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## Genetic approaches for understanding the role of serotonin receptors in mood and behavior

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Serotonin (5-hydroxytryptamine; 5-HT) is an ancient signaling molecule that has a conserved role in modulating mood and behavior. Integral to its pleiotropic actions is the existence of multiple receptors, expressed in distinct but often overlapping patterns within the brain and the periphery. The existence of ~14 mammalian receptor subtypes, many of which possess similar pharmacological profiles, has made assigning functional roles for these receptors challenging. This challenge has been further compounded by the revelation that a single receptor can have several different functions depending upon where and when it is expressed and activated, that is, in brain versus periphery, or at different developmental time points. This review highlights the contribution of genetic techniques to dissect the specific function of distinct serotonin receptor populations across the life course, with an emphasis on the contribution of different serotonin 1A receptor populations to mood and behavior. Similar approaches hold the promise to elucidate the functional roles of other receptors, as well as the interaction of serotonin with other neuroendocrine modulators of mood and behavior.

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### Introduction

The complexity of the serotonergic system is both exciting and daunting from a scientific perspective. There are 14 known human 5-HT receptors, more than in any other neuromodulatory system; receptor diversity is further amplified by alternative splicing, RNA editing, and heterodimerization [1,2]. Multiple receptor subtypes can be expressed in the same brain regions, and even within the same cells [2]. Functionally, 5-HT receptors often have

similar pharmacological profiles, making it difficult to identify specific antagonists or agonists for each receptor. Likewise, there is a dearth of selective antibodies to effectively label the receptors. Many studies therefore depend upon autoradiographic techniques, which lack cellular resolution and can also, in some cases, lack specificity.

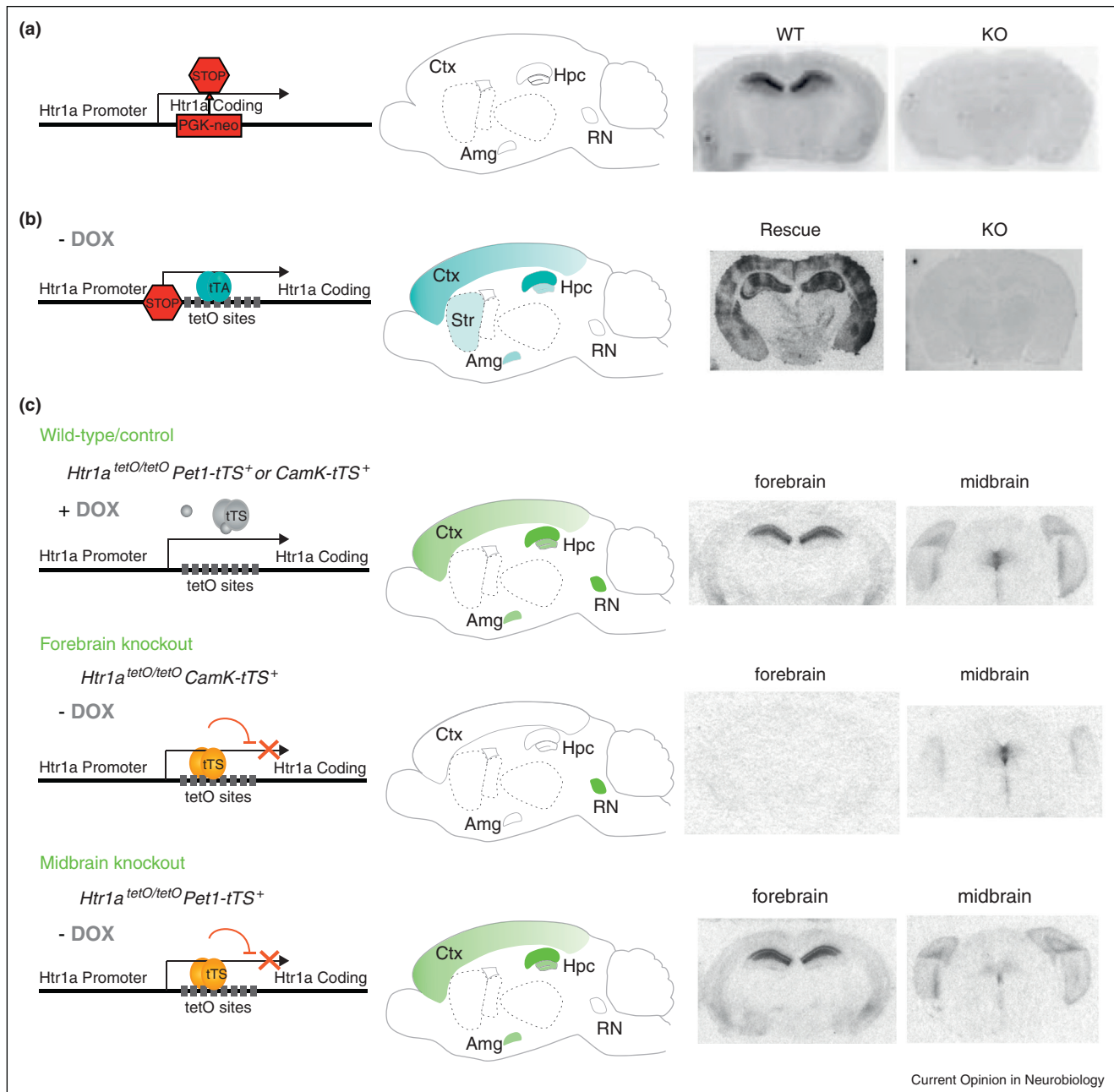
Given this complexity, genetic techniques have proven instrumental in determining the role of different 5-HT receptors in the brain. In particular, refined techniques have moved beyond general questions of receptor function to address how variation in levels of a particular receptor in different brain regions and/or at different developmental time points modulate mood and behavior.

In this review we discuss progress in genetic techniques used to develop mouse models of neuropsychiatric disorders over the last 15 years, beginning with global gene knockout. Using the serotonin 1A receptor (5-HT1A) as an example, we will discuss the benefits and pitfalls of different approaches and provide an overview of how refinement of genetic techniques has contributed to our understanding of the modulatory effects of 5-HT1A on mood and behavior. We also discuss the role of 5-HT1A in the neural circuits involved in anxiety and stress response and briefly highlight future opportunities for use of advanced techniques to investigate complex social behaviors, including mating and aggression.

### Serotonin 1A receptors

5-HT1A was the first serotonin receptor to be cloned, and has been intensively studied due to its potential involvement in psychiatric illness — anxiety disorders in particular [3–6]. 5-HT1A is expressed in the brain, spleen, and neonatal kidney [7–9]. Within the brain, it is expressed on neuronal somas and dendrites both as an autoreceptor and as a heteroreceptor. Its activation leads to hyperpolarization and reduced firing rates. As such, autoreceptors gate the firing of the dorsal and median raphe, impacting 5-HT release in forebrain projection areas [10]. Heteroreceptors exist in high density in limbic regions, including the hippocampus, septum, and cortex, as well as the hypothalamus [11,12]. Given the broad distribution of these receptors in behaviorally relevant brain regions, it has been difficult to determine which subpopulation(s) of 5-HT1A are responsible for anxiety and other behavioral phenotypes.

Figure 1



Evolution of genetic strategies for examination of 5-HT<sub>1A</sub> function. **(a) Germline gene knockout.** To constitutively eradicate 5-HT<sub>1A</sub> gene expression, the PGK-*neo* gene was inserted into an *Ascl* site located after the third transmembrane domain of the 5-HT<sub>1A</sub> gene [15]. This eliminated 5-HT<sub>1A</sub> expression in the whole brain throughout development. Ctx: cortex; Hpc: hippocampus; Amg: amygdala; RN: raphe nuclei; WT: wild-type; KO: knockout. **(b) Forebrain-specific 5-HT<sub>1A</sub> rescue on a knockout background.** A cassette containing a stop codon followed by a tetO binding site was inserted upstream of the mouse 5-HT<sub>1A</sub> gene, halting its transcription globally (KO). Upon addition of a second transgene encoding the tetracycline-transactivator protein (tTA), the stop codon was bypassed and expression of 5-HT<sub>1A</sub> was restored (Rescue). Because tTA was driven by the forebrain-specific  $\alpha$ -calcium-calmodulin-dependent protein kinase II ( $\alpha$ CaMKII) promoter, rescue of 5-HT<sub>1A</sub> was mainly restricted to the cortex and hippocampus, with low levels of expression in the striatum and amygdala (blue shading). The result was a mouse that lacked 5-HT<sub>1A</sub> everywhere except forebrain regions. Temporal regulation could be achieved through administration of doxycycline (DOX) in the food, which binds to tTA and inhibits its ability to activate transcription [19]. **(c) Tissue-specific knockout of 5-HT<sub>1A</sub>.** A dual transgene system was created by initially placing a tetO binding site into the 5' leader sequence of the 5-HT<sub>1A</sub> gene [20\*,21\*]. This was then bred with different lines expressing a tetracycline-controlled transcriptional suppressor (tTS) protein under the control of various promoters. Like tTA, temporal control of this system occurs through administration of doxycycline. In the presence of DOX, tTS fails to bind the tetO site, and normal transcription occurs. As a result, 5-HT<sub>1A</sub> transcription is suppressed only where the tTS is present, while WT patterns exist either if tTS is absent or if tTS is prevented from binding tetO with DOX. *Wild-type*: tetO insertion between the 5-HT<sub>1A</sub>R promoter and coding regions did

### Germline gene knockout

The use of homologous recombination to eliminate genes from the mouse genome provided an opportunity to selectively study the role of individual serotonergic receptors. Using this technique (Figure 1a), three groups independently discovered that deletion of the serotonin 1A receptor gene in mice yielded increased anxiety levels. In addition, an 'anti-depressed' phenotype was observed, characterized by increased active coping in the face of a stressor, such as increased struggling in the forced swim and tail suspension tests [13–15]. These studies are possibly consistent with the anxiolytic effects observed after 5-HT<sub>1A</sub> agonist administration, and led to further investigation of the potential role of 5-HT<sub>1A</sub> receptors in the pathophysiology and treatment of anxiety and depression [6].

While induction of null mutations through the use of knockout technology has been a pivotal development, it also has significant limitations. Specifically, knockout of a gene from the germline results in its complete deletion. Thus, the technique cannot be used to assess the role of different regional populations of a given receptor, or whether its phenotypic impact differs across the life course. Additionally, instances of complete gene deletion in humans are rare, limiting the translational impact of findings from mice to humans. Many of these limitations have been subsequently addressed with newer genetic technologies.

### Genetic rescue

Perturbations that affect the serotonin system have different effects during development versus adulthood, indicating that sensitive windows may exist for the effects of serotonin on various behavioral phenotypes. Transient pharmacological blockade of the serotonin transporter or of 5-HT<sub>1A</sub> during post-natal development has long lasting behavioral effects, demonstrating a critical role for serotonin in the maturation of emotional function [16–18]. As such, genetic techniques that enable not only spatial, but also temporal control of gene expression, were essential for identifying the necessity and sufficiency of different receptor populations at different time points in the modulation of serotonin-mediated behaviors.

Among the first approaches to parse the role of specific 5-HT<sub>1A</sub> subpopulations across development were selective forebrain rescue techniques. Specifically, Gross and colleagues generated a tetracycline operator (tetO) mouse that enabled both spatial and temporal control of expression of 5-HT<sub>1A</sub> on a knockout background (Figure 1b) [19]. Studies of these mice demonstrated that selective

expression of 5-HT<sub>1A</sub> in the forebrain, with the tetracycline-controlled transactivator (tTA) under the control of the  $\alpha$ CaMKII promoter, was sufficient to reverse the anxiety phenotype observed in 5-HT<sub>1A</sub> knockout mice. In addition, they found that rescue of the anxiety phenotype required forebrain 5-HT<sub>1A</sub> expression during development but not during adulthood. Specifically, blocking 5-HT<sub>1A</sub> transcription until post-natal day (PND) 21 produced increased anxiety levels despite fully rescued forebrain receptors in adulthood. In contrast, inhibiting 5-HT<sub>1A</sub> production in adulthood beginning on PND80 did not influence anxiety, further indicating that the effects of 5-HT<sub>1A</sub> on anxiety represented a developmental phenotype [19].

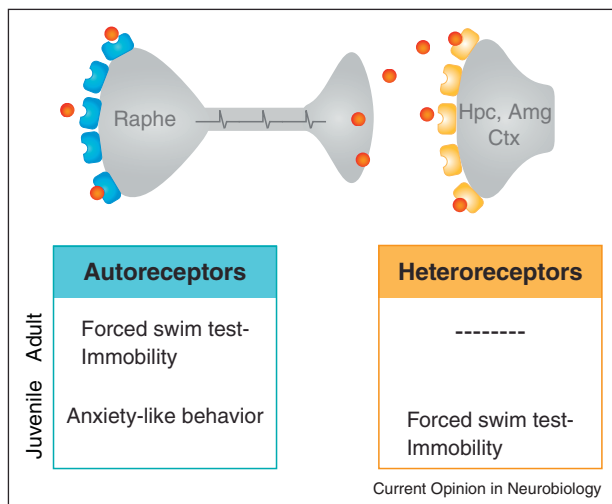
Experiments in which receptor levels are rescued in a subset of cells or brain regions are an ideal way to determine the behavioral sufficiency of different receptor subpopulations. However, there are a number of limitations to the specific approach employed here. Specifically,  $\alpha$ CaMKII-tTA-driven 5-HT<sub>1A</sub> expression resulted in forebrain patterns that were different from endogenous forebrain 5-HT<sub>1A</sub> receptor expression. This raises two complementary concerns: ectopic expression resulting in aberrant 5-HT<sub>1A</sub> mediated inhibition of cells that would not ordinarily express the receptor, and lack of 5-HT-mediated inhibition in cells that would normally express 5-HT<sub>1A</sub>. Therefore, another interpretation of these studies is that developmental inhibition of forebrain structures has an important impact on anxiety-related phenotypes, but the observed 5-HT<sub>1A</sub> mediated effects may not be prominent under natural conditions.

### Determining the necessity of receptor subpopulations

Newer strategies are currently being used to determine the necessity of different receptor subpopulations in the development and expression of anxiety behavior. Similar to the strategy outlined above, Richardson-Jones *et al.* [20<sup>••</sup>,21<sup>•</sup>] developed a tetracycline-sensitive transgenic system in which selected receptor populations could be *suppressed* in a temporally selective manner (Figure 1c). This technique was used to assess the role that both 5-HT<sub>1A</sub> auto-receptors and hetero-receptors play in anxiety and depression-related behaviors. These experiments revealed that whole life suppression of autoreceptors increased anxiety but did not affect helplessness behavior in the forced swim test, which is used as a measure of behavioral despair. On the other hand, whole life suppression of heteroreceptors increased learned helplessness behavior in the forced swim test, implicating heteroreceptors in depressive-like behaviors. Further, the increased

( **Figure 1 Legend Continued** ) not affect protein expression. 5-HT<sub>1A</sub> levels in forebrain and midbrain are thus comparable to wild-type levels. *Forebrain knockout*: The  $\alpha$ CaMKII promoter was used to drive expression of tTS. In the absence of DOX, tTS binds to the tetO sites between the 5-HT<sub>1A</sub> promoter and coding region, leading to specific knockout of forebrain 5-HT<sub>1A</sub> receptors. *Midbrain knockout*: The Pet-1 promoter was used to drive expression of the tTS, leading to raphe-specific knockout of 5-HT<sub>1A</sub> receptors in the absence of DOX.

Figure 2



Schematic diagram depicting developmental and adult impact of 5-HT<sub>1A</sub> receptors on anxiety and depression-related behavior. Autoreceptors (blue) are located in raphe nucleus, and heteroreceptors (yellow) are localized to forebrain regions including hippocampus (Hpc), amygdala (Amg), and cortex (Ctx). Endogenous autoreceptors, which limit levels of serotonin released by raphe neurons, affect anxiety-related circuitry in development, and circuitry mediating forced swim test behavior in the adult. Heteroreceptors affect forced swim test in development, but not anxiety-related behavior. Adapted from [21\*].

behavioral despair or anxiety exhibited by mice with knockdown of the heteroreceptor or autoreceptor populations, respectively, was not evident when the receptors were reduced only in adulthood, indicating that these are developmentally mediated phenotypes (Figure 2) [21\*].

The complementarity of the  $\tau$ TA and  $\tau$ TS systems provide valuable insights via their ability to determine sufficiency and necessity, respectively. Synthesis of the above findings indicates that developmental 5-HT<sub>1A</sub> autoreceptors are necessary for the establishment of normal anxiety behaviors. Likewise, endogenous hippocampal/cortical 5-HT<sub>1A</sub> heteroreceptors are necessary for normal stress coping. While ‘rescuing’ forebrain 5-HT<sub>1A</sub> appears to be sufficient to rescue the heightened anxiety levels in the 5-HT<sub>1A</sub> KO, these populations are not necessary for the establishment of normal anxiety patterns when other 5-HT<sub>1A</sub> populations are intact. This may suggest that there is an important balance of auto-receptors and hetero-receptors in the development of normal anxiety behaviors. Alternatively, as discussed above, forebrain rescue may be mediated by ectopic expression of 5-HT<sub>1A</sub> receptors and may be unrelated to the normal function of these receptors.

### Genetic strategies to model natural variation

Null mutations in serotonergic genes in the human population are rare. Manipulations of gene levels within physio-

logically relevant limits may therefore yield more insight into individual differences and disease risk, and thus provide greater translational impact. Since some natural human genetic variants lead to lower expression levels of genes of interest, the effects of these variants have been indirectly modeled by decreasing, but not eliminating, expression of target genes. In early studies, this involved use of mice that were heterozygous for the serotonin transporter as a model for the low-expressing human allelic variant [22]. More recently, partial 5-HT<sub>1A</sub> autoreceptor suppression has been used as a model for natural variation in 5-HT<sub>1A</sub> receptor levels. To achieve partial suppression, Richardson-Jones *et al.* [20\*\*] used the relatively weak Pet-1-driven  $\tau$ TS to decrease adult autoreceptor levels by ~30% beginning at PND50. This attenuated suppression results in 5-HT<sub>1A</sub> autoreceptor knockdown mice that mimic the natural variation observed in human 5-HT<sub>1A</sub> levels [23]. The authors found that animals with lower expression of autoreceptors were less vulnerable to stress and more responsive to antidepressant treatment. This would suggest that inherent differences in human autoreceptor levels may be an important predictor of both antidepressant efficacy and resilience to depression. Additionally, their results may account for the ‘anti-depressed’ phenotype observed in the 5-HT<sub>1A</sub> constitutive knockout mice. Their findings further suggest that augmentation of classical antidepressant therapies with drugs that antagonize 5-HT<sub>1A</sub> autoreceptor function may be a promising treatment option [24,25].

New work is also beginning to directly model human genetic variation in mouse models to determine the complex effects of specific polymorphisms. To date, such work has focused primarily on variants that alter protein structure. For example, introduction of the human SERT Gly56Ala mutation into mice impacts homeostatic regulation of 5-HT [26\*\*]. Mice carrying this variant exhibit elevated p38 Map kinase-dependent transporter phosphorylation, enhanced 5-HT clearance rates, and peripheral hyperserotonemia. These mice thus represent a model of hyperserotonemia observed in ~30% of autism spectrum disorder cases.

With advances in various genetic technologies, we are now moving beyond variants that affect protein structure and function to investigate human regulatory variants that have been implicated in mood and behavior. This includes the well-known serotonin transporter polymorphism (5-HTTLPR) [27], and the 5-HT<sub>1A</sub> G(-1019)C regulatory polymorphism (rs6295), which has been associated with differences in 5-HT<sub>1A</sub> levels in the raphe, depression risk, and antidepressant responsiveness [23,28]. Mouse models of such polymorphisms will elucidate their specific temporal and regional effects on gene expression, revealing the impact of common genetic variation in shaping individual differences in behavior and disease susceptibility.

### Circuitry underlying effects of 5-HT<sub>1A</sub> on mood and anxiety

Genetic techniques have also begun to shed light on how 5-HT<sub>1A</sub> affects the circuits underlying mood and anxiety. A more in-depth description of these circuits has been reviewed in detail elsewhere [29]. Substantial work has focused on structural and functional connections between the dorsal raphe nucleus (DRN), medial prefrontal cortex (mPFC) including the anterior cingulate cortex, and the amygdala, although the mesencephalic tectum system and the hippocampus have also been implicated in the modulation of stress responses and anxiety [29–31]. Variation in serotonergic modulation of this corticolimbic circuitry is associated with individual differences in personality measures and sensitivity to environmental threat, reflecting a related risk for psychopathology [32\*].

Serotonergic neurons within the raphe, especially the dorsal raphe, project to diverse forebrain regions, including the key corticolimbic structures involved in the regulation of stress, such as the mPFC, septum, extended amygdala, and hippocampus. Within the DRN, further topological organization suggests that specific subregions are preferentially involved in stress and anxiety [33]. Serotonergic tone, established through firing of serotonergic raphe neurons, is under tonic inhibitory control via somatodendritic 5-HT<sub>1A</sub> autoreceptors [34], and constitutive deletion of all 5-HT<sub>1A</sub> receptors increases basal extracellular 5-HT levels in some brain structures [35–37]. Specific reduction or removal of 5-HT<sub>1A</sub> from raphe neurons leads to heterogeneous, region-specific effects on both basal 5-HT levels and fluoxetine-induced 5-HT release [20\*\*,21\*]. For example, whole life knockout of the 5-HT<sub>1A</sub> autoreceptors increases basal 5-HT in the frontal cortex but not the ventral hippocampus [21\*]. More subtle reductions in adult autoreceptor levels, which may more accurately model naturally occurring variation, increase tonic firing rates of DRN neurons but do not affect basal levels of 5-HT in either the mPFC or ventral hippocampus [20\*\*]. In addition, activation of 5-HT<sub>1A</sub> heteroreceptors in the frontal cortex results in inhibition of serotonergic neurons in the raphe while activation of 5-HT<sub>1A</sub> in the hippocampus does not [34,38,39]. In 5-HT<sub>1A</sub> autoreceptor knockout or knock-down, fluoxetine challenge results in much greater elevation of 5-HT in the vHPC than mPFC [20\*\*,21\*]. This suggests that heteroreceptor feedback contributes substantially to buffering local 5-HT homeostasis in the mPFC, but autoreceptor feedback plays a greater role in the hippocampus. These findings exemplify the importance of genetic techniques in elucidating complex circuit level effects of specific receptor populations.

The role of forebrain 5-HT<sub>1A</sub> heteroreceptors is less well understood, partially due to their complexity of action and a lack of region-specific knockouts. As previously mentioned, mPFC heteroreceptors serve an important role in

negative feedback on the DRN [40], but heteroreceptor activation also affects glutamate-receptor mediated firing of other major mPFC neuron projections, including those to the amygdala [41–45]. Within the basolateral amygdala (BLA), an area critical for anxiety and fear processing, 5-HT<sub>1A</sub> activation inhibits both excitatory neurons and GABA interneurons [46]. As a result, the net effect of 5-HT release in this area is thought to be determined by the relative balance of 5-HT<sub>1A</sub> actions on excitatory and inhibitory neurons. Human studies have also suggested that 5-HT<sub>1A</sub> levels can modulate amygdala reactivity, although it remains unclear which population(s) of receptors mediate this effect [47,48]. Finally, 5-HT<sub>1A</sub> activation in the hippocampus is hypothesized to gate environmental information regarding the precise stimulus features of threatening stimuli [49–51], and many parts of this circuit can also affect stress-related phenotypes via indirect actions on the hypothalamic–pituitary–adrenal axis [52]. We have also shown that 5-HT<sub>1A</sub> is required for the effects of SSRIs on behavior and hippocampal neurogenesis [53], and our preliminary results indicate that 5-HT<sub>1A</sub> receptors located in the dentate gyrus are critical for these effects.

It is important to keep in mind that the effects of 5-HT<sub>1A</sub> activation on DRN–mPFC–amygdala circuitry are further influenced by both developmental plasticity and environmental sensitivity. In particular, neonatal blockade leads to increased anxiety during adulthood [17,18]. This suggests that activation of 5-HT<sub>1A</sub> during a developmental sensitive period plays a long-term role in establishment of anxiety circuitry. In addition, 5-HT<sub>1A</sub> receptor levels and sensitivity are influenced by exposure to stressful environments. For instance, social stressors, such as isolation, maternal separation, or peer rearing, have been associated with alteration of 5-HT<sub>1A</sub> levels in both rodents and primates [54–56]. In addition, chronic mild stress desensitizes 5-HT<sub>1A</sub> autoreceptor control of DRN firing via a glucocorticoid receptor-dependent mechanism [57–60]. Chronic antidepressants such as SSRIs have also been shown to desensitize 5-HT<sub>1A</sub> autoreceptors, and this desensitization has been proposed to contribute to the delayed onset of therapeutic efficacy of these compounds [24,25,61]. An active area of investigation is determining how these modifications to 5-HT<sub>1A</sub> contribute to complex, circuit level effects on behavior.

### Expanding the scope of genetic studies

Although advanced genetic examination of the serotonin system has focused primarily on its role in mood and anxiety, serotonin is implicated in a wide range of behaviors [62]. Refinement of these techniques [63\*] will be instrumental for identifying the receptor populations and temporal dynamics of serotonin's effects on complex behaviors, such as aggression and sexual behaviors. For instance, substantial pharmacological evidence suggests

that activation of 5-HT<sub>1A</sub> receptors facilitates male sexual behavior, and genetic techniques will elucidate the role of different 5-HT<sub>1A</sub> populations within the neural circuits underlying sexual behavior [64,65]. Conversely, germ-line knockout of the serotonin 1B receptor (5-HT<sub>1B</sub>) results in a highly aggressive phenotype [66]; the genetic techniques discussed above can now be applied to dissect the spatial and temporal role of 5-HT<sub>1B</sub> receptors in the expression of aggressive behavior.

## Conclusions

To date, we have made key advances towards understanding the necessity and sufficiency of various serotonin receptor populations that impact mood and anxiety, and have gained insight into the importance of investigating sensitive periods of gene action. However, these technological and scientific advances remain only a subset of those required to decode the complex evolutionary history underlying 5-HT's myriad roles. We still know little about the role of different receptor populations in modulating complex behaviors, or how 5-HT interacts with other neuromodulatory systems, including the endocrine and immune systems. Such work has implications not only for targeted therapies and our understanding of complex diseases, but also for providing policy guidelines for the use of serotonin-related drugs in pregnant mothers and young children.

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