

Altered Emotional States in Knockout Mice Lacking 5-HT1A or 5-HT1B Receptors

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Dysfunctions of the serotonergic system have been implicated in a number of psychiatric disorders including depression, anxiety and disorders of impulse control. To model these disorders we have generated mice with altered serotonergic systems. Specifically, we have created mice that lack or express reduced levels of two serotonin receptors: 5-HT1A and 5-HT1B receptors. These receptors are localized both on serotonergic neurons where they act as autoreceptors and on non-serotonergic neurons. As a result, the 5-HT1A and 5-HT1B receptors control the tone of the serotonergic system and mediate some of the postsynaptic effects of serotonin. Agonists of these receptors are currently used in the treatment of migraine and anxiety disorders. Mice lacking these receptors develop, feed, and breed

normally and do not display any obvious abnormalities. However, when analyzed in a number of behavioral paradigms, the 5-HT1A and 5-HT1B knockout mice display a number of contrasting phenotypes. While the 5-HT1B knockout mice are more aggressive, more reactive, and less anxious than the wild-types, the 5-HT1A knockouts are less reactive, more anxious, and possibly less aggressive than the wild-types. We are currently investigating with tissue-specific knockout mice which neural circuits are responsible for these phenotypes. [Neuropsychopharmacology 21:52S–60S, 1999] © 1999 American College of Neuropsychopharmacology. Published by Elsevier Science Inc.

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We will first review briefly the role of serotonin, specifically of the 5-HT1A and 5-HT1B receptors, in the modulation of aggressiveness and anxiety-related behaviors. Next, we will use our recently published data to propose that 5-HT1A and 5-HT1B receptors have opposite effects on mood control. Finally, we will present a strategy that may enable us to identify the neural circuits underlying the effects of the 5-HT1A and 5-HT1B receptors on emotional states.

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SEROTONIN AND AGGRESSIVE BEHAVIOR

Serotonin appears to play a key role in determining the vulnerability of humans and other animals to aggression and violence. An inverse relation between the activity of the serotonergic system and aggressive behavior has been found. In humans, low brain serotonin turnover rates and blunted neuroendocrine responses to serotonergic agonists (which might reflect a low central serotonin activity) have been found in individuals who engage in impulsive violent behaviors (Coccaro 1989; Coccaro et al. 1989; Linnoila et al. 1983). Antisocial personality disorder and type II alcoholism, two conditions displaying elevated levels of impulsivity and aggression, are associated with low levels of CSF 5-HIAA (the major metabolite of 5-HT) (Brown et al. 1982; Coccaro et al. 1997; Virkkunen et al. 1995; Virkkunen and Linnoila 1993). Likewise, impulsive, self-directed vio-

lence in the form of suicide has also been associated with low levels of brain 5-HT, CSF 5-HIAA (Asberg et al. 1976; Linnoila and Virkkunen 1992; Mann et al. 1989, 1990, 1996), and altered serotonin receptor binding (Arranz et al. 1994; Lowther et al. 1994; Pandey et al. 1995). Polymorphisms of the tryptophan hydroxylase enzyme (the rate-limiting enzyme for 5-HT synthesis) have also been associated with increased violence and suicide (Nielsen et al. 1994, 1998). Non-human primates with low CSF 5-HIAA exhibit increased impulsive and aggressive behavior, poorer social functioning, increased risk taking, and shorter life span. Critical to our understanding of impulsivity and aggression are the questions of how 5-HT can modulate these behavioral states, how 5-HT abnormalities are produced, and what are the effects of 5-HT dysregulation on the functioning of other systems. Clues to these questions in humans can perhaps be found in the study of aggressive and impulsive behavior in simpler model systems such as laboratory rodents in which serotonergic function can be modified. Depletion of brain serotonin in rats and mice increases aggressive behavior (Vergnes et al. 1986) whereas manipulations that increase brain serotonin reduce natural aggressive behaviors (Molina et al. 1987).

Pharmacological studies have pointed toward an involvement of specific serotonin receptors in modulating aggressive behavior. In particular, certain mixed agonists of the 5-HT_{1A} and 5-HT_{1B} receptors, have been termed "serenics" because of their selective ability to inhibit aggressive behavior without sedation (Mos et al. 1992; Olivier and Mos 1990; Olivier et al. 1989). The anti-aggressive effects of eltopazine (a prototypical serenic and a mixed 5-HT_{1A}/1B agonist) appear to be mediated primarily by 5-HT_{1B} receptors.

SEROTONIN IN DEPRESSION AND ANXIETY

Serotonin has been implicated not only in the control of aggressive behavior but also in the modulation of sleep, appetite, sexual behavior, motor functions, analgesia, and stress responses. The role of serotonin in modulating stress and anxiety responses has important clinical ramifications. A number of psychiatric disorders are thought to be caused by dysfunctions in the serotonergic system (Owens and Nemeroff 1994). For example, low levels of 5-HT and its metabolite, 5-HIAA, have been found in the brains and CSF of depressed subjects (Brown and Linnoila 1990; Mann et al. 1990; Risch and Nemeroff 1992; Widerlov et al. 1988). Abnormal endocrine responses to serotonergic releasing agents (e.g., fenfluramine) have also been reported for individuals with depression (Dinan 1996; Flory et al. 1998; Mann et al. 1995; O'Keane and Dinan 1991), panic disorder (Targum 1990a, b; Targum and Marshall 1989), and obses-

sive compulsive disorder (Hewlett et al. 1992; Lucey et al. 1992a, b). Depletion of serotonin (via dietary tryptophan restriction) provokes relapse of previously recovered depressed subjects (Delgado et al. 1991, 1993). Evidence for the involvement of serotonin in anxiety comes from the demonstration that 5-HT receptor agonists, such as m-CPP, can induce acute states of panic and anxiety in psychiatric patients and normal volunteers (Murphy et al. 1989), and that antidepressant drugs, especially selective serotonin reuptake inhibitors (SSRI), have therapeutic effects in multiple anxiety disorders (Lucki 1996). SSRIs enhance 5-HT neurotransmission by preventing the reuptake of 5-HT and increasing extracellular levels of 5-HT. However, the original hypothesis implicating serotonin in anxiety arose from observations that the inhibition of 5-HT release can produce anxiolytic-like behavioral effects (Tye et al. 1979) and may mediate some of the anti-anxiety effects of benzodiazepines in animals. More recent evidence for serotonin's involvement in anxiety derives from the development of buspirone as the clinical prototype of a series of compounds (azapirones: gepirone, ipsapirone, and tandospirone) that appear to be effective at treating generalized anxiety disorder. These compounds are 5-HT_{1A} receptor partial agonists (Perroutka 1985; Tunnicliff 1991).

5-HT_{1B} RECEPTOR

The 5-HT_{1B} receptor is expressed in many brain structures and has been shown to play a role in movement, sensory motor gating, and appetite control. Highest levels of receptor protein are found in the substantia nigra, globus pallidus, dorsal subiculum of the hippocampus, central grey, superior colliculus, lateral geniculate nucleus, and in the deep nuclei of the cerebellum (Boschert et al. 1994). The 5-HT_{1B} receptor is also found on the terminals of serotonergic neurons where it acts as an autoreceptor.

Important clues into the role of 5-HT_{1B} receptor in behavior come from studies of 5-HT_{1B} receptor KO mice (1BKO) (for a review see Stark and Hen in press). These mice are more aggressive than wild-type mice (Saudou et al. 1994), confirming the proposed link between 5-HT_{1B} receptors and aggressive behavior (Olivier and Mos 1990). 5-HT_{1B} KO mice are also more motivated to self-administer cocaine, suggesting a modulatory role of serotonin on the dopaminergic reward pathways (Rocha et al. 1998). A number of polymorphisms have been identified in the human 5-HT_{1B} gene (Huang et al. in press). One of these polymorphisms appears to be associated in two independent populations with antisocial personality disorder and alcoholism (Lappalainen et al. 1998).

5-HT_{1A} RECEPTOR

The 5-HT_{1A} receptor mRNA and protein are expressed primarily in the dentate gyrus and CA1 regions of the hippocampus, amygdala, entorhinal cortex, lateral septum, and raphe nuclei (Laporte et al. 1994). The existence of specific 5-HT_{1A} ligands has made it possible to study the function of this receptor (Hamon et al. 1990; Fletcher et al. 1996).

One problem that has plagued investigations of 5-HT_{1A} receptors and their behavior is the difficulty in determining whether presynaptic or postsynaptic receptors mediate these behavioral effects. Current pharmacological approaches to this question require examining the behavioral effects of 5-HT_{1A} agonists after the depletion of 5-HT by neurotoxic lesions or inhibition of 5-HT synthesis, or local intracerebral injections of 5-HT_{1A} agonists in different brain regions. However, all these approaches have demonstrated limitations. This is the reason why we are currently using a genetic strategy to assess the role of the pre- and postsynaptic 5-HT_{1A} receptors.

The current working hypothesis is that anxiolytic effects of 5-HT_{1A} agonists are mediated, at least in part, by presynaptic receptors. However, there are also reports that injections of 5-HT_{1A} agonists in postsynaptic structures have various effects on anxiety-related behaviors. In addition there are suggestions that 5-HT_{1A} agonists have different effects depending on the models of anxiety that are used.

The proposed role of 5-HT_{1A} receptors in modulating anxiety-related behaviors is also supported by behavioral studies of 5-HT_{1A} receptor knockout mice (1AKO). these mice show increased anxiety in the elevated plus maze, open field, and novelty-suppressed feeding paradigms (Heisler et al. 1998; Parks et al. 1998; Ramboz et al. 1998; our unpublished results).

Differential Localization of the 5-HT_{1A} and 5-HT_{1B} Receptors

The 5-HT_{1A} and 5-HT_{1B} receptors share the property of being "inhibitory receptors" expressed by both serotonergic and non-serotonergic neurons. They are, therefore, both autoreceptors which inhibit the activity of serotonergic neurons and heteroreceptors which inhibit the activity of non-serotonergic neurons (Figure 1). However, the analogy stops there since these two receptors are expressed in distinct subcellular compartments and are inhibitory via distinct mechanisms. Specifically, the 5-HT_{1A} receptor is expressed on somas and dendrites of neurons and its activation results in a decrease of neuronal firing, presumably via an interaction with G-protein-gated ion channels (McAllister-Williams and Kelly 1995). In contrast, the 5-HT_{1B} receptor

is transported toward axon terminals (Boschert et al. 1994) and its activation has been shown to result in an inhibition of transmitter release. The intracellular effectors of the 5-HT_{1B} receptor have been suggested to be ion channels (Ghavami et al. 1997). Recently, we have shown that the coding sequences of the 5-HT_{1A} and 5-HT_{1B} receptors are responsible for the specific addressing of these proteins toward somato-dendritic and axonal compartments, respectively (Ghavami et al. 1999).

1AKO AND 1BKO MICE EXHIBIT OPPOSING BEHAVIORAL PHENOTYPES

We have completed a basic behavioral characterization of the 5-HT_{1A} and 5-HT_{1B} knockout mice. The 1AKO and 1BKO mice were characterized in several tests of locomotion/exploratory behavior (open field), anxiety (ultra-sonic vocalizations, open field, and elevated plus maze), and aggressive behavior (resident-intruder test and maternal aggression test) (Saudou et al. 1994; Ramboz et al. 1998; Brunner et al. in press; Stark et al. 1999; our unpublished observations). Interestingly, these two knockout strains displayed opposite behavioral phenotypes in all of these tests. These results are summarized in Table 1.

Reactivity to Novel Environments and Anxiety

1BKO mice seem to be more reactive in almost all the behavioral tests we have performed to date, but they do not show hyperactivity in base-line conditions (activity in home cage). For instance, in open field tests the 1BKO are more active than the WT (Figure 2). However, this difference in activity appears to result from a difference in reactivity to the novel environment or in explor-

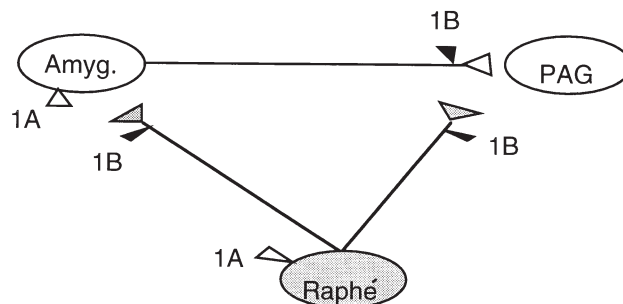


Figure 1. Localization of the 5-HT_{1A} and 5-HT_{1B} receptors. We have represented brain circuits that contain 5-HT_{1A} (1A) and 5-HT_{1B} (1B) receptors and that may be responsible for the effects of these receptors on mood control. Neurons projecting from the amygdala (amyg) to the periaqueductal gray matter (PAG) and serotonergic neurons projecting from the Raphé nuclei to the amygdala and PAG.

Table 1. 5-HT1B and 5-HT1A KO mice show opposite behavioral phenotypes

	1B KO	1A KO
Anxiety	Decreased	Increased
Reactivity	Increased	Decreased
Aggression	Increased	Decreased

Anxiety was assessed in the elevated plus maze, in the novelty suppressed feeding paradigm, and in the open field.

Reactivity was assessed in the open field and aggression was assessed in the resident intruder paradigm.

The aggressive behavior of the 5-HT1A knockout has not yet been fully characterized.

atory activity, since the difference disappears after repeated open field exposures (not shown). The 1AKO mice, on the contrary, show decreased reactivity/exploratory activity in the open field (Figure 2) (Ramboz et al. 1998).

In the open field, mice face a conflict between avoidance and exploration of the center which is more aversive than the peripheral area proximal to the walls. Therefore, the open field is sometimes also used as an anxiety test. We and others have shown that measures of behavior in the open field fall in two main factors: 1) time and distance traveled in the center, which may be related to anxiety; and 2) total distance traveled and rearings, which reflect general behavioral activation or exploration (Ramboz et al. 1998). The behaviors of the 1BKO and 1AKO appear to be opposite, with the 1BKO displaying more exploratory activity and less anxiety-related behavior, and the 1AKO displaying less exploratory activity and more anxiety-related behaviors. We have confirmed this interpretation by performing a number of additional anxiety tests: the elevated plus maze and the novelty-suppressed feeding test. Like in the open field, in these tests the 1AKO appears more anxious than the WT, whereas the 1BKO are less anx-

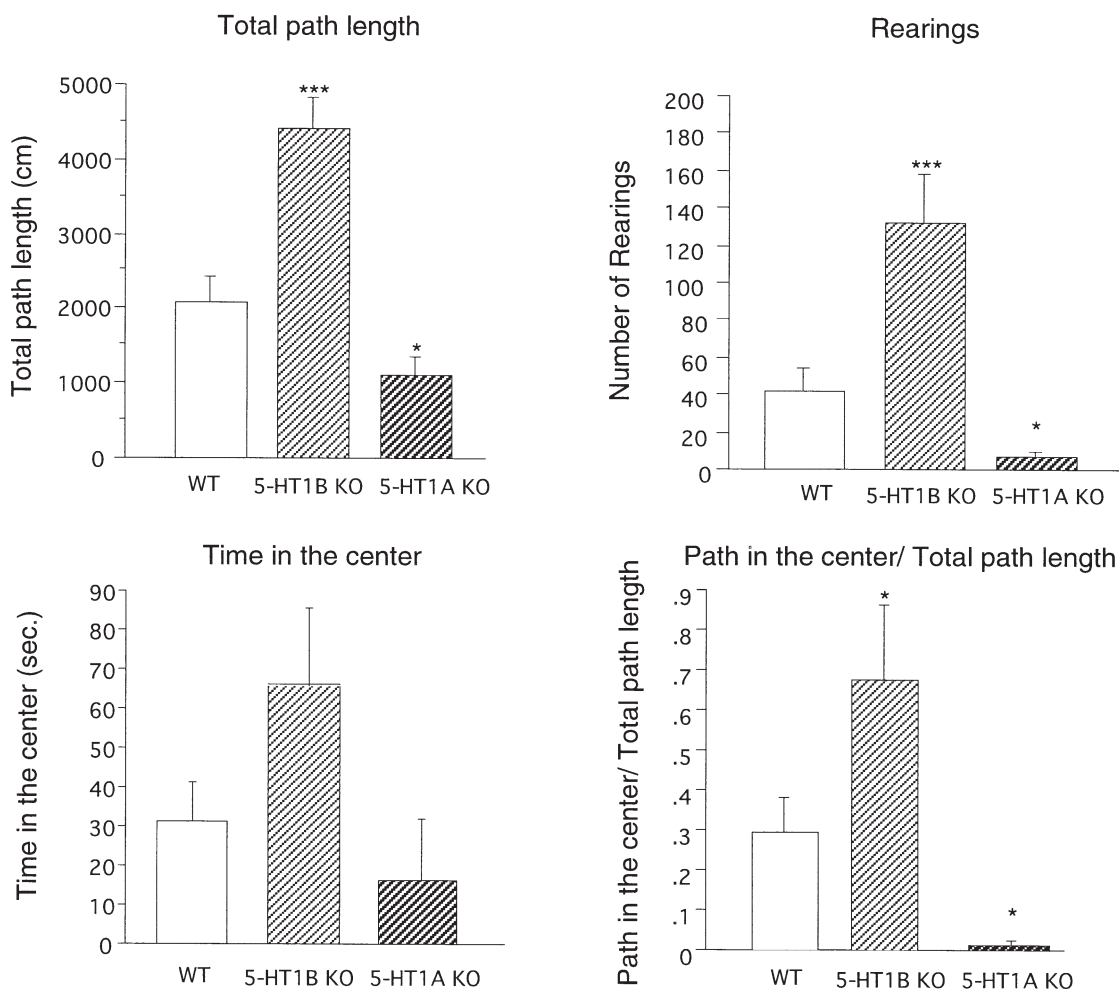


Figure 2. Activity in an open-field. Open-field activity was analyzed as described in Ramboz et al. 1998. Significant ANOVAs were found for total path length ($F_{2, 32} = 24.49$ $p < .001$), rearings ($F_{2, 32} = 13.40$ $p < .0001$), and for path in the center over total path length ($F_{2, 32} = 6.37$ $p < .01$). Post hoc test, * $p < .05$ and *** $p < .001$ when compared to WT group.

ious than the WT (Ramboz et al. 1998; our unpublished observations).

Increased Aggressive Behavior in 1BKO Mice

Our laboratory's studies with the 1BKO mice indicate that the mutant males are more aggressive in the resident intruder aggression test (Saudou et al. 1994). Isolation of male mice for one or more weeks results in increased aggression towards an intruder in their home cage (Brain 1975). In this test, the 1BKO mice attack more vigorously and more rapidly than the WT. A qualitative analysis of the attacks during the 3 min. test period, revealed marked differences between WT and 1BKO mice (Ramboz et al. 1996). Twenty-nine percent of the 1BKO residents attacked the intruder within less than 10 sec. after bringing the intruder into the cage, whereas no WT mice attacked the intruder during the same time interval. Conversely, 75% of the WT mice and only 21% of the 1BKO mice did not attack during the entire 3 min. test period. These results indicate that the 1BKO mice are more aggressive and perhaps more impulsive than the WT mice (Brunner and Hen 1997).

We, then, tested whether this difference in attack latency was due to a differential response of WT and 1BKO mice to isolation, or whether it represented a difference in territorial aggression. Males of both genotypes were either isolated or housed for one week with one female. On the day of testing, the female was removed and a male intruder that had been group-housed was introduced into the cage. In both paradigms, male mice attacked faster than WT mice (our unpublished observations).

In addition, we found that aggressive behavior was also higher in 1BKO females. However, 1BKO females, like wild-type females, displayed aggressive behavior toward intruders only in the presence of their pups (unpublished observations).

Gene Expression Changes in 1AKO and 1BKO Mice

In addition to changes in aggressive behavior, the 1BKO mice also display differences in other traits including increased vulnerability to cocaine and alcohol consumption (Crabbe et al. 1996; Rocha et al. 1997, 1998). Cocaine elicits a larger locomotor response and appears to be more rewarding in KO than in WT mice. These effects appear to result from both pre- and post-synaptic changes in the dopaminergic pathways. Specifically, cocaine elicits larger increases in extracellular dopamine levels in the nucleus accumbens of the KO than in the WT. In addition, levels of D1 receptor mRNA and protein, as measured by in situ hybridization and autoradiography, are increased in the KO.

Other changes in gene expression have also been identified in the 1BKO. In particular, the *fosB* protein, which is induced by repeated cocaine exposure, was up-regulated in the nucleus accumbens of the 1BKO mice *before* they had ever been exposed to cocaine (Rocha et al. 1998). We interpreted these changes in gene expression that occurred in the 1BKO mice to mean that the congenital absence of the 5-HT1B receptor results in compensatory or adaptive changes. Variations in gene expression in the nucleus accumbens may be correlated to increased vulnerability to substance use. Other compensatory changes in gene expression in distinct neural circuits may underlie the differences in aggressive and anxious-like behaviors displayed by 1BKO and 1AKO mice. The 1BKO and 1AKO mice may therefore be a useful model to identify candidate genes that are involved in vulnerability towards aggressive and/or anxious-like behaviors.

TISSUE SPECIFIC RESCUE OF THE 5-HT1B AND 5-HT1A RECEPTORS

In order to identify which neuronal circuits are responsible for the phenotypes of 1AKO and 1BKO mice, we have developed a strategy that will enable us to re-express in the knockout mice the missing receptor, but only in a subset of the structures where that receptor is normally found. This strategy is based on the ability of the Cre recombinase to excise a transcriptional stop DNA sequence flanked by loxP sites (Tsien et al. 1996). When designing the 5-HT1B and 5-HT1A targeting vectors, we flanked the neo-stop cassette containing the *pGK-neo* gene and transcriptional stop sequences with loxP sites so that these sequences could be removed by the Cre recombinase either at the ES cell stage or in the knockout mice (Figure 3) (Stark et al. 1998). We found that, in both cases, removing the neo-stop sequences resulted in reactivation of the knocked out receptor in the animal. This set the stage for using various Cre-expressing transgenic lines to restore receptor expression in specific tissues. We have successfully performed such an experiment in the case of the neo-stop 5-HT1A KO mice. Specifically, after breeding 5-HT1A KO mice with transgenic mice expressing the Cre recombinase under the control of the CaM Kinase II promoter 5-HT1A receptor expression was rescued in the CNS (Figure 3). Additional Cre lines exist which function specifically in hippocampus and amygdala (I. Mansuy and X. Zhuang, unpublished results). We are also currently generating a line that expresses the Cre recombinase under the control of the promoter of the serotonin transporter. Such a line should enable us to selectively rescue the 5-HT1A or 5-HT1B autoreceptors.

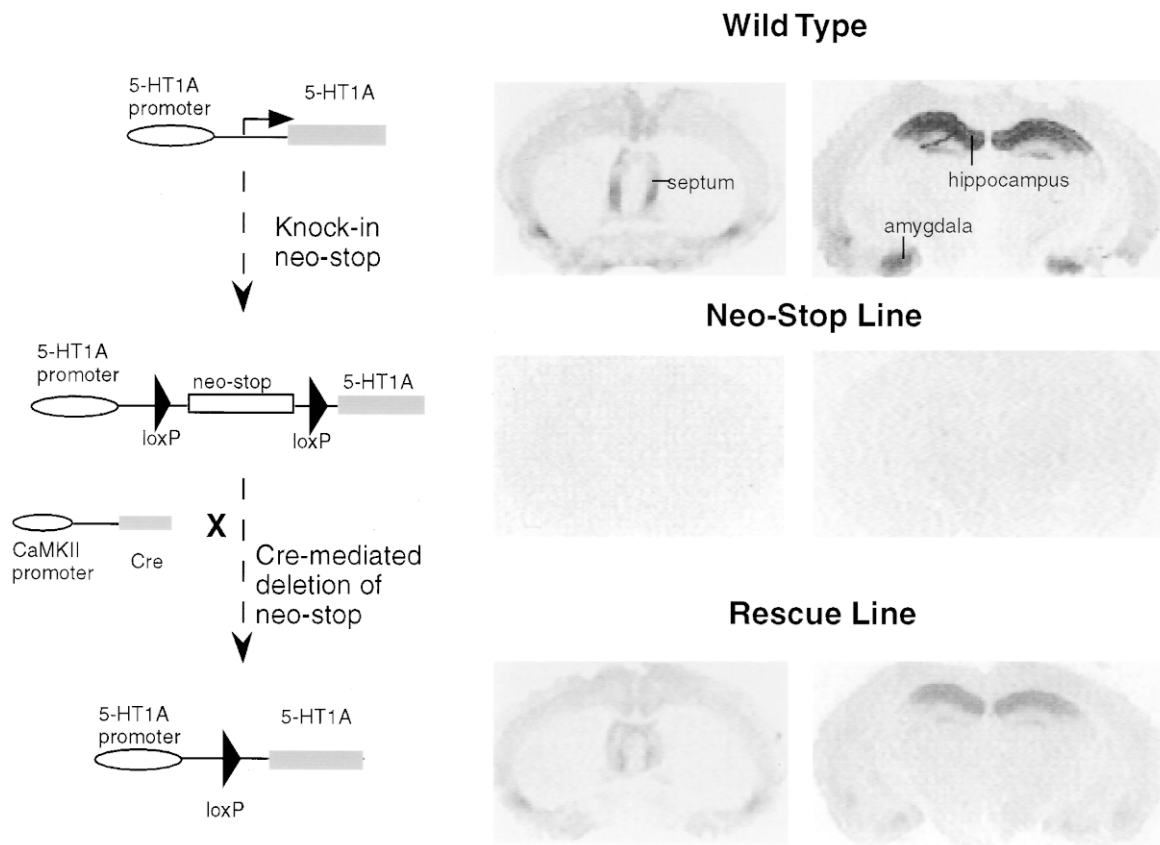


Figure 3. Tissue-specific rescue of the 5-HT1A receptor. Knockout mice were generated by inserting a removable stop cassette in the 5'-untranslated sequence of the 5-HT1A gene (Stark et al. 1998). These Neo-stop mice did not express any detectable level of 5-HT1A receptor as assessed by autoradiography with ^{125}I -MPPI (Ramboz et al. 1998). To rescue 5-HT1A receptor expression the Neo-stop mice were bred with transgenic mice that express the Cre recombinase under the control of the CaMKII promoter (a gift from I. Mansuy and E. Kandel). The resulting rescue mice re-express the 5-HT1A receptor in hippocampus as well as in a number of other brain regions.

Models That Can Be Tested with the Tissue-Specific Rescues

Our goal is to identify the neural circuits that are responsible for the phenotype of the 1AKO and 1BKO mice. The fact that at least three different phenotypes are associated in each of these KO mice and the fact that all three phenotypes are opposite in the 1AKO and 1BKO mice, might suggest a causal link between these phenotypes. One example of such a causal link is illustrated in Model 1 (Figure 4). In this scenario, 5-HT1A receptors in brain area A and 5-HT1B receptors in brain area B (A and B can be different or identical) are involved in the modulation of reactivity and/or anxiety which may be expressed by brain area C (again C can be different from A and B, or identical). A consequence of the hyper-reactive or less anxious phenotype (5-HT1B KO mice) may be an increase in aggressive behavior (which could result in activation of brain area D). Alternatively, a model without causal links between the reactive/anxious phenotype and the aggressive one is also possible (see Model 2). In the second model, in-

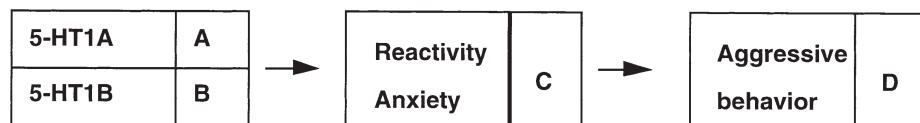
dependent circuits are responsible for the different phenotypes. These two models are, of course, not mutually exclusive and it is conceivable that a causal relation exists in one of the knockouts and not in the other.

The tissue-specific rescue strategy should enable us to discriminate between these models. Specifically, if we can rescue one of the behaviors but not the others, we will favor Model 2. If, on the other hand, we rescue all behaviors at once we will favor Model 1.

CONCLUSIONS

Using the gene targeting technology, we have generated new strains of mice lacking specific serotonin receptors and displaying a variety of phenotypes. Some of these phenotypes appear to reflect the known functions of these receptors which, until recently, have been investigated only with classical pharmacological tools. For example, agonists of the 5-HT1A and 5-HT1B receptors have been shown to decrease anxiety and aggres-

Model 1.



Model 2.

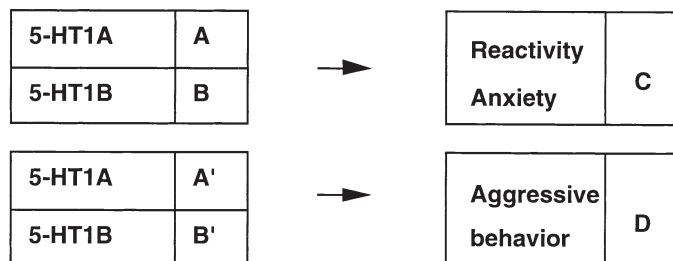


Figure 4. Two hypothetical models accounting for the multiple phenotypes of the 5-HT1A and 5-HT1B knockout mice. Model 1: Changes in aggressive behavior are a consequence of changes in reactivity and/or anxiety. Model 2: Changes in aggressive behavior are independent of the changes in reactivity and/or anxiety. A, A', B, B': brain structures expressing the 5-HT1A and/or 5-HT1B receptors. C, D: brain circuits involved in reactivity/anxiety and aggressive behavior, respectively.

sive behavior. At first glance, the aggressive and anxious phenotype of the 5-HT1B and 5-HT1A knockout mice might, therefore, seem predictable. However, a closer inspection of the relevant pharmacological studies reveals that the effects of these receptors on anxiety or aggressive behavior depend on where they are located in the brain. For example, activation of the 5-HT1A autoreceptor may decrease anxiety, whereas, activation of postsynaptic 5-HT1A receptors has been suggested to increase anxiety.

Classical knockouts lack both auto- and heteroreceptor; it is, therefore, not possible to predict which effect will predominate. Furthermore, antagonists often do not appear to mimic the phenotypes of knockout mice. For example, an acute administration of the 5-HT1B antagonist GR127935 has no effect on aggressive behavior or on cocaine self-administration (our unpublished results). Such differences between knockouts and antagonists might result from the fact that in knockout mice the receptor is absent throughout development. As a result, compensatory changes might occur, which will influence the phenotype of the adult animal.

To draw conclusions from the analysis of the mutant phenotype, it is, therefore, necessary to know the state of other receptors and systems that might have been up- or down-regulated to compensate for the absence of a particular receptor. To circumvent the issue of changes that might take place during the development of "classical" knockouts, we are currently developing an inducible knockout strategy (Stark et al. 1998). However, it is worth remembering that the inducible strategy remains fundamentally different from the acute administration of an antagonist, since this technique blocks mRNA production rather than protein function. Depending on the half-life of the protein of interest, it will take varying amounts of time before the protein is removed. For example, in the case of the 5-HT1B recep-

tor which has a half-life of eight days (Pinto and Battaglia 1994), it will take at least one month to lower receptor levels by more than 10-fold. Inducible knockouts are, therefore, more comparable to chronic than acute antagonist treatment.

We have also developed a new strategy that will allow to re-express in KO mice, the missing protein in only a subset of regions where that protein is normally found. Such "rescue" mice should allow us to determine which neural circuits are involved in specific effects of 5-HT receptors.

Although the inducible and rescue mice will soon be available for investigation, the classic knockout remains worthy of study because it mimics the situation found in genetic disorders where the mutation is present throughout development. For example, 5-HT1B or 5-HT1A knockout or heterozygous mice may be models of vulnerability to psychiatric disorders such as impulse control or anxiety disorders. These models will also enable us to characterize some of the molecular adaptations that may underlie altered emotional states.

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