Serotonin receptor 4 in mature excitatory hippocampal neurons modulates mood and anxiety

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Abstract

Serotonin receptor 4 (5-HT₄R) plays an important role in regulating mood, anxiety, and cognition, and drugs that activate this receptor have fast-acting antidepressant (AD)-like effects in preclinical models. However, 5-HT₄R is widely expressed throughout the central nervous system (CNS) and periphery, making it a poor therapeutic target and difficult to pinpoint the cell types and circuits underlying its effects. Therefore, we generated a Cre-dependent 5-HT₄R knockout mouse line to dissect the function of 5-HT₄R in specific brain regions and cell types. We show that the loss of functional 5-HT₄R specifically in the mature excitatory neurons of hippocampus led to robust AD-like behavioral responses and an elevation in baseline anxiety. 5-HT₄R was necessary to maintain the proper excitability of mature dentate gyrus (DG) granule cells and cell type specific molecular profiling revealed a dysregulation of genes necessary for normal neural function and plasticity in cells lacking 5-HT₄R. These adaptations were accompanied by an increase in the number of immature neurons in ventral, but not dorsal, dentate gyrus, indicating a broad impact of 5-HT₄R loss on the local cellular environment. This study is the first to use conditional genetic targeting to demonstrate a direct role for hippocampal 5-HT₄R signaling in modulating mood and anxiety. Our findings also underscore the need for cell type-based approaches to elucidate the complex action of neuromodulatory systems on distinct neural circuits.

Introduction

Mood and anxiety are governed by a complex neural circuit comprised of anatomically and functionally distinct components that are distributed throughout the CNS. Functional imaging and postmortem analyses of brains of patients suffering from depression and/or anxiety disorders suggest a dysfunction in regions of the brain that control emotion, including limbic structures such as the hippocampus, amygdala, prefrontal cortex and hypothalamus^{1,2}. Each of these areas is strongly modulated by the neurotransmitter

serotonin (5-HT) while drugs that target 5-HT signaling, such as selective serotonin reuptake inhibitors (SSRIs), are a first line treatment for affective disorders³. The widespread influence of 5-HT on numerous cognitive and behavioral processes leads to many unwanted side effects due to off-target engagement of AD drugs⁴ as well as functional and anatomical separation of the neural substrates underlying pathology from those mediating therapeutic responses^{5,6}. This notion is reinforced by regional differences in the expression and regulation of genes and gene networks across the emotion circuit in disease-related contexts^{7,8}. Improved treatments for these diseases are dependent on a more

precise understanding of cell types and molecular pathways mediating 5-HT-dependent signaling.

The 5-HT₄ receptor (5-HT₄R) is one of over 14 known 5-HT receptors in mammals and is strongly linked to AD responses⁹. 5-HT₄R is an excitatory G_s-coupled receptor that activates the cyclic AMP (cAMP)-PKA pathway and promotes the excitability of neurons^{10,11}. Changes in 5-HT₄R binding was observed in several brain regions in depressed patients^{12,13} while polymorphisms in *HTR4* (the gene that encodes 5-HT₄R) were associated with a susceptibility to unipolar depression¹⁴. In preclinical studies, short-term treatment with 5-HT₄R agonists, including RS67333, has anxiolytic and antidepressant properties and mimics the cellular and molecular AD responses achieved after chronic SSRI administration¹⁵⁻¹⁹. In addition, 5-HT₄R agonists potentiate the effects of SSRIs²⁰ while constitutive *Htr4* knockout mice have reduced behavioral responses to acute stress and novelty²¹. 5-HT₄R has also been linked to the regulation of adult neurogenesis in the dentate gyrus (DG)^{15,18} which is a reliable readout of AD efficacy and may underlie some therapeutic AD effects²².

In the brain, 5-HT₄R is highly expressed in many of the regions linked to mood and anxiety, namely the hippocampus, amygdala, prefrontal cortex, and striatum^{10,23}. It is also expressed in peripheral tissues, including the gut, heart, and adrenal glands²⁴. Such widespread expression in the CNS and periphery complicates the use of 5-HT₄R as a viable therapeutic target, as its activation can lead to significant gastrointestinal and cardiac complications^{25,26}. To date, most in vivo studies investigating 5-HT₄R function have relied on systemic pharmacological approaches or constitutive deletion. Therefore, employing a cell type centered approach to elucidate distinct roles for 5-HT₄R signaling in discrete circuits and/or cell types will facilitate the development of more precise and efficacious therapies by identifying novel targets.

To achieve this, we generated mice harboring a conditional mutant allele to delete functional 5-HT₄R from genetically targeted cell populations expressing Cre recombinase. We then utilized this line to investigate the role of 5-HT₄R signaling in excitatory hippocampal neurons on mood since the hippocampus plays a central role in affective behaviors, is highly connected to other brain regions that encode other aspects of emotion²⁷, and 5-HT₄R is expressed at higher levels compared to other brain regions²⁸. We report that the loss of 5-HT₄R specifically from the mature excitatory neurons of the hippocampus led to AD-like effects at the behavioral, cellular, and molecular levels which was accompanied by an anxiogenic phenotype. Cell type specific molecular profiling revealed that the ventral dentate gyrus (vDG) granule cells (GCs) underwent robust molecular adaptations in the absence of 5-HT₄R. In addition, we observed enhanced neurogenesis in the vDG and reduced excitability of mature vDG GCs in the absence of 5-HT₄R. Our data reinforce a direct role for hippocampal 5-HT₄R in mediating affective behaviors, likely through the regulation the excitability of individual neurons and relevant intracellular pathways, and uncovers an unexpected complexity within the hippocampal circuit due to the differential effect on depression- and anxiety-related behaviors.

Results

Generation and validation of an *Htr4*^{Floxed} mouse line

To study the function of the 5-HT₄R in specific cell types, we generated a novel mouse line (*Htr4*^{Floxed}) to conditionally drive the deletion of functional 5-HT₄R from genetically defined cell populations using the Cre/loxP system (**Fig. 1a**). Homologous recombination was used to insert loxP sites flanking exon 5 of the mouse *Htr4* gene (**Fig. S1a**). In silico translation analysis predicted that the deletion of exon 5, which encodes the fourth transmembrane domain of 5-HT₄R,

Fig. 1 | Generation and validation of an *Htr4*^{Floxed} mouse line. a, Schematic of *Htr4*^{Floxed} allele. LoxP sites (red triangles) were inserted to flank *Htr4* exon 5 (light blue bar) for Cre-mediated excision. **b**, Low magnification anti-EGFP immunohistology of coronal brain sections from a *Drd3-Cre::Rosa*^{fsTRAP} mouse showing Cre expression in the hippocampus. dCA, iCA, vCA1/3: dorsal, intermediate, ventral CA1/3 fields, respectively; A: amygdala; TH: thalamus; HY: hypothalamus. **c**, Schematic of the TaqMan probe spanning *Htr4* exons 4 and 5 (Probe 4-5) is shown a top. Below, qRT-PCR (mean $\pm$ SEM) quantification showing diminished expression of *Htr4* transcripts containing exon 5 along the dorsoventral axis of the hippocampus in Cre-negative (WT, gray) and cKO (blue) mice. Two-way ANOVA: genotype factor: $F(1,24) = 439.0$, $p < 0.0001$ followed by post hoc Fisher's LSD test. $n = 2-6$ per group. **d**, Quantification (mean $\pm$ SEM) of cAMP induction HEK293T cells expressing EGFP (Control), intact 5-HT₄R (WT), or *Htr4*^{delE5} (del5) in the presence or absence of the 5-HT₄R agonist, zacopride. One-way ANOVA followed by post hoc Fisher's LSD test, $n = 4$ per group. **e**, Representative traces of whole-cell voltage clamp recordings from DG GC in WT and cKO mice with bath administration of 10 mM BIMU-8. Scale bar: 1 min, 20 pA. Fisher's LSD test, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$, n.s. $p > 0.05$.

would result in a frame shift mutation and the introduction of stop codons downstream, including one right after the deletion site (**Fig. S1b**), leading to a truncated, non-functional, and/or unstable protein tagged for degradation. This should result in animals harboring a null *Htr4* allele.

Because *Htr4* is highly expressed in the hippocampal formation²⁸, we validated the deletion of exon 5 and the loss of functional 5-HT₄R by generating a hippocampus-specific *Htr4* conditional knockout. The BAC transgenic *Drd3-Cre* driver line (founder KI198) was utilized since it expressed Cre recombinase specifically in the

hippocampus²⁹ (gensat.org; **Fig. 1b**). Homozygous *Htr4*^{Floxed} mice (*Htr4*^{loxP/loxP}) were crossed to *Drd3-Cre* mice to generate *Drd3-Cre::Htr4*^{loxP/loxP} (cKO) mice (**Fig. S1c**). To determine relative *Htr4* transcript levels following Cre-mediated recombination, RNA was extracted from dorsal and ventral subdivisions of the CA fields and DG of cKO mice and Cre-negative littermates (WT; **Fig. S1d**). Quantitative RT-PCR (qRT-PCR) using probes spanning exons 4 and 5 (4-5) of *Htr4* revealed negligible expression of exon 5 in cKO samples compared to WT along the dorsoventral axis of the hippocampus (**Fig. 1c**), while probes spanning exons 3-4 and 6-7 showed comparable expression between genotypes (**Fig. S1e**). These data confirm the generation of a mutant *Htr4* transcript (*Htr4*^{delE5}) following Cre-mediated recombination in cKO mice.

Since 5-HT₄R is a G_s-coupled receptor that activates intracellular cAMP upon ligand binding¹⁰, we employed a cell culture-based cAMP induction assay to test the signaling ability of the mutant receptor. Expression vectors for mutant *Htr4*^{delE5} (delE5) and intact *Htr4* (WT) were made by cloning each transcript from total RNA samples isolated from the hippocampus of a cKO mouse or a WT littermate, respectively. The constructs were transfected into HEK293T cells which were stimulated in the presence of IBMX, a phosphodiesterase inhibitor to allow accumulation of newly converted cAMP. Intact WT expression led to five-fold higher baseline levels of cAMP than of mutant delE5 or EGFP (control) (**Fig. 1d**). Stimulation with zacopride, a potent 5-HT₄R agonist induced an 88% increase in cAMP production in cells expressing WT but did not significantly alter cAMP levels in those expressing delE5. These results suggest that the deletion of exon 5 leads to a non-functional receptor in vitro.

To test the functionality of mutant 5-HT₄R in intact hippocampal neurons, we performed whole-cell voltage clamp recordings from dentate gyrus (DG) granule cells (GCs) in acute hippocampal slices from cKO mice and WT littermates. The addition of the 5-HT₄R agonist,

BIMU-8, led to an inward current in WT GCs indicating a depolarizing effect due to the activation of 5-HT₄R (**Fig. 1e**), consistent with previous reports¹¹. In contrast, cKO GCs did not respond to BIMU-8, validating the absence of functional 5-HT₄R in DG GCs in cKO mice at the electrophysiological level. Together with the cAMP activation assay, these results indicate that the Cre-dependent deletion of exon 5 in cKO mice results in a functionally null receptor.

Drd3-Cre expression is restricted to mature excitatory neurons in the hippocampus

To determine the identity of the Cre-expressing cells in the hippocampus of *Drd3-Cre* mice, we crossed them to the *Rosa26*^{fsTRAP} Cre-dependent reporter line³⁰, in which EGFP is fused to ribosomal protein L10a (EGFPL10a). Anti-GFP immunofluorescent staining of coronal brain sections from *Drd3-Cre::Rosa26*^{fsTRAP} mice revealed labeled cells along the dorsoventral axis of the hippocampus. GFP expression was visible in many cells in the dorsal CA1 (dCA1), dorsal CA3 (dCA3), and ventral CA3 (vCA3) although there were also many unstained cells in these areas (**Fig. 2a**). Few GFP expressing cells were visible in ventral CA1 (vCA1). All of the GFP-expressing cells expressed the neuronal marker, NeuN, but not the GABAergic interneuron marker, glutamate decarboxylase (GAD67; **Fig. S2a**), suggesting *Drd3-Cre* primarily labels excitatory pyramidal neurons in the CA fields.

In the DG, GFP expression was visible in the granule cell layer (GCL) and the hilus along the dorsoventral axis (**Fig. 2a, c**). Co-labeling of GFP with markers for the major DG cell types was used to refine the identity of the *Drd3-Cre* cells. GFP was detected in all of the calbindin (CBD)-expressing mature GCs and calretinin (CRT)-expressing mossy cells (MCs) in the hilus (asterisks), whereas it was absent from doublecortin (DCX)- and CRT-expressing immature neurons in the GCL and GAD67-expressing GABAergic interneurons throughout the DG (**Fig. 2c**). Together, we conclude

Fig. 2 | See next page for caption.

Fig. 2 | Drd3-Cre expression is restricted to mature excitatory neurons in the hippocampus. **a**, Anti-EGFP (green) and anti-NeuN (red) immunofluorescent confocal images of coronal hippocampal sections at the level of dorsal (top left) and ventral (bottom left) hippocampus from a *Drd3-Cre::Rosa^{tsTRAP}* mouse. Dorsal and ventral CA1 and CA3 fields (white boxes) are shown at higher magnification on the right. Scale bars: 500 μ m (left panels) or 100 μ m (right panels). **b**, Anti-EGFP (green) and anti-NeuN (red) immunofluorescent confocal images of coronal sections at the level of dorsal (top left) and ventral (bottom left) hippocampus from an *Htr4-bacTRAP* mouse. Dorsal and ventral CA1 and CA3 fields (white boxes) are shown at higher magnification on the right. Scale bars: 500 μ m (left panels) or 100 μ m (right panels). **c**, Immunofluorescent confocal images showing neuronal-type specific marker (red) and EGFP (green) expression in coronal sections through the DG of *Drd3-Cre::Rosa^{tsTRAP}* mice. Cells labeled with a neuron-type marker but not EGFP (arrowheads) and cells double-labeled with hilar mossy cell marker CRT and EGFP (asterisks) are indicated. ml: molecular layer, gcl: granule cell layer. Scale bars, 50 μ m. **d**, Immunofluorescent confocal images of coronal sections through the DG of *Htr4-bacTRAP* mice labeled with anti-NeuN (red) and anti-EGFP (green). ml: molecular layer, gcl: granule cell layer. Scale bars, 50 μ m.

that *Drd3-Cre* targets mature excitatory neurons in the DG, including GCs and hilar mossy cells, but not immature neurons, inhibitory interneurons, or non-neuronal cells.

To determine how the distribution of *Drd3-Cre* cells compared to that of 5-HT₄R expressing cells in the hippocampus, we generated *Htr4-bacTRAP* mice where the EGFP10a transgene was driven by a *Htr4* BAC promoter to recapitulate endogenous *Htr4* expression (**Fig. S2b**). Like *Drd3-Cre* mice, GFP+ cells were seen in a subset of cells in the CA fields in the *Htr4-bacTRAP* mice, although the bacTRAP transgene labeled many more cells in vCA1 (**Fig. 2b, d**). Also, there was a notable gradient in the number of cells labeled in dorsal and ventral CA3 with the density of labeling decreasing from CA2 to the DG. This pattern can also be seen by ISH (**Fig. S2b** and the Allen Brain Atlas, www.brain-map.org). Interestingly, *Drd3-Cre* expression shows an opposite gradient with denser labeling near the DG and fewer cells approaching CA2. None of the GFP+ cells co-labeled with GAD67 (**Fig. S2c**). *Htr4-bacTRAP* expression matched the *Drd3-Cre* expression in that the vast majority of CBD+ GCs and CRT+ mossy cells co-stained with GFP, but not markers for immature neurons, GABAergic interneurons, progenitor cells, or non-neuronal cells (**Fig. S2d**). Therefore, crossing the *Drd3-Cre* mice with the *Htr4^{Floxed}* line results in the loss of functional 5-HT₄R throughout the DG, but only partial deletion in the CA fields.

The effect of hippocampus specific loss of 5-HT₄R on mood and anxiety related behaviors

Given the direct role of serotonin in the regulation of mood³¹ and that 5-HT₄R has been shown to mediate AD responses¹⁵, we investigated the impact of the conditional ablation of 5-HT₄R from hippocampus on mood-related behaviors. We first employed the tail suspension (TST) and forced swim tests (FST) which assess responses to an acute inescapable stressor as a measure of behavioral despair^{32,33}. Immobility was decreased in cKO mice compared to WT littermates in both TST and FST (**Fig. 3a, b**), indicating reduced despair-like behavior and mimicking an AD-like response. It is unlikely that these results were due to a general hyperactivity of the cKOs since they did not differ from WT in locomotor behavior in an open field arena (**Fig. S3e**).

The splash test and sucrose consumption are commonly used as indicators of hedonic state in depression models and rely on the animal's motivation for self-grooming and the rewarding properties of a sweet, palatable sucrose solution, respectively³⁴⁻³⁶. In the splash test, cKO mice spent more time grooming following the application of a sucrose solution compared to WT littermates, mimicking the effect of antidepressant administration (**Fig. 3c**). During the sucrose consumption, both cKO and WT mice drank approximately 5-10-fold more sucrose than water each day over the three-day test (**Fig. S3a, b**), implying no difference in hedonic capacity between genotypes.

Fig. 3 | cKO mice exhibit altered affective behaviors and reduced firing in vDG granule cells. **a**, Quantification of the time spent immobile in the tail suspension test (TST) in cKO mice (cKO, blue) and Cre-negative littermates (WT, black). ** $p = 0.0012$, $n_{WT} = 19$, $n_{cKO} = 16$. **b**, Quantification of the time spent immobile in the forced swim test (FST) for each genotype. *** $p = 0.0005$, $n_{WT} = 19$, $n_{cKO} = 16$. **c**, Quantification of grooming time in the splash test for each genotype. ** $p = 0.001$, $n_{WT} = 19$, $n_{cKO} = 16$. **d**, Mean time spent in the center in the open field (OF) for each genotype. * $p = 0.0139$, $n_{WT} = 19$, $n_{cKO} = 17$. **e**, Quantification of the time spent in the open (left) and closed (right) arms in the elevated plus maze (EPM). * $p = 0.0239$, $n_{WT} = 18$, $n_{cKO} = 16$. **f**, Mean latency to feed in the novelty suppressed feeding (NSF) paradigm for each genotype. ** $p = 0.0033$, $n_{WT} = 20$, $n_{cKO} = 16$. **g**, Sample traces from whole-cell current-clamp recordings of GCs in hippocampal slices from WT and cKO mice showing spiking in response to different steps of injected current. **h**, Quantification of the AP frequency of GCs in WT and cKO mice across current steps. **i**, Histogram of rheobase measurements of GCs from WT and cKO mice. All data are represented as mean $\pm$ SEM and two-tailed unpaired t-tests were performed for all panels. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Interestingly, cKO mice consumed cumulatively more sucrose after 72 hours compared to WT. This result was unlikely due to a change in caloric need since body weight was unaltered in the cKOs (**Fig. S3c**). The significance of the increased sucrose consumption is difficult to interpret given the strong sucrose preference exhibited by both groups but may reflect a subtle rise in pleasure-seeking drive. Together with the FST and TST results, these data indicate that cKO mice displayed AD-like behaviors in despair- and hedonic-based assays.

As anxiety is closely associated with mood disorders and AD treatment, and has been linked to hippocampal and 5-HT₄R function^{18,37,38}, we next assessed how hippocampus-specific loss of 5-HT₄R affected a variety of anxiety-related behaviors. The open field test (OF), elevated plus maze (EPM) and novelty suppressed feeding (NSF) paradigms all rely on mice having an innate aversion to well-lit, open spaces while NSF adds the conflict of engaging in feeding behavior in a novel environment³⁹⁻⁴¹. In the OF, cKO mice spent less time in the center of the arena during the initial 10 min compared to WT littermates while showing similar overall locomotor activity (**Fig. 3d, Fig. S3d, e**). Similarly, in the EPM, the cKOs spent more time in the closed arms compared to WT (**Fig. 3e**). Although the time spent in the open arms did not differ between genotypes, the cKOs displayed a trend towards higher velocity in EPM compared to WT (**Fig. S3f**) suggesting that higher exploratory activity of cKO mice was not accompanied by more exploration of the open arms. The cKO mice also displayed a greater latency to feed in NSF (**Fig. 3f**) yet consumed more food on average when placed back in their familiar home cages following NSF (**Fig. S3g**), conveying that the lack of motivation to feed was not the source of their prolonged latency. Together, these conflict-based behavioral analyses indicate that cKO mice have an anxiety-like phenotype.

We also tested behaviors that may be affected by anxiety levels such as social interaction and acoustic startle response (ASR). Mice were tested in the three-

chamber social interaction test to measure sociability since a reduced amount of time spent interacting with a novel conspecific has been associated with abnormal anxiety-like behaviors⁴². We observed that cKO mice interacted less with a novel mouse compared to WT (**Fig. S3h**). Enhanced startle response has also been linked to increased anxiety and its physiological features^{43,44} while blunted startle was observed in patients with anhedonia⁴⁵⁻⁴⁷. The acoustic startle reflex, a defensive motor response to an intense sudden auditory stimulus, is measured to assess ASR in mice. We found that ASR was significantly enhanced in cKO mice compared to WT (**Fig. S3i, j**). On the other hand, the pre-pulse inhibition (PPI), the suppression of ASR when a startling stimulus is preceded by an immediate, weaker pre-stimulus, was not altered (**Fig. S3k**). Together, these anxiety-regulated responses reflect the anxiogenic phenotype observed in the cKOs.

Loss of 5-HT₄R reduces the excitability of dentate gyrus granule cells

Activation of 5-HT₄R leads to depolarizing currents via second messenger mechanisms in excitatory cells of the hippocampus¹¹, including DG GCs (**Fig. 1d**). To test if the cell type specific loss of functional 5-HT₄R had any electrophysiological consequences, we performed whole-cell current clamp recordings from vDG GCs in acute hippocampal slices from WT and cKO mice. We found that the firing frequency of GCs was significantly reduced in cKO slices compared to WT (**Fig. 3g, h**). In addition, rheobase, the amount of current necessary to be injected to elicit an action potential, was larger in GCs from cKO mice (**Fig. 3i**). On the contrary, there was no difference in the properties of single action potentials between genotypes, including threshold, amplitude, half-amplitude width and fast after-hyperpolarization as well as the input resistance and slow after-hyperpolarization (**Fig. S4a-g**). These data show that the loss of 5-HT₄R from DG GCs leads to specific physiological changes resulting in a reduced excitability in these neurons.

Molecular adaptations in the vDG of cKO mice highlight cellular and functional roles for 5-HT₄R

We next investigated global differences in gene expression that occurred following the deletion of hippocampal 5-HT₄R to identify molecular mechanisms that may underlie the behavioral and electrophysiological phenotypes observed in cKO mice. We focused on the ventral DG since nearly all 5-HT₄R expressing cells in the DG, but not CA fields, are targeted by the *Drd3*-Cre line (**Fig. 2a-c**), and there is a strong link between ventral hippocampus and the regulation of depression and anxiety related behaviors²⁷. The translating ribosome affinity purification (TRAP) method^{48,49} was used to isolate cell type specific transcripts by expressing the EGFP10a TRAP transgene in WT and cKO *Drd3*-Cre cells by crossing *Drd3*-Cre with *Rosa26*^{fsTRAP}, and *Drd3*-Cre::Htr4^{loxP/loxP} with *Rosa26*^{fsTRAP}::Htr4^{loxP/loxP} mice, respectively (**Fig. 4a**). Polysome-bound mRNAs were extracted following anti-EGFP immunoprecipitations (IP) on vDG homogenates and analyzed by RNA-seq alongside mRNAs extracted from whole tissue (input) (**Fig. S5a**). The quality of dissection was confirmed by qRT-PCR which showed that markers for DG (*Dsp*) and vDG (*Trhr*) were highly expressed in vDG inputs but markers for CA fields (*Tyro*) and dDG (*Lct*) were not (**Fig. S5b-c**). Visualization of RNA-seq reads mapped to the *Htr4* locus confirmed the deletion of exon 5 in cKO tissue (**Fig. 4b**), although overall expression of *Htr4* was increased in cKO TRAP mRNA compared to WT, likely reflecting an attempt by the cells to compensate for the non-functional protein (**Fig. 4c**). There did not appear to be a broader compensation in 5-HT signaling since there was no difference in the expression of other 5-HT receptor transcripts between genotypes (**Fig. 4c**). Consistent with previous reports of the distribution of 5-HT receptors in the DG²⁸, the receptors with highest expression in the vDG TRAP were *Htr1a*, *Htr2a*, *Htr4*, and *Htr5a*.

A direct comparison of gene expression between TRAP mRNA (IP) and whole tissue mRNA (input) revealed a substantial depletion of markers for interneurons, neurogenesis, and other non-neuronal cells in the TRAP data set (**Fig. S5d, e**), consistent with our immunohistology showing the *Drd3*-Cre line primarily labels mature GCs and hilar MCs in the DG (**Fig. 2c**). While markers for GCs were highly expressed in TRAP mRNA, their levels of enrichment in IP over input were less robust compared to the depletions of markers of cells not labeled by *Drd3*-Cre (**Fig. S5e, f**), likely because mature GCs constitute the vast majority of all cells in the DG. A similar distribution of marker genes was observed for the cKOs (**Fig. S5d**) and genes enriched or depleted in IP compared to input were similar between WT and cKO (**Fig. S6a**) indicating that the mutant allele did not noticeably alter cellular identity.

Differential expression analysis of TRAP mRNA between WT and cKO mice identified 80 upregulated and 290 downregulated genes following the loss of 5-HT₄R function (**Fig. 4d**). Heatmap visualization based on normalized expression values showed that the significant gene expression differences between genotypes were consistent across all replicates (**Fig. 4e**). Over 40% of the differentially expressed (DE) genes (17 up- and 136 downregulated) were enriched in the IP of *Drd3*-Cre cells (**Fig. 4f**), illustrating a strong cell type specific adaptation. To gain a general understanding of the gene pathways dysregulated in the cKOs, we performed gene set enrichment analysis (GSEA) on a ranked list of genes differing between WT and cKO samples (**Fig. 4g**). We found positive enrichment in signaling pathways linked to translation, neuroplasticity and cytoskeletal regulation, including mTOR, CDC42, TGFβ, neurotrophin signaling and regulation of actin cytoskeleton, all of which have been shown to be regulated by AD treatment as well. Interestingly, there was an upregulation in ubiquitin proteolysis and 3'UTR mediated translation, and a downregulation in pre-mRNA processing, implying

Fig. 4 | See next page for caption.

Fig. 4 | TRAP profiling of vDG neurons in the absence of 5-HT₄R. **a**, Schematic depicting the cell types targeted for TRAP. Green represents EGFPL10a expressing cells and blue indicates conditional 5-HT₄R deletion. The area dissected is outlined in red. **b**, Browser view of mapped reads showing the lack of expression of *Htr4* exon 5 in IP and input samples in the cKO. **c**, Bar graph of normalized expression values (mean $\pm$ SEM) of all 5-HT receptors (Htrs) in IP samples. Dashed line is drawn at normalized expr. = 50. Only *Htr4* has FDR < 0.1. **d**, MA-plot highlighting differentially expressed (DE) genes (FDR < 0.1) between the cKO and WT TRAP mRNA. Complete list of IP DE genes can be found in **Table S1**. **e**, Heatmap visualization of DE genes between genotypes. **f**, Venn diagram showing the overlap of up- and down-regulated DE genes from **e** with genes enriched in the *Drd3*-Cre-expressing cells (IP enriched, **Table S2**). **g**, Summary of gene set enrichment analysis (GSEA) performed on the fold change (FC)-ranked gene list ($p < 0.05$) between cKO and WT IP data sets. The normalized enrichment score (NES) shows the direction of enrichment in the cKO and FC-ranks are arranged from down-regulated (blue) at left to up-regulated (yellow) at right. Complete list of pathways can be found in **Table S3**. **h**, Summary of gene ontology (GO) analysis of DE from **e**. All categories shown have FDR < 0.01, full analysis is provided in **Table S4**.

a possible response to the handling of mutant *Htr4* transcript and 5-HT₄R protein. We also found negative enrichment in pathways related to 5-HT₄R function including GPCR ligand binding, calcium signaling, neuroactive ligand receptor interaction, and GPCR downstream signaling, paralleled by decreased expression of genes involved in cAMP signaling (**Fig. 4g, S6b**). In addition, there was a downregulation in pre-mRNA processing and transmembrane transport of various classes of ions (**Fig 4g, S6c**). Functional annotation analysis of the list of genes that were significantly differentially expressed between genotypes further showed alterations in a variety of processes underlying neuronal function including synaptic vesicle localization, neuron development, synaptic signaling, ion homeostasis, and metabolism while unfolded protein response, protein transport, and protein catabolism imply a change in proteostasis (**Fig. 4h**). Together, our differential expression analysis of the vDG from cKO and WT mice identified substantial transcriptional changes in the absence of 5-HT₄R that are related to neuronal function and intracellular 5-HT₄R receptor signaling.

The ablation of 5-HT₄R resulted in enhanced adult hippocampal neurogenesis specifically in the vDG

Up to this point, we have investigated the effects of 5-HT₄R loss on the intrinsic molecular and electrophysiological properties *Htr4*-expressing cells.

We next asked how these adaptations impacted the overall cellular environment within the DG. To do so we took advantage of the whole tissue mRNA (input) since it was enriched with transcripts expressed in non-neuronal cells, progenitors, and interneurons (**Fig. S5e**) allowing us to examine broad molecular responses in non-*Htr4*-expressing cells. Differential expression analysis between WT and cKO tissue revealed a robust molecular response (625 DE genes) in the whole vDG and a relatively small response in the whole dDG (179 genes) (**Fig. 5a, S7a**). Only 25 genes were shared between regions (**Fig. S7b, c**). Functional annotation of DE genes revealed remarkably distinct cellular adaptations between the two regions. Processes related to neural function and plasticity, including neurogenesis, neuron development and differentiation, synaptic signaling, ion transport and homeostasis, and regulation of action potential were uniquely enriched in the whole vDG mRNA but not dDG (**Fig. 5b, S7d**). The genes altered in the whole dDG mRNA were primarily related to protein and RNA processing. Furthermore, disease-gene association analysis of DE genes in the whole vDG also showed significant enrichment for a variety of diseases, including affective disorders such as depressive, bipolar and anxiety disorders, along with other psychiatric disorders (**Fig. S7e**). No significant disease-gene associations were found in the dDG.

To identify the gene expression changes that occurred primarily in non-*Htr4*-expressing cells in the cKOs, we directly compared the DE genes from the vDG TRAP (IP) and whole vDG mRNA (input), and found 430

Fig. 5 | See next page for caption.

Fig. 5 | Loss of 5-HT₄R led to changes in neurogenesis-related genes and increased number of immature neurons in the vDG. **a**, MA-plots indicating differentially expressed (DE) genes (FDR < 0.05) between cKO and WT mRNA from whole dDG (top) and vDG (bottom). Complete lists can be found in Table S5 and S6. **b**, Comparison of significantly enriched gene ontology (GO) terms among whole dDG and vDG DE genes from **a**. **c**, Venn diagram comparing DE transcripts shared and unique to vDG TRAP (dark blue) and whole vDG tissue (light blue) samples. **d**, Top ten GO terms enriched in the whole vDG-specific gene list from **c**. Terms related to neurogenic tone are in bold. **e**, Representative images of anti-DCX immunofluorescence in coronal sections through dDG and vDG from cKO and WT mice. Arrowheads indicate DCX⁺ cells. DAPI is shown in blue. Scale bar, 150 μ m. **f**, Quantification (mean $\pm$ SEM) of DCX⁺ cells per granule cell layer area in the dorsal (dDG), intermediate (iDG), and ventral (vDG) DG. Two-way ANOVA: genotype $\times$ region interaction, $F(2,24) = 6.328$, $p = 0.0062$ followed by post hoc Fisher's LSD test, $^{**}p = 0.002$. $n = 5$ per genotype.

genes that were altered in the whole vDG but not TRAP (**Fig. 5c**). These whole vDG-specific genes were predominantly related to neurogenesis and neurodevelopment, likely reflecting the fact that progenitors and immature neurons comprised many non-Cre-labeled cells (**Fig. 5d**). It should also be noted that many of the cell intrinsic processes, such as ion homeostasis and transport, synaptic signaling, and protein processing appeared to be unique to the TRAP (**Fig. 4e, f and 5b**).

Our genome-wide transcriptional analysis revealed significant alterations in gene expression related to neurogenesis in the whole vDG and an upregulation of factors known to promote neurogenesis and plasticity specifically in the *Drd3*-Cre cells (**Fig. 4e, f and 5b, d**). This is particularly interesting given that the cKOs exhibit AD-like behavioral responses and the link between adult neurogenesis of DG granule cells and antidepressants²⁷. We therefore examined whether we could detect any change in hippocampal neurogenesis in the cKOs. The number of doublecortin-positive (DCX⁺) immature neurons along the dorsoventral axis of the subgranular zone (SGZ) and GC layer of the DG was quantified in WT and cKOs (**Figs. 5e and S7f**). There was a 67% increase in the number of DCX⁺ neurons in the vDG of cKO mice compared to WT littermates, while no difference in the number of DCX⁺ neurons was detected in the dDG or intermediate DG (**Fig. 5f**). These results suggest that loss of functional 5-HT₄R led to enhanced neurogenesis in the vDG, which likely occurred through a non-cell-autonomous manner since *Drd3*-Cre does not target progenitor cells or immature neurons. Together, these data

demonstrate a remarkable amount of region-specific cellular and molecular adaptations along the dorsoventral axis of DG in response to loss of 5-HT₄R function.

Discussion

In the current study, we generated conditional 5-HT₄R knockout mice to examine the role of this receptor in discrete cell populations in the hippocampal formation at behavioral, cellular, and molecular levels. The loss of 5-HT₄R primarily from mature excitatory neurons in the DG and hilus led to AD-like performance in behavioral tests of despair and anhedonia, and elevated anxiety. DG granule cells were less excitable and showed altered expression of genes related to GPCR function, ion transport, and neuroplasticity in the absence of 5-HT₄R. In addition, cKO mice showed increased neurogenesis in the vDG but not dDG, indicating regional differences in adaptations to 5-HT₄R loss in the local environment along the dorsoventral axis. Taken together, these results strongly suggest that the removal of functional 5-HT₄R from mature excitatory cells in the DG mimics chronic AD treatment and offers insight into hippocampal circuits modulating mood and anxiety.

Decreased functionality of hippocampal 5-HT₄ receptors is consistent with reports describing cellular responses to antidepressants. Chronic SSRI treatment has been shown to reduce the density of 5-HT₄R binding and decrease 5-HT₄R-dependent cAMP signaling and postsynaptic excitability in hippocampal

pyramidal cells in rats⁵⁰. Moreover, short-term treatment with the partial 5-HT₄R agonist, RS67333, leads to the desensitization of the receptors in hippocampus¹⁷. These findings together with our results paint the picture of a multifaceted role for 5-HT₄R in the regulation of neural circuits underlying mood. 5-HT₄R is expressed in many limbic structures besides the hippocampus including prefrontal cortex, ventral striatum, and amygdala¹⁶. Indeed, the mPFC has been implicated as a site mediating the fast-acting anxiolytic/antidepressant effects of RS67333^{19,51} and the overexpression of 5-HT₄R specifically in the mPFC led to AD-like behavior⁵². In addition, we previously found that chronic SSRI administration led to an induction of 5-HT₄R expression in corticostriatal neurons in sensorimotor cortex⁵, although it does not appear to underlie SSRI-mediated changes in 5-HT responses in those cells⁵³. The lack of impact of constitutive deletion of 5-HT₄R on baseline mood²¹ further attests to the complex interplay between different circuit components that express the receptor.

Our results showed that the absence of 5-HT₄R led to a reduced electrophysiological excitability of DG GCs. It is not clear whether the changes we observed in the cKOs were due to a deficiency of 5-HT₄R signaling directly or a result of long-term cellular adaptations in the absence of the receptor. 5-HT₄R has been shown to contribute to the excitability of pyramidal cells through PKA-mediated closure of Ca²⁺-activated K⁺ channels underlying sAHP current^{11,54-56}, however we did not see any change in slow or fast AHPs in the cKOs. A direct coupling to both voltage-sensitive calcium channels⁵⁷ and the extracellular signal-regulated kinase pathway⁵⁸ has also been proposed for 5-HT₄R function, both of which are known to modulate neuronal function^{59,60}. The TRAP data revealed a dysregulation of Ca²⁺ signaling, ion homeostasis, and/or ion channel expression in the 5-HT₄R cKOs, although more work is needed to understand the significance of these changes on the excitability of the GCs.

The AD-like phenotype we observed following the deletion of 5-HT₄R in the hippocampus is possibly mediated by the decreased excitability of the vDG GCs observed in the cKOs. Growing evidence supports a link between suppressed activity of DG GCs and AD-like behavior. Decreased excitability of vDG GCs through local GABAergic inhibition produces behavioral AD action⁶¹, and indirect inhibition of mature GCs of the vDG by young adult-born GCs (abGCs), which are increased by AD treatment, or by chemogenetic methods promotes resilience to stress⁶². In addition, SSRI-mediated inhibition of mature GCs through 5-HT_{1A}R is necessary for AD responses in preclinical models⁶³. Blockade of GC-CA3 glutamate release and NMDA receptor activity reduces behavioral despair⁶⁴, and the therapeutic effects of antidepressants and 5-HT₄R agonists in rodents are dependent on the tonic inhibition of CA3 pyramidal cells via the activation of 5-HT_{1A} receptors¹⁵. Finally, chronic AD administration attenuates excitatory physiological effects of 5-HT₄R throughout the hippocampal circuit^{50,65}. Although we did not detect a compensatory change in the expression of 5-HT_{1A} or other 5-HT receptor transcripts in GCs following 5-HT₄R deletion, it is possible that the AD-like phenotype of the cKOs resulted from a shift in the balance of excitatory and inhibitory 5-HT signaling. 5-HT₄ and 5-HT_{1A} receptors are both highly expressed in DG GCs and clearly cooperate to mediate cellular responses to 5-HT despite activating opposing second messenger pathways and electrophysiological properties^{9,63}. The broad downregulation of cAMP-related genes and GPCR signaling detected in the TRAP data supports this idea.

Behavioral analysis of the cKOs also reinforced a central role for hippocampal 5-HT₄R in modulating anxiety. Previous studies using systemic administration of pharmacological agents demonstrated robust anxiolytic properties of 5-HT₄R agonists while 5-HT₄R antagonists blocked the anxiolytic effects of diazepam and SSRIs^{16,18,66}. Although there is a discrepancy in early reports also showing anxiolytic effects of 5-HT₄R

antagonists in rodents^{66,67}, no obvious anxiety-like phenotype was observed in constitutive 5-HT₄R KO mice²¹. The anxiogenic phenotype observed in our 5-HT₄R cKO mice implies hippocampal 5-HT₄R directly moderates anxiety, possibly through its role in modulating the excitability of vDG GCs. Acute optogenetic activation of vDG GCs was shown to suppress innate anxiety, although increased anxiety was not observed when vDG GCs were acutely inhibited³⁸. Long-term inhibition of GCs due to chronic loss of 5-HT₄R functionality in the cKOs may have amplified this effect in our study. The activity of discrete subsets of vCA1 projection neurons have differing effects on anxiety-related behaviors depending on their postsynaptic targets^{68,69}, indicating a complexity of cell specific mechanisms even within the hippocampal circuit. Although Drd3-Cre was absent from vCA1 and 5-HT₄R function was likely preserved in most vCA1 pyramidal cells in the cKOs, reduced activity of vDG GCs has a significant impact on the overall trisynaptic hippocampal circuit and the outputs of vCA1 pyramidal cells.

5-HT₄R deletion also had a palpable effect on molecular and cellular neuroplasticity in the vDG. Cell type specific molecular profiling of vDG GCs with TRAP was able to detect a clear downregulation of GPCR signaling and an increase in protein degradation pathways, reflecting direct intrinsic adaptations to the loss of functional 5-HT₄R and processing of the unstable mutant protein. However, it also provided evidence for an alteration in the neuroadaptive state of the cells highlighted by a change in genes linked to processes such as neuronal development, differentiation, and synaptic signaling. These pathways have been shown to be differentially regulated in the hippocampus with AD treatment, deep brain stimulation, and exposure to stress^{7,8,70-73} and likely underlie the changes in synaptic plasticity, spine density, and dendritic architecture of hippocampal neurons observed after AD administration^{3,74,75}.

The molecular adaptations in mature GCs were accompanied by heightened generation of abGCs

selectively in the vDG in the cKOs. Hippocampal neurogenesis is a reliable readout of AD activity²² and mediates some of the behavioral effects of AD treatments and responses to stress^{35,62,76} but does not produce AD-like behavior on its own⁷⁷. The absence of 5-HT₄R and Cre from neural progenitors and immature GCs suggests the enhanced neurogenesis seen in the cKOs was due to non-cell autonomous mechanisms, likely resulting from a pro-neurogenic change in the local environment within the vDG. This notion is strongly supported by the enrichment of neurogenesis-related pathways being highly regulated in the vDG niche of the cKOs. The lack of change in neurogenesis at the molecular and cellular level in the dDG underscores the functional divergence of the DG along the dorsoventral axis^{27,78} and the regional specificity of 5-HT₄R function. A similar anatomical discrepancy in neurogenesis was observed in patients and mice following chronic SSRI treatment^{79,80} and the ablation of abGCs in the vDG, but not dDG attenuated some of the behavioral responses to AD treatment, stress, and chronic pain^{81,82}.

The present study begins to refine the role of 5-HT₄R in a key circuit that mediates mood and anxiety and reinforces the complexity of neuromodulatory signaling in arbitrating emotion. This notion is underscored by the fact that the AD-like phenotype of the conditional deletion of 5-HT₄R from mature granule cells in the DG is accompanied by an increase in anxiety. SSRIs and other AD drugs are commonly used to treat anxiety disorders so the anxiogenic behavior of the 5-HT₄R cKO mice seems to oppose their AD-like phenotype, indicating a dissociation between the mechanism underlying each process. Indeed, antidepressants can promote suicidality, anxiety and nervousness when given to healthy subjects⁸³, and acute and chronic SSRI treatment in rodents can induce anxiety-like behaviors depending on the baseline anxiety/stress/depression levels, strain, age, dose and delivery method of AD treatment⁸⁴⁻⁸⁷. Our data justify further inquiry to determine if these phenomena are moderated by a disengagement of hippocampal 5-HT₄R. Such observations and the widespread

influence of the therapeutic of targeting neuromodulatory systems underscore the need to tease apart regional and cell type specific mechanisms to develop better drugs with diminished side effects.

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Author contributions

R.K., N.H., and E.F.S designed the study. R.K. performed all experiments unless noted otherwise. L.M. conducted electrophysiology experiments. M.N.R performed FISH experiments. E.B.H assisted with behavior and RNA-seq. R.K., L.M., J.L.W-S, P.G., N.H., and E.F.S. analyzed data. R.K., L.M., and E.F.S. wrote the manuscript.

Competing interests

The authors declare no competing interests.

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Fig. S1 | Gene targeting strategy to generate $Htr4^{Floxed}$ mouse line. Related to Fig. 1. **a**, Schematic of *Htr4* gene targeting steps. Targeting vector was designed to drive the homologous recombination via long (LA, blue) and short (SA, green) arms to insert the loxP-flanked exon 5 (vertical black bar) and Neomycin resistance cassette (Neo, yellow rectangle). Relative positions of FRT (flippase recognition target) sites (grey half circle) and loxP sites (red triangle) are shown. FLPe-mediated recombination was used to excise the Neo cassette and lead to *Htr4* exon 5 flanked with loxP sites ($Htr4^{Floxed}$ allele). gP, genotyping primers (purple arrowheads). Scale bar for magnified schematics, 1kb. **b**, In silico translation analysis predicting that the loss of exon 5 from *Htr4* mRNA would lead to immediate early stop codons. Green amino acid sequence represents the translation up to exon 5. Red squared X represents the translation of the stop codons. **c**, Sample genotyping results using gP from mice that are $Htr4^{+/+}$ (+/+), $Htr4^{+/loxP}$ (loxP/+) and $Htr4^{loxP/loxP}$ (loxP/loxP) showing the shift in amplicon size in the targeted allele. **d**, Quantitative RT-PCR (mean $\pm$ SEM) of the expression of marker genes for each hippocampal region in all samples used in Fig. 1c. Data points represent biological replicates of WT (empty circles, n = 2) and cKO (filled circles, n = 6) samples. One-way ANOVA followed by post hoc Fisher's LSD test, ***p < 0.001, ****p < 0.0001. **e**, Top shows a schematic of TaqMan probes spanning *Htr4* exons 3-4 (Probe 3-4), and 6-7 (Probe 6-7). Bottom is quantitative RT-PCR results (mean $\pm$ SEM) showing the persistent expression of exon 3, 4, 6 and 7 in *Htr4* mRNA in cKO mice compared to WT along the dorsoventral axis of the hippocampus.

Fig. S2 | Drd3-Cre and Htr4-bacTRAP expression is restricted to mature excitatory neurons in the hippocampus. Related to Fig. 2. **a**, Anti-EGFP (green) and anti-GAD67 (red) immunofluorescent confocal images of coronal hippocampal sections from a Drd3-Cre::Rosa^{fsTRAP} mouse. Drd3-Cre driven EGFP expressing cells were GAD67-negative, both in the CA1 (left), and CA3 (right) fields. Arrowheads, GAD67-positive; asterisks, GAD67-negative. Scale bar, 100 μ m. **b**, Above, FISH images showing the spatial expression profile of *Htr4* in the mouse brain. Below, Anti-EGFP DAB immunohistochemistry of a Htr4-bacTRAP mouse brain showing that the expression of the BAC transgene EGFP^{L10a} under the regulatory elements of *Htr4* recapitulate the expression profile of *Htr4* in the brain. **c**, Anti-EGFP (green) and anti-GAD67 (red) immunofluorescent confocal images of coronal hippocampal sections from a Htr4-bacTRAP mouse. EGFP expressing cells were GAD67-negative, both in the CA1 (left), and CA3 (right) fields. Arrowheads, GAD67-positive; asterisks, GAD67-negative. Scale bar, 100 μ m. **d**, Immunofluorescent confocal images showing neuronal-type specific markers (red) and EGFP (green) expression in coronal DG sections of Htr4-bacTRAP mice. Arrowheads, cells expressing a specific neuron-type marker but not EGFP. Asterisks, cells coexpressing hilar mossy cell marker CRT and EGFP. ml: molecular layer, gcl: granule cell layer. Scale bars, 50 μ m.

Fig. S3 | Additional behavioral assessment of cKO mice. Related to Fig. 3. **a**, Quantification of the amount of 1% sucrose solution consumed over 72 hours normalized to body weight for *Drd3-Cre::Htr4^{loxP/loxP}* (cKO) mice and *Htr4^{loxP/loxP}* control littermates (WT) during the sucrose consumption test (SCT). Data were analyzed by repeated measures (RM) two-way ANOVA: time $\times$ genotype interaction, $F(3,90) = 2.879$, $p = 0.0403$; genotype factor, $F(1,30) = 2.931$, $p = 0.0972$ followed by post hoc Fisher's LSD test, $**p = 0.0077$. $n_{WT} = 16$, $n_{cKO} = 16$. **b**, Quantification of water consumption during the SCT for each genotype. RM two-way ANOVA: time $\times$ genotype interaction, $F(3,90) = 0.4320$, $p = 0.7306$; genotype factor, $F(1,30) = 0.09834$, $p = 0.7560$. $n_{WT} = 16$, $n_{cKO} = 16$. **c**, Body weight of the mice of each genotype at the end of the SCT. Two-tailed unpaired t-test, $p = 0.682$. $n_{WT} = 16$, $n_{cKO} = 16$. **d**, Time spent in the center in the OF test over 60 min. Two-tailed unpaired t-test, $p = 0.1443$. $n_{WT} = 20$, $n_{cKO} = 17$. **e**, Quantification of locomotor activity (total distance traveled) for each 10-min bin in the OF test. RM two-way ANOVA: genotype factor, $F(1,35) = 0.9715$, $p = 0.3311$. $n_{WT} = 20$, $n_{cKO} = 27$. **f**, cKO mice showed a trend towards higher velocity (cm/s) in the EPM compared to WT. Two-tailed unpaired t-test, $p = 0.0761$. $n_{WT} = 19$, $n_{cKO} = 16$. **g**, Home cage food intake was measured for 30 min following the NSF. Two-tailed unpaired t-test, $**p = 0.0044$. $n_{WT} = 20$, $n_{cKO} = 16$. **h**, Quantification of time spent interacting with an unfamiliar mouse (Mouse) or a novel inanimate object (Object) in the three-chamber social interaction test for cKO or WT controls. Schematic of the testing apparatus is shown at left. RM two-way ANOVA: genotype factor, $F(1,44) = 4.572$, $p = 0.0381$ followed by post hoc Fisher's LSD test, $**p = 0.0038$; $n.s.p = 0.9750$. $n_{WT} = 12$, $n_{cKO} = 12$. **i**, cKO mice displayed larger acoustic startle responses (ASR) compared to WT to increasing levels of acoustic pulses. dB, decibel. RM two-way ANOVA: dB $\times$ genotype interaction, $F(4,84) = 2.618$, $p = 0.0407$; genotype factor, $F(1,21) = 3.409$, $p = 0.0790$ followed by post hoc Fisher's LSD test, $**p = 0.0012$. $n_{WT} = 11$, $n_{cKO} = 12$. **j**, During the pre-pulse inhibition (PPI) test, cKO mice consistently exhibited higher startle responses to 120 dB acoustic stimuli preceded by any pre-pulse (PP) magnitude. RM two-way ANOVA: genotype factor, $F(1,21) = 10.20$, $p = 0.0044$ followed by post hoc Fisher's LSD test, $**p < 0.01$, $*p < 0.05$. $n_{WT} = 11$, $n_{cKO} = 12$. **k**, cKO mice displayed similar levels of PPI as WT. RM two-way ANOVA: genotype factor, $F(1,21) = 0.02151$, $p = 0.8848$. $n_{WT} = 11$, $n_{cKO} = 12$. All data are presented as mean $\pm$ SEM.

Fig. S4 | Effect of the loss of 5-HT₄R on the baseline electrophysiological properties of DG GCs.

Related to Fig. 3. **a**, Representative single action potentials (APs) from WT (black) and cKO (blue) DG GCs. **b-e**, No significant differences in AP properties were observed between genotypes: AP threshold (**b**); AP amplitude (**c**); HAW, half-amplitude width (**d**); fAHP, fast afterhyperpolarization potential (**e**). **f**, Input resistance (R_{input}) was not different between genotypes. **g, h**, Slow afterhyperpolarization potential (sAHP) was similar between genotypes after both 50 Hz and 90 Hz trains of AP in 1 s. All data are presented as mean $\pm$ SEM. For both genotypes, 11 neurons from 3 mice were used for each experiment. Two-tailed unpaired t-test was used for all statistical analyses.

Fig. S5 | Assessment of regional and cell type specificity of vDG TRAP samples. Related to Fig. 4. **a**, Schematic representation of the dissection of hippocampal regions. **b**, In situ hybridization on sagittal sections through hippocampus showing the expression of region-specific genes. dDG: dorsal dentate gyrus, dCA: dorsal CA fields, vDG: ventral dentate gyrus, vCA: ventral CA fields. Arrowheads show region-specific expression for each gene. All in situ hybridization images in are © 2007 Allen Institute for Brain Science and available from: <http://www.mouse.brain-map.org>. **c**, qRT-PCR (mean $\pm$ SEM) showing relative expressions of region-specific genes from b in whole tissue mRNA samples. Two-way ANOVA followed by post hoc Fisher's LSD test comparing region means. *Dsp*: genotype $\times$ region Interaction, $F(3,12) = 0.2140$, $p=0.8848$; genotype factor, $F(1,12) = 0.6292$, $p = 0.4431$; region factor, $F(3,12) = 12.10$, $p = 0.0006$; post hoc, *** $p=0.0003$, ^{ns} $p = 0.2501$. *Tyro3*: genotype $\times$ region Interaction, $F(3,12) = 1.916$, $p = 0.1809$; genotype factor, $F(1,12) = 0.005819$, $p=0.9405$; region factor, $F(3,12) = 34.25$, $p < 0.0001$; post hoc, **** $p < 0.0001$. *Lct*: genotype $\times$ region interaction, $F(3,12) = 0.2134$, $p = 0.8852$; genotype factor, $F(1,12) = 0.05478$, $p = 0.8189$; region factor, $F(3,12) = 279.0$, $p < 0.0001$; post hoc, **** $p < 0.0001$. *Trhr*: genotype $\times$ region interaction, $F(3,12) = 0.4318$, $p=0.7340$; genotype factor, $F(1,12) = 0.002429$, $p=0.9615$; region factor, $F(3,12) = 44.02$, $p < 0.0001$; post hoc, **** $p < 0.0001$. **d**, Scatterplot comparing gene expression between input and IP in the vDG. Cell type marker genes are highlighted as vDG GC-specific (blue), non-neuronal (red), interneuron specific (yellow) and neurogenic (green). **e**, MA-plots showing differential expression analysis between IP and input samples from the vDG of WT (left) and cKO (right). Differentially expressed genes (FDR < 0.05) colored. Selected cell type marker genes are labeled. **f**, Genome browser view of RNA-seq reads mapped to selected cell type marker genes shows that IP samples, compared to input counterparts, were enriched in granule cell specific genes (*Tdo2*, *Calb1*), and depleted for genes specific for neuronal precursors (*Dcx*, *Nr2e1*), interneurons (*Gad2*, *Slc32a1*), astrocytes (*Gfap*, *Aldh1l1*), oligodendrocytes (*Olig2*), microglia (*Olfml3*), pericytes (*Cldn5*) and endothelial cells (*Pdgfrb*).

Fig. S6 | Genes related to 5-HT₄R signaling and neuronal function were altered following 5-HT₄R loss. Related to Fig. 4. **a**, Heatmap comparing normalized expression of IP enriched and depleted genes for each genotype in each sample. Detailed gene lists and values can be found in **Table S2**. **b**, Heatmap showing normalized expression values of calcium and cAMP signaling pathway-related genes in each TRAP IP mRNA from each genotype. Only genes with $p < 0.05$ are represented, red gene symbols indicate $FDR < 0.05$. **c**, Differential expression of ion channels in TRAP IP cKO/WT ($\log_2[\text{fold change}]$). Ion channels with normalized expression < 10 in the TRAP IP were excluded. Size of the bubbles are based on normalized mean expression of each gene across genotypes.

Fig. S7 | Comparison of the molecular properties of the dDG and vDG following 5-HT₄R loss.

Related to Fig. 5. **a**, Heatmaps of normalized expression values showing differentially expressed (DE) genes (FDR < 0.05) between WT and cKO in the whole tissue from dDG (top) and vDG (bottom). **b**, Venn diagram showing the number of overlapping DE genes between WT and cKO in the two regions. **c**, Heatmap showing 25 genes that were significantly altered in the cKO in both the dDG and vDG. Note that three genes (*Nectin3*, *Sept6*, and *Atf1*) were regulated in the opposite directions between two regions. **d**, Summary of shared gene ontology (GO) terms enriched in dDG and vDG (related to Fig. 5b). **e**, Summary of disease enrichment analysis of the DE genes in the vDG. For complete list, see **Table S9**. Note that bipolar disorder, depressive disorder and anxiety were among the significantly enriched diseases. No diseases showed significant association in DE genes of the dDG. **f**, Schematic depicting the experimental approach for quantification of neurogenesis. Left, the hippocampus was divided into three subdivisions (dorsal, intermediate, ventral) depending on the relative position of each coronal section to bregma. DCX+ cells were counted for every sixth 40 µm section. Right, example images of dorsal and ventral DG showing the area (highlighted in yellow) used for analysis. See **Materials and Methods** for details.

Supplementary Material Legends

Table S1. vDG IP cKO vs. WT DE genes. Related to Fig. 4. List of differentially expressed (DE) genes between cKO and WT in vDG IP samples.

Table S2. vDG IP vs. input enriched and depleted genes. Related to Figs. 4 and S6. List of genes enriched and depleted in the IP compared to input for both WT vDG and cKO vDG samples.

Table S3. vDG IP GSEA. Related to Fig. 4. Gene set enrichment analysis results for vDG IP data sets.

Table S4. vDG IP GO analysis. Related to Fig. 4. Gene ontology analysis results for DE genes between genotypes in vDG IP samples.

Table S5. dDG input cKO vs. WT DE genes. Related to Figs. 5 and S7. List of DE genes between cKO and WT in dDG input samples.

Table S6. vDG input cKO vs. WT DE genes. Related to Figs. 5 and S7. List of DE genes between cKO and WT in vDG input samples.

Table S7. dDG and vDG input GO analysis. Related to Figs. 5 and S7. Gene ontology analysis results for DE genes between genotypes for both dDG input and vDG input.

Table S8. vDG input only GO analysis. Related to Figs. 5. Gene ontology analysis results for whole vDG (input)-specific DE genes.

Table S9. vDG input disease enrichment. Related to Figs. 5 and S7. Disease enrichment analysis results for DE genes between genotypes for vDG input.

Document S1. *Htr4* Targeting Vector (TV). Related to Figs. 1 and S1. Annotated TV sequence for the generation of the *Htr4*^{Floxed} line.

Materials and Methods

Animals

All procedures involving animals were approved by The Rockefeller University Institutional Animal Care and Use Committee and were in accordance with National Institutes of Health guidelines. Drd3-Cre (Drd3-Cre KI198) mice were generated by the GENSAT Project¹ and were purchased from the Mutant Mouse Regional Resource Center (MMRRC) Repository (Stock #031741-UCD). Rosa26^{fsTRAP} mice were purchased from the Jackson Laboratories (Stock #022367). Htr4^{Floxed} and Htr4-bacTRAP mice were generated as described below. All animals were bred on a C57BL/6J background, group-housed, maintained on a 12-hr light-dark cycle at The Rockefeller University, and given ad libitum access to food and water. Animals used in the study were male, except for RNA sequencing experiments for which samples from male and female mice were pooled.

Generation of Htr4^{Floxed} mouse line. Targeting vector (TV) was designed in collaboration with inGenious Targeting Laboratory Inc., Ronkonkoma, NY. For annotated TV sequence (in full), see **Document S1**. A 7.92 Kb region used to construct the targeting vector (TV) was first subcloned from a positively identified C57BL/6 BAC clone (RP23:358G18) into a ~2.4kb backbone vector (pSP72, Promega) containing an ampicillin selection cassette for retransformation of the construct prior to electroporation. A pGK-gb2 loxP/FRT neomycin resistance (Neo) cassette was inserted into the gene as depicted in **Fig. S1**. The region was designed such that the short homology arm (SA) extended 2347 base pairs (bp) 3' to the Neo cassette. The long homology arm (LA) ended 5' to the target region and was 4820 bp long. The loxP/FRT flanked Neo cassette was inserted 252 bp downstream of exon 5. The single loxP site, containing engineered MfeI and ApaI sites for southern blot analysis, was inserted 347 bp upstream of exon 5. The target region was 752 bp and included exon 5. The total size of the targeting construct (including vector backbone and Neo cassette) was 13.69 Kb. The targeting vector was confirmed by restriction analysis after each modification step, and finally, by sequencing with the following primers: P6 (5'- GAG TGC ACC ATA TGG ACA TAT TGT C -3'), T73 (5'- TAA TGC AGG TTA ACC TGG CTT ATC G -3'), N1 (5'- TGC GAG GCC AGA GGC CAC TTG TGT AGC -3'), and N2 (5'- TGC GAG GCC AGA GGC CAC TTG TGT AGC -3'). The P6 and T73 primers anneal to the backbone vector sequence and read into the 5' and 3' ends of the BAC sub-clone and the N1 and N2 primers anneal to the 5' and 3' ends of the loxP/FRT flanked Neo cassette and sequence the 5' side of SA and 3' side of the target region, respectively. The TV was linearized using NotI prior to electroporation into C57BL/6 mouse embryonic stem cells. Three lines of chimeric mice were generated at the Janelia Research Campus Gene Targeting and Transgenic Facility. One of these lines (1G12) showed germline transmission of the mutation. The 1G12 line was crossed to a germline Flpe recombinase line to remove the Neo cassette, leaving only two loxP sites flanking exon 5 of Htr4. We established this subsequent line, which are referred to as "Htr4^{Floxed} mice" in our laboratory at Rockefeller University.

Generation of Htr4-bacTRAP mouse line. To generate the Htr4-bacTRAP mice, the RP23-53D24 BAC, which contained the Htr4 locus, was modified using the two-plasmid/one recombination protocol as described previously^{2,3}. Briefly, a homology arm corresponding to the region immediately upstream of the ATG translation initiation site of the Abcb1a gene was cloned into the pS296 targeting vector⁴ containing EGFP10a using the AscI and NotI restriction sites. Recombination was performed by electroporating the pS296-Htr4 vector into electrocompetent DH10β bacteria containing pSV1.RecA plasmid and the BAC. Successful recombination was determined by screening cointegrates by PCR and Southern blot

analysis of HindIII digested BAC DNA, using the homology region as a probe. The modified BAC was prepared by double acetate purification with CsCl centrifugation followed by membrane dialysis and microinjected into the pronuclei of fertilized FVB/N mouse oocytes at a concentration of 0.5 ng/μl. Five transgenic founder mice were generated and crossed to C57BL/6J mice. F1 progeny were screened for proper transgene expression by EGFP immunohistochemistry. Founder line ES1299 showed accurate and robust expression of the transgene and was therefore selected for colony expansion.

Genotyping. At age 15-18 days, pups were marked with an ear punch for identification and tail biopsies were performed. Each tail sample was lysed for DNA extraction in 200 μl tail lysis buffer (100 mM Tris.HCl-pH8.8, 1 mM EDTA, 0.5% Tween20, 100 ug/mL proteinase K) at 56 °C for at least 24 hours. The tail DNA samples were analyzed via PCR using 1-2 μl of lysis solution, GoTaq DNA Polymerase (Promega) and corresponding primer pairs as listed below.

Genotyping primers.

Genotyping Primers			
Mouse line	Primer*	Sequence	Expected results (bp)
Htr4-floxed	Htr4 loxP F	TCGAGGCATTCCTCGATTCA	WT: 172 loxP: 239
Htr4-floxed	Htr4 loxP R	TAACACCTGGCCGAAACGTT	
Any Cre	Cre F	CCGGGCTGCCACGACCAA	WT: n/a Cre: 150
Any Cre	Cre R	GGCGCGGCAACACCATT TTT	
Any EGFP10a	EGFP F	GCACGACTTCTTCAAGTCCGCCATGCC	WT: n/a EGFP: 265
Any EGFP10a	EGFP R	GCGGATCTTGAAGTTCACCTTGATGCC	
Rosa ^{fsTRAP}	Rosa WT F	AAGGGAGCTGCAGTGGAGTA	WT: 297
Rosa ^{fsTRAP}	Rosa WT R	GAGCGGGAGAAATGGATA G	
*F: forward, R: reverse			

cAMP induction assay

Intact *Htr4* (WT) and mutant *Htr4*^{delE5} (delE5) cDNA coding sequences (CDSs) (inserts) were cloned into pCMV6-Entry-Myc-DDK tagged mammalian expression vector (OriGene, CAT#: PS100001) by homologous recombination-based In-fusion HD cloning kit (Clontech). The inserts were amplified by PCR using CloneAmp HiFi PCR Premix (Clontech) with primers “Htr4 infusion forward” (5'- AGA TCT GCC GCC GCG ATC GCC ATG GAC AAA CTT GAT GCT AAT GTG -3') and “Htr4 infusion reverse” (5'- GCG GCC GCG TAC GCG TAG TAT CAC TGG GCT GAG CAG -3') from the cDNA samples generated from the dentate gyrus of either Drd3-Cre::Htr4^{loxP/loxP} (cKO) mice for delE5, or Htr4^{loxP/loxP} mice for WT expression. The primers were designed using “Online In-Fusion Tools” (<https://bit.ly/1eFKzsP>) to include 5' and 3' homologous sequences to the ends of the linearized vector for In-Fusion HD cloning, and the *Htr4* CDS start codon, yet exclude the stop codon to allow for the expression of the MYC-DDK tag sequences. pCMV6-EGFP was used as the control. HEK293T cells (ATCC) cultured in 24-well plates (1.9 cm² culture area, Corning) in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) were transfected with the delE5, WT or EGFP plasmids using FuGENE 6 transfection reagent (Promega). Forty-eight hours after the transfection, all cells were incubated in stimulation buffer consisting of DMEM + 0.5mM IBMX (Sigma Aldrich) for 30 min. For cAMP induction, cells were further incubated in either stimulation buffer or 100μM Zacopride (Tocris Bioscience)

in stimulation buffer for 45 min. The cells were then lysed with lysis buffer (0.1 M HCl, 0.1% Triton-X) for 20 min, and the lysates were centrifuged for 10 min at 21,500 x g. cAMP levels in the supernatants were measured by monoclonal anti-cAMP antibody based direct cAMP ELISA kit according to the manufacturer's guidelines (Non-acetylated version, NewEast Biosciences). Four biological replicates and two technical replicates were performed for each experimental group. The cAMP level of each biological replicate was normalized to its mean protein concentration measured using Qubit protein assay kit (Invitrogen) and Qubit 3.0 Fluorometer (Invitrogen). Statistical analysis of normalized cAMP levels was performed in GraphPad Prism 7 using one-way ANOVA followed by Fisher's LSD.

Immunohistochemistry

For fixed brain serial slice preparations, mice were deeply anesthetized by ketamine/xylazine and transcardially perfused with 10 ml of phosphate buffered saline (PBS) followed by 30 ml of 4% paraformaldehyde (PFA) in PBS. Brains were post-fixed in 4% PFA overnight at 4°C and cryoprotected by 30% sucrose in PBS. Coronal sections (40 µm) were acquired using a freezing microtome (Leica SM2010 R Sliding Microtome). The sections were then transferred to PBS, or cryoprotection buffer (25% glycerol and 25% ethylene glycol in PBS, pH 7.4) for long term storage at -20°C. For both 3,3'-Diaminobenzidine (DAB) immunohistochemistry (IHC) and all immunofluorescence (IFC) procedures, free-floating serial sections were blocked for 60 min at room temperature in PBS containing 3-5% normal donkey serum (NDS, Jackson ImmunoResearch) or normal goat serum (NGS, Vector Laboratories) and 0.1% Triton X-100. Sections were then incubated in the same blocking buffer overnight at 4°C with primary antibodies listed below at corresponding dilutions. On the next day, the sections were washed three times with PBS each for 5 min, and then incubated for 2 h at room temperature with the appropriate Alexa dye-conjugated (for IFC) or horseradish peroxidase (HRPT)-conjugated (for DAB IHC) secondary antibodies (Invitrogen) diluted 1:500 in the blocking buffer. The sections were then washed three times with PBS each for 5 min. For IFC, the sections were counterstained with DAPI for nuclear staining at 1:10000 dilution followed by three times PBS wash each for 5 min. For DAB IHC, the staining was developed using SIGMAFAST™ DAB tablets (Sigma-Aldrich) following the manufacturer's protocol. The sections were mounted on the Superfrost slides (VWR) and sealed with Prolong Gold mounting reagent (Invitrogen). The slides were imaged on a Zeiss LSM700 confocal microscope for Fig. 2a-d, S2a-d and 5e, or a Zeiss Axioskop 2 microscope for Fig1b, Fig S2b and Fig S7f. Brightness was optimized using ImageJ software post-acquisition.

Primary antibodies used in this study.

Antibody Name	Species	Source	Catalog #	Dilution
GAD67, clone 1G10.2	Monoclonal	Millipore	MAB5406	1:500
GFP	Chicken	Abcam	ab13970	1:1000
NeuN, clone A60	Monoclonal	Millipore	MAB377	1:1000
Calbindin-D-28K	Rabbit	Sigma	C7354	1:500
Calretinin	Monoclonal	Swant	6B3	1:500
Doublecortin (C-18)	Goat	Santa Cruz	sc-8066	1:200

Quantification of neurogenesis

An experimenter blinded to genotype counted number of DCX-positive (DCX+) cells in the DG granule cell layer (GCL) in every sixth 40 μ m thick coronal sections through the entire hippocampus of WT or cKO mice (n = 5 per genotype). One hemisphere per animal was selected based on section quality and cells were counted at any depth through the entire section. Full size images of each section were then acquired and the area of GCL was calculated for each section by manually tracing the perimeter of the GCL as region of interest (ROI) in ImageJ. The hippocampus was divided into three subdivisions depending on the position of the coronal sections from bregma⁵: dorsal (-0.96 to -2.06), intermediate (-2.06 to -3.08) and ventral (-3.08 to -4.04). Each tissue section was registered to a subdivision (4 $\pm$ 1 sections per animal per subdivision). Neurogenesis was calculated as the number of DCX+ cells per area of GCL within a subdivision and values for each animal (cKO and WT) was normalized to the WT mean for each subdivision.

Fluorescent in-situ hybridization (FISH)

Mice were transcardially perfused first in PBS and then 4% paraformaldehyde in PBS. After dissection, brains were post-fixed overnight at 4°C. They were then cryopreserved in 30% sucrose and sectioned using a cryostat (14 μ m sections). Antigen retrieval was performed on fixed frozen tissue using an Oster Steamer, followed by FISH (RNAscope® Technology). The RNAscope® Multiplex Fluorescent Reagent Kit V2 (Advanced Cell Diagnostics) was used to target *Htr4* RNA using RNAscope® Probe Mm-Htr4 (Advanced Cell Diagnostics). Both antigen retrieval and FISH were performed according to manufacturer's guidelines. Slides were coverslipped and imaged on an LSM700 confocal microscope.

Total RNA isolation and quantitative RT-PCR (qRT-PCR)

Hippocampus tissue samples were dissected with fine tip forceps in ice-cold HBSS containing 2.5 mM HEPES-KOH (pH 7.4), 35 mM glucose, 4 mM NaHCO₃ and total RNA was isolated using RNeasy Micro Kit (Qiagen) with on-column DNase digestion. RNA quantity was measured with a Nanodrop 1000 spectrophotometer (Thermo Scientific). cDNA samples were generated from 300-1000 ng of total RNA using qScript cDNA SuperMix (QuantaBio). All qRT-PCR experiments were performed on the LightCycler 480 System (Roche), using TaqMan assays (Applied Biosystems) and LightCycler 480 Probes Master mix (Roche). TaqMan assays used in this study are listed below. Default cycling conditions were followed (pre-incubation: one cycle, 95°C, 5 min; amplification: 45 cycles, 95°C for 10 s, 60°C for 30 s, 72°C for 1 s; ramp rate: 4.4°C/s). 10-20 ng of cDNA were used for each qRT-PCR reaction and three technical replicates were run for every sample. The mean C_T for each technical replicate was used for the quantification. Data were normalized to *Gapdh* as the endogenous control by the comparative C_T (2^{- $\Delta\Delta$ CT}) method⁶.

TaqMan gene expression assays used for qRT-PCR.

Gene Symbol	Assay ID	Dye
Htr4 (3-4)	Mm00434129_m1	FAM
Htr4 (4-5)	Mm01258807_m1	FAM
Htr4 (5-6)	Mm01258808_m1	FAM
Htr4 (6-7)	Mm01258809_m1	FAM
Tyro3	Mm00444547_m1	FAM
Lct	Mm01285112_m1	FAM

Dsp	Mm01351876_m1	FAM
Trhr	Mm00443262_m1	FAM
Gapdh	Mm99999915_g1	FAM

Translating ribosome affinity purification (TRAP)

Affinity purification of EGFP-tagged polysomes was performed as previously described⁷, with minor modifications. Drd3-Cre::Rosa^{fsTRAP} (WT) and Drd3-Cre::Htr4^{loxP/loxP}::Rosa^{fsTRAP} (cKO) mice were sacrificed, followed by rapid dissection of dorsal and ventral dentate gyrus with fine tip forceps in ice-cold HBSS containing 2.5 mM HEPES-KOH (pH 7.4), 35 mM glucose, 4 mM NaHCO₃, and 100 µg/ml cycloheximide. Tissue from one male and one female mouse was pooled for each biological replicate. Pooled dentate gyrus samples were then homogenized in 1 ml extraction buffer containing 10 mM HEPES-KOH (pH 7.4), 150 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 100 µg/ml cycloheximide, RNasin (Promega) and SUPERas-In (Life Technologies) RNase inhibitors, and Complete-EDTA-free protease inhibitors (Roche). Homogenates were cleared by centrifugation at 2000 x g at 4 °C. IGEPAL CA-630 (NP-40, Sigma) and DHPC (Avanti Polar Lipids) were both added to the supernatants (S2) to a final concentration of 1% for each, followed by centrifugation at 20,000 x g. Polysomes were immunoprecipitated from these supernatants (S20) using 100 µg monoclonal anti-EGFP antibodies (50 µg each of clones 19C8 and 19F7⁴) bound to biotinylated-Protein L (Pierce, Thermo Scientific) coated streptavidin-conjugated magnetic beads (Life Technologies), and washed in low salt buffer containing 10 mM HEPES-KOH (pH7.4), 150 mM KCl, 5 mM MgCl₂, 1% IGEPAL CA-630, 0.5 mM DTT, 100 µg/ml cycloheximide, and RNasin RNase inhibitors (Promega). IPs were carried out overnight at 4°C and beads were washed with and washed in high salt buffer containing 10 mM HEPES-KOH (pH7.4), 350 mM KCl, 5 mM MgCl₂, 1% IGEPAL CA-630, 0.5 mM DTT, 100 µg/ml cycloheximide, and RNasin RNase inhibitors (Promega). Bound RNA was purified using the RNeasy Micro Kit (Qiagen) with on-column DNase digestion. RNA was also purified from a fraction of the pre-IP S20 supernatant to serve as whole-tissue (input) samples. RNA quantity was determined using the Qubit RNA HS Assay kit (Invitrogen) and RNA quality was determined using Agilent 2100 Bioanalyzer with RNA 6000 Pico chips. Only samples with RNA integrity values ≥ 7.0 were used for RNA-seq.

RNA-sequencing (RNA-seq)

For each sample, 15 ng of purified RNA was converted to cDNA and amplified using the Ovation RNA-Seq System V2 Kit (NuGEN) following manufacture's guidelines. cDNA was fragmented to an average size of 250 bp using a Covaris C2 sonicator with the following parameters: intensity 5, duty cycle 10%, cycles per burst 200, treatment time 120 seconds. RNA-seq libraries were prepared from 10 µg amplified RNA using the TruSeq RNA Sample Preparation Kit v2 (Illumina) following manufacturer's protocols and libraries were sequenced at The Rockefeller University Genomics Resource Center on the Illumina NextSeq 500 platform to obtain 75 bp paired-end reads. The RNA-seq datasets generated in this study are listed below.

Overview of RNA-seq datasets.

Sample*	FastQ Name*
vDG_WT_IP_1	Drd3-Cre_vDG_WT_IP_Rep1
vDG_WT_IP_2	Drd3-Cre_vDG_WT_IP_Rep2
vDG_WT_IP_3	Drd3-Cre_vDG_WT_IP_Rep3

vDG_cKO_IP_1	Drd3-Cre_vDG_Htr4_cKO_IP_Rep1
vDG_cKO_IP_2	Drd3-Cre_vDG_Htr4_cKO_IP_Rep2
vDG_WT_Input_1	Drd3-Cre_vDG_WT_input_Rep1
vDG_WT_Input_2	Drd3-Cre_vDG_WT_input_Rep2
vDG_WT_Input_3	Drd3-Cre_vDG_WT_input_Rep3
vDG_cKO_Input_1	Drd3-Cre_vDG_Htr4_cKO_input_Rep1
vDG_cKO_Input_2	Drd3-Cre_vDG_Htr4_cKO_input_Rep2
dDG_WT_Input_1	Drd3-Cre_dDG_WT_input_Rep1
dDG_WT_Input_2	Drd3-Cre_dDG_WT_input_Rep2
dDG_WT_Input_3	Drd3-Cre_dDG_WT_input_Rep3
dDG_cKO_Input_1	Drd3-Cre_dDG_Htr4_cKO_input_Rep1
dDG_cKO_Input_2	Drd3-Cre_dDG_Htr4_cKO_input_Rep2
* brain_region_genotype_RNA source_replicate	
‡ All data sets have been deposited in NCBI's Gene Expression Omnibus (GEO) ⁸ : accession number pending.	

RNA-seq read mapping, analysis, and visualization

RNA-seq read quality was assessed by FastQC (0.11.4). Sequences were trimmed for trailing adaptors from the Illumina sequencing process by Trim Galor (0.4.1). The trimmed reads were then aligned to annotated exons using the mm10 mouse reference genome (UCSC) with STAR (2.4.2a)⁹ using default settings. SAMtools (v0.1.19-444) was used for indexing and removal of duplicates. The numbers of raw and mapped reads (bases), and the percentage of mapping for each sample were calculated via Picard (1.123) using the CollectRNASeqMetrics program and listed below. The quantification of aligned, sorted and indexed reads was done using the htseq-count module of the HTSeq framework (0.6.0)¹⁰, using the “union” mode with default settings to generate raw counts for each sample. Aligned bam files were converted to tdf format using igvtools (2.3.61) for Integrative Genomics Viewer (IGV) visualization. Differential expression analyses were performed using DESeq2¹¹, R-package version 1.4.5. Differentially expressed (DE) genes for each analysis were determined based on adjusted p-values (p-adj), also termed as false discovery rate (FDR), and reported in Results or the figure legends. IP enriched genes were determined as IP over input fold change ≥ 1.25 with FDR < 0.05, and IP depleted genes as IP over input fold change < 0.5 with FDR < 0.05. For Fig. 4d-f, when determining DE genes between the cKO and WT TRAP mRNA, nine genes were detected as genes normally depleted in IP (IP depleted in WT) and excluded from further analysis (red genes in Table S2). For MA-plots, $\log_2$ of the fold change between genotypes ($\log_2$ [fold change]) and $\log_2$ of the mean of the normalized expression values in all samples ($\log_2$ [mean expression]) for each gene were used. Scatter plots were generated using normalized expression values ($\log_2$ [norm. expr.]). Heatmap visualizations (one minus Pearson correlation) of normalized expression values were generated using web-based Morpheus software (Broad Institute, <https://software.broadinstitute.org/morpheus>) and z-scores were calculated using mean subtracted and SD normalized values by gene. Nominal p-values (p) were also given when normalized expressions of specific genes were reported. Full lists of DE genes can be found in **Tables S1** (vDG IP cKO vs. WT), **S2** (IP vs. input for cKO and WT), **S5** (dDG input cKO vs. WT), and **S6** (vDG input cKO vs. WT). Gene set enrichment analysis (GSEA) was performed on the fold change-ranked gene list of IP samples comparing cKO and WT (p < 0.05, $\log_2$ [norm. expr.] > 1). We also excluded genes strongly depleted in the IP (WT IP/Input enrichment FDR < 0.05, $\log_2$ [fold change] < -1) and *Htr4* as GSEA accounts for the

direction of the regulation of gene expression and *Htr4* expression seemed upregulated although it was non-functional. GSEA desktop v3.0 software (Broad Institute) was used with the following parameters: Gene sets database = Molecular Signature Database (MSigDB), C2, Canonical Pathways (CP, c2.cp.v6.2.symbols.gmt); number of permutations = 1000; enrichment statistics = classic; set size, max = 100, min = 10; normalization = meandiv. Gene sets with FDR ≤ 25 are reported as advised¹². Gene ontology (GO) and disease enrichment analyses were performed using ToppGene Suite (<http://toppgene.cchmc.org>)¹³. “GO: Biological Process” results were reported for GO analyses. FDR (Benjamini-Hochberg) < 0.05 was considered statistically significant. Network view in Fig. 4h was generated using the EnrichmentMap plugin (3.1.0) for Cytoscape (3.7.0) using enriched GO terms (FDR < 0.01, nodes) and the percentage of overlapping genes in among those terms (overlap > 50%, edges).

Quality control of RNA-seq alignments.

Sample	Raw Reads	Mapped Reads	Ribosomal%	Coding %	UTR %	Intronic%	Intergenic %	mRNA* %
dDG_WT_Input_1	4976974951	4898777541	11.00	26.15	34.18	11.65	17.48	60.33
dDG_WT_Input_2	4114194145	4048376030	9.60	28.97	36.85	9.12	15.87	65.82
dDG_WT_Input_3	6348807839	6241867530	13.52	30.73	36.91	7.14	12.29	67.64
dDG_KO_Input_1	5318465410	5237452256	9.40	29.55	37.17	8.97	15.33	66.72
dDG_KO_Input_2	4494618359	4433172922	7.08	30.48	37.47	8.40	16.89	67.95
vDG_WT_Input_1	5274266386	5200347558	7.32	29.76	37.30	9.69	16.25	67.07
vDG_WT_Input_2	5908160994	5817160131	8.02	31.64	36.97	8.64	15.09	68.61
vDG_WT_Input_3	5200024888	5110416182	12.45	30.12	36.81	7.70	13.47	66.93
vDG_KO_Input_1	5390357255	5295752262	11.57	27.52	35.93	8.45	17.04	63.45
vDG_KO_Input_2	5347253361	5264487834	10.03	29.78	38.46	8.79	13.38	68.24
vDG_WT_IP_1	5055676715	4963214738	15.25	35.93	39.45	3.22	6.83	75.37
vDG_WT_IP_2	5200875692	5096531166	18.77	34.24	37.98	2.97	6.88	72.22
vDG_WT_IP_3	5176647321	5088146609	11.74	39.38	38.93	3.07	7.41	78.31
vDG_KO_IP_1	4386834142	4294720920	18.98	33.93	37.81	3.40	6.72	71.74
vDG_KO_IP_2	5041820503	4938661168	18.97	34.77	37.26	3.27	6.56	72.04
* mRNA is the percentage of total bases mapping to Coding + UTR.								

Electrophysiological recordings

Eight-week-old mice were euthanized with CO₂. After decapitation and removal of the brains, transverse slices (400 μ m thickness) were cut using a Vibratome 1000 Plus (Leica Microsystems) at 2 °C in a NMDG-containing cutting solution (in mM): 105 NMDG (N-Methyl-D-glucamine), 105 HCl, 2.5 KCl, 1.2 NaH₂PO₄, 26 NaHCO₃, 25 Glucose, 10 MgSO₄, 0.5 CaCl₂, 5 L-Ascorbic Acid, 3 Sodium Pyruvate, 2 Thiourea (pH was around 7.4, with osmolarity of 295–305 mOsm). After cutting, slices were left to recover for 15 min in the same cutting solution at 35 °C and for 1 h at room temperature (RT) in recording solution (see below). Whole-cell patch-clamp recordings were performed with a Multiclamp 700B/Digidata1550A system (Molecular Devices). Dentate gyrus granule neurons were selected for recording based on their size, shape and position in the granular layer using an upright Olympus BX51WI microscope. The extracellular solution used for recordings contained (in mM): 125 NaCl, 25 NaHCO₃, 2.5 KCl, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂ and 25 glucose (bubbled with 95% O₂ and 5% CO₂). The slice was placed

in a recording chamber (RC-27L, Warner Instruments) and constantly perfused with oxygenated aCSF at 24 °C (TC-324B, Warner Instruments) at a rate of 1.5–2.0 ml/min. Whole-cell patch-clamp recordings were obtained from granule neurons using recording pipettes (King Precision Glass, Inc, Glass type 8250) pulled in a horizontal pipette puller (Narishige) to a resistance of 3–4 MΩ, filled with an internal solution containing (in mM): 126 K-gluconate, 4 NaCl, 1 MgSO₄, 0.02 CaCl₂, 0.1 BAPTA, 15 glucose, 5 HEPES, 3 ATP, 0.1 GTP (pH 7.3). For whole-cell recordings in the voltage clamp configuration, BIMU-8 (10 mM, Tocris Bioscience) was added to the bath for 1 minute and subsequently washed. Tetrodotoxin (TTX, 1 mM, Tocris Bioscience) was added previously to the bath to avoid activation of neighboring neurons by BIMU-8. To measure the firing of the DG granule neurons, steps of 10 pA current were injected from a set starting membrane potential of -80mV. Data were acquired at a sampling frequency of 50 kHz and filtered at 1 kHz and analyzed offline using pClamp10 software (Molecular Devices).

Behavior

Behavioral tests were performed on male mice from eight weeks up to four months of age. All mice were age-matched for each test, and control groups always consisted of WT littermates. For each test, mouse order was randomized, and the experimenter was blinded to genotype during the tests and data analyses. Mice were brought into the procedure room in their home cages and habituated to the room for one hour. Tests were performed within the light period of the light-dark cycle unless otherwise noted. The number of animals per group (n) and the statistical analyses are reported in the figure legends. For analysis of each behavioral tests, data points outside two standard-deviations of the mean for each group were determined as outliers and excluded. Statistical analyses were performed using GraphPad Prism 7 and 8 software, and $p < 0.05$ was considered significant.

Tail suspension test. Adhesive tape was used to suspend mice by their tail (0.5-1 cm from the tip of the tail) to the end of a piece of flexible plastic tubing hanging from the top center of one of the four compartments of a white acrylic box. Four mice were tested at the same time in each compartment and were separated by white acrylic walls. While suspending, mice were about 30 cm above the floor of the box. Test sessions lasted 6 min and were videotaped. Mice that held on to their bodies using their front limbs or started climbing up their tails were gently repositioned using a long stick without distracting other subjects. The floors and the walls of the box were cleaned with Clidox sterilizer and water in between trials. The amount of time spent immobile in the last 4 min of the test was quantified, which was extrapolated by scoring as mobile or immobile in every 5 s. In case of clasping to body parts or climbing up the tail, scoring was not applicable, and those mice were excluded from the analysis

Forced swim test. Mice were individually placed into glass cylinders (15 cm diameter, 35 cm height) filled with tap water (23-25 °C) to a height of 15.7 cm. Four mice were tested at the same time in separate cylinders placed as 2x2 adjacently. The cylinders were visually separated by white acrylic sheets. To start a trial, mice were gently placed into the cylinders keeping their head above the water surface. Test sessions lasted 6 min and were videotaped from above. The amount of time spent immobile, defined as the absence of all motions except floating and those required to keep a mouse's head above the surface, was measured for the last 4 min of the trial, which was extrapolated by scoring as mobile or immobile in every 5 s.

Splash test. These experiments were performed in the dark cycle (20:00 – 23:59) to observe a higher amount of activity. The procedure room was illuminated with red light. Each mouse was sprayed with 200 µl 10% sucrose in tap water on their lower back using a 1 ml syringe with a needle tip attached.

Immediately after, mice were placed in clean small mouse cages (22.2x30.80x16.24 cm) with bedding and filter top (no wire top). Four mice were tested at the same time in adjacent cages visually separated by white acrylic sheets. Test sessions lasted 5 min and were videotaped from the side. Grooming time was measured for a total 5 min, which was extrapolated by scoring as grooming or non-grooming in every 5 s.

Sucrose consumption test. Each mouse was placed in a clean cage with bedding and food, containing two water bottles, one filled with 1% sucrose in tap water and another filled with just tap water. Bottles were 50 ml Falcon conical tubes with sipper caps composed of 2.5 cm straight stainless steel ball-point sipper tubes (Ancare), inserted into rubber stoppers. Consumption of sucrose solution and water was monitored for 72 hours by measuring the decrease in the weight of the bottles every 12 h. At the each 12-hour mark, the bottles were switched to avoid side preference. At the end of the test, animals were weighed and amount of sucrose and water consumed (g) was normalized the weight per animal (g).

Open field test. Open field behavior was assayed in a square, acrylic arena (50x50x22.5 cm) with white floor and clear walls, equipped with two rows of infrared photocells placed 20 and 50 mm above the floor, spaced 31 mm apart. The procedure room was brightly and homogenously lit with fluorescent ceiling lamps. Each animal was placed in the open field arena for 60 min. Photocell beam interruptions were recorded on a computer using the Superflex software (Accuscan Instruments). The floors and the walls of the arenas were cleaned with Clidox sterilizer and water in between trials. The data was exported as time spent and distance traveled in total, peripheral and center areas in 10 min bins.

Elevated plus maze. The elevated plus maze consisted of two opposite open arms without sidewalls (35x5 cm) and two enclosed arms with black 14 cm high sidewalls (35x5x14 cm) connected by a common central platform (5 cm²). The entire plus-maze apparatus was elevated 30 cm off the ground. The experimental area was isolated from the experimenter with black curtains running down from the ceiling to the floor on four-sides and homogenously lit by ceiling mounted LED lamps on four sides, ensuring minimal shadows. The illumination was adjusted so the middle of each open arm was at 30-lux. Testing began by placing an animal on the central platform of the maze facing the same open arm. Trials lasted 5 min and recorded by a ceiling mounted camera connected to a computer. EthoVision XT 7.0 software (Noldus) was used to track the mouse and record the total time spent in each arm, total distance traveled, and velocity. The maze was wiped with 30% Ethanol in between trials. Mice that fell off the open arms were excluded from the analysis.

Novelty suppressed feeding. Mice were food deprived for 24-hour prior to testing. Body weight was monitored to determine efficiency of deprivation. Testing was performed in a brightly lit arena similar to the open field (50x50x22.5 cm) where a food pellet (1.8 cm length of 1.58x0.95 cm diameter oval pellets) was placed in the center on a circular filter paper (20 cm diameter, Fisherbrand). A white noise source was active throughout the habituation and testing. Four subjects were tested at the same time in adjacent arenas. The latency to bite the food within a 15 min trial was recoded. Following NSF, each animal was put in a clean vivarium cage for 30 min with food ad libidum after each trial. The weight of the food was measured before and after the 30 min test.

Acoustic startle response and pre-pulse inhibition. Startle and PPI testing were performed in a startle response systems (39x38x58 cm, SR-LAB system, San Diego Instruments) consisting of a non-restrictive clear Plexiglas cylinders (inner diameter 4 cm, length 13 cm) resting on a white Plexiglas platform and placed in a ventilated, sound-attenuated chamber. High frequency speakers, controlled by

SR-LAB software, were mounted 33 cm above the cylinders and used to present all acoustic stimuli. Piezoelectric accelerometers mounted under the cylinders transduced movements of the animals which were digitized and stored by an interface and computer assembly. A dynamic calibration system was used to ensure comparable sensitivities across four separate chambers. The test mouse was placed into the startle chamber and allowed to acclimatize for 5 min. The time between each trial was 7-23 s. The trial consisted of four blocks. All sound intensities were presented in a pseudo-random order within a block. Beginning at startling stimulus onset, 65 consecutive 1-ms readings were recorded to obtain the peak amplitude of the animal's startle response (V_{max}). Five seconds after each stimulus, another 65 ms were measured which constitutes the no-stimulus trials. Background noise was 65 dB. In the first block (pre-test), mice were presented with 120 dB acoustic stimuli (65 ms) for five trials. V_{max} were recorded per trial and averaged per subject. In the second block (ASR), acoustic stimuli were presented in five different intensities (120, 110, 100, 90 and 80 dB, 65 ms), each for four trials. V_{max} was recorded for each trial and averaged for each stimulus intensity per subject. In the third block (PPI), a 120 dB acoustic stimuli (40 ms) was preceded by four different pre-pulse (PP) intensities (0, 3, 6, 12 dB, 20 ms) for ten trials. The time between the prepulse and the pulse was 100 ms. V_{max} was recorded for each trial and averaged for each prepulse intensity per subject. PPI was calculated as the percent decrease in startle response in PP3, PP6 and PP12 compared to PP0 per subject. In the fourth block (post-test), 120 dB stimuli (65 ms) were presented for five trials. V_{max} was recorded per trial, averaged per subject, and compared to the pre-test responses to assess the habituation of ASR during the testing.

Social interaction. Social behavior was measured using the three-chamber social interaction test. A rectangular test arena (58.5x43x22.7 cm) was made of white acrylic and two inner walls divided it equally into three chambers. Each inner wall had an open middle section with a removable sliding door, allowing free access between the middle and the two side chambers when the doors were removed. The social interaction test was comprised of two parts: a habituation session and a trial session. During the habituation session, two empty wire cups were placed in the left and right chambers. The test mouse was placed in the center chamber and allowed to explore all three chambers for 10 min. Next, the doors to the side chambers were closed, and the test mouse was placed in the middle chamber for the trial session. A stranger mouse of the same sex/appearance and a novel inanimate object (plastic building toy) of similar size were placed inside the left and right wire cups in a random order to balance for side preference. Doors between chambers were opened to allow the test mouse to explore the three chambers freely for 10 min. During all sessions, the arena was videotaped from above and the interaction time with each cup, defined as sniffing or touching cup, was manually measured from the recorded videos. Stranger mice were habituated to the wire cups for 10 min each day for three days prior to testing. The arena and the wire cups were thoroughly cleaned with Clidox sterilizer and water between subjects.

Statistical analysis

All data analysis was performed using GraphPad Prism 7 and 8, Microsoft Excel or R¹⁴. Statistical parameters including the exact value of n, precision measures (mean $\pm$ SEM) and statistical significance are reported within the Results or the figure legends. Data were determined to be statistically significant when $p < 0.05$ by two-way ANOVA (ordinary or repeated measures [RM]) followed by post hoc Fisher's LSD test, one-way ANOVA followed by post hoc Fisher's LSD test, or two-tailed unpaired t-test. Statistical methods to analyze RNA-seq data are discussed in the "RNA-seq read mapping, analysis, and visualization" section.

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