Behavioral Characterization of Mice Packing the 5-HT_{1B} Receptor

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INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is a neuromodulator that is involved in a number of mood disorders such as depression, anxiety, and impulsive violence. In an attempt to dissect the contribution of individual 5-HT receptor subtypes to behavior, the authors have generated, by homologous recombination, mutant mice lacking the 5-HT1B receptor. These mice did not exhibit any obvious developmental or behavioral defect; however, the hyperlocomotor effect of the 5-HT1A/5-HT1B agonist RU24969 was completely absent in mutant mice, indicating that this effect is mediated by 5-HT1B receptors. Moreover, when confronted with an intruder, isolated mutant mice attacked the intruder faster and more intensely than wild-type mice, suggesting an involvement of 5-HT1B receptors in the modulation of aggressive behavior. These data might be related to the fact that a class of 5-HT1 agonists, termed serenics, have antiaggressive properties, and with the findings that certain impulsive aggressive behaviors are associated with deficits in central serotonin.

5-HT is a biogenic amine that is involved in a wide range of physiological functions including sleep, appetite, pain perception, sexual activity, memory, and mood control (for a review see Wilkinson and Dourish 1991). A central serotonin deficit has been associated with behaviors such as suicidality, impulsive violence, depression, and alcoholism (Eichelmann 1992; Roy et al. 1991), and serotonergic drugs are used in the treatment of a number of pathological states including migraine, depression, and anxiety (Sleight et al. 1991). The multiple actions of serotonin are mediated by the interaction of this amine with at least 14 receptors (for a review see Saudou and Hen, in press), most of which belong to the guanosine triphosphate binding protein (G-protein)-coupled receptor family.

The purpose of this study was to determine the contribution of one of these receptors, the 5-HT1B subtype, to the various behavioral responses elicited by serotonin and serotonergic drugs. The 5-HT1B receptor, which is the rodent homolog of the human 5-HT1Db receptor, is expressed in a variety of brain regions, including motor control centers such as the basal ganglia, as well as structures involved in mood control such as the central gray, hippocampus, and raphe nuclei (Boschert et al. 1993; Bruinvels et al. 1993; Maroteaux et al. 1992). Pharmacological studies using poorly specific agonists have suggested that activation of 5-HT1B receptors might lead to an increase in anxiety and locomotion (Griebel et al. 1990; Pellow et al. 1987) and to a decrease in food intake, sexual activity, and aggressive behavior (Fernandez-Guasti et al. 1992; Kennett et al. 1987; Koe et al. 1992; Olivier et al. 1987). In particular, a class of 5-HT1 agonists have been termed serenics because of their antiaggressive properties in several rodent aggression models (Flannelly et al. 1985; Mos et al. 1992; Olivier et al. 1986, 1989). However, it is not clear to what extent the effects of the serenics are mediated by 5-HT1B receptors because these drugs also activate 5-HT1A receptors and possibly some of the recently discovered 5-HT receptors. In addition, the consequences of a blockade of 5-HT1B receptors, or of their human counterpart the 5-HT1Db receptors, are unknown, since there are no specific antagonists for these receptors.

In order to study the function of the 5-HT1B receptor, the authors have generated by homologous recombination homozygous mutant mice lacking both copies of the gene encoding this receptor. These mice are viable and fertile, and they were analyzed for a variety of behaviors that are thought to be modulated by 5-HT1B receptors such as locomotion, anxiety, and aggression.

5-HT1B RECEPTOR GENE TARGETING

The 5-HT1B receptor gene was disrupted by homologous recombination (Capecchi 1989; figure 1). The JA construct consisted of 6.0 kilobase-pairs (kb) of genomic sequence in which part of the 5-HT1B coding sequence was replaced by a neomycin phosphotransferase gene (neo) under the control of the GTI-II enhancer (Lufkin et al. 1991; figure 1A). In the JB construct, the neocassette was inserted in the coding sequence of the 5-HT1B gene (figure 1B). The two linearized targeting vectors were electroporated into D3 embryonic stem (ES) cells and G418 resistant colonies were screened by Southern blotting (figure1). The EX400 probe identified

positive clones with the expected 10 and 8.5 kb Kpn I fragments for the JA and JB constructs respectively (figure 1). Four positive clones were obtained with both constructs yielding a targeting frequency of 1/15 (JA) and 1/12 (JB) (table 1). Southern analyses using Xba I digests and the E2A1 probe or the neo probe confirmed that accurate targeting occurred and that no additional integration took place (data not shown). Cells from the positives clones JA7 and JB13 were microinjected into 3.5-day C57BL/6 mouse blastocysts. The two clones gave rise to highly chimeric mice that were bred with C57BL/6 females in order to test for germline transmission of the mutated 5-HT1B receptor gene (table 1). The positive chimeras were bred with females from the 129/Sv-ter inbred strain to obtain heterozygotes on the 129/Sv-ter genetic background. Heterozygous mice were phenotypically normal and fertile. Homozygous animals deriving from both cell lines were generated by heterozygote crossings. In 243 JA offspring and 213 JB offspring, the expected 1:2:1 ratio of wild-type, heterozygous, and homozygous mutant progeny was observed. The homozygous mutants did not display any obvious developmental or behavioral abnormality and were fertile. Although the average lifespan has not yet been determined, no spon-taneous deaths occurred during the first 12 months of life. All the analyses presented here were performed on animals having a pure 129/Sv genetic background. There were no differences between the mice derived from the JA7 and JB13 targeted cell lines.

EFFECTIVENESS OF 5-HT1B RECEPTOR ABLATION

In order to ensure that disruption of the 5-HT1B receptor gene was effective, the authors performed autoradiographic studies (figure 2) on brains of wild-type, heterozygous, and homozygous mutants using the radiolabeled ligand 3[125I]iodocyanopindolol ([125I]CYP). When used in the presence of appropriate masking agents, this radioligand binds specifically to the 5-HT1B receptor (Hoyer et al. 1985; Offord et al. 1988; Pazos and Palacios 1985). In wild-type mice, [125I]CYP binding sites were found in the globus pallidus, substantia nigra, cerebellar nuclei, subiculum, lateral geniculate nucleus, central gray, and colliculi, while no specific binding was observed in homozygous mutants (figure 2). These results demonstrate that effective disruption of the 5-HT1B gene occurred and that in these experimental conditions, [125I]CYP binding sites correspond exclusively to 5-HT1B receptors. Heterozygous mice displayed the same level of binding sites as wild-type mice (figure 2), although they have only one functional allele of the 5-HT1B gene.

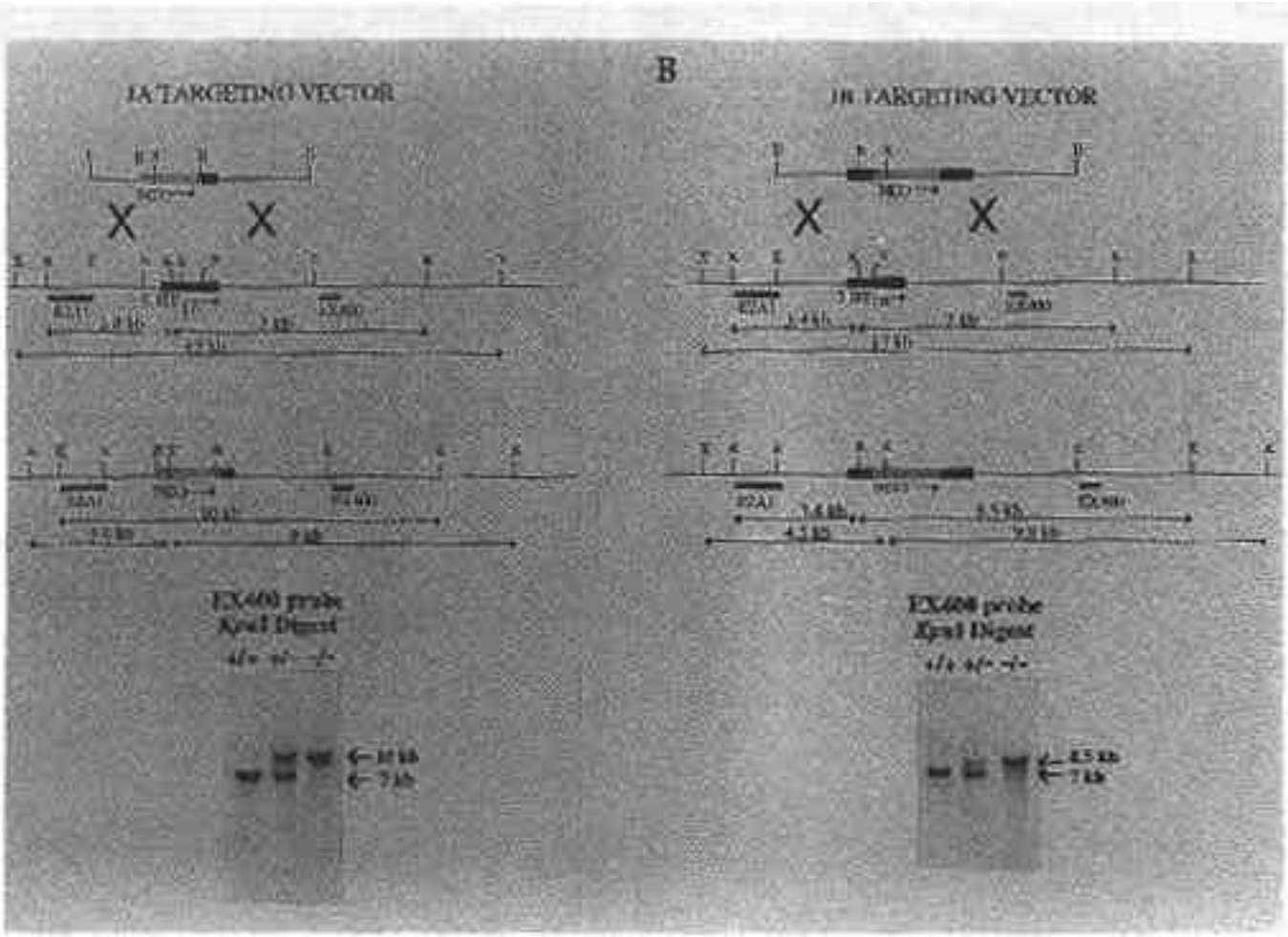
B
1A TARGETING VECTOR
1B TARGETING VECTOR
EX400 probe
XpnI Digest
EX400 probe
XpnI Digest

FIGURE 1. *Homologous recombination at the 5-HT1B locus. Schematic representation of the targeting event using JA* ***(A)*** *and JB* ***(B)*** *constructs and Southern analysis of JA7* ***(A)*** *and JB13* ***(B)*** *mutant mice. The upper panels correspond to the targeting vectors, genomic structure of the 5-HT1B gene, and predicted structures of the mutated alleles after homologous recombination. The black box corresponds to the coding sequence of the 5-HT1B receptor and the hatched box to the neocassette. Arrows on the neocassette (purified from p 581 (Lufkin et al. 1991)) and on the 5-HT1B gene indicate the direction of transcription (from left to right). The locations of the probes E2A1 and EX400 used in Southern analysis are shown. E2A1 and EX400 probes were used to screen neomycin resistant clones after XbaI and KpnI digests, respectively. Bottom, Southern blot analysis. Tail DNA from wild-type, heterozygous, and homozygous JA7* ***(A)*** *and JB13* ***(B)*** *mutant mice were cut by KpnI and hybridized with the 3' end EX400 probe.*

KEY: E=EcoRI; B=BalI; X=XbaI; K=KpnI; V=EcoRV; +/+=wild-type; +/-=heterozygous; and -/ =homozygous mutant.

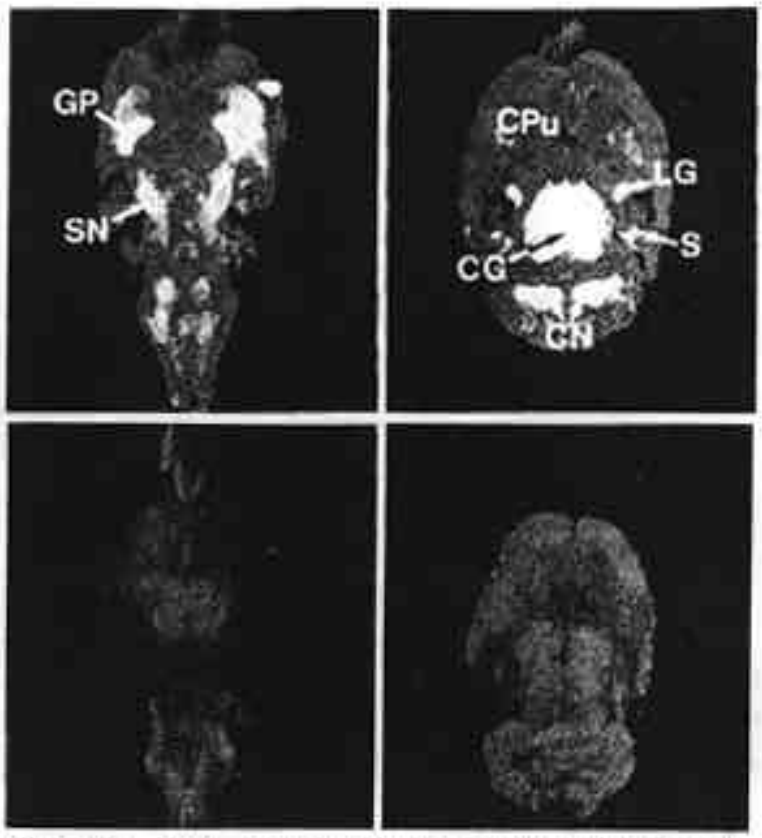

FIGURE 2a. *5-HT$_{1B}$ receptor autoradiography in wild-type and mutant mice. 3[^{125}I]iodocyanopindolol was used to label 5-HT$_{1B}$ receptors in mouse horizontal brain sections. Upper panels correspond to successively more dorsal brain sections of wild type mice and lower pannels correspond to similar levels of sections of homozygous mutant mice (Boschert et al. 1993). Arrows indicate the main sites of 5-HT$_{1B}$ receptor expression. The differences were significant between mutant and wild-type and between mutant and heterozygous mice in all brain regions tested.*

KEY: CG=central gray; CN=cerebellar nuclei; CPu= caudate putamen; GP=globus pallidus; LG=lateral geniculate nucleus; S=subiculum; SN=substantia nigra; **=P<0.001.

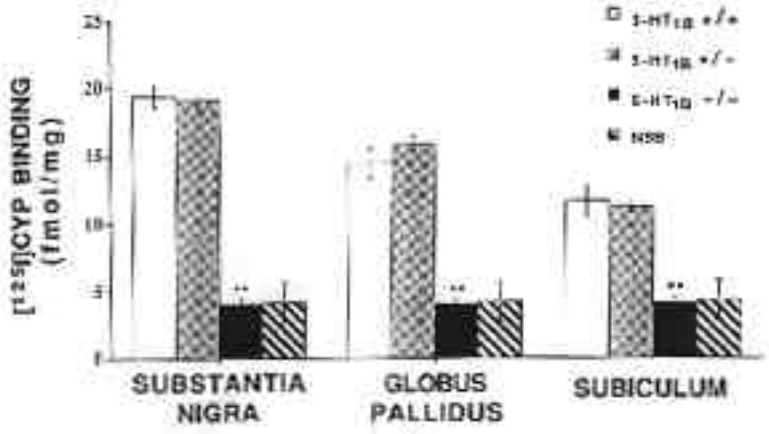

FIGURE 2b. *Graph, density of [^{125}I]CYP binding sites (mean±SEM; n=3) in different brain regions for wild-type (5-HT$_{1B}$ +/+), heterozygous (5-HT$_{1B}$ +/-), and homozygous mutant mice (5-HT$_{1B}$ -/-). Student's t-test revealed no difference between wild-type and heterozygous mice, and between mutant mice and non specific binding (NSB). The differences were significant between mutant and wild-type and between mutant and heterozygous mice in all brain regions tested.*

Locomotion

As shown in figure 2, the 5-HT1B receptor is localized in motor control centers such as the globus pallidus, substantia nigra, and deep cerebellar nuclei. Furthermore, pharmacological studies have suggested an involvement of 5-HT1B receptors in the control of locomotor activity (Green et al. 1984; Oberlander et al. 1986, 1987). The activity of the mice in an open field was analyzed with a video tracking device. No significant differences were detected between the mutant mice and their wild-type littermates (figure 3). Administration of the 5-HT1 agonist RU24969 stimulated locomotor activity in the wild-type mice, while it had no effect in the mutants (figure 3). These results indicate that the hyperlocomotor effect of RU 24969 is mediated by 5-HT1B receptors.

TABLE 1. Homologous recombination of 5-HT1B gene. Analysis of the recombination events and generation of homozygous mutant mice.

Targeting Vector		JA	JB
Length of homology		4.5 kb	6 kb
Neomycin-resistant colonies		62	49
Positives colonies		4	4
Positive colony used		JA7	JB13
Chimeric males		23	2
Chimeric males analyzed		8	2
GLT chimeric males		5	2
Offspring	Total	243	213
from	+ / +	61	54
heterozygote	+ / -	130	99
crosses	- / -	52	60

KEY: GLT = Germline transmission.

Anxiety

5-HT1 agonists such as RU 24969, eltoprazine, fluprazine, or 1-(3-trifluromethylphenyl)piperazine (TFMPP) have been reported to induce anxiogenic responses in rats and mice (Griebel et al. 1990; Pellow et al. 1987). To evaluate the level of anxiety of the 5-HT1B-minus mice, the light/dark choice test (figure 4) was used. The time spent in the lit compartment, as well as the number of transitions between the dark and the lit compartments, have been considered as indices of anxiety since they are increased by anxiolytic drugs. There were no significant differences between mutants and their wild-type littermates for either parameter, suggesting that the mutants have the same level of anxiety as

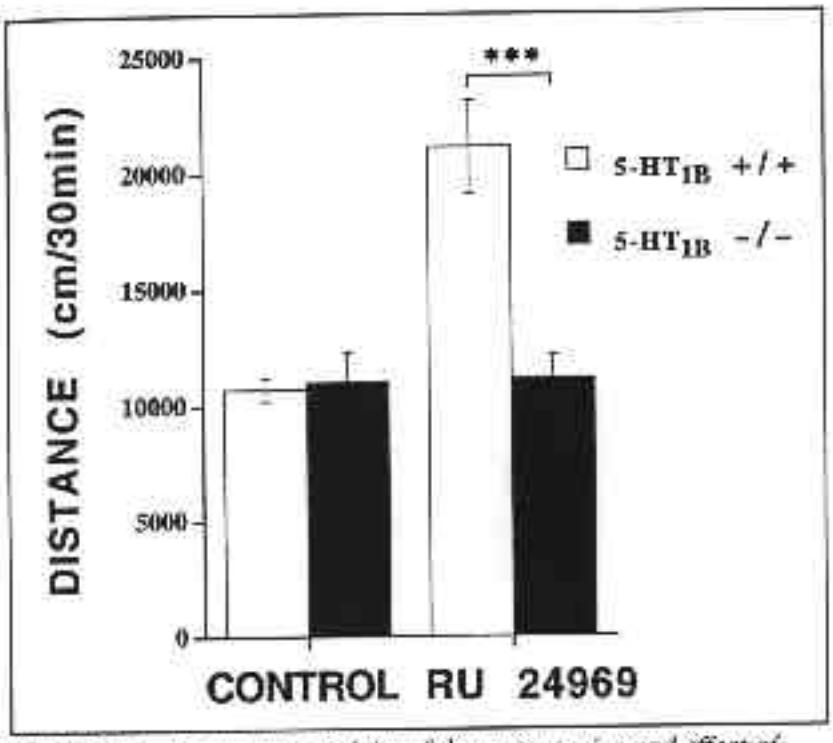

FIGURE 3. *Locomotor activity of the mutant mice and effect of RU 24969 (mean±SEM). Male mice were 12 weeks old at time of testing. They were housed alone in a standard cage with food and water, and kept on a 12/12-hr light dark cycle with light onset at 0700 hr. The mice were tested between 1000 and 1600 hours during the light phase. A video tracking device was used to measure the distance traveled by the animals during a period of 30 min in an open field. The two left columns correspond to the locomotor activity in control conditions of wild-type (N=12) and mutant mice (N=10) and the two right columns to the effect of RU 24969 (5mg/kg bodyweight, 40 min before testing) injected in the same mice 10 days after the first test. There was no significant difference between the wild-type mice and the mutant mice in control conditions as revealed by t-test analysis ($t_{(20)}$=0.22; not significant, NS). However, after RU 24969 treatment there was a significant difference between the two groups ($t_{(20)}$=4.18; ***=P<0.001).*

the wild-type mice in this test (figure 4). In the wild-type mice, RU4969 increased significantly the number of transitions between the dark box and the lit box, but it had no significant effect on the time spent in the lit box (figure4). In the mutants, RU24969 did not modify these parameters. The larger number of transitions displayed by the wild-type mice are probably a result of the increase in locomotion induced by RU-24969. The lack of response of the mutants demonstrates that the effect of RU24969 is mediated by 5-HT1B receptors. In contrast with earlier reports, RU 24969 had no anxiogenic effect. Such an effect might have been masked in the present experiment by the hyperlocomotor effect of this drug. A dose-response curve, as well as other anxiety tests, might reveal an effect of RU24969.

Aggression

A class of 5-HT1 agonists including eltoprazine and fluprazine have been termed serenics because of their antiaggressive properties (Flannelly et al. 1985; Mos et al. 1992; Olivier et al. 1986, 1989) and their effects have been suggested to be mediated by 5-HT1B receptors. The authors therefore investigated the aggressiveness of 5-HT1B-minus mice in a classical aggression test. After an isolation period of 4 weeks, test mice (resident) were analyzed for intermale aggression after introduction in their cage of a wild-type mouse that had been reared in a group (intruder) (figure 5). In this test, the latency of attack and the number of attacks performed by the resident during a 3-minute period were used as aggression indices. The mutant residents attacked the intruder faster than the wild-type or heterozygous residents (figure 5A). Furthermore, the number of attacks in the mutant group was significantly higher than in the wild-type or heterozygote groups (figure 5B). In addition, the intensity of attacks of the mutant residents was higher, as well as the number of tail rattlings preceding the attacks (not shown). Similar results were obtained in two tests performed 1 week apart. The level of aggressiveness was higher in the second test with both the wild-type and the mutant animals, in good agreement with previous reports showing that aggression increases with fighting experience (Lagerspetz and Lagerspetz 1971). A qualitative analysis of the attacks during the 3-minute test period revealed additional marked differences between wild-type and mutant mice (figure6). In the first test, 29 percent of the mutant residents attacked the intruder within less than 10 seconds after introduction of the intruder in the cage (impulsive attacks, figure 6A), while no wild-type or hetero-zygous mice attacked the intruder during that time interval. Conversely, 75 percent of the wild-type mice and only 21 percent of the mutants did

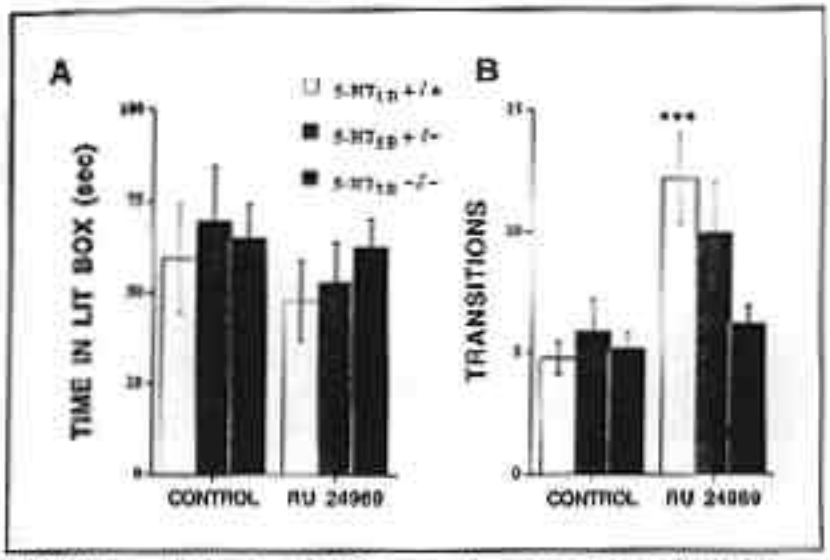

FIGURE 4. *Behavior of wild-type and mutant mice in the light-dark choice test. The mice were tested during the dark phase. (A) time spent by mice in the lit box (time in lit box: mean±SEM) (B) number of tunnel crossings from the dark box to the lit box (transitions: mean±SEM). The left columns (control) correspond to wild-type (N=22), heterozygous (N=12), and homozygous mutant mice (N=26) injected with saline vehicle and the right columns correspond to a different series of wild-type (N=23), heterozygous (N=12), and mutant mice (N=26) injected with RU 24969. ANOVA (factors=genotype and treatment) revealed no significant differences among groups for time spent by mice in the lit box (A): genotype [$F_{(2,111)}$=0.9, NS]; treatment [$F_{(1,111)}$=1.31, NS]; genotype x treatment interaction [$F(2,115)$=0.25, NS]. In contrast, there were significant differences among groups for the number of transitions (B): genotype [$F_{(2,111)}$=3.38, $P<0.05$]; treatment [$F_{(1,111)}$=15.62, $P<0.0001$]; genotype x treatment interaction [$F_{(2,111)}$=4.12, $P< 0.05$]. There were no significant differences among control groups for the transitions [$F_{(2,70)}$=0.49, NS] but the treatment with RU 24969 increased significantly the number of transitions in wild type mice [$t_{(42)}$=3.54, $P< 0.001$], not quite significantly in heterozygous mice [$t_{(22)}$=1.63, $P= 0.11$], and not in homozygous mutant mice [$t_{(50)}$=1.01, NS].*

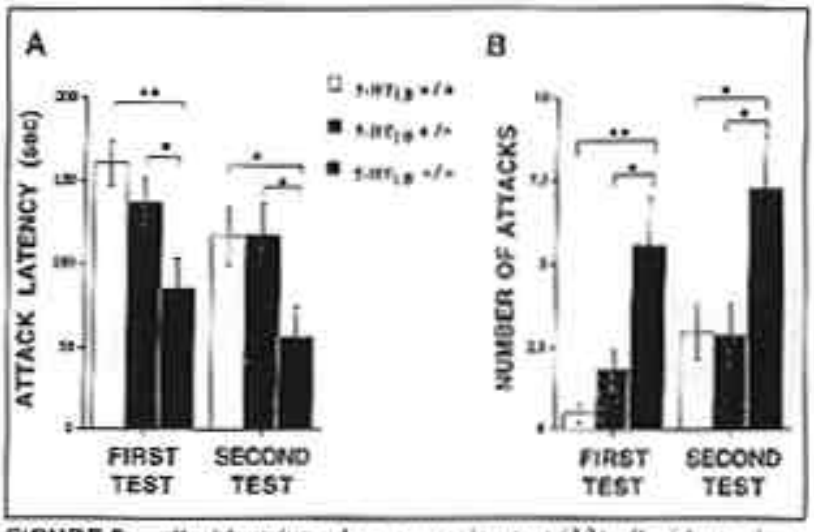

FIGURE 5. *Resident-intruder aggression test (13). Resident mice were: wild-type (N=12), heterozygotes (N=16), and mutant mice (N=14) and isolated during 4 weeks in transparent cages. (A) Attack latency (mean ± SEM): time between the introduction of the intruder and the first attack by the resident. ANOVA revealed significant differences for the attack latency both in the first test [$F_{(2,39)}$=5.28, P<0.01] and in the second test [$F_{(2,37)}$=3.49, P<0.05]. Further statistical analyses revealed significant differences between wild-type and mutant mice (first test, [$t_{(26)}$=3.19, P<0.01]; second test, [$t_{(23)}$=2.38, P<0.05]), heterozygotes and mutant mice (first test, [$t_{(28)}$=2.17, P<0.05]; second test, [$t_{(26)}$=2.26, P<0.05]), but not between wild-type and heterozygous mice (first test, [$t_{(26)}$=1.10, NS]; second test, [$t_{(23)}$=0.01, NS]). (B) Number of attacks (means ± SEM) during the 3-min session. ANOVA: (first test, [$F_{(2,39)}$=7.39, P<0.01]; second test, [$F_{(2,37)}$=4.48, P<0.02]. T-tests: wild-type versus mutants (first test, [$t_{(24)}$=3.19, P<0.01]; second test, [$t_{(22)}$=2.32, P<0.05]), heterozygotes versus mutants (first test, [$t_{(28)}$=2.16, P<0.05]; second test, [$t_{(26)}$=2.44, P<0.05]), wild-type versus heterozygotes (first test, [$t_{(26)}$=1.72, NS]; second test, [$t_{(25)}$=0.99, NS]).*

KEY: *=P<0.05; **=P<0.01.

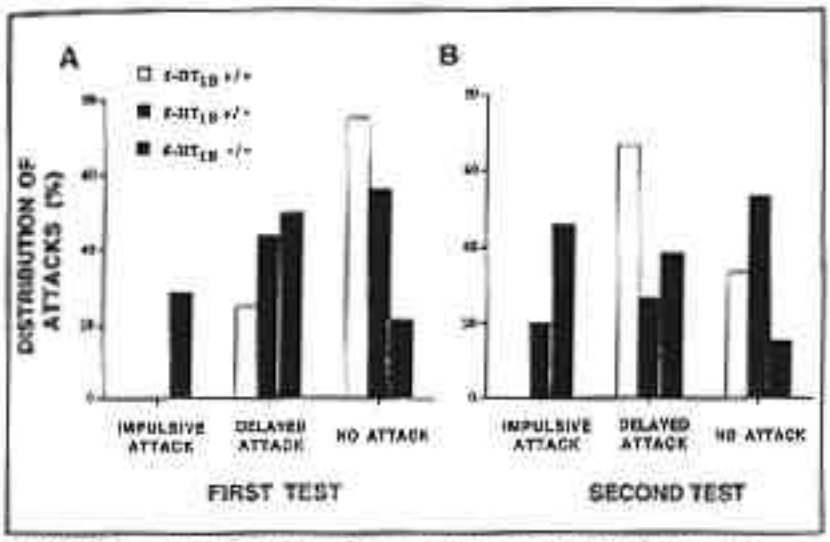

FIGURE 6. *Resident-intruder aggression test: distribution of attacks. Attacks were categorized as impulsive attacks (attacks within less than 10 sec), delayed attacks (attacks displayed between 10 and 180 sec), and no attacks during the 3-min test session. (A) correspond to the first test and (B) to the second test. The values presented here derive from the experiment described in figure 5.*

not attack during the 3-minute test. In the second test, the percentage of mutants displaying impulsive attacks was even higher (46percent), while still no wild-type animals performed such short latency attacks (figure6B). These results indicate that the 5-HT1B-minus mice are more aggressive than their wild-type or heterozygous littermates.

DISCUSSION

The authors have generated, by homologous recombination, mice lacking the 5-HT1B receptor. Autoradiographic data demonstrated the absence of 5-HT1B receptors in the homozygous mutants. Such mice develop and live apparently normally. Preliminary histological analyses of their central nervous systems did not reveal any obvious defect (not shown). A number of behaviors were analyzed that were thought to be modulated by activation of 5-HT1B receptors, such as locomotion, anxiety, and aggression (Fernandez-Guasti et al. 1992;

Green et al. 1984; Griebel et al. 1990; Kennett et al. 1987; Koe et al. 1992; Oberlander et al. 1986, 1987; Olivier et al. 1987; Pellow et al. 1987). Surprisingly, in the authors' test conditions, no differences were detected in the levels of basal locomotor activity or anxiety in the mutant mice. However, the hyperlocomotor effect of the 5-HT1 agonist RU 24969 was totally absent in the mutants, demonstrating that this effect is mediated by 5-HT1B receptors. A decrease in locomotor activity in the 5-HT1B-minus mice might therefore have been expected. The absence of such a motor effect in these experimental conditions suggests either that compensatory mechanisms occurred during development or, alternatively, that in normal "baseline" conditions the 5-HT1B receptor is not activated. Preliminary results indicated that the levels of the 5-HT1A and 5-HT1Da receptors were not altered in the mutants (not shown). The levels of 5-HT and catecho-lamines as well as the levels of aminergic receptors that are involved in motor control and which might have compensated for the absence of the 5-HT1B receptor are currently being analyzed. An alternative possibility, that 5-HT1B receptors are activated in response to environmental changes such as stressful situations, is appealing in light of the results obtained in the aggression test. When the mutants are group housed they do not appear to be more aggressive than grouped wild-type mice. However, after a month of isolation and in the presence of an intruder, the mutants are significantly more aggressive than the wild-type mice. In male mice, isolation and the presence of a conspecific male intruder have been shown to increase aggressive behavior (Lagerspetz and Lagerspetz 1971). The authors' results indicate that 5-HT1B-minus mice are more responsive to the isolation and the stress or fear generated by the intruder and suggest that 5-HT1B receptors might be activated in stressful situations such as those encountered in this test.

The increased aggressiveness of 5-HT1B-minus mice is in good agreement with the fact that a family of 5-HT1B agonists termed serenics have anti-aggressive properties (Olivier et al. 1987). These compounds were shown to decrease aggressive behavior in several animal models including isolation-induced aggression in mice (Olivier et al. 1989), resident-intruder aggression in rats (Flannelly et al. 1985; Mos et al. 1992), and maternal aggression in rats (Mos et al. 1992). The authors' results suggest that the 5-HT1B receptor is at least in part responsible for the antiaggressive properties of the serenics, but do not rule out a participation of other receptors with a high affinity for these compounds such as the 5-HT1A receptor. The effects of the serenics in the 5-HT1B-minus mice are currently being tested in order

to determine whether 5-HT1B receptors are the preferential target of these drugs.

Several studies have revealed an association between aggressive behavior and a reduction in the activity of the serotonergic system. In rodents and primates, aggressiveness is increased after inhibition of 5-HT synthesis (Higley et al. 1992; Vergnes et al. 1986) or destruction of serotonergic neurons (Molina et al. 1987). Mouse strains that display increased aggressiveness have low brain 5-HT levels (Maas 1962). In humans, impulsive aggressive behaviors have been associated with a deficit in central serotonin (Coccaro 1989). Cerebrospinal fluid (CSF) concen-trations of the 5-HT metabolite 5-hydroxyindole acetic acid (5-HIAA) is reduced in the brain of violent offenders (Brown et al. 1979), arsonists (Virkkunen et al. 1987), and people who committed violent suicide (Coccaro 1989; Mann et al. 1989). Interestingly, in one recent study, impulsive violent offenders had low CSF 5-HIAA levels, while offenders who premeditated their acts had high CSF 5-HIAA levels (Virkkunen et al. 1994). Similarly, in studies of suicide victims, only those who committed violent suicides exhibited low CSF 5-HIAA levels (Olivier et al. 1987; Pellow et al. 1987). These findings suggest a link between low serotonin levels and a lack of impulse control. The aggressive behavior displayed by the 5-HT1B-minus mice might be considered impulsive, since the mutants attacked much faster than the wild-type mice. In light of these results it is tempting to speculate that low serotonergic activity would result in a decreased activation of 5-HT1B receptors, which might trigger aggressive behavior.

The 5-HT1B receptor is localized both presynaptically on serotonergic terminals, where it inhibits the release of 5-HT, and postsynaptically on other nerve endings, where it might inhibit the release of various neuro-transmitters (for a review see Bruinvels et al. 1993). The antiaggressive effects of serenics are most likely mediated by postsynaptic receptors since they are not affected by lesions of serotonergic neurons (Sijbesma et al. 1991). Such postsynaptic receptors might be localized in the central gray, a brain structure involved in defensive behavior and response to fear (Fanselow 1991) and containing moderate densities of 5-HT1B receptors (Boschert et al. 1993; Bruinvels et al. 1993). Activation of the 5-HT1B receptor might be a component of the adaptation to fearful stimuli. Interestingly, several behavioral responses elicited by fear such as those observed in a flight situation (increased locomotion and decreased aggressiveness, sexual activity, and food intake) are also induced by 5-HT1B agonists. These behaviors as well as the level of

stress hormones are currently being analyzed in order to determine whether adaptation to stress or fear is altered in the absence of the 5-HT1B receptor.

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