cells were exposed to galactose (Fig. 3E). Occupancy at these galactose-inducible genes was dependent on gene activation because it was not detected in strains lacking the transcriptional activator Gal4p (Fig. 3E). These results confirm that Tpk1p generally becomes physically associated with actively transcribed genes and that occupancy occurs throughout the transcribed portions of these genes.

We then investigated whether Tpk2p occupies specific portions of the genome. Tpk2p was found almost exclusively associated with the promoters of ribosomal protein genes (Fig. 3F, fig. S3, and table S6). Gene occupancy by Tpk2p did not correlate with transcription rates throughout the genome, and Tpk2p remained associated with its target genes when cells were exposed to oxidative stress, which leads to reduced transcription of ribosomal protein genes (Fig. 3F). We did not detect Tpk3p occupancy on chromatin under the conditions used here (rich media, oxidative stress, and pheromone exposure). Although we have not shown that occupancy of genes by Tpk1p and Tpk2p regulates gene expression, previous studies have shown that PKA phosphorylates the Srb9 subunit of the Mediator complex (*19*) and that PKA activity regulates ribosomal gene expression (*20–22*). The idea that some PKA family members might operate, at least in part, through occupancy of actively transcribed genes is attractive because it might provide an efficient means for cells to respond to the nutrient environment at the level of gene expression.

Our finding that most activated MAPKs and PKAs in yeast become associated with distinct target genes changes our perception of the sites at which signaling pathways act to regulate gene expression. With the exception of Hog1p and p38, studies of the effect of signal transduction pathways on gene expression have not implied that the activities of MAPKs or PKAs involve genome occupancy. Although it is still possible that the phosphorylation of transcriptional regulators also occurs elsewhere in the cell, the detection of kinases by ChIP-Chip analyses at target genes suggests a model in which regulation by signal transduction kinases often occurs at the genes themselves. In this model, kinases become physically localized at specific sites in the genome by association with transcription factors, chromatin regulators, the transcription apparatus, nucleosomes, or nuclear pore proteins that are associated with subsets of actively transcribed genes (*5–10*, *19*, *23–25*) (fig. S4).

The kinases studied here associate with target genes in at least three different patterns, suggesting that there are different mechanisms involved in their association with genes. Tpk2p was found only at the promoter regions of its target genes. Hog1p occupancy was greatest at the promoters but also occurred to a limited extent within the transcribed regions of genes. Fus3p, Kss1p, and Tpk1p showed the greatest occupancy over the transcribed regions of genes. ChIP-Chip experiments show that DNA binding transcription factors and promoter-associated chromatin regulators generally occupy the promoters of genes, whereas transcription elongation factors, gene-associated chromatin regulators, certain histone modifications, and nuclear pore proteins are found enriched along the transcribed regions of genes (figs. S2 and S4). Preferential binding to these factors could explain the localization of the kinases.

Many features of signal transduction pathways are highly conserved in eukaryotes, so it is reasonable to expect that MAPKs and PKAs of higher eukaryotes may also be found to occupy genes that they regulate. Indeed, a human homolog of Hog1p, p38, occupies and activates the myogenin (*MYOG*) and muscle-creatine kinase (*CKM*) promoters during human myogenesis (*10*). The observation that components of many signal transduction pathways physically occupy their target genes upon activation should facilitate the mapping of the regulatory circuitry that eukaryotic cells use to modify gene expression in response to a broad range of environmental cues.

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SOM Text
Figs. S1 to S5
Tables S1 to S7
References

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Cortical 5-HT$_{2A}$ Receptor Signaling Modulates Anxiety-Like Behaviors in Mice

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Serotonin [5-hydroxytryptamine (5-HT)] neurotransmission in the central nervous system modulates depression and anxiety-related behaviors in humans and rodents, but the responsible downstream receptors remain poorly understood. We demonstrate that global disruption of 5-HT2A receptor (5HT2AR) signaling in mice reduces inhibition in conflict anxiety paradigms without affecting fear-conditioned and depression-related behaviors. Selective restoration of 5HT2AR signaling to the cortex normalized conflict anxiety behaviors. These findings indicate a specific role for cortical 5HT2AR function in the modulation of conflict anxiety, consistent with models of cortical, "top-down" influences on risk assessment.

The neurotransmitter serotonin modulates a diverse array of functions related to homeostasis and responses to the environment (*1–11*). Despite the importance of these observations, little is known about the brain structures or the postsynaptic receptors that mediate these effects of 5-HT.

The cortex, ventral striatum, hippocampus, and amygdala are highly enriched in 5HT2AR expression. These structures and their connecting circuits modulate the behavioral response to novelty and threat—behaviors that are typically thought to reflect the anxiety state of the organism (*12*). Given the importance of 5-HT

in modulating anxiety states, we sought to determine whether 5HT2AR signaling mediates 5-HT effects on anxiety-related behaviors. We therefore generated genetically modified mice with global disruption of 5HT2AR signaling capacity ($htr2a^{-/-}$ mice; fig. S1).

We examined anxiety-related behaviors of $htr2a^{-/-}$ mice in several paradigms. The open field (OF) is an arena that presents a conflict between innate drives to explore a novel environment and safety. Under brightly lit conditions, the center of the OF is aversive and potentially risk-laden, whereas exploration of the periphery provides a safer choice. We found that $htr2a^{-/-}$ mice explored the center portion of the environment (as a percentage of total exploratory activity) more than their intact $htr2a^{+/+}$ littermates did (Fig. 1A; $P < 0.01$). The $htr2a^{-/-}$ mice also exhibited more rearing—a maneuver that raises the animal onto its hind limbs, allowing greater visual perspective of the environment but also exposing the animal to greater risk (Fig. 1B; $P < 0.05$).

We examined the behavior of $htr2a^{-/-}$ mice in three other conflict paradigms: the dark-light choice test (DLC), the elevated plus-maze (EPM), and the novelty-suppressed feeding (NSF) paradigm. The DLC provides the chance to explore an arena consisting of dark (safe) and brightly lit (risky) areas. The total time of exploratory activity did not differ between genotypes (Fig. 1F); however, $htr2a^{-/-}$ mice explored the lit compartment to a greater extent than their $htr2a^{+/+}$ littermates, as measured by the percentage of total exploratory time spent in the light compartment (Fig. 1D; $P < 0.05$) and the percentage of total time spent in the light compartment (Fig. 1E; $P < 0.01$). The EPM has two "risk-laden" arms (open without sidewalls) and two "safe" arms (closed by sidewalls). The $htr2a^{-/-}$ mice explored the riskier portions of the EPM to a greater extent than the $htr2a^{+/+}$ mice, as measured by the percentage of entries made into the open arms (Fig. 1G; $P < 0.05$) and the percentage of time spent in the open arms (Fig. 1H; $P < 0.01$). As in the other tests, total locomotor activity was comparable between genotypes (Fig. 1I). We also examined the effect of $htr2a^{-/-}$ mice in the NSF test, which depends less on locomotor activity and is driven by hunger rather than exploratory drive. Consistent with other conflict tests, $htr2a^{-/-}$ mice exhibited a shorter latency to begin feeding in a novel environment (Fig. 1J) than the $htr2a^{+/+}$ mice ($P < 0.05$), with no differences in feeding activity in the home cage (Fig. 1L) or differences in weight loss (Fig. 1K).

In humans, anxiety and depression often coexist, and altered serotonin signaling has been implicated in the etiology of both disorders (*13*). Therefore, we examined the role of reduced 5HT2AR signaling in depression-related behaviors, as measured by the forced swim test (FST) and the tail suspension test (TST). These paradigms reflect the behavioral response to inescapable stress, not conflict, and are sensitive to antidepressant but not anxiolytic treatments (*14*, *15*). In both tests, rodents usually struggle to escape from these situations, interspersed with periods of immobility that has been interpreted as "behavioral despair" (*16*). When we used these tests to assess $htr2a^{-/-}$ mice, we found no difference in immobility when compared to their $htr2a^{+/+}$ littermates in either test

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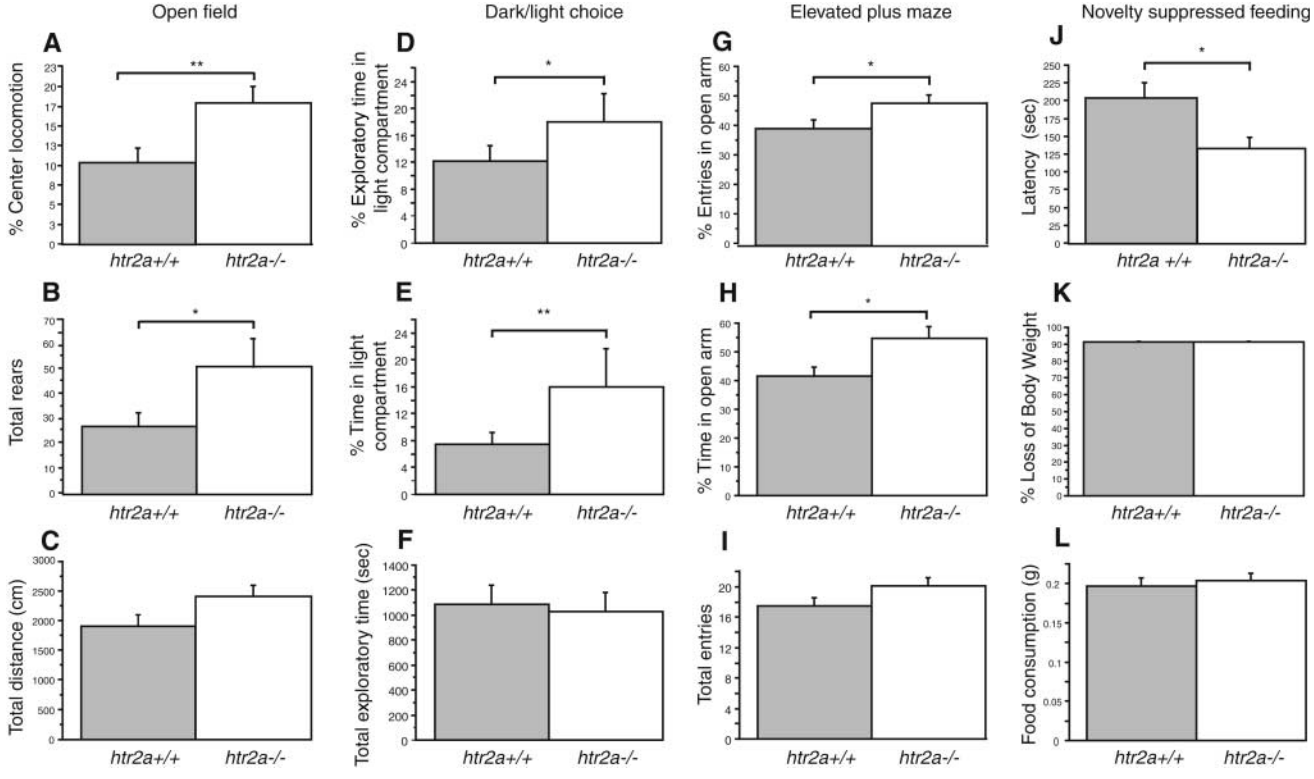

Fig. 1. $htr2a^{-/-}$ mice show decreased inhibition in conflict anxiety paradigms. (**A** to **C**) OF measures. (A) percentage of total locomotor activity occurring in the center of the arena. (B) Rearing. (C) Total distance traveled in the periphery and center. (**D** to **F**) DLC measures. (D) Percentage of total exploratory time spent in the light compartment. (E) Percentage of total time spent in the light compartment. (F) Total exploratory time (s). (**G** to **I**) EPM measures. (G) Percentage of total entries made into the open arms. (H) Percentage of time spent in the open arms. (I) Total number of entries into any arm. (**J** to **L**) NSF measures. (J) Latency to approach the food pellet (s). (K) Percentage of body weight lost after deprivation. (L) Amount of food consumed in home cage during 5-min period. *$P < 0.05$; **$P < 0.01$. Mean ± SEM, n = 26 to 39 mice per group.

(Fig. 2, A and B). These findings dissociated the low-anxiety phenotype of *htr2a*$^{-/-}$ mice from depression-related behaviors.

To assess the specificity of these findings, we examined other parameters that might influence their outcome. The effect of genotype on exploratory activity was specific to conflict tests because home cage activity did not differ between genotypes. Motor coordination, strength, and sensory processing were unimpaired. We also assessed whether anxiety differences might be due to abnormal hypothalamic-pituitary-adrenal function. Baseline concentrations of plasma corticosterone were comparable in each genotype. Likewise, following novel OF or FST exposure, the rise in corticosterone release was the same in each genotype (fig. S2). We surveyed the content of bioamines and their metabolites in several different brain regions to determine whether the absence of 5HT2AR signaling may have altered the functioning of these systems that are known to influence anxiety-related behaviors. We found no evidence of altered content or turnover of these transmitters as a function of genotype (fig. S5). We assessed the cortical expression of 30 different neurotransmitter receptors using quantitative real-time polymerase chain reaction and found no differences between *htr2a*$^{+/+}$ and *htr2a*$^{-/-}$ mice (with the exception of 5HT2AR expression; table S1).

Although we did not find differences at the mRNA level, differences of receptor expression or coupling might still exist in *htr2a*$^{-/-}$ mice. Because the $5HT_{2C}$ receptor (5HT2CR) has been implicated in anxiety (*17*), we quantified the amount of agonist-coupled 5HT2CR in *htr2a*$^{+/+}$ and *htr2a*$^{-/-}$ mice using [I^{125}]-DOI [1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane] autoradiography. No differences in the level of expression of 5HT2CR were observed (fig. S3).

Finally, we also investigated the cellular structure of the cortex, given the high level of expression of 5HT2AR in this brain area. No differences in cell number, mantle thickness, barrel field formation, or the expression of GABA (γ-aminobutyric acid)–containing neuronal markers were seen (fig. S4).

The relation between anxiety and fear is complex because each construct depends on partially overlapping circuitry. Acquisition of fear conditioning requires functional integrity of the hippocampus and the amygdala (*18*), whereas conflict anxiety behaviors implicate the hippocampus, amygdala, cortex, and periaquaductal grey (PAG) (*7*, *19*). To examine whether impaired 5HT2AR signaling in the hippocampus or amygdala disrupts fear-related behaviors, we performed cued and contextual fear-conditioning experiments using an aversive foot-shock stimulus (unconditioned stimulus) paired with a tone (conditioned stimulus). Before the tone-shock pairing, fear-related behavior (i.e., freezing) in the conditioning context was comparable between genotypes (Fig. 2C). After pairing of the conditioning context with the foot shock, we observed increased freezing in response to the context alone with no differences between genotypes (Fig. 2C). When presented with the conditioned tone in an unfamiliar context, mice of both genotypes (previously exposed to paired presentations of tone and foot shock) froze to a greater extent during the tone presentation than during the first minute spent in the new environment (Fig. 2D) and more than control mice previously exposed to unpaired presentation of these stimuli.

The dissociation from learned fear in these studies indicates that the low conflict anxiety shown by *htr2a*$^{-/-}$ mice is not affected by abnormal conditioned fear learning and consequently does not result from altered 5HT2AR signaling in the hippocampus or amygdala. If hippocampal and amygdala functioning is intact, this finding suggests that impaired 5HT2AR signaling in PAG or cortex might underlie their conflict anxiety phenotype. However, the PAG acts to modulate "escape" or freezing behaviors (*20*), which appear to be unaffected in *htr2a*$^{-/-}$ mice. This led us to reason that reduced cortical 5HT2AR signaling may underlie our observed phenotype. We thus attempted to rescue normal conflict behavior in *htr2a*$^{-/-}$ mice by selective restoration of 5HT2AR function to the cortex.

To restore 5HT2AR signaling in the cortex, we capitalized on the methodology used to create our global knockout—namely, an insertion mutation between the promoter and the coding region that blocks transcription and translation of the *htr2a* gene (Fig. 3A and fig. S1). Unidirectional lox-P sites flank the insertion mutation, and under the action of the bacteriophage P1 recombinase, Cre, the inserted sequence can be removed, thus restoring receptor expression under the control of its endogenous promoter (Fig. 3A).

The gene *Emx1* is expressed in the forebrain during early brain maturation (*21*) and has been used to drive *Cre* expression and control forebrain gene expression in other systems (*22*). We crossed *htr2a*$^{-/-}$ mice with mice expressing *Emx1-Cre* to selectively restore 5HT2AR expression to the forebrain while leaving other sites of 5HT2AR expression blocked (*htr2a*$^{-/-}$ × *Emx1-Cre*).

Receptor autoradiography was performed using the agonist [^{125}I]-DOI. In *htr2a*$^{-/-}$ × *Emx1-Cre* mice, we observed that 5HT2AR expression was restored principally in layer V of the cortex and in a closely associated structure, the claustrum (*23*). No measurable expression was seen in the hippocampus, a structure expressing *Emx1*. We found no significant 5HT2AR mRNA expression in the striatum of *htr2a*$^{-/-}$ × *Emx1-Cre* mice as compared to *htr2a*$^{-/-}$ mice (fig S6A). Likewise, the thalamus and other subcortical structures that express 5HT2AR, but not *Emx1*, were devoid of expression (Fig. 3C).

To determine whether compensatory alterations in 5HT2CR expression were present in *htr2a*$^{-/-}$ mice or *htr2a*$^{-/-}$ × *Emx1-Cre* mice, we assessed 5HT2CR mRNA expression (fig. S6B). We found no evidence of 5HT2CR alterations in *htr2a*$^{-/-}$ × *Emx1-Cre* mice.

To verify the functionality of the restored cortical 5HT2AR, we assessed the electrophysiological response of cortical slices to 5-HT. We performed whole-cell recordings of layer V pyramidal neurons in cortical slices from *htr2a*$^{+/+}$, *htr2a*$^{-/-}$, and *htr2a*$^{-/-}$ × *Emx1-Cre* mice. There were no significant differences among these groups in resting potential, input resistance, and spike amplitude. However, 5-HT

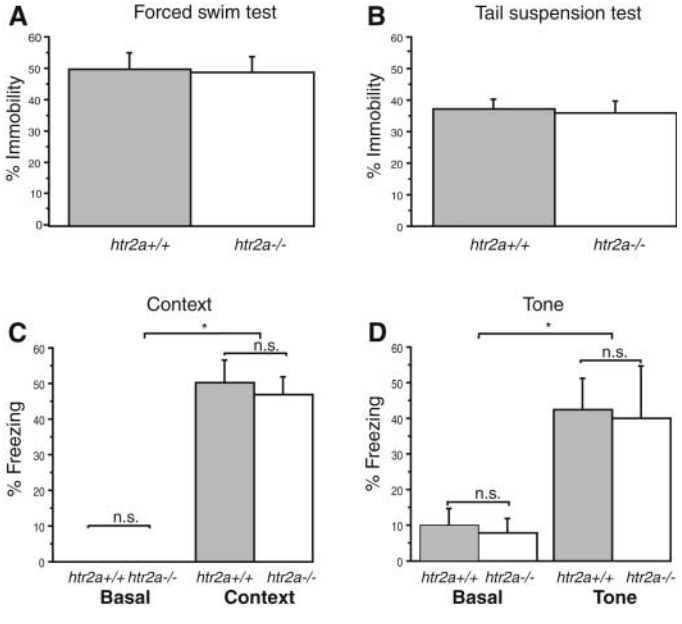

Fig. 2. Depression and fear-related measures are not affected in *htr2a*$^{-/-}$ mice. (**A**) FST: Percentage of time spent immobile during the 4-min test. (**B**) TST: Percentage of time spent immobile during the 7-min test. (**C** and **D**) Fear-conditioned learning. (C) Mean percentage of freezing in basal condition measured during the first 60 s in the first day of exposure and mean percentage of freezing time during context test. (D) Percentage of freezing time in new context without and during the presence of the cue test. Mean ± SEM, $n = 12$ to 40 mice per group for all tests.

produced robust increases in spontaneous excitatory postsynaptic currents (sEPSCs) in pyramidal neurons from *htr2a*$^{+/+}$ and *htr2a*$^{-/-}$ × *Emx1-Cre* mice, but not in *htr2a*$^{-/-}$ mice (Fig. 3D; $P < 0.0001$). The selective 5HT2AR antagonist, MDL 100907, blocked the 5-HT–elicited increases in sEPSC frequency, but had no effect in *htr2a*$^{-/-}$ mice. Norepinephrine (NE) increased sEPSCs to an equal extent in all

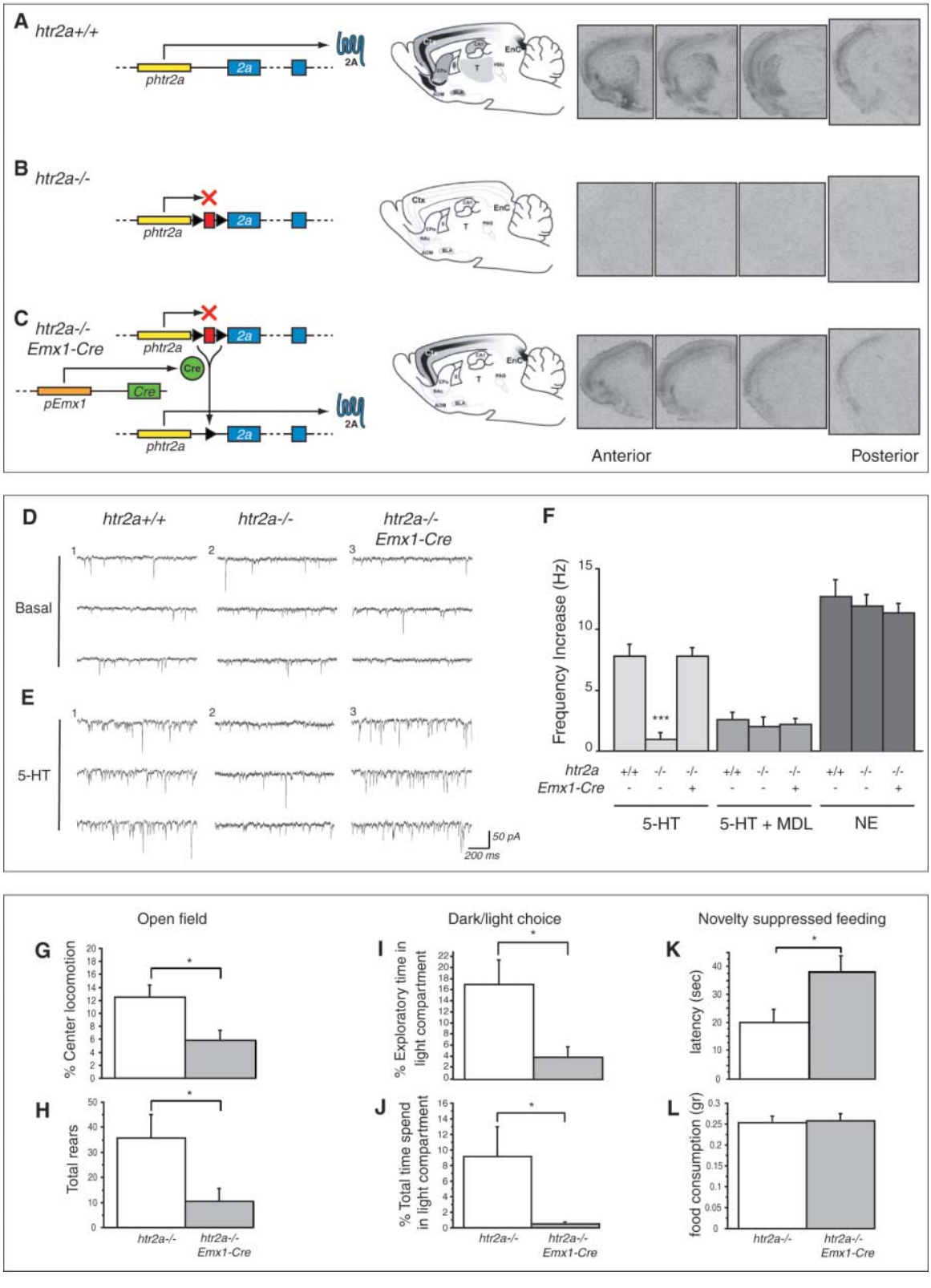

Fig. 3. Cortical restoration of 5HT2AR function normalizes conflict anxiety in *htr2a*$^{-/-}$ mice. (**A** to **C**) Filled blue boxes represent exons of *htr2a* gene. Narrow boxes labeled with p*htr2a* or p*Emx1* represent the endogenous promoters for each gene. Serpentine symbol indicates the *htr2a* gene product. (Left) (A) Schematic of the wild-type *htr2a* locus. (B) Lox-p (triangles)–flanked cassette (red box) inserted upstream from the first initiation codon of the *htr2a* gene blocks transcription and translation. (C) Expression of Cre under the control of the *Emx1* promoter interacts with the lox-p sequences to remove the cassette and restore expression of *htr2a* gene. (Middle) Schematic representation of the pattern of expression of 5HT2AR in *htr2a*$^{+/+}$ (A), *htr2a*$^{-/-}$ (B), and *htr2a*$^{-/-}$ × *Emx1-Cre* (C) mice. Abbreviations: CTX, cortex; T, thalamus; CA1, CA1 region of hippocampus; PAG, periaquaductal grey; CPu, caudate-putamen; NAc, nucleus accumbens; BLA, basolateral nucleus amygdala; AOM, anterior olfactory nucleus (medial); EnC, entorhinal cortex. (Right) Autoradiography with [^{125}I]-DOI in *htr2a* $^{+/+}$ (A), *htr2a* $^{-/-}$ (B), *htr2a*$^{-/-}$ × *Emx1-Cre* (C) mice shown at representative anterior and posterior slices. (**D**) Voltage-clamp recordings under basal conditions from (1) *htr2a*$^{+/+}$, (2) *htr2a*$^{-/-}$, and (3) *htr2a*$^{-/-}$ × *Emx1-Cre* mice. (**E**) Voltage-clamp recordings of the peak response to bath-applied 5-HT (100 μM, 1 min) in the same neurons. (**F**) Bar graph showing changes in sEPSC frequency in neurons from *htr2a*$^{+/+}$ and *htr2a*$^{-/-}$ × *Emx1-Cre* mice, using 5-HT (100 μM), 5-HT (100 μM) + MDL 100907 (100 nM), and NE (100 μM). (**G** and **H**) OF measures. (**I** and **J**) DLC measures. (**K** and **L**) NSF. See Fig. 1 for explanations. *$P < 0.05$, ***$P <$ 0.0001. Mean ± SEM, $n = 10$ to 12 neurons per genotype, $n = 13$ to 14 mice per group for behavioral experiments.

groups, indicating that the loss of 5HT2AR signaling had no effect on the response to other bioamines (Fig. 3E).

To determine whether restored cortical 5HT2AR signaling was sufficient to normalize conflict behavior, we used three paradigms that previously elicited a robust phenotype in *htr2a*$^{-/-}$ mice: OF, DLC, and NSF. In the OF, mice with cortical restoration of 5HT2AR signaling exhibited wild-type levels of anxiety-like behavior as measured by the percentage of exploratory activity in the center of the field (Fig. 3G; $P < 0.05$) and rearing (Fig. 3H; $P < 0.05$). Similar effects of the cortical 5HT2AR rescue on anxiety were seen in the DLC [decreased percentage of exploratory time (Fig. 3I; $P < 0.05$) and decreased percentage of total time (Fig. 3J; $P < 0.05$) in the light compartment as compared to *htr2a*$^{-/-}$ mice] and the NSF (increased latency; Fig. 3K, $P < 0.05$ compared to *htr2a*$^{-/-}$ mice). Corroborating the specificity of these anxiety-related findings, behavioral responses in depression-related paradigms, such as the FST and TST, were unchanged in *htr2a*$^{-/-}$ × *Emx1-Cre* mice (fig. S7) as compared with *htr2a* $^{-/-}$ littermates. A similar strategy when used to restore 5HT2AR expression to a subcortical region (i.e., thalamus) produced no difference between rescue and *htr2a*$^{-/-}$ mice in the DLC (see supporting online material), supporting the specificity of the cortex in the normalization of anxiety-related behaviors.

The tissue-specific restoration of an endogenous gene product to a knockout animal provides a precise method for assessing the role of specific circuits in modulating behavior. In addition, when a tissue-restricted rescue normalizes the lost function of a global knockout, such a finding offsets many of the interpretive problems that arise with loss-of-function mutations. In our study, the absence of measurable adaptations in the *htr2a*$^{-/-}$ mice, combined with the reversal of their phenotype by a selective reactivation of *htr2A* gene expression in the cortex, suggests that nonspecific developmental alterations are unlikely to explain our findings.

The precise role of 5-HT signaling in anxiety appears to be complex. Mice with mutations of the 5-HT plasma membrane transporter or 5-HT$_{1A}$ receptor exhibit elevated anxiety levels, but the effects of these mutations on anxiety have been attributed to altered brain development (*24*, *25*). In contrast, the low-anxiety phenotype of *htr2a*$^{-/-}$ mice does not appear to be related to altered brain development, but it may be related to the chronic nature of the mutation in the adult mice. Attempts to reduce conflict anxiety with acute pharmacological administration of 5HT2AR antagonists have been unsuccessful (*26*) or mixed (*27*), whereas chronic reduction of 5HT2AR signaling through the use of antisense receptor down-regulation methods has proven quite effective (*28*). The need for chronic blockade or down-regulation of 5HT2ARs is consistent with the properties of serotonergic anxiolytics that require several weeks to achieve therapeutic effects.

The cortex has been hypothesized as a "top-down" modulator of anxiety-related processes, given the extensive interconnections between the cortex and structures such as the hippocampus and amygdala. Recent functional imaging data in human subjects support this notion (*29*–*31*). Thus, it is intriguing that 5-HT signaling in the cortex can exert pronounced effects on behavior in conflict anxiety tests. A primary role of cortical 5HT2AR signaling in risk or threat assessment may explain the specificity of *htr2a* disruption on conflict anxiety and the absence of effects on conditioned fear and depression-related behaviors. Indeed, modulation of layer V pyramidal neuron glutamate release by 5HT2AR signaling is a likely mechanism by which these cortical projection neurons could modify the activity of subcortical structures. Given the complex effects of 5-HT on a variety of central nervous system functions, a better understanding of the receptor and neural substrates that mediate them may lead to a more nuanced view of 5-HT function and improved therapeutics for anxiety and affective disorders.

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Materials and Methods
Figs. S1 to S7
References

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