HHS Public Access

Author manuscript

Neuropharmacology. Author manuscript; available in PMC 2018 November 01.

Published in final edited form as:

Neuropharmacology. 2017 November ; 126: 224–232. doi:10.1016/j.neuropharm.2017.09.013.

Serotonin gating of cortical and thalamic glutamate inputs onto principal neurons of the basolateral amygdala

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Abstract

The basolateral amygdala (BLA) is a key site for crossmodal association of sensory stimuli and an important relay in the neural circuitry of emotion. Indeed, the BLA receives substantial glutamatergic inputs from multiple brain regions including the prefrontal cortex and thalamic nuclei. Modulation of glutamatergic transmission in the BLA regulates stress- and anxiety-related behaviors. Serotonin (5-HT) also plays an important role in regulating stress-related behavior through activation of both pre- and postsynaptic 5-HT receptors. Multiple 5-HT receptors are expressed in the BLA, where 5-HT has been reported to modulate glutamatergic transmission. However, the 5-HT receptor subtype mediating this effect is not yet clear. The aim of this study was to use patch-clamp recordings from BLA neurons in an *ex vivo* slice preparation to examine 1) the effect of 5-HT on extrinsic sensory inputs, and 2) to determine if any pathway specificity exists in 5-HT regulation of glutamatergic transmission. Two independent input pathways into the BLA were stimulated: the external capsule to mimic cortical input, and the internal capsule to mimic thalamic input. Bath application of 5-HT reversibly reduced the amplitude of evoked excitatory postsynaptic currents (eEPSCs) induced by stimulation of both pathways. The decrease was associated with an increase in the paired-pulse ratio and coefficient of variation of eEPSC amplitude, suggesting 5-HT acts presynaptically. Moreover, the effect of 5-HT in both pathways was mimicked by the selective 5-HT_{1B} receptor agonist CP93129, but not by the 5-HT_{1A} receptor agonist 8-OH DPAT. Similarly the effect of exogenous 5-HT was blocked by the 5-HT_{1B} receptor antagonist GR55562, but not affected by the 5-HT_{1A} receptor antagonist WAY 100635 or the 5-HT_2 receptor antagonists pirenperone and MDL 100907. Together these data suggest 5-HT gates cortical and thalamic glutamatergic inputs into the BLA by activating presynaptic 5-HT_{1B} receptors.

Keywords

serotonin receptor; glutamatergic transmission; basolateral amygdala

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Introduction

The basolateral amygdala (BLA) is a key relay structure in emotional circuitry. The BLA receives substantial glutamatergic input from both the somatosensory cortex through the external capsule, and from the thalamic nuclei through the internal capsule. The BLA in turn projects to multiple downstream targets critically involved in the regulation of stress and anxiety-like behavior, including the central nucleus of amygdala (CeA) and the bed nucleus of the stria terminalis (BNST) (Walker et al., 2003). Significantly, abnormal hyperactivity of the BLA has been implicated in the etiology of several emotional disorders including depression and anxiety. Consistent with this observation, repeated stress increases the activity of BLA neurons *in vivo* (Zhang and Rosenkranz, 2012, Padival et al., 2013). Furthermore, activation or inhibition of neural activity in the BLA respectively enhances or reduces anxiety-like behavior (Davis, 2002, Tye et al., 2011). Additionally, stressful stimuli increase glutamate release in the BLA (Reznikov et al., 2007), and microinjection of glutamate antagonists into the BLA abolishes the expression of conditioned fear (Kim et al., 1993, Lee et al., 2001). Hence, glutamatergic transmission is essential for normal BLA function.

Importantly, glutamatergic input into the BLA can be regulated by neurotransmitters and/or neuromodulators such as serotonin (5-HT) (Bocchio et al., 2016). Indeed, dysregulation of 5-HT transmission is thought to play a major role in the etiology of emotional disorders (Lowry et al., 2005, Asan et al., 2013). For example, the BLA hyperactivity seen in major depressive disorder is normalized after successful pharmacotherapy using selective serotonin reuptake inhibitors (SSRIs). The BLA is heavily innervated by 5-HT terminals originating from the dorsal raphe nucleus (Abrams et al., 2004, Muller et al., 2007), and multiple 5-HT receptor subtypes are expressed by both BLA principal neurons and interneurons (Asan et al., 2013). Indeed, local activation of 5-HT_{2A} or 5-HT_{2C} receptors induces anxiety-like behavior in rodents (Campbell and Merchant, 2003, de Mello Cruz et al., 2005, Cornelio and Nunes-de-Souza, 2007, Christianson et al., 2010), an effect thought to be mediated by activating postsynaptic 5-HT receptors. Far fewer studies have examined 5-HT receptor-mediated modulation of glutamatergic transmission in the BLA. Previous studies reported that activating presynaptic 5-HT receptors inhibits glutamatergic transmission in the BLA (Cheng et al., 1998, Rainnie, 1999, Daftary et al., 2012, Yamamoto et al., 2012). However, the receptor subtype(s) mediating this response have not been clearly established. In this study we used *in vitro* patch clamp recording from BLA slices to 1) determine the identity of the presynaptic 5-HT receptor(s) modulating BLA glutamatergic transmission, and 2) determine if presynaptic 5-HT differentially regulated cortical and thalamic glutamatergic inputs into the BLA.

Methods

Animals

Adolescent male Sprague-Dawley rats (35–49 days old, Charles River, Raleigh, NC) were used throughout this study. Animals were group-housed with 4–5 rats per cage and had access to food and water *ad libitum*. Efforts were taken to minimize both animal suffering and the number of animals used in experiments. Animal care and all procedures used in this

study were performed in accordance with the National Institutes for Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee of Emory University.

Slice preparation

Coronal slices (350 μm) containing the BLA were obtained as previously reported (Li et al., 2011). Briefly, under deep anesthesia (isofluorane, Henry Schein Inc, Melville, NY, USA), the brains of anesthetized rats were rapidly dissected and immersed in a cold (4°C) oxygenated artificial cerebrospinal fluid (ACSF) "cutting solution", of the following composition (in mM): NaCl (130), $NaHCO_3$ (30), KCl (3.50), KH_2PO_4 (1.10), $MgCl_2$ (6.0), $CaCl_2$ (1.0), glucose (10), ascorbate (0.4), thiourea (0.8), sodium pyruvate (2.0), and kynurenic acid (2.0). BLA slices were cut using a Leica VTS-1000 vibratome (Leica Microsystems Inc., Bannockburn, IL, USA). After sectioning, slices were maintained at 32°C in "cutting solution" oxygenated with a mixture of 95% oxygen and 5% carbon dioxide for 1 h prior to recording. Slices were then transferred to a holding chamber containing "regular" ACSF maintained at room temperature, with the following composition (in mM): NaCl (130), NaHCO3 (30), KCl (3.50), KH2PO4 (1.10), MgCl2 (1.30), CaCl2 (2.50), and glucose (10), ascorbate (0.4), thiourea (0.8), and sodium pyruvate (2.0).

Patch clamp recording

For whole-cell patch clamp recording, slices were continuously perfused by gravity-fed oxygenated "regular" ACSF heated to 32°C (2–3 ml per min) in a Warner Series 20 submersion-type slice chamber (0.5 ml volume; Warner Instruments, Hamden, CT). Slices were viewed using differential interference contrast (DIC) optics and infrared (IR) illumination with an IR sensitive CCD camera (Orca ER, Hamamatsu, Tokyo, Japan) mounted on a Leica DMF6000 microscope (Leica Microsystems Inc., Bannockburn, IL). Patch pipettes were fabricated from borosilicate glass (resistance 4–6 MΩ) and filled with a recording solution of the following composition (in mM): K-Gluconate (130), KCl (2), HEPES (10), $MgCl_2$ (3), phosphocreatine (5), K-ATP (2), and NaGTP (0.2). The patch solution was adjusted to pH 7.3 with KOH and had an osmolarity of 280–290 mOsm. Whole-cell recordings were made with a Multiclamp 700B amplifier (Molecular Devices Corporation, Sunnyvale, CA) using pClamp 10.4 software and an Axon Digidata 1550 A-D interface (Molecular Devices Corporation). BLA principal neurons were identified visually by their pyramidal shape and confirmed physiologically by their membrane properties. Whole-cell access resistances measured in voltage clamp were in the range 5 – 20 MΩ and were routinely monitored throughout each experiment; a change of <15% was deemed acceptable.

Evoked EPSCs

Postsynaptic currents onto BLA neurons were evoked by stimulating the external capsule (cortical input) or the internal capsule (thalamic input) with a concentric bipolar stimulation electrode (FHC, Bowdoinham, ME) as previously reported (Li et al., 2011). To isolate evoked excitatory postsynaptic currents (eEPSCs), the $GABA_A$ receptor antagonist picrotoxin (100 μM) was included in the patch solution to block inhibitory postsynaptic currents (IPSCs). Furthermore, the membrane potential was held at −65 mV, which is close

to chloride equilibrium potential, to minimize contamination by residual $GABA_A$ receptor-mediated evoked IPSCs. The selective $GABA_B$ receptor antagonist CGP 53432 (1 μM) was also bath applied to block the slow component of evoked IPSCs. Two stimulation paradigms were used in this study to induce eEPSCs: 1) one train of five single square wave pulses (150 μs, 0.2 Hz) delivered every 2 min, and 2) consecutive single pulse stimulations (150 μs, 0.1 Hz) were delivered throughout the experiment. Baseline amplitude of eEPSCs was adjusted to half maximal stimulation response. For analysis, all eEPSCs values were normalized to the baseline amplitude and expressed as the percentage of baseline.

To examine the potential involvement of presynaptic 5-HT receptors in the attenuation of glutamate release, we employed a paired-pulse paradigm in conjunction with an analysis of the coefficient of variation (CV) of eEPSC amplitude, as previously reported (Guo et al., 2012). Alterations in the paired-pulse ratio (PPR) are thought to represent changes in release probability in the presynaptic terminal (Hess and Ludin, 1987; Manabe et al., 1993). A change of CV is associated with either a change of release probability or the number of release sites (Choi and Lovinger, 1997). For the paired-pulse paradigm, two electrical stimuli were delivered with an inter-stimulus-interval of 50 ms. The PPR was calculated as the mean peak amplitude of the second eEPSC (P2) divided by the first eEPSC (P1). CV was calculated as δ/μ, where δ is the stand deviation of the peak eEPSC amplitude and μ is the mean eEPSC amplitude. Here, we used 10 eEPSCs immediately before drug application and 10 eEPSCs during the maximal drug effect to calculate CV in the baseline and drug application respectively. The selection of 10 sweeps was adequate for analysis based on the low variation of eEPSCs amplitude and ensured that they were captured at the time of the maximum drug response. Including more sweeps would increase the chance that some sweeps may not have reached maximal 5-HT effect and if included in the analysis would erroneously increase CV value.

Drug application

The following drugs were obtained from 1) Sigma-Aldrich (St. Louis, MO): 5-HT, 8-OH-DPAT, and WAY 100635; 2) Tocris Bioscience (Ellisville, MO): serotonin, CP93129, GR55562, picrotoxin, and CGP 53432. Picrotoxin was included in the patch solution with final concentration of 100 μM. All other drugs were applied in the ACSF using a continuous gravity-fed bath application. Agonists were applied for 6 min and antagonists were continuously applied throughout whole recording duration.

Statistical analysis

Statistical analysis was performed using Graphpad Prism 4.0 (Graphpad Software Inc, La Jolla, CA) or Microsoft Excel software (Office 2010). For evoked EPSCs, the amplitudes were normalized and expressed as the percentage of the baseline eEPSC amplitude. Data are presented as mean ± S.E.M. Sample size n refers to numbers of neurons; no more than 2 neurons were sampled per rat. Changes before and during drug applications were analyzed using paired Student's t-test. Two-way repeated measures ANOVA (two-way RM ANOVA) was used to determine the difference between drug treatments during application. For all comparisons, a p value < 0.05 was considered statistically significant.

Results

Monosynaptic evoked postsynaptic currents were elicited in BLA principal neurons by stimulation of either the external- or internal capsule. Evoked excitatory post-synaptic currents (eEPSCs) were isolated by inclusion of picrotoxin (100 μM) in the intracellular solution, and CGP 54532 (1 μM) in the ACSF (see Methods). Any apparent polysynaptic events were excluded from the analysis.

5-HT suppresses glutamatergic transmission in afferent pathways from the cortex and thalamus onto BLA principal neurons

Bath application of 5-HT (10 μM) caused a time-dependent decrease in the amplitude of cortical eEPSCs, with a peak inhibition of 63.8±5.8% of baseline reached 6 min after drug application (baseline 335±29 pA, 5-HT 203±21 pA, $p<0.01$, paired t-test, n=6 from 3 rats, Fig 1A, B). Similarly, bath application of 5-HT (10 μM) decreased the amplitude of thalamic eEPSCs to 52.8±5.4% of baseline after 6 min (baseline 391±47 pA, 5-HT 206±26 pA; n=7 from 4 rats, $p<0.01$, paired t-test, Fig 1C, D). The inhibitory effect of 5-HT could be reversed after a 10-minute washout with ACSF for both cortical (314±32 pA, 94±8.9% of baseline, $p<0.01$, paired t-test, n=6 from 3 rats) and thalamic pathways (374±68 pA; 102.8% ±9.3% of baseline, $p<0.01$ vs 5-HT, paired t-test, n=7 from 4 rats). No significant difference was observed in the effects of 5-HT on the cortical versus thalamic pathway (two-way RM ANOVA, $F(1,55)=2.62$, $p>0.05$). Notably, increasing the concentration of 5-HT to 50 μM failed to further reduce the amplitude of cortical eEPSCs (baseline 251±45 pA; 5-HT 151±29 pA; 65±5% of baseline, n=5 from 3 rats; paired t-test $p<0.05$ vs baseline; $p>0.05$ vs 5-HT 10 μM, unpaired t-test), suggesting that 10 μM 5-HT was close to the asymptote of the dose-response curve. Hence, in subsequent studies, we used 10 μM 5-HT to determine 1) the site of action of 5-HT and, 2) the receptor subtype mediating the 5-HT response.

A change of the eEPSC amplitude induced by 5-HT could be due to either pre- or postsynaptic actions. To identify the locus of action of 5-HT, we examined the paired-pulse ratio (PPR, interval 50 ms) of eEPSCs as well as the coefficient of variation (CV) before and during 5-HT application. In both cortical and thalamic pathways, application of 5-HT decreased the amplitude of both the first and second eEPSC, with the first eEPSC reduced more than the second. At baseline, the cortical PPR of eEPSCs was 1.22±0.10, whereas during 5-HT application the PPR increased to 1.52±0.10 (n=8 from 4 rats, paried t-test, $p<0.01$, Fig 1E,F). Similarly, 5-HT application increased the thalamic PPR compared to baseline (1.10±0.03 baseline; 1.42±0.08 5-HT; n=8 from 4 rats, paried t-test, $p<0.01$, Fig 1 G, H). Together the reduced eEPSC amplitude and the increase in the PPR strongly suggested 5-HT acted presynaptically in both cortical and thalamic pathways.

Consistent with this observation, 5-HT application also increased the CV of cortical and thalamic eEPSCs compared to baseline. Cortical CV increased from 0.15±0.03 to 0.23±0.05 during 5-HT application (n=6 from 3 rats, paired t-test, $p<0.05$), and thalamic CV increased from 0.12±0.02 to 0.22±0.06 (n=7 from 4 rats, paried t-test, $p<0.05$). The increase in CV in both pathways further suggested that presynaptic 5-HT receptors changed the probability of release and/or the number of release sites in glutamatergic terminals. We next investigated

whether the two pathways were regulated by the same, or different, presynaptic 5-HT receptor subtypes.

5-HT$_{1B}$ agonists mimicked the 5-HT effect

Out of the seven 5-HT receptor subfamilies, the Gi-coupled 5-HT$_1$ receptor family has been most widely examined for its inhibitory action on glutamatergic transmission (Pehrson and Sanchez, 2014). Indeed, the 5-HT$_{1B}$ receptor is predominantly expressed on presynaptic terminals (Boschert et al., 1994), and has been shown to decrease glutamate release in multiple brain regions, including the extended amygdala (Guo and Rainnie, 2010). Hence, we first tested whether a selective 5-HT$_{1B}$ receptor agonist CP93129 could mimic the effect of 5-HT on eEPSCs. Bath application of CP93129 (10 μM) significantly decreased cortical eEPSC amplitude to 61.8 ± 5.3% of baseline (baseline 415±33 pA, CP93129 272±36 pA; paried t-test, $p<0.01$, n=6 from 3 rats, Fig 2A,B), and thalamic eEPSC amplitude to 48.2±5.2% of baseline (baseline 295±42 pA, CP93129 163±34 pA; paired t-test, $p<0.01$, n=7 from 4 rats, Fig 2I,J). Similar to the effect of 5-HT on cortical and thalamic pathways, the effect of CP93129 in the thalamic pathway showed no significant difference from that of cortical pathway (two way RM ANOVA, $F(1,55)=2.62$, $p>0.05$). In contrast to 5-HT, the effect of CP93129 on eEPSC amplitude showed little to no reversal after a 10 min washout in ACSF for both the cortical (288±46 pA; 73±8% of baseline, paired t-test, $p>0.05$ vs CP93129, n=6 from 3 rats) and thalamic pathways (201±49 pA, 53±5.2% of baseline, paired t-test, $p>0.05$ vs 5-HT, n=7 from 4 rats). Similar to the 5-HT response, the CP93129-induced decrease of eEPSCs amplitude was associated with an increase of PPR for eEPSCs in both the cortical (baseline 1.15±0.08; CP93129 1.51±0.16, n=7 from 4 rats, paired t-test, $p<0.05$, Fig 2E, F) and thalamic pathways (baseline 1.13±0.06; CP93129 1.44±0.13, paired t-test, $p<0.05$, n=6 from 3 rats, Fig 2K, L). Application of CP93129 also increased the CV of cortical (0.11±0.022 baseline; 0.16±0.024 CP93129, n=6 from 3 rats, paired t-test, $p<0.05$) and thalamic eEPSCs (baseline 0.13±0.02, CP93129 0.25±0.02, n=6 from 3 rats, paired t-test, $p<0.05$). Taken together, these results strongly suggest that presynaptic 5-HT$_{1B}$ receptor activation decreases glutamate release in both cortical and thalamic pathways.

We also examined the effect of the mixed 5-HT$_{1B/D}$ agonist, sumatriptan, on eEPSCs in cortical pathway. Similar to CP93129, sumatriptan (10 μM) decreased the amplitude of eEPSCs (peak inhibition to 45.5±4% of baseline; baseline 361±47 pA; sumatriptan 159±22 pA; n=6 from 3 rats, $p<0.01$ paired t-test, Fig 2E,F). The decreased eEPSCs amplitude was also accompanied by a significant increase of PPR (Baseline 1.44±0.08; sumatriptan 1.73±0.08; n=6 from 3 rats, paried t-test, $p<0.05$, Fig 2 G,H), suggesting this effect was mediated by a presynaptic inhibition of glutamate release. Consistent with the action of CP93129, the sumatriptan-induced inhibition of eEPSCs persisted after 10 min of washout as well.

As there is no apparent difference in serotoninergic inhibition of EPSCs between cortical and thalamic pathways, in following experiments further justifying underlying receptors we only examined drug actions in the cortical pathway.

The inhibitory effect of 5-HT receptor activation on EPSC amplitude was blocked by 5-HT_{1B} receptor antagonists

To verify that presynaptic 5-HT_{1B} receptors mediated the effect of 5-HT on eEPSCs, we tested whether prior application of the selective 5-HT_{1B} receptor antagonist GR55562 could block the effect of exogenous 5-HT. Here, application of GR55562 (20 μM), which had no effect on the amplitude of eEPSCs by itself (baseline 354±55 pA, GR55562 360±53 pA, n=5 from 3 rats, $p>0.05$, paired t-test), markedly attenuated the inhibitory effect of 5-HT (10 μM) on cortical eEPSC amplitude, with a reduction to 82.6±4.5% of baseline (GR55562 alone 334±47 pA, 5-HT in GR55562 276±44 pA, paired t-test, $p<0.05$, n=6 from 3 rats, Fig 3A, B). ANOVA showed that GR55562 caused a significant reduction in the effect of 5-HT application compared to that in ACSF alone (61.2±5.2% of baseline, two-way RM ANOVA, $F(1,66)=7.06$, $p<0.05$).

Because application of GR55562 did not fully block the effects of 5-HT we next examined if additional 5-HT receptor subtypes contribute to the presynaptic 5-HT response. Indeed, presynaptic 5-HT_{1A} receptor activation was reported to attenuate glutamatergic transmission from the lateral amygdala (LA) to the BLA (Cheng et al., 1998). We next tested whether presynaptic 5-HT_{1A} receptor activation contributed to the 5-HT-induced attenuation of glutamate inputs to the BLA. Unlike CP93129, application of the selective 5-HT_{1A} receptor agonist 8-OH DPAT (10 μM) failed to decrease the amplitude of cortical eEPSCs in any neuron tested. Instead, a slight yet non-significant increase was noticed (baseline 260±30 pA, 8-OH DPAT 291±40 pA; 110±7% of baseline, n=7 from 4 rats, paired t-test, $p>0.05$, Fig 4A,B). To further exclude the possibility that 5-HT_{1A} receptor activation contributed to the 5-HT response, we examined the effects of prior application of the 5-HT_{1A} receptor antagonist, WAY100635. Application of WAY 100635 (1 μM) alone had no effect on eEPSC amplitude compared to baseline, and had no effect on the 5-HT(10 μM) induced attenuation of the eEPSC amplitude (WAY 100635 324±60 pA, 5-HT in WAY 100635 237±57 pA, paired t-test, $p<0.01$ vs baseline, n=7 from 4 rats, Fig 4C,D), a response that was not significantly different from the effect of 5-HT application in ACSF alone (two way RM ANOVA, $F(1,288)=0.54$, $p>0.05$).

Finally, a recent study reported that 5-HT_2 receptor activation also contributes to the 5-HT-induced suppression of glutamatergic transmission in the BLA (Yamamoto et al., 2012). To determine whether 5-HT_2 receptor activation contributed to the residual 5-HT-induced EPSCs decrease observed in this study, we examined the effect of 5-HT (10 μM) in the presence of two 5-HT_2 receptor antagonists. First, we tested the effect of the non-selective 5-HT_2 receptor antagonist pirenperone. In the presence of 10 μM pirenperone, a concentration known to fully block 5-HT_2-mediated postsynaptic actions in the BNST (Guo et al., 2009), 5-HT(10 μM) significantly reduced the amplitude of eEPSCs to 60± 9% of baseline (peak inhibition, baseline 256±11 pA, 5-HT 167±17 pA, n=6 from 3 rats, paired t-test, $p<0.05$), which was not different from the 5-HT effect in ACSF alone (two-way RM ANOVA, $F(1,264)=0.50$, $p>0.05$, Fig 5A,C).

Lastly, 5-HT_{2A} receptors are highly expressed in the BLA (Bombardi, 2011). Despite the fact that 5-HT_{2A} receptor activation has been reported to facilitate glutamatergic transmission elsewhere in the CNS (Hasuo et al., 2002), we tested whether the selective 5-

HT_{2A} receptor antagonist MDL 100907 could block the effect of 5-HT on glutamatergic transmission. Prior application of MDL 100907 (10 μM) had no significant effect on the 5-HT(10 μM) induced attenuation of eEPSC amplitude (MDL 100907 293±29 pA, 5-HT 171±27 pA, maximal inhibition to 60±2% of baseline, paired t-test, $p<0.05$, n=6 from 3 rats, Fig 5B, C), which was not different from 5-HT effect in ACSF alone (two-way RM ANOVA, $F(1,264)=0.03$, $p>0.05$).

Taken together, our results strongly suggest that presynaptic 5-HT_{1B} receptors are the predominant 5-HT receptor subtype mediating presynaptic inhibition of glutamatergic input onto BLA principal neurons. Moreover, our data further suggest that the response to 5-HT is common to both cortical and subcortical pathways into the BLA.

Discussion

In this study, we extended earlier observations that reported serotonergic modulation of glutamatergic transmission onto BLA principal neurons. We conclude this effect is consistent across afferent pathways and predominantly mediated by activation of presynaptic 5-HT_{1B} receptors and not by activation of 5-HT_{1A} or 5-HT_2 receptors.

Serotonin is known to play a central role in early brain development, regulation of mood, aggression, stress reactivity, and the risk of developing psychiatric diseases (Lesch et al., 2012; Azmitia, 2007; Sodhi & Sanders-Bush, 2004). However, the exact role of 5-HT in regulating such a diverse array of functions has been complicated due to the existence of 14 receptor subtypes (Palacios, 2016). Moreover, not only does 5-HT regulate neuronal function by direct actions at postsynaptic 5-HT receptors, it also acts as a presynaptic neuromodulator to regulate release of many other neurotransmitters. Of the 14 5-HT receptor subtypes, 5-$HT_{1A/B/D}$ receptors are believed to be the predominant presynaptic 5-HT receptors (Hoyer et al., 2002, Fink and Gothert, 2007). Whereas, 5-HT_{1A} receptors are located both pre- and postsynaptically, mRNA expression studies, binding studies, and electron microscopy studies suggest that 5-HT_{1B} receptors are mainly presynaptic (Boschert et al., 1994, Sari et al., 1997). Similar to 5-HT_{1B} receptors, 5-HT_{1D} receptors are thought to act predominantly as presynaptic receptors, but are only expressed at low levels in the rodent brain (Lanfumey and Hamon, 2004). In this study we showed that the 5-HT_{1B} receptor agonist CP93129 and the mixed 5-$HT_{1B/D}$ receptor agonist sumatriptan had similar effects on eEPSC amplitude, and that the 5-HT_{1B} receptor antagonist GR55562 blocked the effect of 5-HT. Taken together we propose that the 5-HT_{1B} receptor, and not the 5-HT_{1D} receptor, is the major 5-HT receptor subtype acting to reduce glutamate release in the BLA.

In rats the amygdala receives dense and topographically organized serotonergic inputs from the raphe nucleus, with the BLA showing the highest density of 5-HT terminal innervation (Vertes et al., 1999, Muller et al., 2007, Bonn et al., 2013, Linley et al., 2017). The amygdala also shows moderate to high levels of ligand binding for multiple 5-HT receptor subtypes, including 5-$HT_{1A, 1B, 2A, 2C,3}$ and 5-HT_7 receptors (Gustafson et al., 1996, Saha et al., 2010, Bombardi, 2011, Bocchio et al., 2016). As mentioned earlier, 5-HT_{1B} receptors are mainly expressed on axon terminals. Hence, it is possible that the 5-HT_{1B} binding observed in the amygdala (Bonaventure et al., 1998) represents heteroreceptors on axon terminals from

upstream input regions, including cerebral cortex and thalamus, which also express mRNA for 5-HT_{1B} receptors (Bruinvels et al., 1994). These studies offer morphological support for 5-HT_{1B} receptor modulation of glutamate release in the BLA.

Indeed, we have shown that the decreased eEPSC amplitude during 5-HT application was associated with an increase of both the paired-pulse ratio and the coefficient of variation, strongly suggesting a presynaptic mechanism of 5-HT action. Our results are consistent with previous studies showing that 5-HT decreased glutamatergic transmission in multiple brain regions including the BLA (Cheng et al., 1998, Rainnie, 1999, Yamamoto et al., 2012). However depending on the brain region examined, the inhibitory effect of 5-HT was reported to be mediated by 5-HT_{1A} receptors (Costa et al., 2012, Ostrowski et al., 2014), 5-HT_{1B} receptors (Singer et al., 1996, Laurent et al., 2002, Lemos et al., 2006, Choi et al., 2012), or both (Bouryi and Lewis, 2003).

In the current study we found the selective 5-HT_{1B} receptor agonist and antagonist could respectively mimic or block the effect of 5-HT, but the selective 5-HT_{1A} receptor agonist or antagonist had no effect. The latter result is in conflict with an earlier study that reported 5-HT_{1A} receptor-mediated inhibition of glutamate release in the BLA (Cheng et al., 1998). However consistent with our observation, Yamamoto and colleagues reported that application of 8-OH DPAT had no inhibitory effect on eEPSCs amplitude in the BLA (Yamamoto et al., 2012). The discrepancy between our results and that of Cheng and colleagues may result from activation of different afferent pathways. However, this cannot explain the discrepancy between the Cheng and Yamamoto studies in which both groups induced eEPSCs in the BLA by stimulating the LA. Interestingly, in this study application of 8-OH DPAT induced a slight yet non-significant increase of amplitude of eEPSCs. This effect may be due to an indirect activation of 5-HT_{1A} receptors located on NPY-expressing GABAergic interneurons (Bonn et al., 2013). Activation of 5-HT_{1A} receptors could in turn hyperpolarize GABA interneurons and lead to disinhibition of glutamate release. However, to avoid complications arising from bath application of picrotoxin, we isolated eEPSCs using intracellular picrotoxin application. Hence, this indirect gating of glutamatergic transmission mediated by 5-HT_{1A} receptor cannot be excluded.

Interestingly, Yamamoto and colleagues further suggested that 5-HT_2 receptors may contribute to the inhibitory effect of 5-HT (Yamamoto et al., 2012) as the non-selective 5-HT_2 receptor agonist α-methyl-5-HT mimicked the effect of 5-HT. However, we think this conclusion needs reevaluation. Firstly, 5-HT_2 receptors are Gq/11 coupled and are consistently reported to increase excitability (Hasuo et al., 2002, Campbell and Merchant, 2003). Indeed, Daftary and colleagues have reported that 5-HT facilitated glutamatergic transmission in BLA pyramidal neurons through activation of 5-HT_{2A} receptors (Daftary et al., 2012). Secondly, α-methyl-5-HT has a high affinity for 5-HT_{1A} (Ki 42 nM) and 5-HT_{1B} receptors (Ki 85 nM) (Ismaiel et al., 1990). At the agonist concentration used in Yamamoto study, 5 μM, α-methyl-5-HT most likely also activated presynaptic 5-HT_{1B} receptors. Hence, the effect of α-methyl-5-HT on glutamatergic transmission might be due to a spillover effect on presynaptic 5-HT_{1B} receptors rather than through 5-HT_2 receptor activation. Finally, we saw no effects of prior application of the non-selective 5-HT_2 receptor antagonist pirenperone on the inhibitory 5-HT response. While we never observed a

facilitatory effect of 5-HT on the eEPSC amplitude, it is possible that residual 5-HT_{2A} receptor activation is insufficient to overcome the inhibitory effect of 5-HT_{1B} activation. In future studies we will selectively examine the role of 5-HT_2 receptors in modulating glutamatergic transmission in the BLA.

Functional MRI studies have revealed that the amygdala is abnormally active in anxiety disorders, post-traumatic stress disorder (PTSD), and depression (Fredrikson and Faria, 2013). Significantly, treatment of patients with depression using selective serotonin reuptake inhibitors (SSRIs) normalizes amygdala hyper-activation, and this response positively correlates with reduced symptom severity (Sheline et al., 2001, Langenecker et al., 2007). Notably, decreased 5-HT_{1B} receptor binding has been observed in patients with PTSD, which was closely associated with the age of first trauma (Murrough et al., 2011a). A similar decrease of 5-HT_{1B} receptor binding has also been found in patients with MDD (Murrough et al., 2011b). Moreover, a polymorphism of the *HTR1B* gene has been associated with the antidepressant effect of SSRIs (Villafuerte et al., 2009), and the methylation level of the *HTR1B* gene is negatively correlated with clinical improvement after fluoxetine (Gasso et al., 2016). As shown in this study, 5-HT_{1B} acts to decrease glutamate release from cortical and thalamic inputs onto BLA projection neurons. Together the evidence presented above suggests 5-HT_{1B} receptors in the BLA may be a unique target for the treatment of mood and anxiety disorders.

Acknowledgments

This work was supported by NIH MH069852 and MH069056 Sub-Project 6569 to DGR, National Primate Research Center base grant #RR-00165, Animal Resource Program at NIH.

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Highlights

- 5-HT attenuates glutamatergic cortical and thalamic inputs onto BLA principal neurons
- Presynaptic 5-HT receptors gate cortical and thalamic glutamate inputs into the BLA
- Presynaptic gating is mediated by 5-HT_{1B}, but not 5-HT_{1A} or 5-HT_2 receptors

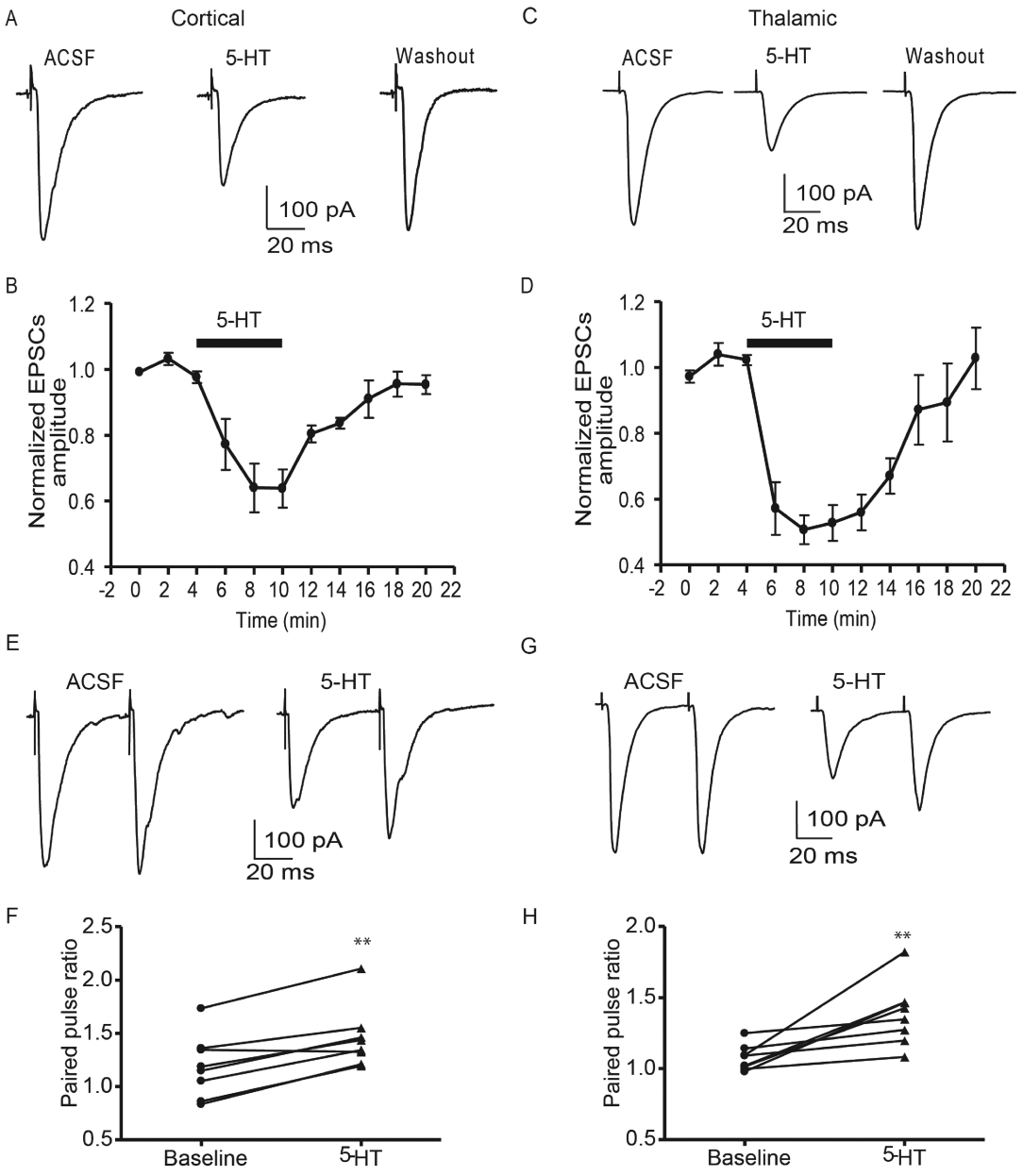

Figure 1. Effects of 5-HT on evoked EPSCs in cortical and thalamic glutamate inputs onto BLA pyramidal neurons

5-HT (10 μM) reversibly decreased the amplitude of eEPSCs in either cortical (A, B) or thalamic pathway (C,D). The decrease of eEPSCs amplitude during 5-HT application was associated with an increase of paired pulse ratio in either cortical (E, F) or thalamic pathway (G, H). **, $p<0.01$ vs baseline.

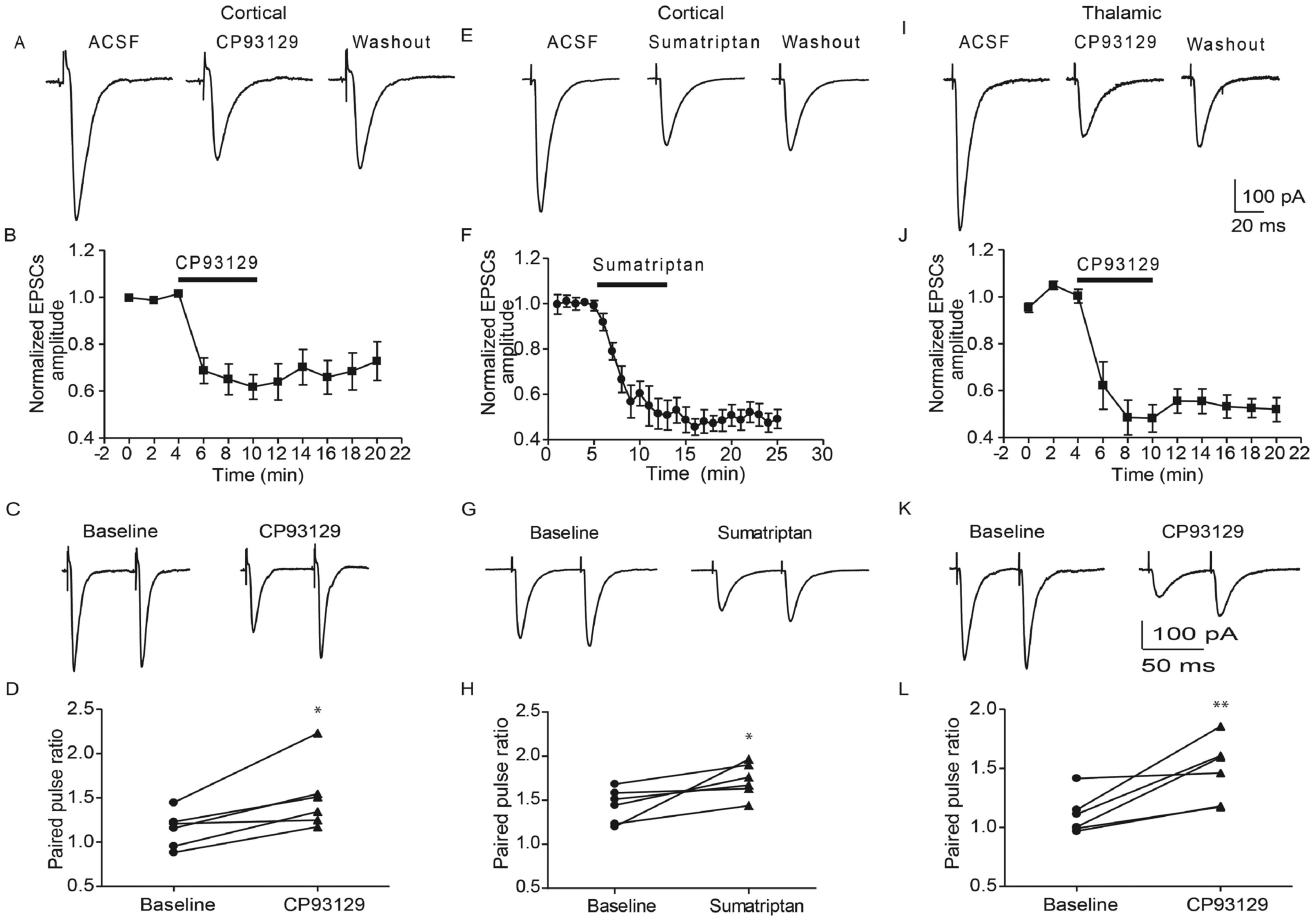

Figure 2. 5-HT$_{1B}$ receptor agonist mimicked the effect of 5-HT on eEPSCs in BLA

A, B) 5-HT$_{1B}$ receptor agonist CP93129 (10 μM) reduced the amplitude of eEPSCs in cortical pathway, which effect was accompanied by an increase of paired pulse ratio (C, D). E,F) Mixed 5-HT$_{1B/D}$ agonist sumatriptan (10 μM) reduced the amplitude of eEPSCs in cortical pathway. The decrease of eEPSCs amplitude was associated with increases of PPR (G,H). I,J) In thalamic pathway CP 93129 (10 μM) decreased the amplitude of eEPSCs, which associated with an increase of paired pulse ratio. *,**, $p<0.05$ and 0.01 respectively vs baseline.

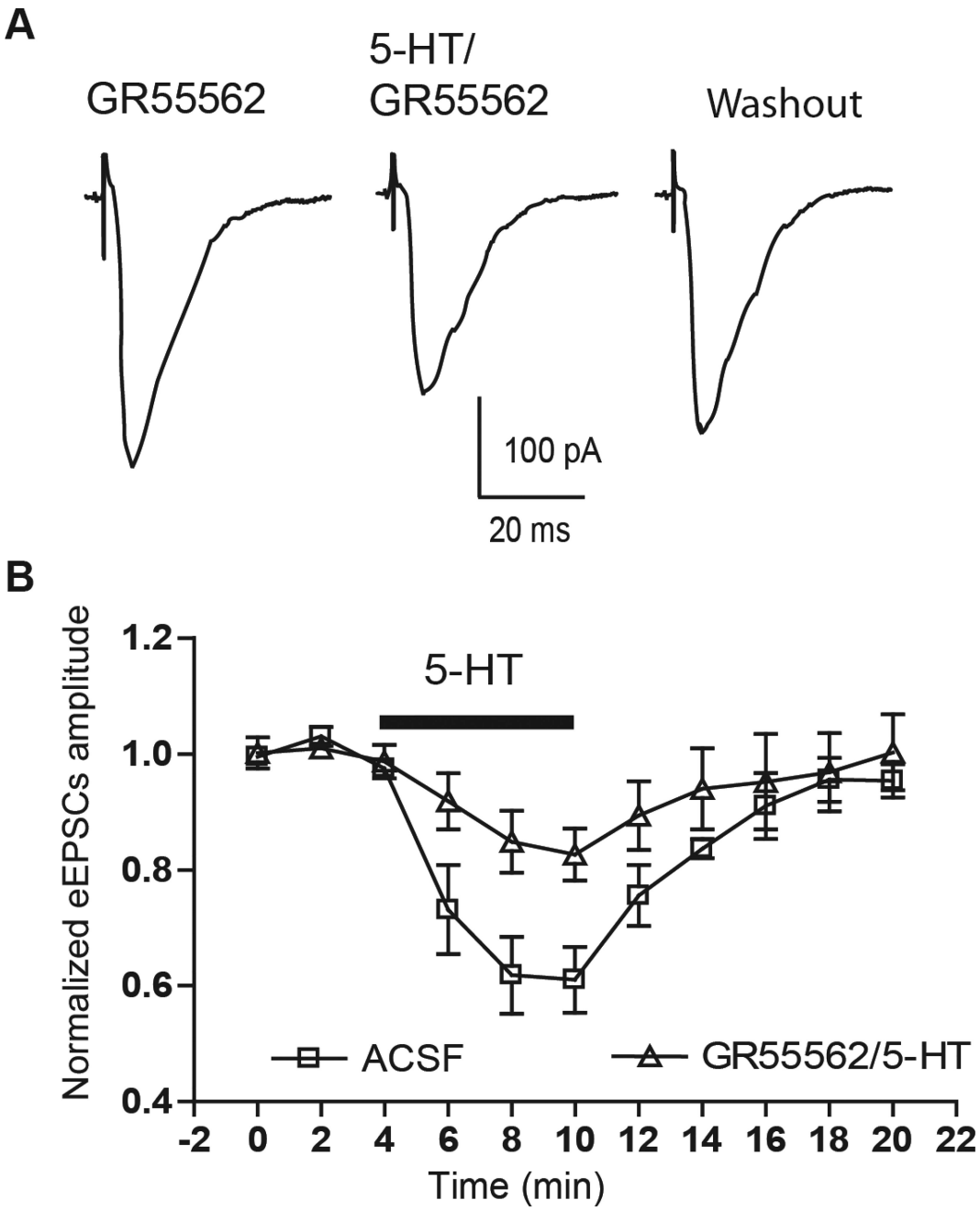

Figure 3. 5-HT$_{1B}$ antagonist blocked the effect of 5-HT on eEPSCs

Selective 5-HT$_{1B}$ antagonist GR55562 (20 μM) significantly attenuated the effect of 5-HT (10 μM) on eEPSCs in cortical pathway(A,B).

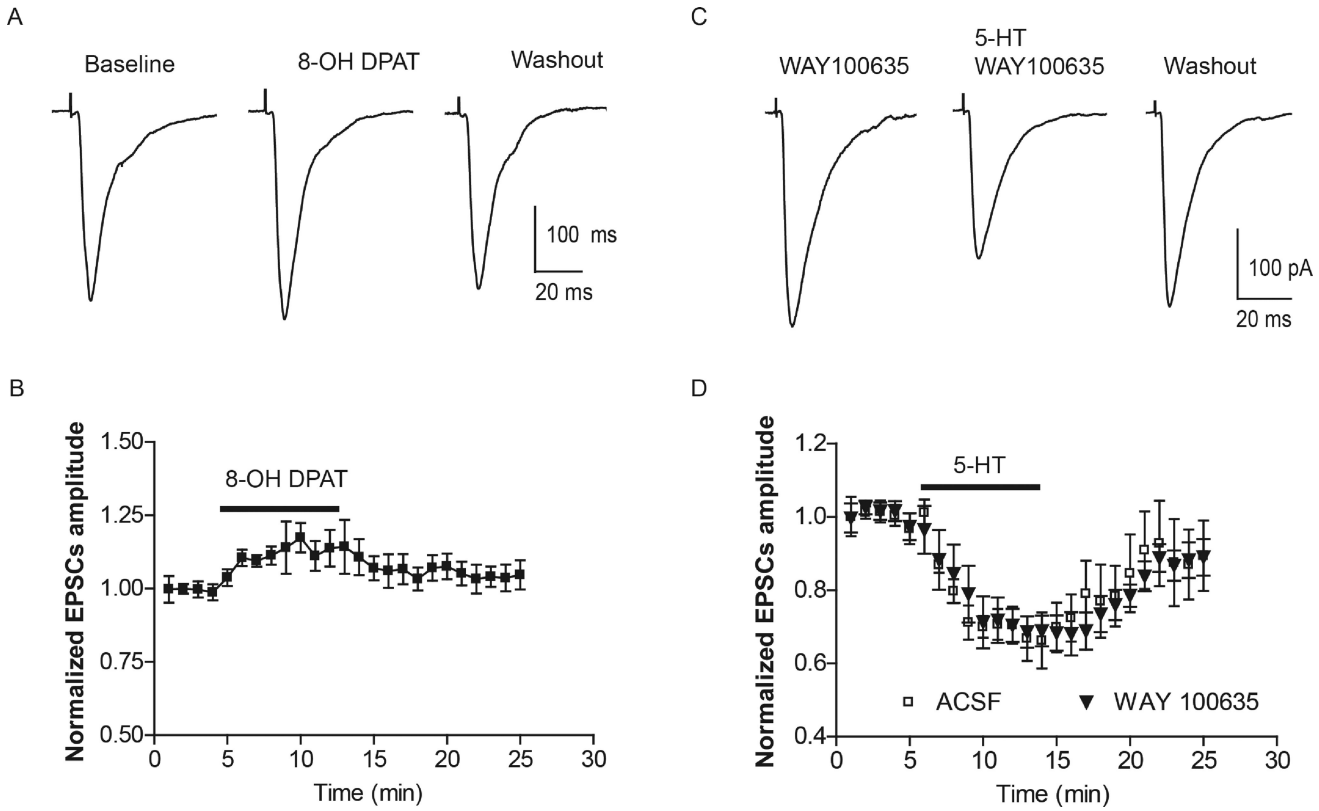

Figure 4. Effect of 5-HT$_{1A}$ activation on cortical eEPSCs

A,B) 5-HT$_{1A}$ agonist 8-OH DPAT slightly increased the amplitude of eEPSCs in cortical pathway. C,D) Blockade of 5-HT$_{1A}$ receptor with WAY 100635 (1 μM) has no effect on 5-HT (10 μM) inhibition of cortical eEPSCs.

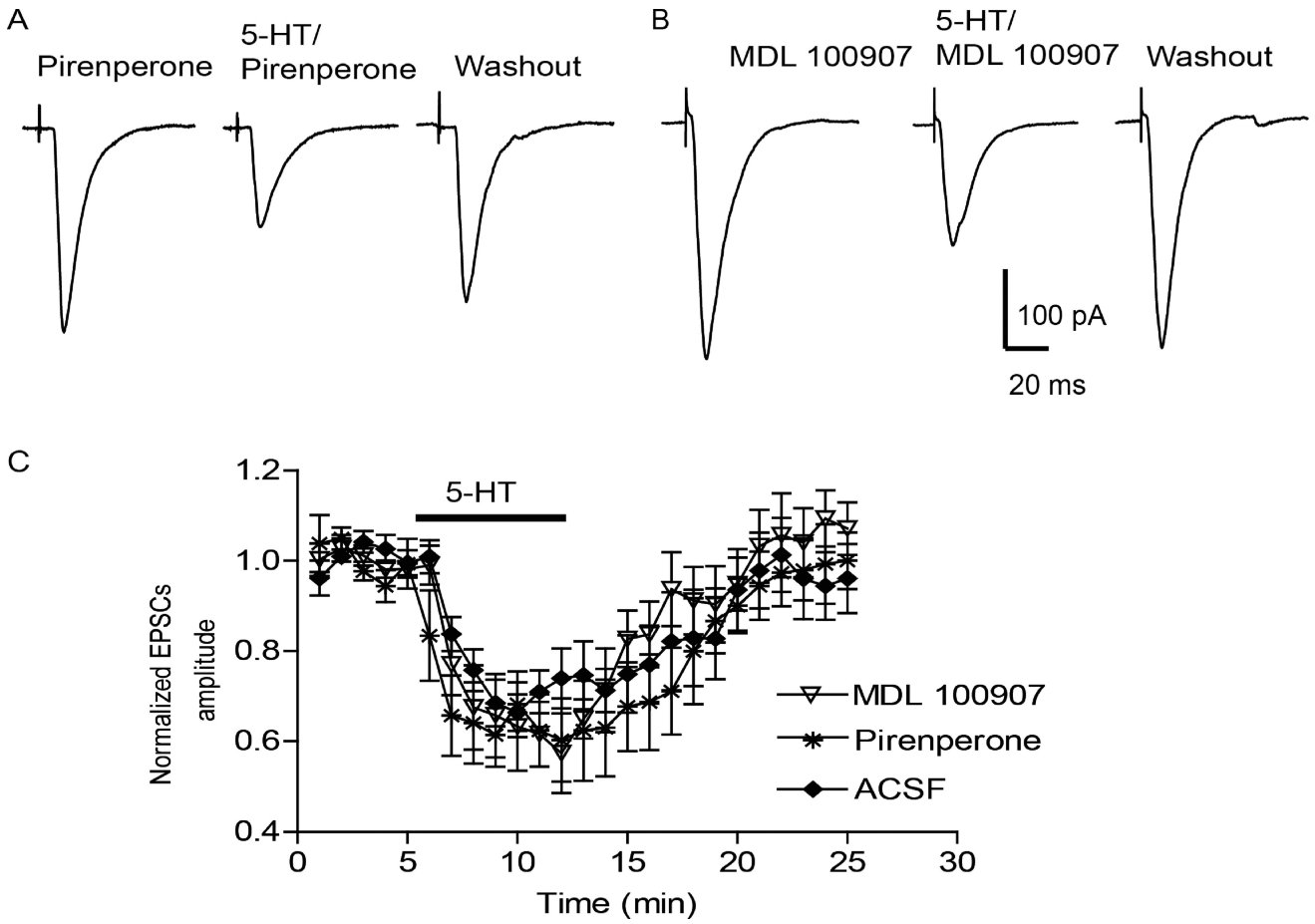

Figure 5. 5-HT$_2$ receptor antagonists have no effect on 5-HT inhibition of EPSCs in cortical pathway

In the presence of 5-HT$_{2A}$ antagonist MDL 100907 (10 μM) (A) or non-selective 5-HT$_2$ antagonist pirenperone (10 μM)(B), 5-HT (10 μM) significantly decreased the amplitude of cortical eEPSCs, which is not different from 5-HT effect in control ACSF (C).