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Research article

Serotonin 5-HT$_4$ receptors modulate the development of glutamatergic input to the dorsal raphe nucleus

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HIGHLIGHTS

- The dorsal raphe nucleus is controlled by glutamatergic inputs.
- Reduced expression of 5-HT$_4$ receptors affects the formation of glutamatergic synapses in the DRN.
- 5-HT$_4$ receptors may modulate the development of cortical control of the DRN.

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ABSTRACT

The dorsal raphe nucleus (DRN) is a major serotonin (5-hydroxytryptamine, 5-HT)-producing region in the central nervous system. It receives glutamatergic inputs from several brain regions, which are reciprocally modulated by serotonergic signals. We investigated whether serotonin 5-HT$_4$ receptors (5-HT$_4$Rs) play a role in the development of glutamatergic control of the DRN, with an emphasis on cortical inputs. Double-label immunohistochemistry and confocal microscopy were used to quantify vesicular glutamate transporter 1 (vGluT1)-immunoreactive terminals in the DRN of mice with a null-mutation in the 5-HT$_4$R gene. We found no significant change in the overall density of vGluT1-positive terminals in homozygous and heterozygous mice, but heterozygous mice had a significantly higher density of vGluT1-positive terminals contacting serotonergic neurons. These results suggest that altered 5-HT$_4$R expression may affect the development of cortical glutamatergic control of the DRN.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT)-producing neurons in the dorsal raphe nucleus (DRN) are modulated by glutamatergic inputs. Some of these inputs are local [1,2], but a number of major, long-range projections originate in forebrain regions, such as the hypothalamus, the lateral habenula, and the prefrontal cortex (PFC) [3–5]. Cortical terminals in the DRN primarily express the vesicular glutamate transporter 1 (vGluT1) and therefore can be distinguished from non-cortical afferents that primarily express other vGluTs (vGluT2 or vGluT3) [4,6,7]. This general expression pattern has been well conserved in vertebrate evolution [8].

The medial PFC (mPFC) has been particularly well studied in relation to the DRN [9,10]. The rodent mPFC is homologous to the human agranular mPFC [11–13] and it dynamically controls behavioral decisions on a fine temporal scale [14,15], with implications for the neurobiology of depression, social avoidance, and other brain states [15,16]. DRN-projecting neurons in the mPFC express several 5-HT receptors, the activity of which has different effects on DRN targets. Activation of 5-HT$_{1A}$ receptors (5-HT$_{1A}$R) in the mPFC decreases the firing rate of DRN neurons [17,18], whereas activation of 5-HT$_{2A}$ and 5-HT$_4$ receptors (5-HT$_{2A}$R and 5-HT$_4$R) has the opposite effect [18–20]. The three receptors (5-HT$_{1A}$R, 5-HT$_{2A}$R, 5-HT$_4$R) are coupled to different G-proteins (G_i, G_q, and G_s, respectively) and can collectively regulate neuronal excitability [21–23], including compensatory changes [24]. Their expression is upregulated prenatally [25–27], before cortical projection neurons can establish synapses with their brainstem targets [28]. Therefore, these receptors are likely to affect the formation and stabilization of synapses between cortical terminals and DRN neurons, and their altered expression in perinatal development may result in an abnormal behavioral dynamic later in life.

To gain a better understanding of these processes, we investigated the structure of synaptic contacts between vGluT1-positive glutamatergic terminals and serotonergic DRN neurons in mice lacking functional 5-HT$_4$Rs.

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2. Materials and methods

2.1. Animals

Mice with a null-mutation for the 5-HT_4R gene (B6.129P2-Htr4^{tm1Dgen}/J) were purchased from The Jackson Laboratory and kept in a colony. They were bred to produce homozygous knockout (−/−), homozygous wild-type (+/+), and heterozygous (+/−) mice on the same genetic background. Pups were genotyped using the protocol recommended by the supplier. Mice were kept on a 12:12 light-dark cycle with free access to water and food. All procedures have been approved by the UCSB Institutional Animal Care and Use Committee.

2.2. Immunohistochemistry and confocal microscopy

Adult 5-HT_4R −/−, +/−, and +/+ mice (males and females) were deeply anesthetized with a mixture of ketamine (200 mg/kg) and xylazine (20 mg/kg) and transcardially perfused with saline, followed by chilled 4% paraformaldehyde. The mice were 8 weeks of age in all groups. Brains were dissected, postfixed overnight in 4% paraformaldehyde at 4 °C, cryoprotected overnight in 30% sucrose at 4 °C, and sectioned at 40 μm thickness on a freezing microtome. Sections through the DRN were rinsed in 0.1 M phosphate-buffered saline (PBS, pH 7.2), blocked in 0.5% bovine serum albumin (BSA) and 0.25% Triton X-100 (TX) in PBS for 30 min, and incubated overnight at 4 °C with guinea pig anti-vGluT1 IgG (Millipore #AB5905, 1:1000) and rabbit anti-5-HT IgG (ImmunoStar #20080, 1:1000) in the blocking solution. These primary antibodies have been validated and used in other studies [6,29]. Sections were rinsed 3 times (10 min each) in PBS, incubated for 90 min at room temperature in Alexa Fluor 633-conjugated goat anti-guinea pig IgG (Life Technologies #A21105, 1:200) and Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Life Technologies #A21206, 1:200) in the blocking solution, rinsed 3 times (10 min each) in PBS, mounted onto gelatin/chromium-subbed slides, allowed to air-dry, and coverslipped with ProLong Gold antifade mountant with DAPI (Life Technologies). Sections were imaged with the Olympus FluoView 1000S confocal system with a 60× objective (NA 1.40) by obtaining *z*-stacks (one per animal) of around 10 optical sections (0.45 μm thick) through the center part of the ventromedial DRN. Next, vGluT1-positive puncta or vGluT1-positive puncta overlapping with 5-HT-signal (referred to as vGluT1/5-HT puncta) were automatically detected and counted in Imaris (Bitplane) by using the Spot Detection function (with the estimated *xy*-diameter of 1 μm and the estimated *z*-length of 2 μm) and a colocalization algorithm that takes into account the spatial statistics of immunosignals and minimizes the false-positive error [30]. We operationally defined "colocalization" as the coincidence of two signals at the used sampling resolution (with NA = 1.40 and λ = 633 nm, the theoretical resolution in the *xy*-plane is around 0.2 μm [31]). This approach can detect the coincidence of two elements in the same synapse but does not imply that these elements are located in the same cell (vGluT1 and 5-HT are presynaptic and postsynaptic, respectively, but the width of a typical synapse is considerably smaller than the size of a single pixel).

2.3. Statistical analysis

ANOVAs were performed in IBM SPSS 19 (IBM, Inc.). The significance level was set at 0.05.

3. Results

We investigated the density of contacts between cortical afferents (positive for vGluT1) and serotonergic neurons in the DRN of 5-HT_4R−/−, 5-HT_4R+/−, and 5-HT_4R+/+ (wild-type) mice (Fig. 1A). Consistent with previous reports [6,32], vGluT1-positive puncta were especially dense in the ventromedial DRN (Fig. 1B, C). Therefore, we focused on this DRN subdivision, in which we automatically detected and counted puncta that were vGluT1-positive or both vGluT1-positive and overlapping with 5-HT-signal (Fig. 1D, E). Immunosignals often vary in intensity at different distances from the section surface due to microdefects, limited antibody penetration, depth-dependent optical properties, and other factors. In order to avoid these artifacts in our high-resolution analyses, we first examined genotype-blind counts of vGluT1-positive puncta through the entire *z*-stacks of all individual mice and eliminated the cases with clearly inconsistent counts (Fig. 2A). The final set included 9 −/− mice, 10 +/− mice, and 12 +/+ mice. In the wild-type (+/+) mice, the mean counts of vGluT1-positive puncta and vGluT1/5-HT-positive puncta per optical section were 54.7 ± 3.5 and 19.1 ± 3.3, respectively. The proportion of the vGluT1-positive puncta colocalized with 5-HT with respect to all vGluT1-positive puncta (∼35%) was consistent with an array tomography analysis of wild-type C57BL/6 mice [6].

A two-way ANOVA showed no significant effects of sex or genotype on the counts of vGluT1-positive puncta ($F(1, 25) = 0.010$, $p = 0.92$ and $F(2, 25) = 0.443$, $p = 0.65$, respectively) (Fig. 2B). A two-way ANOVA revealed a significant effect of genotype on the counts of vGluT1-positive puncta colocalized with 5-HT ($F(2, 25) = 5.727$, $p = 0.009$) (Fig. 2C), but the effects of sex and the genotype-sex interaction were not significant ($F(1, 25) = 0.843$, $p = 0.37$ and $F(2, 25) = 1.292$, $p = 0.29$, respectively). A *post-hoc* analysis confirmed that the heterozygous mice had significantly more vGluT1/5-HT-positive puncta than the two homozygous groups ($p < 0.03$).

We investigated whether the observed change in the vGluT1/5-HT-positive puncta could be due to a higher number of 5-HT-positive profiles (somata and processes) in the heterozygous mice. A one-way ANOVA showed no significant difference among the mean grayscale intensities of the 5-HT channel in the same *z*-stacks (after flattening) ($F(2, 28) = 0.172$, $p = 0.843$). The mean grayscale intensities remained non-significant after the autocontrast correction ($F(2, 28) = 0.650$, $p = 0.530$).

4. Discussion

We found that the absence of functional 5-HT_4Rs had no significant effect on the overall density of vGluT1-immunoreactive terminals in the DRN, but that the density of vGluT1-immunoreactive terminals contacting serotonergic neurons was significantly increased in heterozygous mice. This increase was unlikely to be due to a larger proportion of serotonergic neurons in the DRN, the number of which does not appear to be altered in 5-HT_4R-knockout mice [33]. This finding is generally consistent with our analysis of the 5-HT-optical intensities in the vmDRN. On the other hand, a high performance liquid chromatography analysis has found a significantly lower 5-HT concentration in the DRN of 5-HT_4R-knockout mice [33], which suggests that the detected increase in the vGluT1/5-HT-positive puncta might have been underestimated. In summary, current evidence does not support the possibility that the increase in vGluT1/5-HT contacts was caused by a higher number or intensity of 5-HT-positive profiles.

The main source of vGluT1-immunoreactive terminals in the DRN is the cerebral cortex [4,10]. The hippocampus and the cerebellum also strongly express vGluT1 in the rat and human brains [4,6,34,35], but their direct projections to the DRN are weak or absent [3,19]. A recent analysis of the tree shew brain has shown that vGluT1 can be co-expressed with vGluT2 in non-cortical structures of the image-forming visual system [36], but the direct visual projections to the DRN are primarily retinal and non-

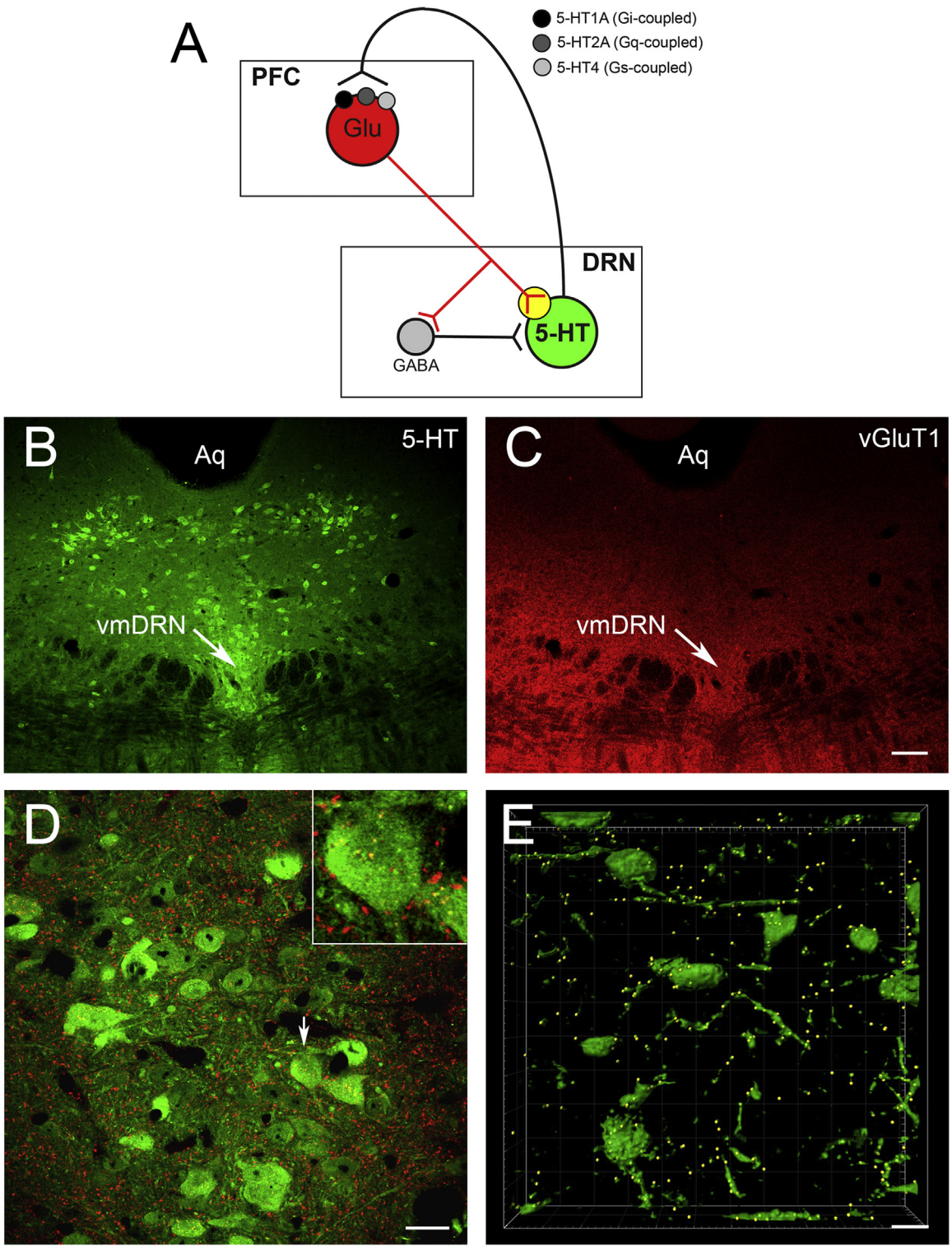

Fig. 1. (A) A schematic representation of the direct projection from the prefrontal cortex (PFC) to the dorsal raphe nucleus (DRN). The glutamatergic PFC terminals express vGluT1 and control serotonergic neurons directly (the yellow synapse) or through GABAergic interneurons. Studies differ in their estimates of the relative densities of these synapse types in the DRN [40,41]. Many vGluT1-positive and GABAergic axons converge onto the same serotonergic neurons, and GABAergic terminals can also form presynaptic contacts with vGluT1-positive axons (not shown) [46]. (B, C) Low-magnification images of a section through the DRN in the 5-HT-channel (B) and the vGluT1-channel (C). Aq, cerebral aqueduct; vmDRN, ventromedial DRN. Scale bar = 100 μm. (D) A high-magnification image of a section through the vmDRN of a 5-HT_4R+/− male. 5-HT-positive neurons and processes are green, vGluT1-positive puncta are red, and overlapping (vGluT1/5-HT) puncta are yellow. The arrow points to the cell that is shown enlarged in the inset (note the discrete puncta). Scale bar = 20 μm. (E) Automatic detection of 3D-colocalization between vGluT1 and 5-HT (pseudo-colored green) immunoreactive profiles. Scale bar = 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

image-forming [37–39]. Generally, the non-cortical brain regions projecting to the DRN express other vGluTs, such as vGluT2 and vGluT3 [4].

A recent study has shown that cortical afferents in the DRN are more likely to target serotonergic neurons than GABAergic neurons [40]. However, cortical afferents can also terminate on GABAergic

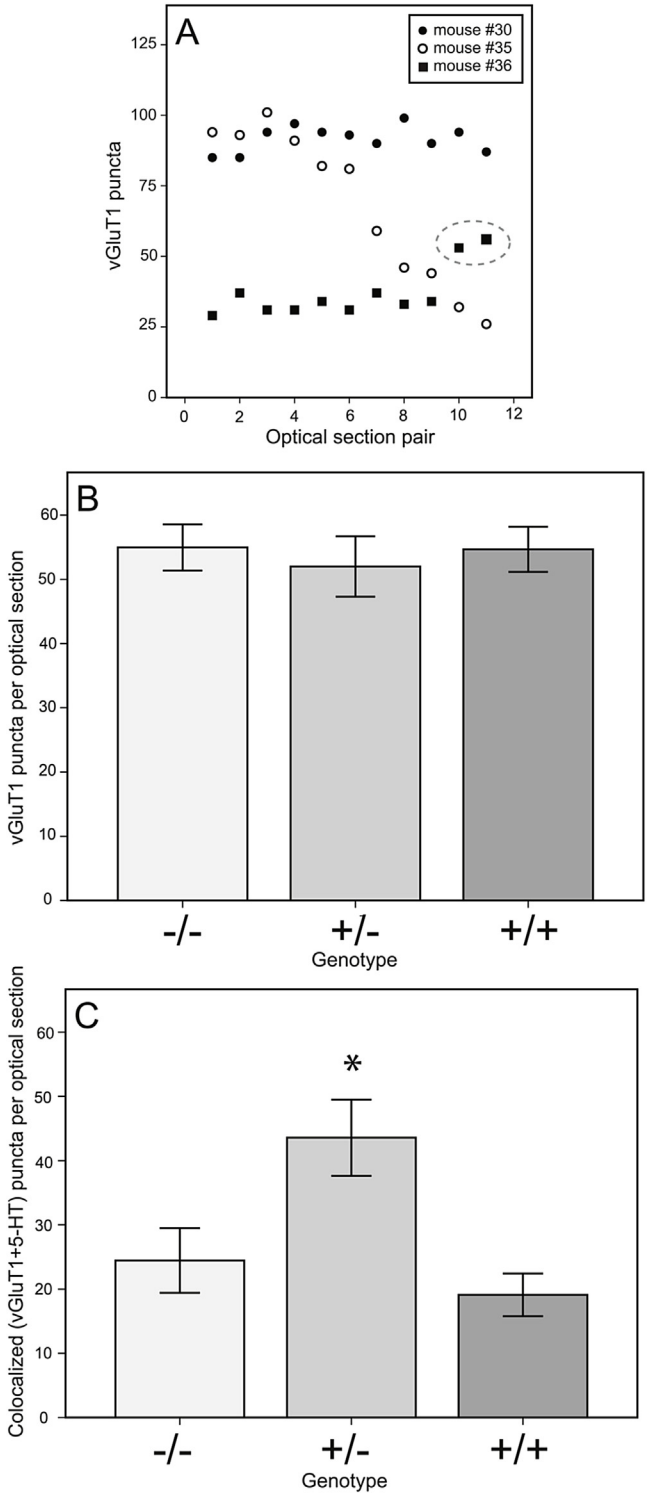

Fig. 2. (A) The reliability analysis of automatically-detected vGluT1-positive puncta in serial optical sections through the same physical section. Cases were included in the analysis only if the counts of puncta were relatively stable across the pairs of adjacent optical sections (mouse #30) or if the counts became relatively stable after the elimination of a small number of optical sections at an extreme end of the *z*-stack (mouse #36; the eliminated points are circled). Cases with unstable counts (mouse #35) or with outlier counts in the middle of the *z*-stack were not included in the analysis. (B) vGluT1-positive puncta in the ventromedial DRN of 5-HT$_4$R −/−, +/−, and +/+ mice. The genotypes were not significantly different (one-way ANOVA: $F(2,28) = 0.165$, $p = 0.85$). (C) Puncta with colocalized vGluT1 and 5-HT immunoreactivity in the ventromedial DRN of 5-HT$_4$R −/−, +/−, and +/+ mice. The heterozygous mice had significantly more contacts between vGluT1-positive terminals and 5-HT-positive profiles (cells bodies and dendrites) than the homozygous mice (one-way ANOVA: $F(2, 28) = 7.542$, $p = 0.002$; Tukey's HSD post-hoc tests for +/− vs. −/− and +/− vs. +/+: $^*p < 0.03$). The error bars are SEMs (B and C).

neurons [41–43]. Therefore, the absence of change in the overall density of cortical terminals in heterozygous mice could be due to a lower density of contacts with GABAergic neurons. These synapses appear to play an important role in real-time behavioral control [15,44]. In addition to the presented data, we attempted to directly quantify the colocalization between vGluT1-positive terminals and

GABAergic DRN neurons with an anti-GAD67 antibody that has been used in other studies [42,45], but could not obtain a sufficiently reliable signal for automatic colocalization detection [30]. A recent study suggests that GAD65, another isoform of glutamic acid decarboxylase (GAD), may be a better alternative [46]. Functionally, a large proportion of GABAergic axons in the DRN form synaptic "triads" with vGluT1-positive terminals at the same postsynaptic target [46]. This suggests that many cortical terminals on GABAergic neurons eventually modulate the same serotonergic neurons that receive direct cortical projections.

The finding that the density of contacts between cortical terminals and serotonergic neurons was affected in heterozygous but not homozygous mice was unexpected, but similar findings have been reported elsewhere. For example, a null-mutation of the parkin gene alters the synaptic activity in the hippocampus of heterozygous but not homozygous knockout mice [47]. Likewise, a null-mutation of the Shank3 gene alters the number of perforated synapses in the hippocampus of only heterozygous mice [48]. These results may be associated with adaptive processes that are well documented in homozygous 5-HT_4R-knockout mice [49,50].

The observed synaptic changes are unlikely to be caused by altered 5-HT_4R signaling in the DRN itself because its 5-HT_4R expression is very low [19,51]. However, there is a strong possibility that they are related to altered activity of cortical neurons that express 5-HT_4Rs [19], produce vGluT1-terminals [3,4], and directly drive DRN neurons [19,20].

5. Conclusions

The study demonstrates that reduced expression of serotonergic 5-HT_4 receptors can result in altered glutamatergic input to the DRN. This may affect the dynamics of serotonergic signaling in response to external and internal stimuli.

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