effect in the FST (unpublished personal data). Taking these evidences into account, we investigated here a putative participation of 5-HT_{2B} receptors in the expression of the 5-HT syndrome, and revealed that this 5-HT receptor has a impeding role in the development of this syndrome.

2. **MATERIALS AND METHODS**

2.1 Animals

5-$HT_{2B}^{-/-}$ mice used in these experiments are in a pure 129/SvPAS background, while $SERT^{-/-}$ mice used have a pure C57Bl/6NCrl background. Adult (7- 9 week-old) male and female $5HT_{2B}^{-/-}$, $SERT^{-/-}$ and their respective wild type (WT) control mice (originally obtained at Charles River Laboratories, L'Arbresle France) were bred at our animal facilities. All mice were maintained on a 12 light: 12 dark schedule (lights on at 8:00) and housed in groups of 3-5 of the same sex and genetic background according to the EC directive 86/609/CEE. Food and water were provided ad libitum. Behavioural studies were carried out in the afternoon (14:00–20:00). Mice were moved to the testing room in their home cage at least 5 days prior to testing to allow for habituation to the environment.

2.2 Forced Swimming Test

Mice were randomly assigned to the different experimental groups receiving various drug treatments or vehicle. Mice FST was conducted essentially as described by Lucki et al, (2001). Briefly, swim sessions were conducted by placing mice individually in a plastic cylinder (26 cm tall × 17 cm in diameter) filled with water (24–26°C) to a depth of 15 cm. The depth was deep enough so that mice could not support themselves by placing their paws or tail on the base of the cylinder. Standard 6-min test duration was employed, and immobility time was only measured during the last 4 minutes of the test period. Mice were judged to be immobile when no additional activity was observed other than that required to keep their head above the water. After removing mice from water, they were dried and placed in their home cage. Each animal was challenged once. The observer was blind to the experimental conditions being measured.

Injections were administered 30 minutes before the test session. In those experiments where two drugs were studied, the injections were administered 45 (i.e., an antagonist) and 30

minutes before the test session.

2.3 Serotonin Syndrome assessment

Experiments were conducted in an open field, consisting of a Plexiglass box (20 x 40 x 15cm). Each animal was challenged once. Fifteen minutes after the injection, mice were placed in one of the corners of the box. Postures and behaviours associated with the rodent 5-HT syndrome (Kalueff et al., 2008; Fox et al., 2007) were recorded for five 1-min periods every 5 min. In each assessment period the acts and postures evaluated were the following: intermittent behaviours including backward gait, tics, tremor and hunched back were scored on a scale of 0 to 4: 0, absent; 1, expressed once; 2, expressed several times; 3, permanently expressed; for continuous behaviors including flat body position, piloerection, Straub tail and hind leg abduction, a value of 1 was assigned each time that they were present. In order to summarize the results obtained, a global score was calculated individually for each mouse by adding each of the five 1-min periods for each sign. Behaviour assessment was performed by an observer blind to both genotype and treatment.

2.4 Plasmatic 5-HT determination

Submandibular bleeding was performed by means of appropriated lancets (Goldenrod™; Golde et al., 2005) in citrated tubes (Sarstedt) 15 min after i.p. drug administration (Flx, Parox or 5-HTP) and kept at room temperature until centrifugation at 2000 rpm for 15 min. Plasma was recovered, purified in 10K filters (Nanoseps®, Pall) at 11000 for 10 min, and dilutions of the filtered plasma were injected without any purification into an HPLC system that consists of a pump linked to an automatic injector (Agilent 1100, Palo Alto, CA, USA), a reverse-phase column (Zorbax SB C18, 3.5 lm, 150 · 4.6 mm; Agilent Technologies, Palo Alto, CA, USA) and a coulometric detector (Coulochem III; ESA Inc., Chelmsford, USA) with a 5011 analytical cell to quantify 5-HT. The first electrode was fixed at -100 mV and the second

electrode at +300 mV. The gain of the detector was set at 50 nA. The signal of the second electrode was connected to an HP Chemstation for HPLC. The composition of the mobile phase was 50 mM NaH2PO4, 0.1 mM Na2EDTA, 0.65 mM octyl sodium sulphate and 14% (v ⁄ v) methanol, pH 3.5. The flow rate was set at 1 ml ⁄ min.

2.5 Statistical analysis

To determine differences between the experimental groups, behavioural parameters (immobility times in the FST; score in the 5-HT syndrome) and 5-HT plasma levels were analyzed by either a single-factor or a two-factor analysis of variance (ANOVA) with genotype and treatment as main factors depending on the experimental design. Previously, normal distribution and homoscedasticity were verified by Shapiro-Wilks test and Levene's test, respectively. Dunnet's or Student Newman-Keuls (SNK) tests were used for *post hoc* comparisons. In all cases, $p < 0.05$ was considered statistically significant.

3. **RESULTS**

3.1 Dose-response curves to SSRIs in 129/SvPAS and 5-HT$_{2B}^{-/-}$ mice

A significant decreased of immobility time in the FST was observed in 129/SvPAS (wild type, WT) mice treated with the prototypical SSRI fluoxetine (Flx) at 3 mg/kg and 5 mg/kg i.p. (Fig. 1.A). Data obtained also indicated that a loss of antidepressant effect occurs at 20 mg/kg, and a significant increase in immobility time takes place at 30 mg/kg. Additionally, WT mice treated with Flx 30 mg/kg experienced hind limb abduction and Straub tail, both signs associated with the 5-HT syndrome (Lucki et al. 2001; Kalueff et al., 2008). In order to analyze if the high sensitivity to Flx could be generalized among SSRIs, paroxetine (Parox), another drug of this pharmacological group, was tested. A significant reduction of immobility time in the FST was induced by Parox 1 mg/kg and 2 mg/kg in WT mice (Fig. 1.B). Likewise, WT mice injected with Parox 20 mg/kg, a dose ten-fold higher than the active dose, induced a significant increase of immobility time, in the same way as Flx 30 mg/kg.

The role of the 5-HT$_{2B}$ receptor in the response to SSRI was then investigated in 5-HT$_{2B}^{-/-}$ mice. Strikingly, 5-HT$_{2B}^{-/-}$ mice did neither respond to the doses of Flx nor to that of Parox which were efficacious in WT mice (Fig. 1.C-D). Moreover, 5-HT$_{2B}^{-/-}$ mice developed characteristic signs of 5-HT syndrome when received Flx 20 mg/kg, a dose that did not induce a syndrome in WT mice or Parox 2 mg/kg, the efficacious dose in WT mice.

Data from our laboratory showed that the preferential 5-HT$_{2B}$ receptor agonist BW723C86 administered to WT mice decreases immobility time in the FST (Suppl Fig 1). These evidences, together with the data described above suggest that the 5-HT$_{2B}$ receptor participates in the acute response to SSRIs. The results obtained at high SSRIs doses in 5-HT$_{2B}^{-/-}$ mice indicate a possible role for this 5-HT receptor in the development of the 5-HT syndrome. In order to validate our results the participation of other 5-HT receptors, previously implicated in both the FST and the expression of the 5-HT syndrome, was studied.

3.2 Evaluation of putative contribution of 5-HT_{1B} receptors in the FST

A significant decrease of immobility time was measured in WT mice treated with the selective 5-HT_{1B} receptor agonist CGS12066A at 2 mg/kg (Fig 2.A). This effect was blocked by pretreating animals with the selective 5-HT_{1B} receptor antagonist SB216641 at 0.5 mg/kg (a dose that did not modify the basal response of mice in the FST). Interestingly, CGS12066A also induced a significant decrease of immobility time in 5-$HT_{2B}^{-/-}$ mice, effect blocked by pretreatment with SB216641. These results imply that the 5-HT_{1B} receptor is involved in the response in the FST as previously reported, and this role does not depend on the presence of 5-HT_{2B} receptors.

3.3 Evaluation of the contribution of 5-HT_{1A} receptors in the FST

WT and 5-$HT_{2B}^{-/-}$ mice treated with the selective 5-HT_{1A} receptor agonist 8-OH-DPAT at 5 mg/kg presented increased immobility time (Fig 2.B), hind limb abduction and Straub tail, as it was the case of mice receiving high doses of SSRIs. Further, the selective 5-HT_{1A} receptor antagonist WAY100635 at 0.5 mg/kg (a dose that did not modify the basal performance of neither genotype in the FST) prevented the increase in immobility time induced by 8-OH-DPAT in WT as well as in 5-$HT_{2B}^{-/-}$ mice.

3.4 Evaluation of the contribution of 5-HT_{2C} receptor in the FST

WT and 5-$HT_{2B}^{-/-}$ mice treated with the preferential 5-HT_{2C} receptor agonist WAY161503 at 5 mg/kg presented increased immobility time (Fig 3.C), hind limb abduction and Straub tail, as it was the case of mice receiving high SSRI doses and the 5-HT_{1A} receptor agonist. Further, blockade of the 5-HT_{2C} receptor by SB242084 at 0.5 mg/kg prevented the WAY161503-induced increase in immobility time in WT as well as in 5-$HT_{2B}^{-/-}$ mice.

3.5 Evaluation of the contribution of 5-HT_{2A} receptor in the FST

WT and 5-$HT_{2B}^{-/-}$ mice treated with the non selective 5-HT_2 agonist DOI at 1 mg/kg presented increased immobility time (Fig 2.D), hind limb abduction and Straub tail. In this case, the selective 5-HT_{2A} antagonist MDL100907 at 0.5 mg/kg significantly blocked the effect induced by DOI in WT mice, but the reduction in 5-$HT_{2B}^{-/-}$ mice did not reach statistical significance.

3.6 Behavioural characterization of the serotonin syndrome

In order to confirm that the signs observed in the FST in mice treated with high doses of SSRIs or certain 5-HT receptors agonists corresponded to a 5-HT syndrome, we characterized its behavioral expression in our experimental conditions by first evaluating it in $SERT^{-/-}$ mice treated with the 5-HT precursor 5-hydroxytryptophane (5-HTP). The absence of SERT makes these mice more prone to develop a 5-HT syndrome (Fox et al., 2007) and we confirmed this finding in our paradigm (Suppl Fig 2). We then studied the 5-HTP-induced 5-HT syndrome in 129/SvPAS and 5-$HT_{2B}^{-/-}$ mice. A significant increase in the syndrome score (see methods) was observed in 5-$HT_{2B}^{-/-}$ mice but not in WT mice receiving 5-HTP at 50 mg/kg, (Fig 3.A). After receiving 5-HTP at 100 mg/kg mice of either genotype showed a significant increase in the score, much higher in mutant mice. Then, and according to the Flx dose-response curve obtained in each genotype Flx 3 and 30 mg/kg was administered to WT mice, and Flx 3 and 20 mg/kg to 5-$HT_{2B}^{-/-}$ mice and demonstrated that high doses of SSRI can induce a 5-HT syndrome in WT as well as in 5-$HT_{2B}^{-/-}$ mice (Fig 3A).

To further study the role of the 5-HT_{2B} receptor, WT mice pretreated with the selective 5-HT_{2B} receptor antagonist RS127445 at 0.5 mg/kg received 5-HTP 50 mg/kg or Flx 20 mg/kg (Fig 3.B), but in opposition to 5-$HT_{2B}^{-/-}$ mice, the score was not significantly different from vehicle-treated mice.

3.7 Attenuation of the Serotonin Syndrome in the FST

Mice injected with high doses of Flx were pretreated with 5-HT_{1A}, 5-HT_{2C} or 5-HT_{2A} receptor antagonists and evaluated in the FST. WAY100635 at 0.5 mg/kg as well as SB242084 at 0.5 mg/kg were able to prevent the expression of 5-HT syndrome signs in either WT or 5-$HT_{2B}^{-/-}$ mice treated with Flx at 30 mg/kg and 20 mg/kg, respectively (doses that induced the 5-HT syndrome in either genotype; Fig 3.C), confirming that this syndrome was due, at least in part, to the activation of 5-HT_{1A} receptors and 5-HT_{2C} receptors. On the contrary, the blockade of the 5-HT_{2A} receptor by means of MDL100907 at 0.5 mg/kg did not attenuate the expression of the 5-HT syndrome in either genotype.

3.8 Determination of plasmatic 5-HT levels

The sensibility to develop the 5-HT syndrome in either genotype at different doses of SSRIs could be due to differences in the increase of extracellular serotonin sufficient to activate several receptors mediating this syndrome. Accordingly, plasmatic 5-HT levels were measured after SSRIs administration in WT and 5-$HT_{2B}^{-/-}$ mice (Fig 4). No significant differences were observed between genotypes in the 5-HT plasmatic levels attained after each treatment.

4. **DISCUSSION**

The results presented herein strongly implicate 5-HT_{2B} receptors in the expression of the 5-HT syndrome. So far, 5-HT_{1A}, 5-HT_{2C} and 5-HT_{2A} receptors have been shown to participate in the development of this acute 5-HT toxicity, but our present data reveal that 5-HT_{2B} receptors hinder the manifestations of this syndrome.

Consistent with a 5-HT_{2B}-dependent regulation over SERT in raphe neurons demonstrated in *in vitro* assays (Launay et al., 2006), previous studies from our laboratory strongly support a presynaptic location of the 5-HT_{2B} receptor (Doly et al., 2008; Banas et al., in press). Indeed, the acute injection of the 5-HT_{2B} preferential agonist BW723C86 into the raphe nuclei induced an increase of 5-HT levels (Doly et al., 2008), which could partially explain the antidepressant-like effect of BW723C86 shown herein in the FST. Even though no FST studies have been formerly conducted with selective 5-HT_{2B} agonists or antagonists, several articles have been published analyzing the role of 5-HT_{2C} receptors (Cryan & Lucki, 2000; Bristow et al., 2000; Nic Dhonnchadha et al., 2005). However, these results were difficult to interpret given that Ro-60-0175, WAY161503, SB 206553 and SB 221284 are non selective 5-$HT_{2B/2C}$ agonists (Banas et al, in press). Therefore, those results could be attributed to combined actions at 5-HT_{2C} as well as at 5-HT_{2B} receptors. Our experiments including the administration of the 5-HT_{2C} agonist WAY 161503 (1) to naïve WT mice, (2) to 5-HT_{2C} receptor antagonist-pretreated WT mice or (3) to 5-$HT_{2B}^{-/-}$ mice clearly showed that selective stimulation of 5-HT_{2C} receptors does not induce a decrease in the response to the FST as previously reported (Cryan et al., 2000), but on the contrary, an increase of immobility time. Additionally, previous data confirmed that selective 5-HT_{2C} receptor antagonists are able to potentiate SSRI-induced extracellular 5-HT elevation (Cremers et al., 2004, 2007), but have no effect on extracellular serotonin levels when administered alone. Besides, no behavioural *per se* effect of 5-HT_{2C} antagonists has been described (Cremers et al, 2004; Cryan & Lucki, 2000; Bristow et al., 2000; Dhonnchadha et al., 2005), consistent with the lack of effect of SB242084

that we observed in WT mice. The increase of the immobility time observed in WT mice treated with WAY161503 was completely abolished when 5-HT_{2C} receptors were previously blocked by SB242084, confirming that stimulation of 5-HT_{2C} receptor mediates this response of increased immobility. Altogether, our results suggest that functions of both 5-HT_{2B} and 5-HT_{2C} receptors are opposite when mediating responses in the FST. This hypothesis is supported by the fact that 5-HT_{2C} receptors present on GABAergic interneurons with a constitutive inhibitory activity on the raphe (De Deurwaerdère et al., 2004).

Another receptor that has been involved in the acute antidepressant effects of SSRI is 5-HT_{1B}. Our results confirm that agonist stimulation of 5-HT_{1B} receptors induced the classical acute response of antidepressants in WT as well as in 5-$HT_{2B}^{-/-}$ mice. Therefore, this mechanism is clearly independent of 5-HT_{2B} receptors, which is consistent with previous results where it was demonstrated that 5-HT_{1B} receptor agonists mimicked SSRI effects in the FST at least in rats (Tatarczynska et al., 2004, 2005) by acting at postsynaptic receptors. Finally, we also demonstrated that the stimulation of the 5-HT_{1A} and 5-HT_{2A} receptors have an effect opposed to that induced by antidepressants in the FST and that this role is independent of the presence of the 5-HT_{2B} receptor. Remarkably, the effect of the non selective 5-HT_2 agonist DOI was not completely blocked in 5-$HT_{2B}^{-/-}$ mice. This particular result could be explained by the fact that DOI activates the three subtypes of 5-HT_2 receptors, with the 5-HT_{2A} and 5-HT_{2C} increasing the immobility time and the 5-HT_{2B} decreasing this parameter. Therefore, when 5-HT_{2A} receptors are blocked with MDL100907, DOI still has an effect on 5-HT_{2C} and 5-HT_{2B} receptors in WT mice, but only in 5-HT_{2C} receptors in 5-$HT_{2B}^{-/-}$ mice. Altogether these data, suggest that the different subtypes of 5-HT receptors are positively (5-HT_{1B} and 5-HT_{2B}) or negatively (5-HT_{2C}, 5-HT_{1A} and 5-HT_{2A}) involved in the response in the FST. Taking into account their participation in the FST, these receptors could also respectively have hindering or facilitating properties in the development of the 5-HT syndrome.

The U-shaped dose-response curve observed in Flx-treated mice could be explained by the fact that a higher extracellular 5-HT concentration attained at a high dose of Flx, exhibits activity at additional 5-HT receptors. Therefore, and taking into account our results with 5-HT_{2C}, 5-HT_{1A} and 5-HT_{2A} receptors agonists, we propose that at high doses of SSRIs, the increased extracellular 5-HT activates some of these receptors, which are as well involved in the expression of the 5-HT syndrome. This hypothesis is also based on our observation of the attenuated behavioral response induced by WAY161503 and SB242084 in WT and 5-$HT_{2B}^{-/-}$ mice treated with high doses of Flx as it has been already reported (Cryan et al., 2005).

The 5-HT syndrome signs that we observed in mice treated with high doses of SSRIs were unexpected, considering that the doses of Flx and Parox employed are usually reported when working with these antidepressants in other mice strain (Lucki et al., 2001; Da-Rocha et al., 1997). Additionally, 129/SvPas mice are frequently described as a non- or weakly-responsive strain in these behavioural tests (Urani et al, 2005; Dulawa et al., 2004). Dose-response curve obtained for SSRI in the FST of 129/SvPAS clearly indicates that these mice have a different sensitivity to this family of pharmacological compounds. Nevertheless, 129/SvPas mice are able to develop classical responses to antidepressants when administered at appropriated dose. It is therefore worthy to insist on the cautious selection of an efficacious dose for the mice strain intended to be used, given that working with incorrect doses could lead to misinterpretation of results. Concerning the 5-HT syndrome, it has been already remarked the importance of mice genetic background which could influence behavioral and neurochemical phenotypes (Kaluef et al., 2008). Accordingly, a study on the sensitivity to antidepressants in different mice strains showed that an increase of immobility time was observed only in 129/SvPas mice receiving Flx 20 mg/kg, but not other strains like C57Bl/6J (Lucki et al., 2001). It is argued that the dose of SSRIs administered apparently interferes with the movement of mice hind limbs, inducing an opposite effect. Likewise, we confirmed that

129/SvPas mice receiving Flx 30 mg/kg experienced hind limb abduction and Straub tail, both characteristic signs of the 5-HT syndrome.

The 5-HT syndrome has been extensively analyzed in humans, but data on animal models is less consistent. Similarly, there is no definite consensus about which receptors mediate the expression of the 5-HT syndrome. In rodents, 5-HT_{2A} receptors have been involved in responses like forepaw treading, head shaking and tremor (Izumi et al., 2006; van Oekelen et al. 2002), 5-HT_{1A} receptors would be linked to neuromuscular responses of the 5-HT syndrome (Zhang et al. 2009), whereas 5-HT_{2C} receptors have been associated to hunched back (van Oekelen et al. 2002). Our results add evidence in favor of a role of these receptors in mediating this syndrome, but most importantly reveal that the 5-HT_{2B} receptors could have a protective role in the triggering of the 5-HT syndrome. Indeed, the absence or pharmacological blockade of 5-HT_{2B} receptors increased the sensitivity to develop the 5-HT syndrome in response to SSRI administration. Surprisingly, 5-$HT_{2B}^{-/-}$ mice responded similarly to WT mice to the 5-HT_{1A} receptor agonist 8-OH-DPAT, the 5-HT_{2C} receptor agonist WAY161503, and the 5-HT_{2A} receptor agonist DOI, suggesting that these post-synaptic pathways are intact in 5-$HT_{2B}^{-/-}$ mice. In addition, plasma 5-HT concentrations determined after administration of high doses of SSRIs were similar in either genotype, and therefore differences in systemic 5-HT levels induced by these compounds could not be invocated for explaining the differential reactivity. Thus, it is conceivable that the increased sensitivity to develop the serotonin syndrome, when the 5-HT_{2B} receptor is not functional, is mainly related to events inherent to 5-HT_{2B}-mediated actions rather than to a role in the modulation of extracellular 5-HT levels.

Taken together the results described herein shed new light on the role of the 5-HT_{2B} receptor on the development of the 5-HT syndrome. Future studies should further dissect the specific mechanisms by which the lack of this 5-HT receptor facilitates the expression of a 5-HT syndrome. The present information is of prime relevance for developing more efficacious pharmacological treatments for this toxicological disorder.

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FIGURE LEGENDS

Figure 1. Dose-response curve to SSRIs in the Forced Swimming Test. Swimming performance was measured 30 minutes after injection (see experimental protocol). The time spent immobile in the FST was determined in 129/SvPAS mice after Flx (A) and Parox (B) and in 5-$HT_{2B}^{-/-}$ mice after Flx (C) and Parox (D). One-way ANOVA, Dunnet's test; **p < 0,01; *p < 0,05; data is expressed as mean ± SEM (n = 8-14 mice for each group).

Figure 2. Effect of 5-HT_{1B}, 5-HT_{1A}, 5-HT_{2C}, and 5-HT_{2A} receptors agonists and antagonists in the Forced Swimming Test. The role of the 5-HT_{1B} receptor was analyzed in WT and 5-$HT_{2B}^{-/-}$ mice (A). Two-way ANOVA revealed neither a significant interaction between factors F(2, 40)=0.37, ns, nor a significant effect of genotype F(1, 40) = 3.61, ns, but a significant effect of the treatment F(2, 40) = 11.97, p <0.001. Post hoc analysis (SNK) indicated that the 5-HT_{1B} agonist, CGS12066A at 2 mg/kg induced a significant increased in the immobility time in both WT and 5-$HT_{2B}^{-/-}$ mice. This effect was abolished by the pretreatment with the selective 5-HT_{1B} antagonist SB216641 at 0.5 mg/kg in WT as well as in 5-$HT_{2B}^{-/-}$ mice. The role of the 5-HT_{1A} receptor was analyzed in WT and 5-$HT_{2B}^{-/-}$ mice (B). Two-way ANOVA revealed neither a significant interaction between factors F(2, 45)=0.53, ns, nor a significant effect of genotype F(1, 45) = 1.39, ns, but a significant effect of the treatment F(2, 45) = 16.74, p <0.001. Post hoc analysis (SNK) indicated that the 5-HT_{1A} agonist, 8-OH-DPAT at 5 mg/kg induced a significant increased in the immobility time in both WT and 5-$HT_{2B}^{-/-}$ mice. This effect was abolished by the pretreatment with the selective 5-HT_{1A} antagonist WAY100635 at 0.5 mg/kg in WT as well as in 5-$HT_{2B}^{-/-}$ mice. The role of the 5-HT_{2C} receptor was analyzed in WT and 5-$HT_{2B}^{-/-}$ mice (C). Two-way ANOVA revealed neither a significant interaction between factors F(2, 25)=0.71, ns, nor a significant effect of genotype F(1, 25) = 0.01, ns, but a significant effect of the treatment F(2, 25) = 20.62, p <0.001. Post hoc analysis (SNK) indicated that the 5-

HT_{2C} agonist, WAY161503 at 5 mg/kg induced a significant increased of the immobility time in both WT and 5-$HT_{2B}^{-/-}$ mice. This effect was abolished by the pretreatment with the selective 5-HT_{2C} antagonist SB242084 at 0.5 mg/kg in WT as well as in 5-$HT_{2B}^{-/-}$ mice. The role of the 5-HT_{2A} receptor was analyzed in WT and 5-$HT_{2B}^{-/-}$ mice (D). Two-way ANOVA revealed neither a significant interaction between factors $F(2, 33)=2.19$, ns, nor a significant effect of genotype $F(1, 33) = 2.35$, ns, but a significant effect of the treatment $F(2, 33) = 43.31$, $p < 0.001$. Post hoc analysis (SNK) indicated that the preferential 5-HT_{2A} agonist, DOI at 1 mg/kg induced a significant increased of the immobility time in both WT and 5-$HT_{2B}^{-/-}$ mice. This effect was abolished by the pretreatment with the selective 5-HT_{2A} antagonist MDL100907 at 0,5 mg/kg in WT but not in 5-$HT_{2B}^{-/-}$ mice. *$p<0.05$; ** $p < 0.01$; *** $p < 0.001$ comparing to Veh-treated groups; # $p<0.05$; ## $p < 0.01$; ### $p < 0.001$ comparing to agonist-treated groups; data are expressed as mean ± SEM (n = 6–12 mice for each group).

Figure 3. Behavioural assessment of the Serotonin Syndrome. The 5-HT syndrome has been studied in WT and 5-$HT_{2B}^{-/-}$ mice after administration of the 5-HT precursor and SSRI (A). Two-way ANOVA revealed no significant interaction between factors $F(4, 30)=5.40$, ns, but a significant effect of genotype $F(1, 30) = 9.06$, $p<0.01$, and of the treatment $F(4, 30) = 57.41$, $p < 0.001$. Post hoc analysis (SNK) indicated that 5-HTP induced a significant increased of the score at 50 mg/kg only in 5-$HT_{2B}^{-/-}$ mice and in both WT and 5-$HT_{2B}^{-/-}$ mice at 100 mg/kg. In addition, the score of the 5-HT syndrome significantly increased in WT and 5-$HT_{2B}^{-/-}$ mice receiving Flx 30 and 20 mg/kg, respectively. 5-HT syndrome in RS127445-pretreated WT mice (B). Two-way ANOVA revealed a significant interaction between factors $F(4, 31)=14.75$, $p<0.05$, a significant effect of genotype/antagonist treatment $F(2, 31) = 19.61$, $p<0.01$, and of the treatment $F(2, 31) = 27.46$, $p < 0.001$. Post hoc analysis (SNK) indicated that 5-HTP at 50 mg/kg or Flx at 20 mg/kg induced a significant increased of the score only in 5-$HT_{2B}^{-/-}$ mice, but not in WT or RS127445-pretreated WT mice. Effect of 5-HT_{1A}, 5-HT_{2C}, and 5-HT_{2A}

receptors antagonists in the FST (C). Two-way ANOVA revealed neither a significant interaction between factors F(4, 85)=0.74, ns, nor a significant effect of genotype F(1, 85) = 2.91, ns, but a significant effect of the treatment F(4, 85) = 30.84, p <0.001. Post hoc analysis (SNK) indicated that the high dose of Flx induced a significant increased of the immobility time in both WT and 5-$HT_{2B}^{-/-}$ mice, and that these effects were abolished by the pretreatment with the selective 5-HT_{1A} or 5-HT_{2C} receptors antagonist in WT as well as in 5-$HT_{2B}^{-/-}$ mice, but not by the 5-HT_{2A} receptor antagonist in neither genotype. *p<0.05; ** p <0.01; *** p<0.001 comparing to Veh-treated groups; data are expressed as mean ± SEM (n = 4 mice in A and B; 5-8 mice in C, for each group).

Figure 4. Plasma 5-HT levels after SSRIs administration. Two-way ANOVA revealed neither a significant interaction between factors F(2, 35)=0.17, ns, nor a significant effect of genotype F(1, 35) = 0.16, ns, but a significant effect of the treatment F(1, 35) = 35.3, p <0.001. Post hoc analysis (SNK) indicated no significant differences in plasma 5-HT concentrations between WT and 5-$HT_{2B}^{-/-}$ mice, either after the administration of Flx or Parox; data are expressed as mean ± SEM (n = 6-11 mice for each group).

Supplementary Figure 1. Effect of BW723C86 in the Forced Swimming Test. The 5-HT_{2B} agonist, BW723C86 was administered to WT and 5-$HT_{2B}^{-/-}$ mice. Two-way ANOVA revealed neither a significant interaction between factors F(1, 43)=0.12, ns, nor a significant effect of genotype F(1, 43) = 3.88, ns, or the treatment F(1, 43) = 0.15, ns. Post hoc analysis (SNK) indicated that BW723C86 significantly decreased immobility time in WT but not in 5-$HT_{2B}^{-/-}$ mice. * p <0.05 comparing to Veh-treated group. Data is expressed as mean ± SEM (n = 10–15 mice for each group).

Supplementary Figure 2. Behavioural assessment of the Serotonin Syndrome. The 5-HT syndrome has been characterized in $SERT^{-/-}$ mice after administration of the 5-HT precursor (5-HTP). Two-way ANOVA revealed a significant interaction between factors $F(2, 18)=8.31$, $p<0.05$, a significant effect of genotype $F(1, 18) = 7.55$, $p<0.05$, and of the treatment $F(2, 18) = 65.79$, $p <0.001$. Post hoc analysis (SNK) indicated that 5-HTP induced a significant increased of the 5-HT syndrome score at 50 mg/kg only in $SERT^{-/-}$ mice and in both WT and $SERT^{-/-}$ mice at 100 mg/kg. ** $p <0.01$; *** $p <0.001$ comparing to Veh-treated groups; data are expressed as mean ± SEM (n = 4 mice for each group).

Figure1

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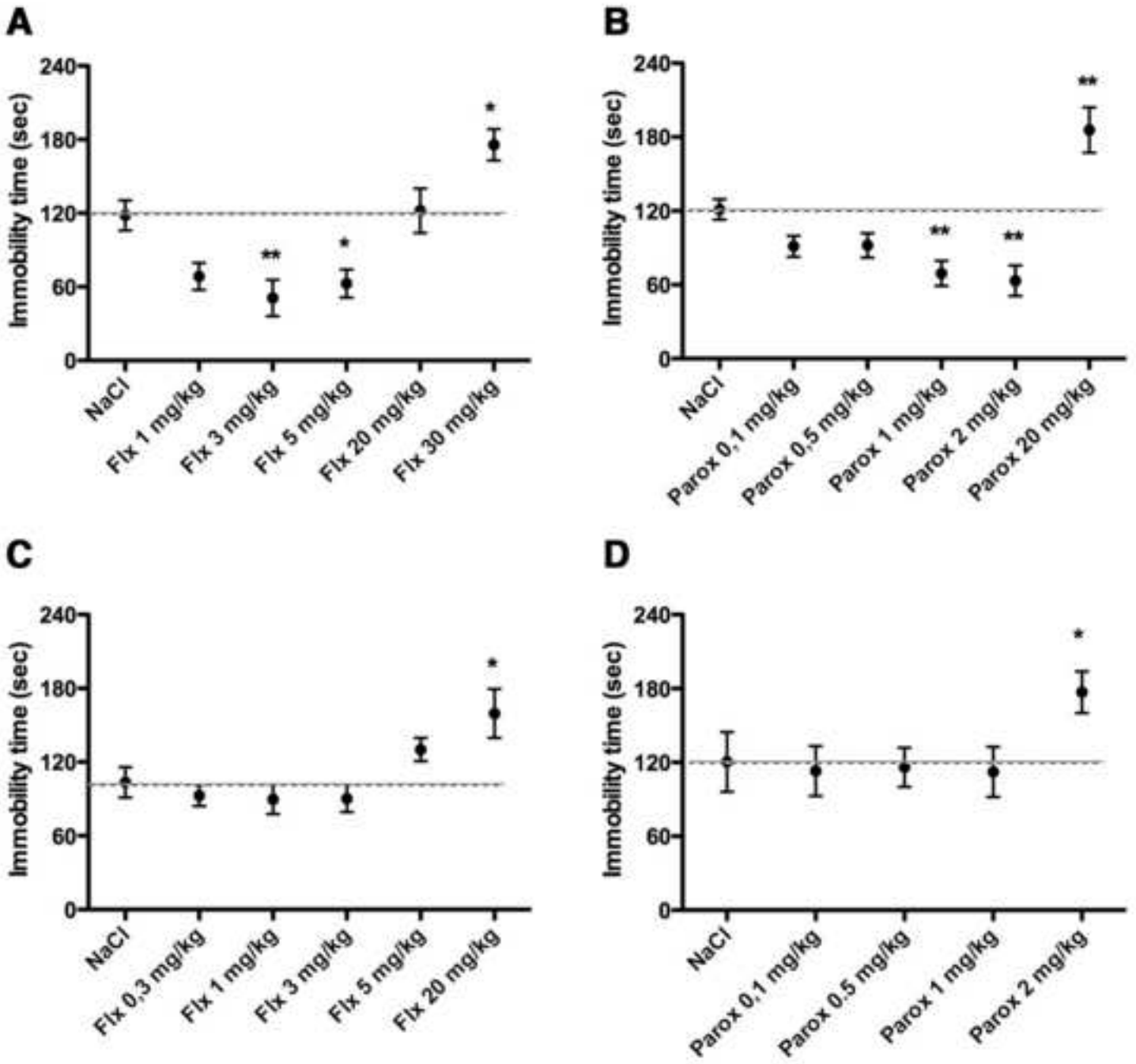
Figure 1



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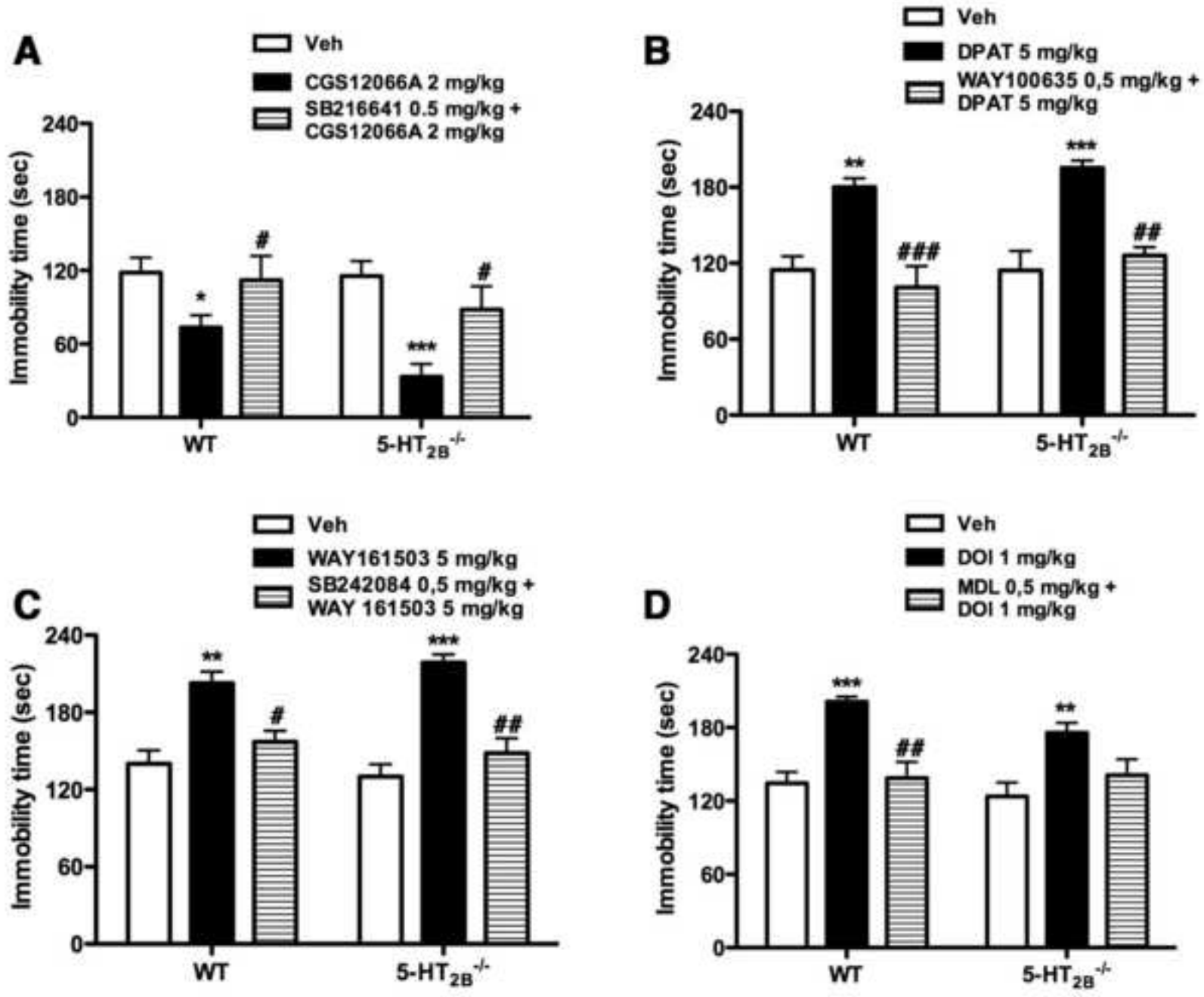

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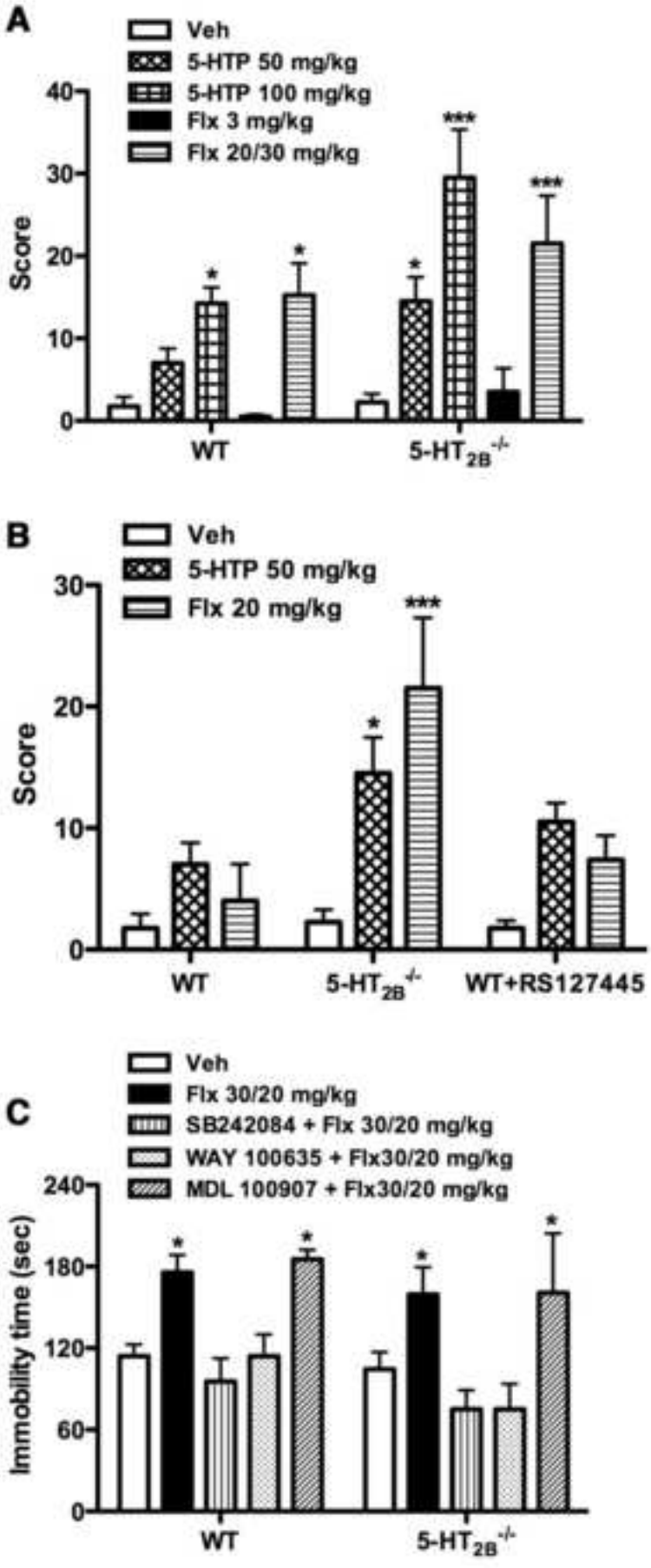

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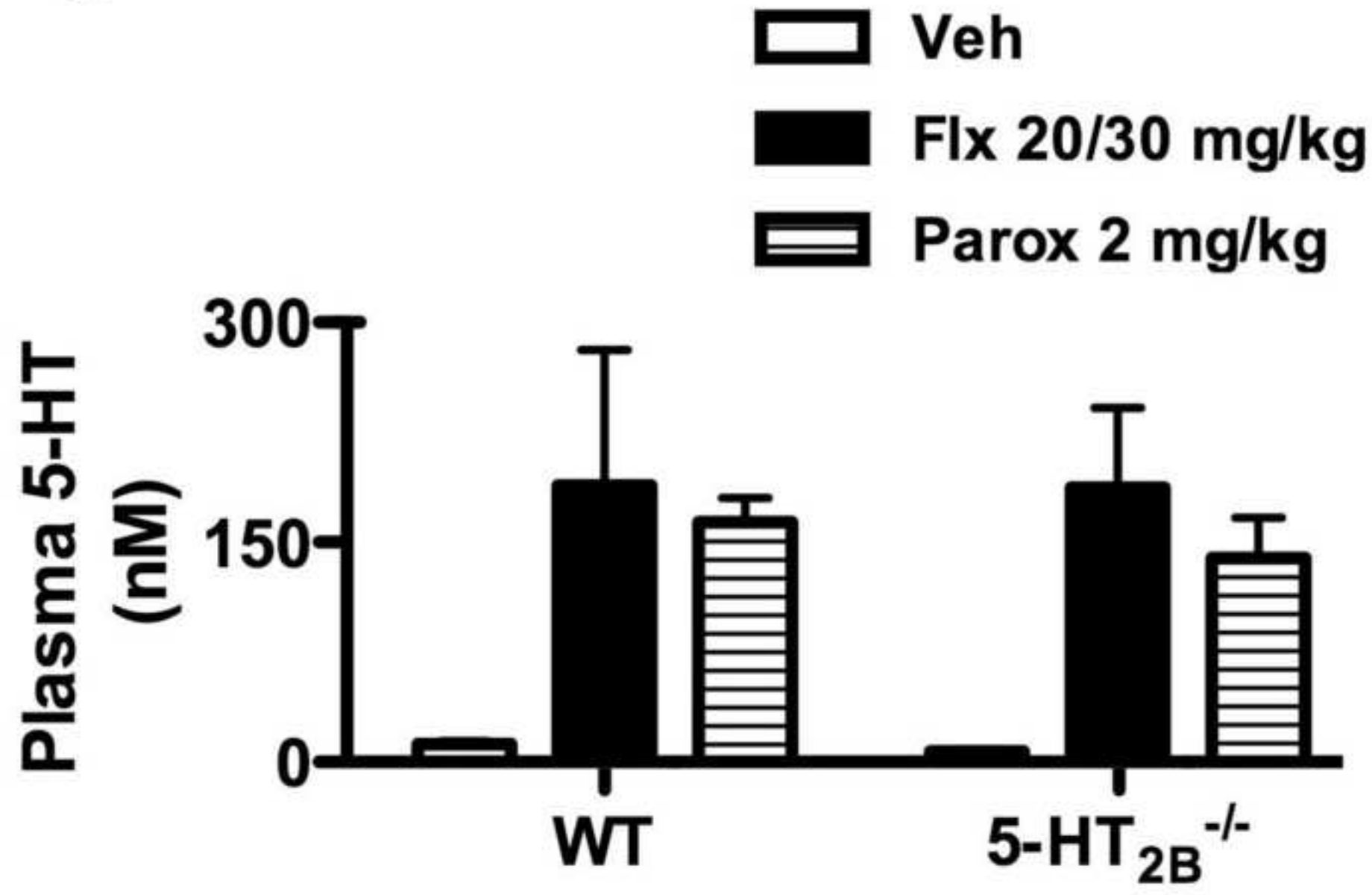

Supplementary Fig1

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Supplementary Fig2

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