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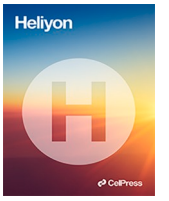

Research article

Median raphe glutamatergic neuron-mediated enhancement of GABAergic transmission and suppression of long-term potentiation in the hippocampus

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A B S T R A C T

The ascending neuromodulatory pathway from the median raphe nucleus (MRN) extends widely throughout midline/para-midline regions and robustly innervates the hippocampus. This neuromodulatory pathway is believed to be critical for regulating emotional and affective behaviors. Although the MRN primarily contains serotoninergic (5-HTergic), GABAergic, and glutamatergic neurons, glutamatergic neurons expressing vesicular glutamate transporter 3 (VGLUT3) form the primary MRN input to the hippocampus. Despite the earlier demonstration of the robust MRN VGLUT3 innervation of the hippocampus, little is known about how this MRN glutamatergic input modulates synaptic transmission and plasticity in the hippocampus. Our studies show that MRN VGLUT3 neurons activate serotonin 3a receptor (5-HT3aR)-expressing GABAergic neurons, including VGLUT3-expressing neurons, at the stratum radiatum (SR)/stratum lacunosum moleculare (SLM) border. This MRN VGLUT3 neuron-mediated glutamatergic transmission onto SR/SLM 5-HT3aR neurons is negatively regulated by 5-HT through 5-HT1B receptors. In agreement with the MRN VGLUT3 neuron-mediated activation of the 5-HT3aR GABAergic neurons, activation of MRN VGLUT3 projections induces a long-lasting increase in GABAergic transmission but not glutamatergic transmission in CA1 pyramidal neurons from male but not female mice. Consistent with the MRN VGLUT3 neuron-mediated enhancement of GABAergic transmission in male mice, activation of MRN VGLUT3 projections suppresses Schaffer collateral (SC)-CA1 long-term potentiation (LTP) in male but not female mice. Thus, our results show that MRN VGLUT3 neurons modulate the dorsal hippocampus by augmenting synaptic inhibition of CA1 pyramidal neurons and by suppressing SC-CA1 LTP in a sex-specific manner.

1. Introduction

The ascending neuromodulatory pathways are critical for regulating hippocampus-dependent behaviors, including cognitive and emotional behaviors [1–4]. The median raphe nucleus (MRN), a major midbrain neuromodulatory structure involved in cognitive and emotional behaviors, robustly innervates the hippocampus [5–15]. Consistent with the MRN modulation of the hippocampus, it has been shown that MRN is critical for regulating hippocampal sharp wave-associated field oscillations, which are believed to be critical for the consolidation of explicit memory [16]. Also, MRN suppresses hippocampal theta oscillations during rapid eye movement sleep [17]. These findings suggest the critical role of the MRN in regulating hippocampal activity and hippocampus-mediated behaviors. Consistently, earlier studies have shown MRN modulation of fear behaviors and their potential role in anxiety-related behaviors [9–11]. Therefore, understanding how the MRN modulates the hippocampus might open avenues for the management of disorders involving altered cognitive and emotional behaviors. The MRN is primarily comprised of serotonergic (5-HTergic), GABAergic, and glutamatergic neurons, which express vesicular glutamate transporters VGLUT2 or VGLUT3 [18–21]. Interestingly, VGLUT3 neurons form the primary MRN input into the hippocampus [7,15]. Consistently, an earlier study has shown MRN neuron-mediated glutamatergic responses in GABAergic neurons in the hippocampus [6]. The limited expression of 5-HT in the hippocampus-projecting MRN

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VGLUT3 neurons suggests that the 5-HT input to the hippocampus is from 5-HT neurons and not the MRN VGLUT3 neurons [15]. Despite the predominant role of VGLUT3 neurons in the MRN modulation of the hippocampus, little is known about how this midbrain glutamatergic input modulates synaptic transmission and plasticity in the hippocampus [6,7,15]. Also, it is unknown whether the MRN VGLUT3 neuron modulation of the hippocampus is sex-specific. Our recent study shows sex-specific MRN VGLUT3 neuron modulation of the medial prefrontal cortex [14].

The forebrain GABAergic neurons are comprised of parvalbumin (PV)-, somatostatin (SST), and serotonin 3a receptor (5-HT3aR)-expressing GABAergic neurons [22,23]. Given the abundant presence of serotonin 3a receptor (5-HT3aR)-expressing GABAergic neurons at the stratum radiatum (SR)/stratum lacunosum moleculare (SLM) border, a hippocampal region that receives strong MRN input, we examined whether the MRN VGLUT3 neurons activate 5-HT3aR-expressing GABAergic neurons at the SR/SLM border of the dorsal hippocampus [6,7,15,24,25]. Our analysis also involved MRN VGLUT3 neuron activation of SR/SLM VGLUT3-expressing GABAergic neurons, a major subclass of 5-HT3aR neurons which release both glutamate and GABA [14,26]. Our studies show that MRN VGLUT3 neurons form monosynaptic glutamatergic connectivity with SR/SLM 5-HT3aR GABAergic neurons, including VGLUT3 neurons, but not pyramidal neurons in the hippocampus. Furthermore, we show that MRN VGLUT3 neurons selectively enhance synaptic inhibition and suppress LTP in the dorsal hippocampus in a sex-specific manner.

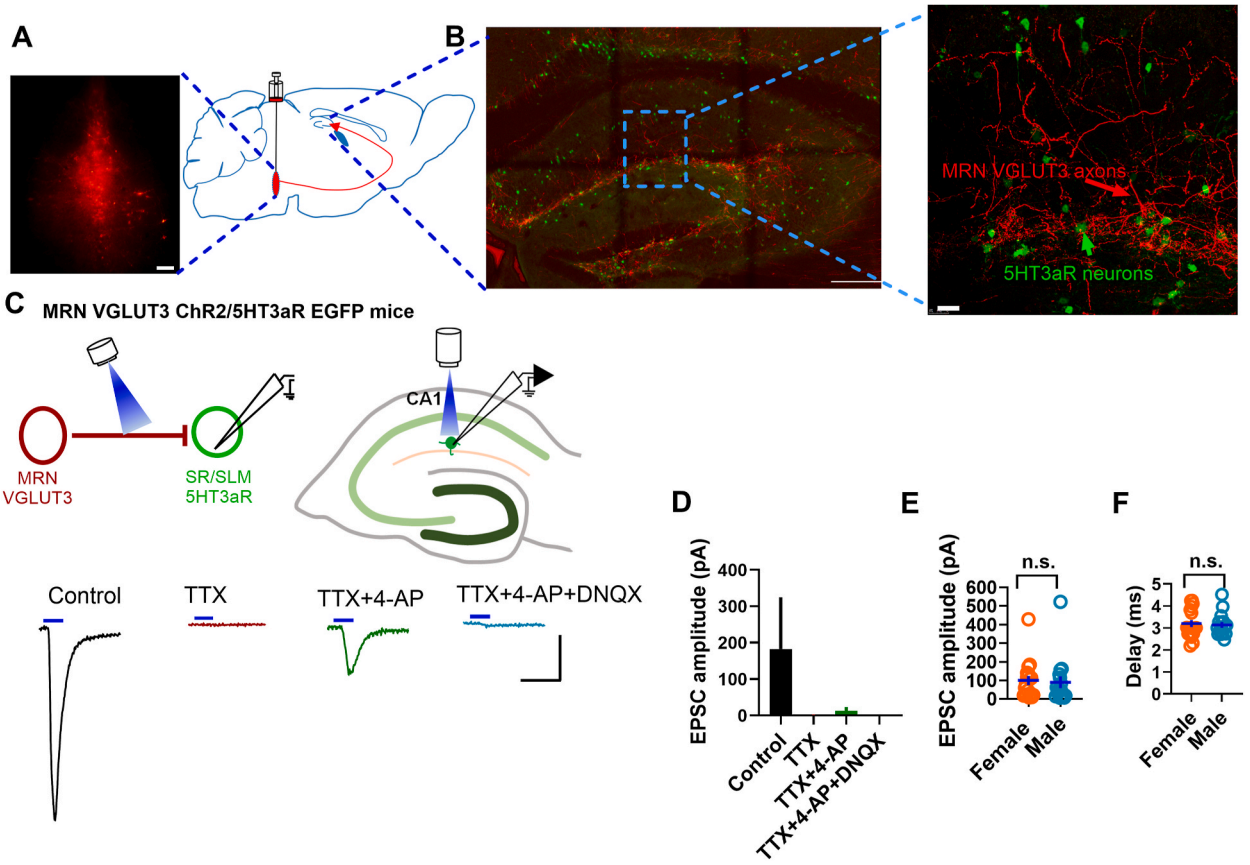


Fig. 1. MRN VGLUT3 neuron-mediated glutamatergic transmission in SR/SLM 5-HT3aR neurons. A) A schematic of AAV injection into the MRN. The left panel shows an image of the MRN from VGLUT3 Cre/5-HT3aR-EGFP mouse which received an AAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA injection into the MRN. Scale 150 μm. B) An image of the hippocampus from MRN VGLUT3 ChR2/5-HT3aR-EGFP mouse. The inset shows EGFP-expressing SR/SLM 5-HT3aR neurons and mCherry-expressing MRN VGLUT3 axons. Scale 300 μm/25 μm (inset). C) The upper panel shows a schematic for recording MRN VGLUT3 neuron-mediated transmission in SR/SLM 5-HT3aR neurons. The bottom panel shows example traces for MRN VGLUT3 neuron-mediated EPSCs in SR/SLM 5-HT3aR neurons without drug application, in the presence of tetrodotoxin (TTX, 1 μM), TTX+4-aminopyridine (4-AP, 100 μM) and TTX+4-AP + DNQX (10 μM). Scale 50 pA/10 ms. The blue line indicates light application. D) EPSC amplitude in SR/SLM 5-HT3aR neurons before the drug application, in the presence of TTX, TTX+4-AP, and TTX+4-AP + DNQX (6 neurons from 3 female mice and 6 neurons from 3 male mice). $F(1,11) = 22.01$, $P = 0.001$. Data are presented as mean ± SEM. E) EPSC amplitude in female (17 neurons/5 mice) and male (16 neurons/5 mice) groups. Female and male groups did not show statistically significant difference in EPSC amplitude ($P = 0.8$). The horizontal line in each group represents the mean and the vertical line represents SEM. F) The delay between the onset of the light stimulation and the onset of EPSCs in female (17 neurons/5 mice) and male (16 neurons/5 mice) groups. Female and male groups did not show a statistically significant difference in the delay between the onset of light stimulation and the onset of EPSCs ($P = 0.77$). n.s. not statistically significant.

2. Results

2.1. MRN VGLUT3 neurons form monosynaptic connectivity with SR/SLM 5-HT3aR neurons through glutamatergic synapses

Based on the earlier studies showing MRN innervation of GABAergic neurons at the SR/SLM border and the abundant presence of 5-HT3aR neurons at the SR/SLM border, which receives strong MRN input [6,7,15,24,25], we have investigated whether MRN VGLUT3 neurons form synapses onto 5-HT3aR neurons at the SR/SLM border of the dorsal hippocampus. We employed cell type-specific expression of channelrhodopsin (ChR2) to study the MRN VGLUT3 neuron innervation of the hippocampus. Light-evoked currents were recorded in EGFP-expressing SR/SLM 5-HT3aR neurons in hippocampal slice preparations from MRN VGLUT3 ChR2/5-HT3aR EGFP mice [generated by stereotactic injection of AAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA injection into the MRN of B6; 129S-*Slc17a8*$^{tm1.1(cre)Hze}$/J mice (VGLUT3 Cre mice) crossed with 5-HT3aR-BAC EGFP mice] [14,27–30] (Fig. 1A and B). We observed that light stimulation of MRN VGLUT3 axonal fibers produced synaptic currents in most of the recorded SR/SLM 5-HT3aR neurons (33 out of 42 5-HT3aR neurons) (Fig. 1C–F). Next, we examined whether MRN VGLUT3 neuron-mediated glutamatergic transmission in SR/SLM 5-HT3aR neurons is monosynaptic. We observed that the average delay between the onset of the light stimulation and the onset of synaptic currents was 3.17 ± 0.1 ms (mean ± SEM), suggesting monosynaptic transmission between MRN VGLUT3 neurons and SR/SLM 5-HT3aR neurons. To confirm the monosynaptic nature of this pathway, we examined the effect of 4-aminopyridine (4-AP, 100 μM), a blocker of voltage-gated potassium channels, on tetrodotoxin (TTX, 1 μM)-induced suppression of light-evoked currents in SR/SLM 5-HT3aR neurons (Fig. 1C and D) [14,31,32]. We observed a statistically significant effect of 4-AP on the TTX block of EPSCs, confirming the monosynaptic nature of MRN VGLUT3 transmission onto SR/SLM 5-HT3aR neurons (Fig. 1C and D). We did not observe a statistically significant difference in EPSC amplitude between the female and male groups (Fig. 1E). Similarly, we did not observe a statistically significant difference in the delay between the onset of light stimulation and the onset of EPSCs in the female and male groups (Fig. 1F). These MRN VGLUT3 neuron-mediated currents were blocked by DNQX (10 μM), an AMPA receptor antagonist, confirming the glutamatergic nature of these currents (Fig. 1C and D).

Since VGLUT3-expressing GABAergic neurons are a major subtype of 5-HT3aR neurons, we asked whether VGLUT3-expressing GABAergic neurons at the SR/SLM border of the dorsal hippocampus also receive glutamatergic input from MRN VGLUT3 neurons [23,24,33–35]. We examined whether light stimulation of MRN VGLUT3 axon terminals produces synaptic responses in tdTomato-expressing SR/SLM VGLUT3 neurons from MRN VGLUT3 ChR2/VGLUT3-tdTomato mice (generated by stereotactic injection of AAV-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA injection into the MRN of B6; 129S-*Slc17a8*$^{tm1.1(cre)Hze}$/J mice crossed with B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J mice) (Fig. 2A and B). We observed that light stimulation of MRN VGLUT3 axonal fibers produced synaptic currents in most of the recorded SR/SLM VGLUT3 neurons (35 out of 39 neurons) (Fig. 2C–E).

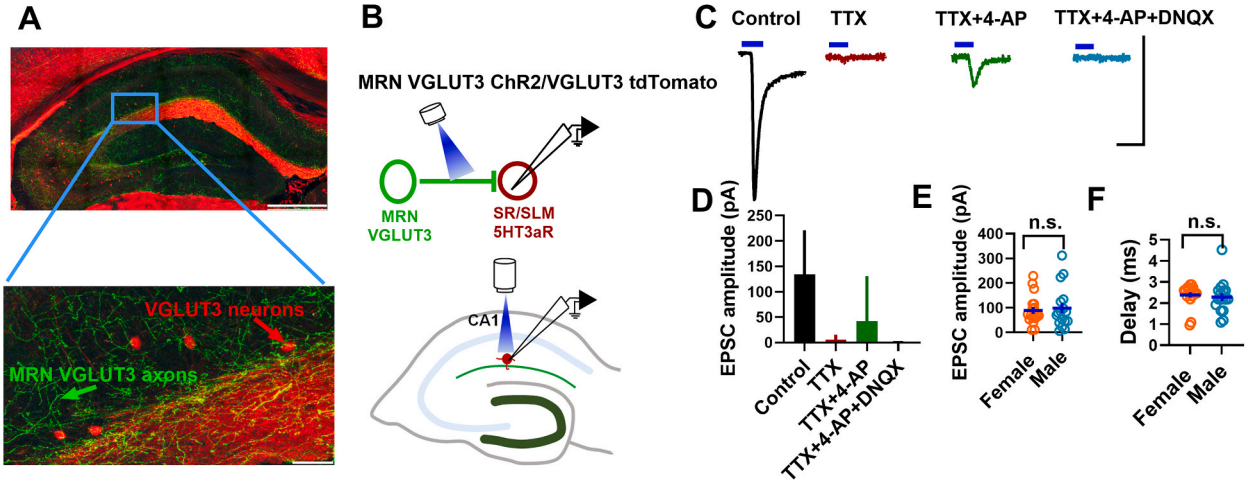


Fig. 2. MRN VGLUT3 neuron-mediated glutamatergic transmission in SR/SLM VGLUT3 neurons. A) An image of the hippocampus from VGLUT3-tdTomato mouse which received AAV-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA injection into the MRN. The inset shows tdTomato-expressing SR/SLM VGLUT3 neurons and EYFP-expressing MRN VGLUT3 axons. Scale 500 μm/50 μm (inset). B) A schematic for recording MRN VGLUT3 neuron-mediated transmission in SR/SLM VGLUT3 neurons. C) Example traces for MRN VGLUT3 neuron-mediated EPSCs in SR/SLM VGLUT3 neurons without drug application, in the presence of TTX, TTX+4-AP, and TTX+4-AP + DNQX. Scale 50 pA/10 ms. The blue line indicates light application. D) EPSC amplitude in SR/SLM VGLUT3 neurons before the drug application, in the presence of TTX, TTX+4-AP, and TTX+4-AP + DNQX (5 neurons/4 female mice and 5 neurons/4 male mice). F (1,9) = 11.25, P = 0.008. Data are presented as mean ± SEM. E) EPSC amplitude in female (18 neurons/6 mice) and male (17 neurons/7 mice) groups. Female and male groups did not show statistically significant difference in EPSC amplitude (P = 0.7). The horizontal line in each group represents the mean and the vertical line represents SEM. F) Average delay between the onset of light stimulation and the onset of EPSCs in female (18 neurons/6 mice) and male (17 neurons/7 mice) groups. Female and male groups did not show a statistically significant difference in delay between the onset of light stimulation and the onset of EPSCs (P = 0.64). n.s. not statistically significant.

The MRN VGLUT3 neuron-mediated EPSCs in SR/SLM VGLUT3 neurons were monosynaptic in nature as 4-AP rescued TTX block of EPSCs (Fig. 2C and D). Similar to MRN VGLUT3 neuron-mediated currents in SR/SLM 5-HT3aR neurons, DNQX completely blocked MRN VGLUT3 neuron-mediated currents in SR/SLM VGLUT3 neurons (Fig. 2C and D). We did not observe a statistically significant

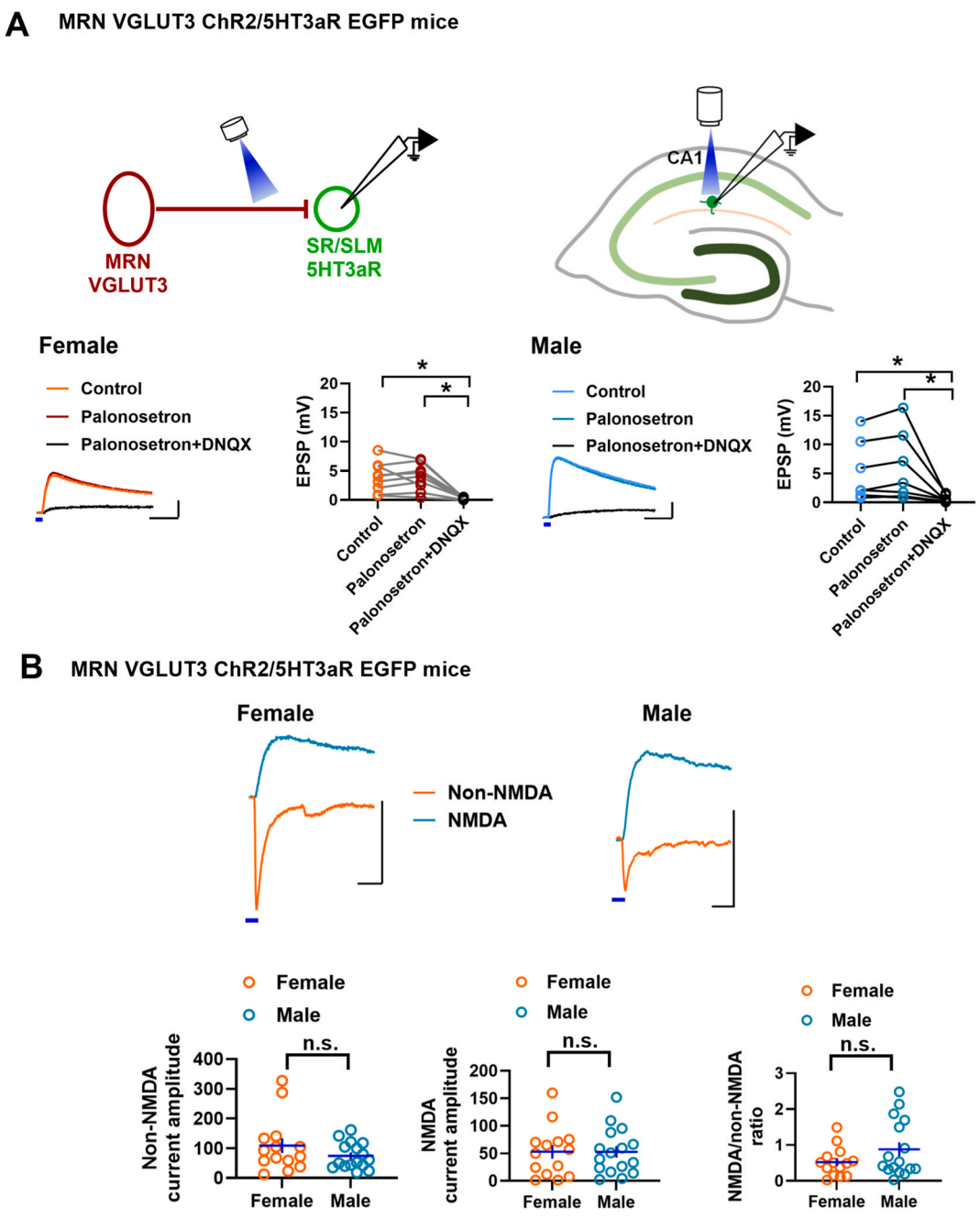


Fig. 3. MRN VGLUT3 neuron-mediated transmission in SR/SLM 5-HT3aR neurons is mediated through glutamate receptors. A) MRN VGLUT3 neuron-mediated EPSPs in SR/SLM 5-HT3aR neurons were blocked by DNQX, an AMPA receptor blocker, but not palonosetron, a 5-HT3 receptor antagonist, in female [9 neurons/3 mice, $F(1,8) = 15.7$, $P = 0.004$, control vs palonosetron: $P = 1$, control vs palonosetron + DNQX: $P = 0.006$, palonosetron vs palonosetron + DNQX: $P = 0.003$] and male [9 neurons/3 mice, $F(1,8) = 6.9$, $P = 0.03$, control vs palonosetron: $P = 0.12$, control vs palonosetron + DNQX: $P = 0.028$, palonosetron vs palonosetron + DNQX: $P = 0.033$] groups. The left panels show example traces. Scale 2mV/25 ms. The asterisk denotes statistical significance. B) Both female and male groups show MRN VGLUT3 neuron-mediated NMDA currents in SR/SLM 5-HT3aR neurons. We did not observe sex difference in non-NMDA receptor transmission ($P = 0.19$), NMDA receptor transmission ($P = 0.98$), and NMDA/non-NMDA ratio ($P = 0.19$). The horizontal line in each group represents the mean and the vertical line represents SEM. The upper panels show example traces. Scale 100pA/10 ms. The blue line indicates light application. n.s. not statistically significant.

difference in either EPSC amplitude or the delay between the onset of the light stimulation and the onset of EPSCs in the female and male groups (Fig. 2E and F). Thus, these results confirm MRN VGLUT3 neuron-mediated monosynaptic glutamatergic transmission in SR/SLM 5-HT3aR neurons, including the VGLUT3 GABAergic neurons. Unlike the effect of light activation of MRN VGLUT3 projections on SR/SLM 5-HT3aR and VGLUT3 neurons, light activation of MRN VGLUT3 axon terminals did not produce any response in dorsal CA1 pyramidal neurons (N = 41 neurons/12 female+10 male mice).

A small proportion of MRN VGLUT3 neurons express 5-HT [14,15,18]. Therefore, we asked whether MRN VGLUT3 neuron-mediated responses in SR/SLM 5-HT3aR neurons depend upon the activation of 5-HT3 receptors, an ionotropic 5-HT receptor [36,37]. We have recorded light-evoked excitatory post-synaptic potentials (EPSPs) in EGFP-expressing SR/SLM 5-HT3aR neurons from hippocampal slices of MRN VGLUT3 ChR2/5-HT3aR EGFP mice. We observed robust light-evoked EPSPs in female and male groups (Fig. 3A). Perfusion of palonosetron (10 nM), a 5-HT3 receptor antagonist, for 20 min did not affect EPSP amplitude (Fig. 3A). However, a subsequent perfusion of DNQX for 10 min completely blocked light-evoked EPSPs, confirming the exclusive glutamatergic nature of MRN VGLUT3 neuron-mediated transmission in SR/SLM 5-HT3aR neurons (Fig. 3A). Given the glutamatergic nature of this synaptic connectivity, we examined the role of NMDA receptors in MRN VGLUT3 neuron-mediated glutamatergic transmission in SR/SLM 5-HT3aR neurons. First, we recorded light-evoked EPSCs at −60 mV in the presence of $GABA_A$ receptor antagonist bicuculline (10 μM), followed by confirmation of the non-NMDA nature of these EPSCs by the addition of DNQX (Fig. 3B). Then, we recorded NMDA EPSCs at +40 mV from the same neurons in the presence of bicuculline and DNQX (Fig. 3B). NMDA EPSCs were confirmed by blocking with AP5 (50 μM), an NMDA receptor antagonist. We quantified the peak non-NMDA and NMDA EPSC amplitude at −60mV and +40 mV, respectively. We did not observe a statistically significant difference in non-NMDA current amplitude, NMDA current amplitude, or NMDA/non-NMDA ratio in female and male mice (Fig. 3B). These results show the exclusive glutamatergic nature of MRN VGLUT3-SR/SLM 5-HT3aR neuron synapses and the involvement of robust NMDA receptor-mediated transmission at these synapses.

2.2. 5-HT suppresses MRN VGLUT3 neuron-SR/SLM 5-HT3aR neuron glutamatergic transmission through 5-HT1B receptors

Since a minority of MRN VGLUT3 neurons express 5-HT and 5-HT neurons are a major subtype of MRN neurons [14,15,18], we examined whether 5-HT regulates MRN VGLUT3 neuron-mediated glutamatergic transmission in SR/SLM 5-HT3aR neurons. We tested whether 5-HT (10 μM) perfusion for 10 min affects light-evoked EPSCs in SR/SLM 5-HT3aR neurons from MRN VGLUT3 ChR2/5-HT3aR EGFP mice. The concentration of 5-HT was selected based on previous studies [38–40]. 5-HT produced a significant suppression of MRN VGLUT3 neuron-mediated EPSCs in SR/SLM 5-HT3aR neurons (Fig. 4A and B). This suppression of MRN VGLUT3 neuron-mediated transmission was partially reversed following the washout of 5-HT (Fig. 4B). Next, we tested whether the 5-HT1B

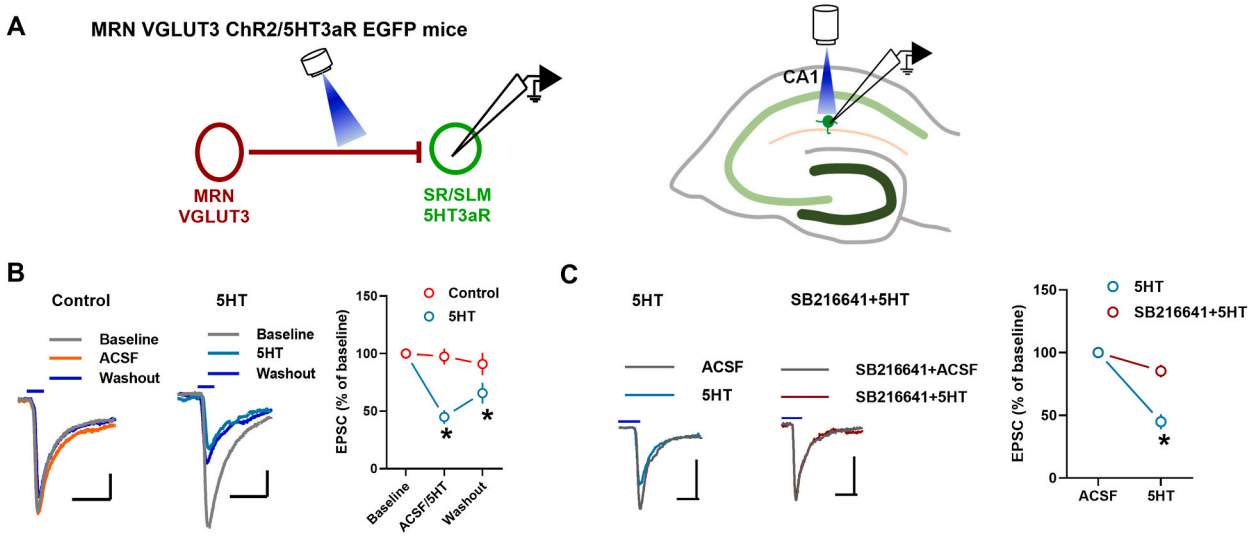


Fig. 4. 5-HT suppresses MRN VGLUT3 neuron-mediated glutamatergic transmission in SR/SLM 5-HT3aR neurons. A) A schematic for recording MRN VGLUT3 neuron-mediated transmission in SR/SLM 5-HT3aR neurons. B) Application of 5-HT (10 μM) for 10 min suppressed the amplitude of MRN VGLUT3 neuron-mediated EPSCs in SR/SLM 5-HT3aR neurons ($F_{1,27} = 9.62$, $P = 0.004$) (control: 8 neurons from 2 female and 2 male mice, 5-HT: 21 neurons from 5 female and 5 male mice). Washout of 5-HT showed statistically significant recovery of EPSC amplitude [before (basline) vs 5-HT: $P = 0.001$, before (baseline) vs washout: $P = 0.001$, 5-HT vs washout: $P = 0.001$). We did not observe an effect of sex on 5-HT-mediated suppression of EPSC amplitude ($P = 0.73$). Data are presented as mean ± SEM. The left panel shows example traces. Scale 50pA/10 ms. C) SB216641 (10 μM), a 5-HT1B receptor antagonist, blocked 5-HT-mediated suppression of MRN VGLUT3 neuron-mediated glutamatergic transmission in SR/SLM 5-HT3aR neurons. The right panel shows the effect of 5-HT in the absence and presence of SB216641 ($F_{1,34} = 23.7$, $P = 0.001$) (5-HT data is same as in Fig. 4B. SB216641+5-HT group: 15 neurons from 3 female and 5 male mice). The left panel shows example traces. Scale 50pA/5 ms. The asterisk denotes statistical significance.

receptor, a 5-HT receptor predominantly present on pre-synaptic sites and known to negatively regulate excitatory synaptic transmission, mediates the effect of 5-HT on MRN VGLUT3 neuron-SR/SLM 5-HT3aR neuron glutamatergic transmission [41,42]. Perfusion of brain slices with a 5-HT1B antagonist, SB216641 (10 μM), for 20 min blocked the effect of 5-HT on MRN VGLUT3 neuron-mediated currents in SR/SLM 5-HT3aR neurons (Fig. 4C) [41]. SB216641 did not show a statistically significant effect on basal EPSC amplitude (79.8 ± 15.6 pA and 68.6 ± 12.3 pA, before and 20 min after the perfusion of SB216641, N = 15/7 mice). These results suggest that

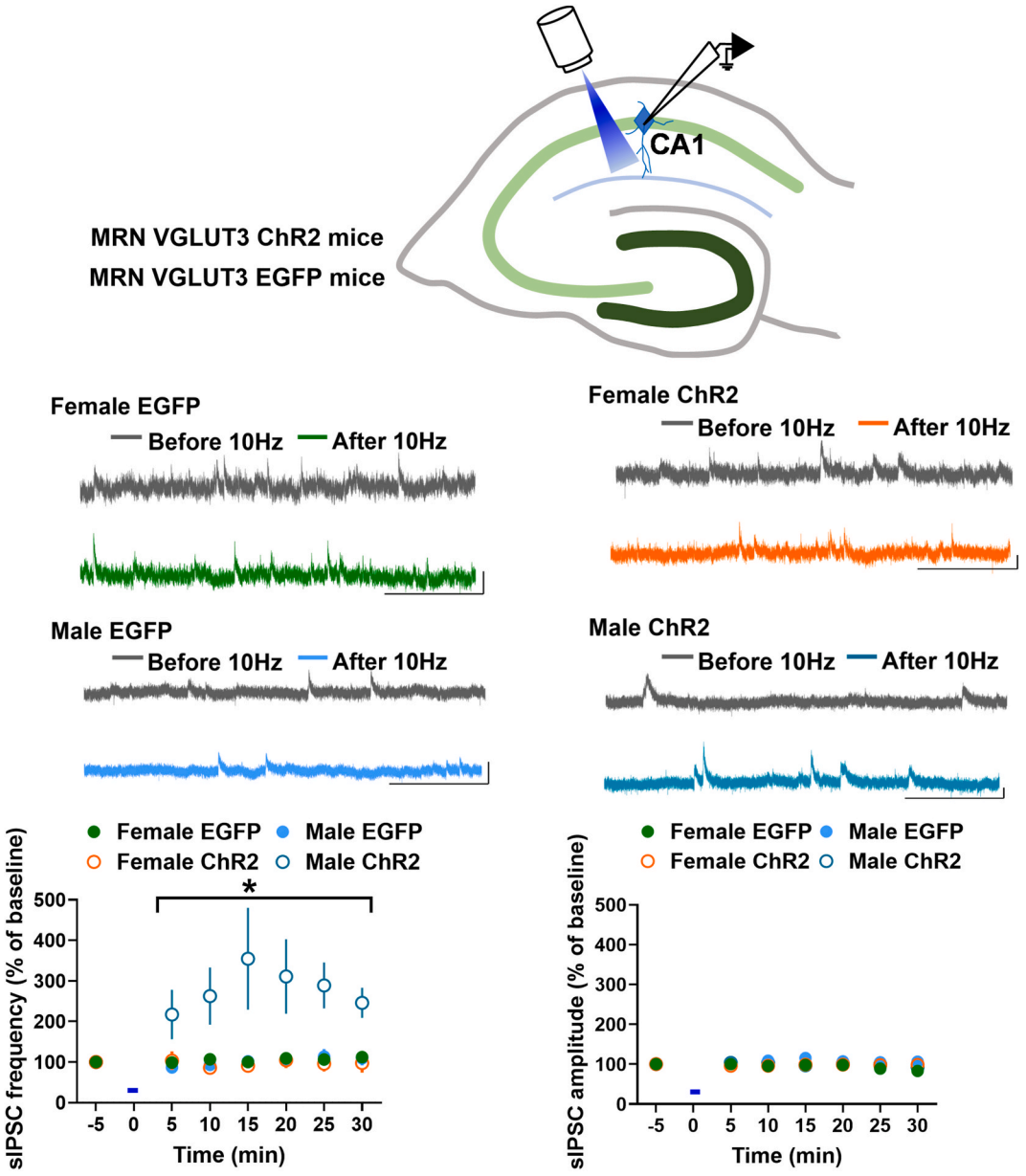


Fig. 5. Activation of MRN VGLUT3 axon terminals at the SR/SLM region enhances GABAergic transmission in hippocampal CA1 pyramidal neurons in male but not female mice. The top panel shows a schematic of whole cell recording of CA1 pyramidal neurons and the application of 10 Hz light stimulation to activate ChR2-expressing MRN VGLUT3 axon terminals. 10 Hz light stimulation produced a significant increase in frequency ($F_{1,19} = 4.8$, $P = 0.04$) but not amplitude ($F_{1,19} = 1.54$, $P = 0.23$) of sIPSCs in CA1 pyramidal neurons from male MRN VGLUT3 ChR2 mice compared to male MRN VGLUT3 EGFP mice. 10 Hz light stimulation did not affect sIPSC frequency ($F_{1,16} = 0.15$, $P = 0.7$) or amplitude ($F_{1,16} = 0.3$, $P = 0.58$) in female MRN VGLUT3-ChR2 mice and MRN VGLUT3-EGFP mice. Consistently, we observed an effect of sex on sIPSC frequency ($F_{3,38} = 5.3$, $P = 0.02$) but not amplitude ($F_{3,38} = 1.8$, $P = 0.18$). The basal sIPSC frequency was significantly higher in female mice (mean ± SEM: 1.74 ± 0.27 Hz) compared to male mice (0.82 ± 0.16 Hz) ($P = 0.003$). However, there was no significant sex difference in basal sIPSC amplitude (female: 55.97 ± 6.2 pA, male: 53.02 ± 5.3 pA) ($P = 0.72$). Female EGFP group: 9 neurons/5 mice, male EGFP group: 9 neurons/5 mice, female ChR2 group: 9 neurons/5 mice, male ChR2 group: 12 neurons/6 mice. Data are presented as mean ± SEM. The upper panel shows example traces. Scale 50 pA/500 ms. The blue line indicates the application of 10 Hz stimulation. The asterisk denotes statistical significance.

5-HT negatively regulates MRN VGLUT3 neuron-mediated glutamatergic transmission in SR/SLM 5-HT3aR neurons through 5-HT1B receptors.

2.3. Activation of MRN VGLUT3 projections enhances GABAergic but not glutamatergic transmission in CA1 pyramidal neurons in a sex-specific manner

Given the robust MRN VGLUT3 neuron-mediated glutamatergic transmission in SR/SLM 5-HT3aR neurons, a major subclass of GABAergic neurons, we asked whether activation of MRN VGLUT3 axon terminals at the SR/SLM region affects GABAergic transmission in CA1 pyramidal neurons [23,35]. To investigate the effect of light activation of MRN VGLUT3 axon terminals on GABAergic transmission, we used MRN VGLUT3 ChR2 mice (B6; 129S-*Slc17a8*$^{tm1.1(cre)Hze}$/J mice which received AAV-EF1a-double floxed-hChR2 (H134R)-mCherry-WPRE-HGHpA injection into the MRN). As controls, we used MRN VGLUT3 EGFP mice (B6; 129S-*Slc17a8*$^{tm1.1(cre)Hze}$/J mice which received AAV-hSyn-DIO-EGFP injection into the MRN). MRN VGLUT3 axon terminals at the SR/SLM region were activated by a 10 Hz light stimulation for 60 s (470 nm, each pulse of 5 ms duration). Spontaneous inhibitory post-synaptic currents (sIPSCs) were recorded at 0 mV before and after the 10 Hz stimulation. The GABAergic nature of sIPSCs was confirmed using bicuculline (10 μM), a $GABA_A$ receptor antagonist. This 10 Hz stimulation produced a long-lasting enhancement of the frequency but not the amplitude of sIPSCs in the CA1 pyramidal neurons of male MRN VGLUT3 ChR2 mice but not MRN VGLUT3 EGFP mice (Fig. 5). However, the female MRN VGLUT3 ChR2 group failed to show this GABAergic plasticity (Fig. 5). Consistently, we observed a statistically significant effect of sex on sIPSC frequency but not amplitude. We observed a sex difference in basal sIPSC frequency, which was significantly higher in female mice than male mice. However, there was no significant sex difference in basal sIPSC amplitude.

To determine whether activation of MRN VGLUT3 axon terminals affects glutamatergic transmission in CA1 pyramidal neurons, we undertook experiments to test the effect of 10 Hz light activation on spontaneous glutamatergic transmission in CA1 pyramidal neurons from MRN VGLUT3 ChR2 and MRN VGLUT3 EGFP mice. Spontaneous excitatory post-synaptic currents (sEPSCs) were recorded at −60 mV before and after the 10 Hz stimulation. The glutamatergic nature of sEPSCs was confirmed using DNQX. Unlike the effect of activation of MRN VGLUT3 projections on GABAergic transmission to CA1 pyramidal neurons, the 10 Hz light activation did not affect sEPSC frequency or amplitude in female or male mice (Fig. S1). Our analysis did not show a statistically significant sex difference in basal sEPSC frequency or amplitude. These results show that activation of MRN VGLUT3 projections induces a selective enhancement of GABAergic transmission in CA1 pyramidal neurons in a sex-specific manner.

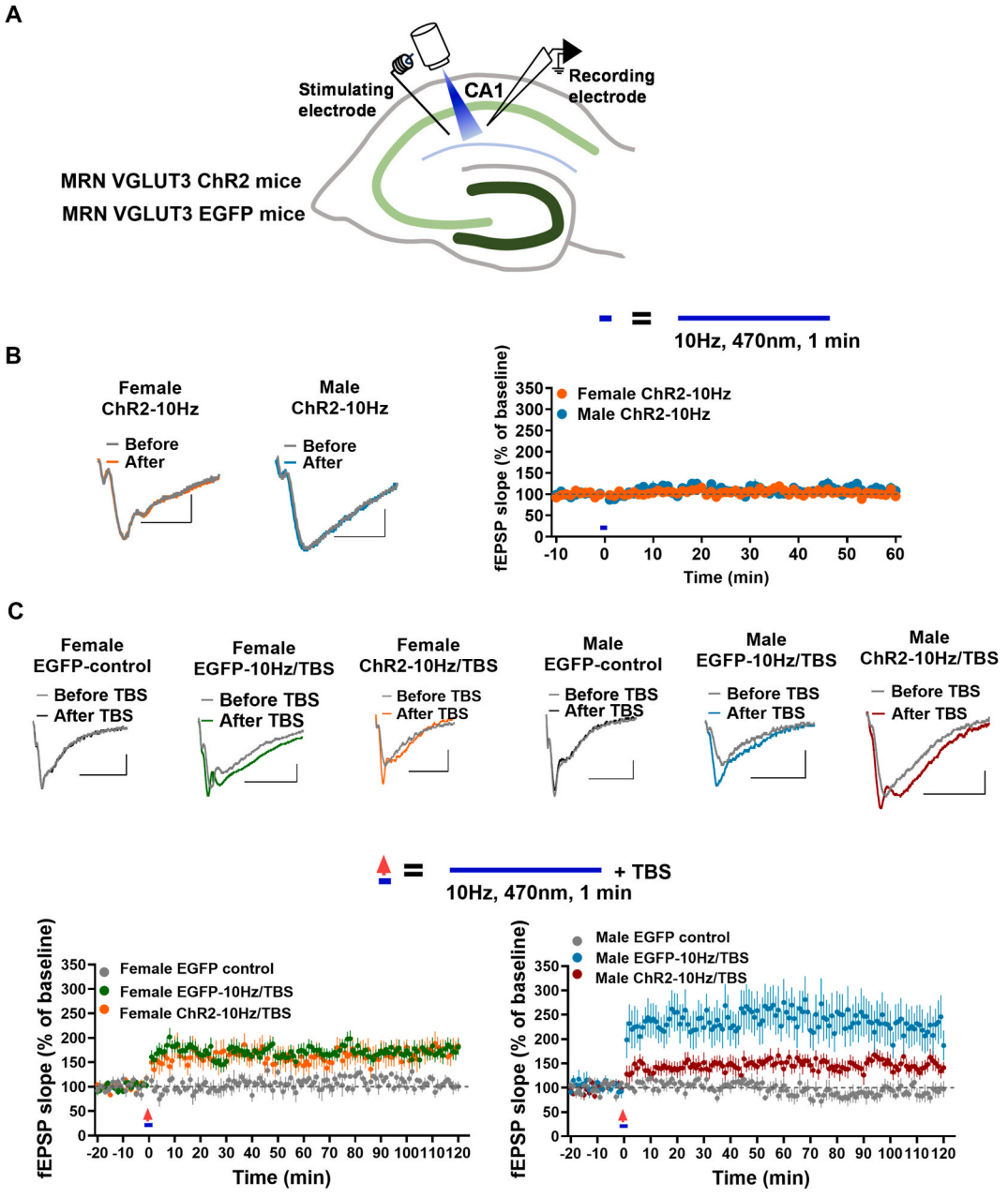
2.4. Activation of MRN VGLUT3 projections suppresses SC-CA1 LTP in a sex-specific manner

Based on the sex-specific effect of activation of MRN VGLUT3 projections on GABAergic transmission in CA1 pyramidal neurons, we asked whether activation of MRN VGLUT3 axon terminals at the SR/SLM region affects synaptic plasticity in the dorsal hippocampus. An increase in GABAergic transmission in CA1 pyramidal neurons alters CA1 LTP [43,44]. First, we examined whether the 10 Hz light activation of MRN VGLUT3 axon terminals affects SC-CA1 pathway transmission. We recorded field excitatory post-synaptic potentials (fEPSPs) from the CA1 SR region by stimulating the SC input with an extracellular stimulating electrode. Experiments in female and male MRN VGLUT3 ChR2 groups did not show a change in fEPSP slope in response to the light activation of MRN VGLUT3 projections in the SR/SLM region (Fig. 6A and B). These results are consistent with the lack of effect of 10 Hz light stimulation on glutamatergic transmission in CA1 pyramidal neurons (Fig. S1). An examination of basal transmission in female and male mice by comparing the input-output relationship of fEPSPs did not show statistical significance (Fig. S2). Next, we investigated whether 10 Hz light activation of MRN VGLUT3 axon terminals at the SR/SLM region immediately before applying theta burst stimulation (TBS) affects TBS-induced long-term potentiation (LTP) at the SC-CA1 pathway. In the control MRN VGLUT3 EGFP mice, 10 Hz light stimulation followed by TBS produced a robust LTP in both female and male mice (Fig. 6C). Although the LTP in the male MRN VGLUT3 EGFP group was higher than that in the female MRN VGLUT3 EGFP group, this effect did not show statistical significance. We did not observe a statistically significant difference in LTP in the female MRN VGLUT3 ChR2 and MRN VGLUT3 EGFP groups that underwent 10 Hz light stimulation followed by TBS (Fig. 6C). However, the 10 Hz light stimulation suppressed TBS-induced LTP in the male MRN VGLUT3 ChR2 group compared to the male MRN VGLUT3 EGFP group (Fig. 6C). Consistently, we observed a statistically significant effect of treatment and sex on LTP. These results show that activation of MRN VGLUT3 projections suppresses LTP selectively in male mice.

3. Discussion

The current study demonstrates the robust monosynaptic connectivity between MRN VGLUT3 neurons and 5-HT3aR-expressing neurons at the SR/SLM border of the dorsal hippocampus. These results are consistent with the high density of MRN axonal projections at the SR/SLM region [5,6,15,25]. Also, MRN VGLUT3 neurons form glutamatergic synapses onto SR/SLM VGLUT3 neurons, a major subclass of 5-HT3aR neurons. Our results show that the connectivity between MRN VGLUT3 neurons and SR/SLM 5-HT3aR neurons is solely mediated through glutamatergic transmission and not the ionotropic 5-HT3aR-mediated transmission [14,15,18]. We show that MRN VGLUT3 neurons selectively enhance synaptic inhibition and suppress LTP in the dorsal hippocampus in a sex-specific manner.

Earlier studies have demonstrated the innervation of the hippocampus by ascending neuromodulatory pathways [1–3,45–48]. These cholinergic, serotonergic, noradrenergic, and dopaminergic pathways modulate hippocampal activity and behavior [1–3,45–48]. Despite the demonstration that the MRN modulation of the hippocampus is primarily mediated through MRN



(caption on next page)

Fig. 6. Activation of MRN VGLUT3 axon terminals at the SR/SLM region suppresses SC-CA1 LTP in male but not female mice. A) A schematic of CA1 fEPSP recording and applying 10 Hz light stimulation to activate ChR2-expressing MRN VGLUT3 axon terminals in the hippocampus slice preparation. B) fEPSP recording before and after the application of 10 Hz light stimulation. Light activation of MRN VGLUT3 axons in the SR/SLM region did not affect SC-CA1 synaptic transmission in female and male mice ($F_{1,22} = 0.26$, $P = 0.6$, $N = 12/5$ mice for both female and male groups). Data are presented as mean ± SEM. The left panel shows example traces. Scale 0.5mV/10 ms. C) 10 Hz light stimulation of SR/SLM MRN VGLUT3 axon terminals just before the application of TBS suppressed TBS-induced LTP in male but not female group. The top panel shows example fEPSP traces. Scale 0.5mV/10 ms. The left bottom panel shows the percentage change in fEPSP slope in female EGFP control (N = 7/5 mice), female EGFP-10Hz/TBS group (N = 16/10 mice), and female ChR2-10Hz/TBS (N = 15/9 mice) groups. Application of 10 Hz stimulation and TBS produced robust LTP in female mice expressing ChR2 or EGFP in MRN VGLUT3 neurons compared to the female EGFP control group that did not receive the 10 Hz light stimulation and the TBS ($F_{2,35} = 4.5$, $P = 0.018$). A comparison of the effect of 10 Hz light stimulation and TBS on female groups expressing ChR2 and EGFP in MRN VGLUT3 neurons did not show a statistically significant difference ($P = 0.93$). The arrowhead and blue bar indicate the light stimulation followed by the TBS. The right bottom panel shows the percentage change in fEPSP slope in Male EGFP control (N = 5/3 mice), male EGFP-10Hz/TBS (N = 13/7 mice), and male ChR2-10Hz/TBS (N = 16/9 mice) groups. A 10 Hz light stimulation before the application of TBS suppressed TBS-induced LTP in the male group expressing ChR2 in MRN VGLUT3 neurons compared to the male group expressing EGFP in MRN VGLUT3 neurons ($F_{2,31} = 6.1$, $P = 0.006$, male EGFP-10Hz/TBS group vs male ChR2-10Hz/TBS group: $P = 0.03$, male EGFP-10Hz/TBS group vs male EGFP control: $P = 0.01$, male ChR2-10Hz/TBS group vs male EGFP control: $P = 0.75$). We observed a statistically significant effect of treatment ($F_{3,59} = 9.7$, $P = 0.003$) and sex ($F_{3,59} = 3.54$, $P = 0.03$) on LTP.

VGLUT3-expressing neurons, little was known about the cellular specificity and the effect of MRN VGLUT3 modulation of the hippocampus [7,15]. Earlier studies have shown that the MRN modulation of the hippocampus involves glutamatergic transmission onto hippocampal GABAergic neurons [6]. Our studies show that MRN VGLUT3 neurons innervate 5-HT3aR-expressing neurons at the SR/SLM border through glutamatergic synapses. While this MRN VGLUT3 innervation of SR/SLM 5-HT3aR neurons is mediated through both non-NMDA and NMDA receptors, we did not find any evidence for the contribution of ionotropic 5-HT3aRs in this connectivity. Our results are consistent with the notable segregation of VGLUT3 and 5-HT neurons in the MRN and the predominant innervation of the hippocampus by MRN VGLUT3 neurons that lack 5-HT expression [14,15]. Interestingly, 5-HT suppresses MRN VGLUT3 neuron-mediated glutamatergic transmission onto SR/SLM 5-HT3aR neurons through 5-HT1B receptors. Given the predominant pre-synaptic nature of 5-HT1B receptors, it is highly likely that 5-HT negatively regulates glutamate release from MRN VGLUT3 neurons [41,42].

In agreement with the MRN VGLUT3 neuron modulation of SR/SLM 5-HT3aR GABAergic neurons, activation of MRN VGLUT3 axon terminals in the SR/SLM region produced a long-lasting increase in GABAergic transmission in CA1 pyramidal neurons in male mice. Although a major subgroup of 5-HT3aR neurons, i.e., VGLUT3 neurons, release both GABA and glutamate, we did not observe any change in glutamatergic transmission in CA1 pyramidal neurons following the activation of MRN VGLUT3 axon terminals in the SR/SLM region [14,26]. Furthermore, we did not observe sex difference in CA1 VGLUT3 neuron-mediated dual release of glutamate and GABA onto pyramidal neurons (Fig. S3). Consistent with the selective enhancement of GABAergic transmission, activation of MRN VGLUT3 axon terminals suppresses LTP in male but not female mice. Given the lack of sex difference in both the MRN VGLUT3 neuron-mediated transmission in SR/SLM 5-HT3aR neurons and the CA1 VGLUT3 neuron-mediated dual release of glutamate and GABA onto pyramidal neurons (Fig. S3), it is likely that the sex difference in the effect of MRN VGLUT3 neurons on GABAergic transmission and LTP is due to mechanisms downstream of SR/SLM 5-HT3aR neurons and upstream of pyramidal neurons. Unlike the male-specific effects in the hippocampus, our recent study has shown a selective effect of MRN VGLUT3 neurons on synaptic plasticity in the medial prefrontal cortex of female mice [14]. These studies suggest that MRN VGLUT3 neurons exert a differential modulation of these two forebrain structures in a sex-specific manner. These sex-specific effects of MRN VGLUT3 neurons are likely due to the sex-dependent development of synaptic connections, neuronal membrane properties, or molecular mechanisms at the target forebrain structures. Also, sex difference in GABAergic transmission and LTP might play a role in the lack of effect of MRN VGLUT3 neuron activation on hippocampal synapses in female mice. Both sex hormones and chromosomes might play a role in the organization of these sex-specific mechanisms [49].

Recent studies from other laboratories as well as our current study show that the raphe modulation of the hippocampus is mediated through 5-HT and glutamate [15,48]. Activation of 5-HT signaling in the hippocampus enhances CA1 LTP and memory formation [48]. Our study shows that activation of MRN VGLUT3 projections to the hippocampus enhances synaptic inhibition and suppresses CA1 LTP. Therefore, 5-HTergic and glutamatergic transmission from the raphe may exert functionally opposing effects in the dorsal hippocampus. The 5-HT-dependent negative regulation of MRN VGLUT3 neuron-mediated glutamatergic transmission in the hippocampus might play a role in the effect of 5-HTergic signaling on LTP and memory. Given that the dorsal hippocampal 5-HT signaling mediates anxiogenic behavior, 5-HTergic and glutamatergic transmission from the raphe may also exert opposing effects on emotional behaviors [11,48,50]. Previous studies have shown sex-specific effects of 5-HT on synapses and behavior [51,52]. Therefore, it is plausible that the sex-specific modulation of the forebrain by raphe neurons might play a role in sex difference in cognitive and emotional disorders [53,54]. Given the robust MRN VGLUT3 innervation of the 5-HT3aR neurons at the SR/SLM border, a region that receives inputs from the entorhinal cortex, the thalamus, and the amygdala, the MRN VGLUT3 neurons, possibly through its modulation of SR/SLM 5-HT3aR neurons might play a critical role in gating these inputs and regulating hippocampus-dependent cognitive and emotional behaviors [55–57].

A limitation of our study is the need to identify the mechanism underlying the sex-specific effect of MRN VGLUT3 neurons on GABAergic transmission and LTP in the hippocampus. Given the lack of sex difference in MRN VGLUT3 neuron-mediated transmission in SR/SLM 5-HT3aR neurons, future studies are needed to determine whether MRN VGLUT3 neuron-mediated effects on GABAergic

neurons other than SR/SLM 5-HT3aR neurons are responsible for the sex-specific effects of MRN VGLUT3 neurons. Also, SST or PV neurons downstream of SR/SLM 5-HT3aR neurons may mediate these sex-specific effects. Future studies are also needed to determine the behavioral relevance of the sex-specific effects of MRN VGLUT3 neurons on the hippocampus.

In conclusion, our studies demonstrate monosynaptic connectivity between MRN VGLUT3 neurons and SR/SLM 5-HT3aR neurons via glutamatergic transmission, which is negatively regulated by 5-HT through 5-HT1B receptors. Activation of MRN VGLUT3 projections produces a long-lasting and selective enhancement of GABAergic transmission and suppression of LTP in the dorsal hippocampus in a sex-specific manner.

4. Materials and methods

4.1. Animals

Female and male mice (3–6 months old) were used in all experiments. B6; 129S-*Slc17a8*$^{tm1.1(cre)Hze}$/J (Stock number: 028534, Jackson Laboratories) mice were used for studying the effect of optogenetic activation of VGLUT3 neurons [28–30]. B6; 129S-*Slc17a8*$^{tm1.1(cre)Hze}$/J mice were crossed with C57BL/6J (Stock number: 000664, Jackson Laboratories) for breeding. We crossed B6; 129S-*Slc17a8*$^{tm1.1(cre)Hze}$/J mice with 5-HT3aR-BAC^{EGFP} mice (Mutant Mouse Resource & Research Centers) to generate mice that express Cre in VGLUT3 neurons and EGFP in 5-HT3aR neurons. VGLUT3-tdTomato mice were created by crossing B6; 129S-*Slc17a8*$^{tm1.1(cre)Hze}$/J with B6.Cg-*Gt(ROSA)26Sor*$^{tm14(CAG-tdTomato)Hze}$/J (Stock number: 007914, Jackson Laboratories) [58]. Mice were maintained on a 12-h light-dark cycle at 23°C with access to food and water ad libitum. The Institutional Animal Care and Use Committee of the University of Toledo approved all the utilized procedures (Approved protocol number 108914-02).

4.2. Stereotactic surgery

AAV injections were performed using a stereotactic apparatus (Stoelting, IL, USA) under isoflurane anesthesia. 200 nl AAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA (AAV9), AAV-EF1a-double floxed-hChR2(H134R)-EYFP-WPRE-HGHpA (AAV9), and AAV-hSyn-DIO-EGFP (AAV2) were injected through a pulled glass injection micropipette (~50 μm tip, Item no: 504949, WPI, FL, USA) at a rate of 100 nl/min using an injector (Nanoliter 2010, WPI, FL, USA). Injection coordinates for the MRN were (from bregma): AP: −3.6 mm; ML: 1.2 mm, DV: −4.6–4.8 mm, 16° angle (ear bars at 18 mm, anesthesia mask bar at 14 mm). Injection coordinates for the hippocampus were (from bregma): AP: −1.5 mm; ML: ±1.5 mm, DV: −1.7 mm, (ear bars at 18 mm, anesthesia mask bar at 14 mm). Fluorescence microscopy was used to image mCherry/EGFP/EYFP-expressing neurons to confirm the accuracy of virus injections. Mice were used for experiments 3 weeks after the surgery.

4.3. Electrophysiology

For whole cell recording experiments, brains were isolated after intracardiac perfusion with oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (118), glucose (10), KCl (2.5), NaH_2PO_4 (1), $CaCl_2$ (1) and $MgSO_4$ (1.5) (325 mOsm, pH 7.4) under pentobarbital (120 mg//kg) anesthesia. Dorsal hippocampal slices (300 μm) were prepared on a vibratome (Campden Instruments). Hippocampal slices were incubated in ACSF at 28°C for a minimum of 1 h. For recording, brain slices were transferred to a recording chamber maintained at 32°C on an upright microscope (Axio Examiner. D1, Zeiss). The slices were perfused with the aforementioned ACSF containing 2.5 mM $CaCl_2$ at 2 ml/min. The dorsal CA1 hippocampus was located using a 4× objective, and recorded neurons were identified using a 40× water immersion objective and video-enhanced differential interference contrast microscopy. Fluorescence microscopy was used to identify EGFP- and tdTomato-expressing neurons. Pyramidal neurons were identified by their morphology, membrane properties, and location. MRN VGLUT3 neuron-mediated transmission in SR/SLM neurons and CA1 VGLUT3 neuron-mediated transmission were recorded using glass electrodes of 3–5 MΩ resistance filled with an internal solution containing (in mM): K-gluconate (130), KCl (10), $MgCl_2$ (2), MgATP (5), NaGTP (0.2), HEPES (5), pH adjusted to 7.4 with KOH. To examine the monosynaptic nature of MRN VGLUT3 neuron-mediated transmission, we tested whether 4-AP (100 μM) rescues TTX (1 μM)-induced suppression of light-evoked currents. MRN VGLUT3 neuron-mediated EPSCs were recorded at −60 mV, and DNQX (10 μM) was used for confirming the glutamatergic nature of MRN VGLUT3 neurons-mediated transmission. Non-NMDA and NMDA receptor transmission in 5-HT3aR neurons were recorded using an internal solution containing CsCl (130 mM), HEPES (10 mM), EGTA (0.5 mM), MgATP (5 mM), sucrose (10 mM), and QX314 (5 mM), pH adjusted to 7.4 with CsOH. Bicuculline was included in the recording solution. DNQX (10 μM) and AP5 (50 μM) were used for confirming non-NMDA and NMDA currents. Non-NMDA and NMDA currents were recorded at the single neuron level. An internal solution containing K-gluconate (130 nM), KCl (10), $MgCl_2$ (2 nM), MgATP (5 nM), NaGTP (0.2 nM), and HEPES (5 nM) (pH adjusted to 7.4) was used for studying sEPSCs and sIPSCs in pyramidal neurons. sEPSCs and sIPSCs were recorded at −60 mV and 0 mV, respectively. DNQX (10 μM) and bicuculline (10 μM) were used for confirming sEPSCs and sIPSCs. Electrophysiological recordings were rejected when series resistance or holding current changed by 10 % or more.

For fEPSP experiments, brains were isolated after intracardiac perfusion with ACSF under pentobarbital anesthesia. After isolating the hippocampi, dorsal hippocampal slices (400 μm) were prepared on a vibratome. Slices were maintained at 28°C in a brain slice keeper for a minimum of 1 h before transferring to a recording chamber at 32°C on an upright microscope (Axio Examiner. D1, Zeiss). Slices were immersed in ACSF using slice anchors. ACSF consisted of (in mM): NaCl (118), KCl (4.5), glucose (10), NaH2PO4 (1), CaCl2 (2), MgCl2 (2) (aerated by 95%O2/5 % CO2, pH 7.4). CA1 fEPSPs were recorded with a glass electrode containing ACSF by stimulating

the SC fibers through a concentric bipolar electrode (FHC). The input-output curves of fEPSPs were generated by plotting stimulus strength (100–900 μA) against the fEPSP slope. For the LTP experiments, a 20-min baseline was recorded every minute at an intensity that evoked a response ~35 % of the maximum response. Following the baseline recording, a 10 Hz light stimulation (5 ms pulse, 470 nm for 60s) was applied at the SR/SLM region of the hippocampus using PE-4000 LED light source (CoolLED) through the 40× water immersion objective. The light stimulation was immediately followed by TBS (4 pulses at 100 Hz, with the bursts repeated 10 times at 5 Hz, three 10-burst trains separated by 15 s) [59]. fEPSPs were recorded for 2 h after the light stimulation and TBS.

4.4. Immunohistochemistry and microscopy

Mice were intracardially perfused with 4 % PFA in PBS under pentobarbital (120 mg/kg) anesthesia. Brains were isolated and transferred to 4 % PFA for maintaining at 4 °C overnight. Brains were washed in PBS three times and transferred to 15 % sucrose for 24 h, followed by 30 % sucrose for 24 h at 4 °C. Brains were frozen on dry ice before preparing 45 μm brain slices on a cryostat. Brain slices were washed in TBS 3 times for 5 min. Washed slices were blocked using 3 % normal goat serum (mCherry) or 10 % horse serum (GFP) in 0.3 % Triton X in TBS for 30 min at room temperature. The slices were then incubated in mCherry polyclonal antibody (Rockland Immunochemicals, Cat. No. 600401P16) or Anti-GFP antibody (Abcam, Cat. No. ab13970, RRID: AB_300798) at a dilution of 1:1000 in 0.3 % Triton-X/TBS for 24 h at 4 °C [60,61]. Slices were rinsed 3 times in TBS for 5 min and transferred to either goat anti-rabbit IgG (H + L) Alexa Fluor™ 594 (Invitrogen, Cat. No. A11037, RRID: AB_2534095) or Goat anti-chicken IgY (H + L) (Alexa Fluor™ 488 (ThermoFisher, Cat. No. A-11039, RRID: AB_2534096) secondary antibody with a dilution of 1:1000 in 0.3 % Triton-X/TBS and 5 % Normal Goat Serum for 2 h incubation at room temperature. Slices were then rinsed 3 times in TBS for 5 min, mounted on Superfrost Plus microscope slides, and coverslipped with Fluoromount-G with DAPI (Southern Biotech, Cat# 0100–20). Confocal images of the hippocampus were taken using a TCS SP5 multiphoton laser-scanning confocal microscope (Leica Microsystems, Buffalo Grove, IL) with a 20× objective lens.

4.5. Data analysis

EPSCs amplitude, EPSP amplitude, and fEPSP slope measurements were analyzed using Clampfit 10.7 (Molecular Devices, CA, USA). Statistical analyses were performed using IBM SPSS statistics (version 27) or GraphPad Prism (version 9) software. Studies on MRN VGLUT3 neuron-mediated monosynaptic transmission in SR/SLM 5-HT3aR and VGLUT3 neurons were analyzed using repeated measures ANOVA. A two-way repeated measures ANOVA was used for analyzing spontaneous currents, fEPSPs, and the effect of drugs on EPSCs/EPSPs. The normality of the data was assessed using the Shapiro-Wilk test. Sex difference in MRN VGLUT3 neuron-mediated non-NMDA and NMDA currents and hippocampal VGLUT3 neuron-mediated glutamatergic and GABAergic currents were compared using Mann Whitney test.

Data availability

The authors confirm that all the data needed to evaluate the conclusions in the article are available within the article.

CRediT authorship contribution statement

Hannah E. Stinson: Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Ipe Ninan:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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AAV-EF1a-double floxed-hChR2(H134R)-mCherry-WPRE-HGHpA (20297-AAV9, Addgene) and AAV-EF1a-double floxed-hChR2 (H134R)-EYFP-WPRE-HGHpA (20298-AAV9, Addgene) were gifts from Dr. Karl Deisseroth. AAV-hSyn-DIO-EGFP (50457-AAV2, Addgene) was a gift from Dr. Bryan Roth. Tg(Htr3a-EGFP)DH30Gsat/Mmnc, RRID:MMRRC_000273-UNC, was obtained from the Mutant Mouse Resource and Research Center (MMRRC) at University of North Carolina at Chapel Hill, an NIH-funded strain repository, and was donated to the MMRRC by Nathaniel Heintz, Ph.D., The Rockefeller University, GENSAT. We thank Dr. Peter Koppensteiner for his comments on the manuscript. We acknowledge the support of Dr. Andrea Nestor-Kalinoski in imaging experiments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38192.

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