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Abstract

Serotonin (5-HT) regulates attention by neurobiological mechanisms that are not well understood. Layer 6 (L6) pyramidal neurons of prefrontal cortex play an important role in attention and express 5-HT receptors, but the serotonergic modulation of this layer and its excitatory output is not well understood. Here, we performed whole-cell recordings and pharmacological manipulations in acute brain slices from wildtype and transgenic mice expressing either eGFP or eGFP-channelrhodopsin in prefrontal L6 pyramidal neurons. Excitatory circuits between L6 pyramidal neurons and L5 GABAergic interneurons, including a population of interneurons essential for task attention, were investigated using optogenetic techniques. Our experiments show that prefrontal L6 pyramidal neurons are subject to strong serotonergic inhibition and demonstrate direct 5-HT-sensitive connections between prefrontal L6 pyramidal neurons and two classes of L5 interneurons. This work helps to build a neurobiological framework to appreciate serotonergic disruption of task attention and yields insight into the disruptions of attention observed in psychiatric disorders with altered 5-HT receptors and signaling.

Significance Statement

While serotonin shapes and biases attention, the mechanisms underlying this phenomenon are not well understood. Layer 6 (L6) pyramidal neurons of medial prefrontal cortex play a critical role in performance on attention tasks, but their serotonergic modulation is unclear. Using electrophysiology and optogenetic techniques, we investigated the effects of serotonin on L6 pyramidal neurons and their local cortical circuits. We discovered a direct and serotonin-sensitive functional link from prefrontal L6 pyramidal neurons to GABAergic interneurons in L5, two populations of neurons essential for attention.

Introduction

The medial prefrontal cortex (mPFC) is critical for "top-down" executive control of attention (Miller and Cohen, 2001; Knudsen, 2007), and disruption of its signaling impairs normal performance on attention tasks (Muir et al., 1996; Miner et al., 1997; Granon et al., 1998). Layer 6 (L6) of mPFC, in particular, plays an important role in attention (Alitto and Usrey, 2003; West et al., 2006; Zikopoulos and Barbas, 2006; Kassam et al., 2008; Bailey et al., 2010; Guillem et al., 2011), and is a major source of cortico-thalamic output (Guillery and Sherman, 2002; Thomson et al., 2002; Mercer et al., 2005; Watts and Thomson, 2005; West et al., 2006; Zikopoulos and Barbas, 2006; Parikh et al., 2007; Sherman, 2007; Kassam et al., 2008; Bailey et al., 2010; Thomson, 2010). Much less, however, is known about the cortico-cortical collaterals of L6 pyramidal neurons in mPFC. This question is increasingly urgent in view of recent work from primary sensory cortex showing L6 pyramidal neurons send robust excitatory projections to fast-spiking cortical interneurons which achieve strong gain control over the cortical column (Olsen et al., 2012), together with recent work from mPFC showing that fast-spiking prefrontal interneurons are essential for attention (Kim et al., 2016). In mPFC, there remains much to be understood about the local targets of prefrontal L6 pyramidal neurons and the susceptibility of these attention circuits to neuromodulators, such as serotonin.

Serotonin is known to shape and bias attention in human and nonhuman primates, with reduction of brain 5-HT enhancing attention (Schmitt et al., 2000; Gallagher et al., 2003; Booij et al., 2005; Wingen et al., 2007) and elevation of brain 5-HT impairing attention task performance (Riedel et al., 2005; Oranje et al., 2008; Watson et al., 2015). Yet, the mechanisms underlying this relationship between 5-HT and attention are not well understood. Like primates, the deep layers of mPFC in rodents are well innervated by serotonin afferents (Wilson and Molliver,

1991; Linley et al., 2013; Goodfellow et al., 2014; Muzerelle et al., 2016). Therefore we can ask in a mouse model whether serotonin modulates prefrontal L6 pyramidal neurons known to play a role in task attention (Bailey et al., 2010; Guillem et al., 2011). In rodents, a substantial proportion of prefrontal L6 pyramidal neurons express 5-HT_{1A} and/or 5-HT_{2A} receptors (Chalmers and Watson, 1991; Pompeiano et al., 1992, 1994; Cornea-Hébert et al., 1999; Amargós-Bosch et al., 2004), with broad similarities in the expression of these 5-HT receptors in prefrontal L6 of human and nonhuman primates (de Almeida and Mengod, 2007; de Almeida and Mengod, 2008; Mengod et al., 2015). Co-expression of 5-HT_{1A} and 5-HT_{2A} receptors is common in mPFC pyramidal neurons (Amargós-Bosch et al., 2004) and is observed in 48% of pyramidal neurons in L6 of mouse mPFC (Table 3 in Amargós-Bosch et al., 2004). Roles for 5-HT_{1A} and 5-HT_{2A} receptors in attentional performance have been suggested (Carli and Samanin, 2000; Koskinen et al., 2000; Winstanley et al., 2003; Wingen et al., 2007). Furthermore, the serotonergic system is dysregulated in several brain disorders that are accompanied by disruptions of attention, including autism (Chugani, 2002; Kane et al., 2012), schizophrenia (Luck and Gold, 2008), and mood disorders (Marvel and Paradiso, 2004; Jans et al., 2007; Murrough et al., 2011).

Here, we investigated how mPFC L6 pyramidal neurons are modulated by 5-HT, how L6 excitation affects the two major classes of L5 interneurons, and how 5-HT modulates these internal mPFC circuits. Our results reveal a strong 5-HT-elicited inhibition of L6 pyramidal neurons mediated by 5-HT_{1A} receptors and a lesser, state-dependent, and somewhat unexpected, contribution by 5-HT_{2A} receptors. We find that L6 pyramidal neurons robustly activate fast-spiking (FS) as well as non-fast-spiking (nFS) interneurons in L5. Lastly, we show that these intra-cortical circuits in mPFC are strongly suppressed by 5-HT.

Methods

Experimental Animals

We employed BAC transgenic Swiss Webster mice with expression of eGFP driven by the synaptotagmin 6 promoter (Syt6-EGFP EL71, MMRRC; RRID:MMRRC 010557-UCD) made by the GENSAT Project (Gong et al., 2003). L6 pyramidal neurons in mPFC strongly express eGFP, and facilitates visual targeting of these neurons for recording (Tian et al., 2014). Syt6 mice were kept heterozygous and there were no significant differences in their 5-HT responses compared to their wildtype littermate controls or wildtype C57BL/6 mice ($F_{(2,64)}$ = 0.58, P = 0.56, one-way ANOVA). To investigate the downstream synaptic connection of prefrontal L6 pyramidal neurons, we crossed GENSAT epiphycan BAC transgenic mice expressing cre-recombinase (Epyc-Cre KR363, a gift from Dr. Nathaniel Heintz at Rockefeller University; RRID:MMRRC_036145-UCD) with Ai:32 mice (Jackson Laboratories; RRID:IMSR_JAX:024109) to achieve eGFP-channelrhodpsin-2 expression in prefrontal L6 neurons (Epyc-ChR2). Wildtype littermates of the Epyc-ChR2 were used as controls to ensure that the UV light did not have effects in brain slices from mice lacking channelrhodopsin-2. Translating Ribosome Affinity Purification and quantitative RT-PCR were used to confirm that Syt6 and Epyc-cre neurons indeed represent an overlapping population of L6 glutamatergic neurons. All experimental animal procedures were performed in accordance with the University of Toronto and The Rockefeller University Institutional Animal Care and Use Committee's regulations.

Electrophysiology

Coronal brain slices (400 µm) for electrophysiological recordings were obtained from adult male mice (postnatal 60 to 170 days; mean ± SEM; 101 ± 4 days; n = 41 mice). Brains were rapidly excised and chilled in 4 °C oxygenated sucrose artificial cerebrospinal fluid (ACSF) (254 mM sucrose, 10 mM D-glucose, 24 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 3 mM KCl, 1.25 mM NaH_2PO_4; pH 7.4). Coronal slices (400 µm thick, 2.34 – 0.74 mm from Bregma) were cut on a Dosaka Linear Slicer (SciMedia, Costa Mesa CA) and recovered in 30°C, oxygenated ACSF (128 mM NaCl, 10 mM D-glucose, 26 mM $NaHCO_3$, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 3 mM KCl, 1.25 mM NaH_2PO_4; pH 7.4) for at least 2 hours.

Recovered slices were transferred to a perfusion chamber on the stage of a BX50W1 microscope (Olympus). ACSF was bubbled (95% O_2, 5% CO_2 at room temperature) and perfused the chamber at a rate of 3-4 ml/min. In addition to recording from L6 pyramidal neurons based on neuronal morphology and anatomical landmarks in wildtype mice, L6 in Syt6 mice was landmarked with fluorescently-identified eGFP-positive neurons (X-cite Series 120; Lumen Dynamics; Tian et al., 2014). Recording electrodes (2-4 MΩ) containing 120 mM potassium gluconate, 5 mM KCl, 2 mM $MgCl_2$, 4 mM K_2-ATP, 0.4 mM Na_2-GTP, 10 mM Na_2-phosphocreatine, and 10 mM HEPES buffer (adjusted to pH 7.3 with KOH) were used to patch L6 pyramidal neurons. Interneurons in L5 were identified visually based on their unique morphology in IR-DIC (small, circular somata) in contrast to L5 pyramidal neurons (oriented, triangular shaped somata, relatively thick apical dendrites towards pia). A subset of patched interneurons was filled with Alexa-594 (20 µM) or Texas red dextran (0.15%) in the patch solution for morphological confirmation of these criteria. Interneurons were further sub-classified as FS or nFS based on their electrophysiological spike pattern and maximal spike frequency. Multiphoton images were acquired with a Ti:sapphire laser (Mai Tai, Spectra

Physics) using an Olympus Fluoview FV1000 microscope and an Olympus XLPlan N 25x water-immersion objective. Neuronal membrane potential and holding current were recorded with an EPC10 (HEKA Electroniks), and corrected for the liquid junction potential (14 mV). All data were acquired at 20 kHz and low-pass filtered at 3 kHz with pClamp software (Molecular Devices). Threshold potential for action potentials were detected using a derivative threshold of at least 20 mV/ms, and action potential amplitude was calculated as the change in membrane potential from threshold to the peak of the action potential. Intrinsic properties of L6 pyramidal neurons, as well as L5 FS and nFS interneurons are summarized in **Table 1**.

To examine the effects of 5-HT on L6 pyramidal neurons near rest and during spiking, we performed whole cell patch clamp recording in voltage clamp at -75 mV and in current clamp with current injections to elicit either constant spiking (2-3 Hz) at baseline or an initial membrane potential of -75 mV before depolarizing current injections (1 sec, 25 pA steps, 15 s intervals) were used to assess input-output relationships. For the latter experiment, the frequency of action potential firing was measured for each depolarizing current step, and plotted against the magnitude of the injected current step.

Pharmacology

Acute responses to 5-HT were probed by bath application of 5-HT (serotonin creatinine sulphate, Sigma; 10 µM; 30 s) in ACSF. To examine the effect of 5-HT on the excitability of L6 pyramidal neurons, 5-HT (10 µM) was bath applied until a steady state response is reached, and remained in bath throughout the duration of the input-output test protocols (~2 min total application). Selective antagonists and agonist were from Tocris, except where mentioned. Antagonists for 5-HT_{1A} receptors (30 nM WAY100635, 10 µM NAN-190) and 5-$HT_{2A/C}$

receptors (30 nM MDL100907; 2 μM ketanserin; 300 nM – 3 μM ritanserin) were applied in bath for 10 minutes before further experiments with 5-HT. There were no significant differences between effects of 300 nM and 3 μM ritanserin, and results were grouped for analysis. TCB-2 was used as a specific agonist of 5-HT_{2A} receptors (300 nM – 1 μM). Other agonists and antagonists used for characterization of the 5-HT response in L6 neurons were as follows: 2 μM TTX (Alomone), 20 μM CNQX, 50 μM D-APV, 100 μM picrotoxin, CGP52432 1 μM, and 8-OH-DPAT 10 μM.

Optogenetic stimulation

Channelrhodopsin-expressing neurons in Epyc-ChR2 mice were stimulated by blue LED light (473 nm) delivered by optic fiber (Thorlabs) mounted on a mechanical micromanipulator (Narishige). Light stimulation was directed directly to L6 of mPFC by targeted positioning of the optic fiber. Twenty light pulses (2-5 ms each) were delivered at 20 Hz to stimulate L6 neurons. This stimulation profile was sufficient to elicit robust activation of L6 pyramidal neurons expressing channelrhodopsin. In control experiments with brain slices from littermate mice lacking channelrhodopsin, light stimulation did not elicit a response in either L6 pyramidal neurons or L5 interneurons. Responses to light stimulation in L6 pyramidal neurons and L5 interneurons were measured in current-clamp from a baseline membrane potential of -75 mV held by continuous injection of depolarizing current. Response latency in L6 pyramidal neurons expressing channelrhodopsin was calculated from the time of light-on to the onset of the corresponding membrane potential change. Time-to-spike for L6 neurons from light-on was also calculated using the peak of the first resulting action potential. In L5 interneurons, the latency to response from L6 activation was calculated in voltage clamp as the time taken from light-on to

the onset of the post-synaptic current, then corrected by the time-to-spike in L6 pyramidal neurons. Pairwise analysis of the effects of 5-HT on the excitation of L5 interneurons by optogenetic activation of L6 were performed using light stimulus that was able to elicit at least 4 action potentials in patched L5 interneurons. Light stimulus intensity to elicit a baseline of at least 4 action potentials did not differ between FS and nFS interneurons ($t_{14} = 1.4$, $P = 0.18$, unpaired *t*-test).

Statistical analysis

All recordings were analyzed using Clampfit software (Molecular Devices). Statistical analyses were performed with GraphPad Prism (GraphPad Software). Analyses performed were: one-sample *t*-test, unpaired Student's *t*-test, paired Student's *t*-test, one-way ANOVA, two-way ANOVA, two-way repeated measures ANOVA. All tests were two-sided. Dunnett's multiple comparison tests were performed *post hoc* to compare changes in action potential firing in L6 neurons elicited by 5-HT. Sidak's multiple comparison tests were used to compare differences in spike frequency at individual injected current steps in the presence of 5-HT. All data are presented as mean ± SEM.

Translating Ribosome Affinity Purification and Quantitative RT-PCR

Adult (8-12 weeks old) Epyc-Cre mice under ketamine/xylazine (100/10 mg/kg) anesthesia received single bilateral stereotaxic injections of 0.25 µl AAV-FLEX-EGFPL10a virus ($3.75x10^{12}$ gc/ml) into the mPFC (+1.54 AP from bregma, +0.4 ML, -1.80 DV from dura). Animals were sacrificed in a controlled CO_2 chamber three weeks after surgery, brains were rapidly dissected in ice-cold HBSS containing 2.5 mM HEPES-KOH (pH 7.4), 35 mM glucose,

4 mM NaHCO3, and 100 µg/ml cycloheximide. The cortex was isolated from the rest of the brain and each hemisphere was split along the coronal plane at the level of the genu of the corpus collosum (~1.6 mm AP from bregma). The rostral portion was saved as the "PFC" and used for TRAP. Tissue from three mice (male and female) was pooled for each sample and three biological replicates were collected. Polysome immunoprecipitations were carried out as previously described (Schmidt et al., 2012; Heiman et al., 2014). Briefly, the tissue was homogenized in extraction buffer containing 10 mM HEPES-KOH (pH 7.4), 150 mM KCl, 5 mM MgCl2, 0.5 mM DTT, 100 µg/ml cycloheximide, RNasin (Promega, Madison, WI) and SUPERas-InTM (Life Technologies) RNase inhibitors, and Complete-EDTA-free protease inhibitors (Roche), and then cleared by centrifugation at 2000 x g. IGEPAL CA-630 (NP-40, Sigma) and DHPC (Avanti Polar Lipids, Alabaster, AL) were both added to the S2 supernatant for a final concentration of 1% for each, followed by centrifugation at 20,000 x g. Polysomes were immunoprecipitated from the S20 supernatant using 100 µg monoclonal anti-EGFP antibodies (50 µg each of clones 19C8 and 19F7; see ref. 2) bound to biotinylated-Protein L (Pierce, Thermo Fisher, Waltham, MA) coated streptavidin-conjugated magnetic beads (Life Technologies), and washed in high salt buffer containing 10 mM HEPES-KOH (pH7.4), 350 mM KCl, 5 mM MgCl2, 1% IGEPAL CA-630, 0.5 mM DTT, 100 µg/ml cycloheximide, and RNasin RNase inhibitors (Promega). IPs were carried out overnight at 4°C. Bound RNA was purified using the Absolutely RNA Nanoprep kit (Agilent, Santa Clara, CA). RNA was also purified from the pre-IP supernatant to serve as whole-PFC "input" samples. RNA quantity was measured with a Nanodrop 1000 spectrophotometer and quality was assayed on an Agilent 2100 Bioanalyzer. Only samples with RNA integrity values >7.0 were used for qRT-PCR analysis.

cDNA was synthesized from 15 ng of IP or input total RNA using the Ovation qPCR System (NuGEN Technologies) following manufacturer's protocol. Quantitative RT-PCR was performed on an Applied Biosystems StepOnePlus Fast Real-Time PCR System using commercially available Taqman assays (**Table 2**) and following standard cycling conditions (50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min). Ten nanograms of cDNA were used for each qRT-PCR reaction and technical triplicates were run for each of the biological triplicates from TRAP IP and input samples. The mean C_T for technical replicates was used for quantification. Data were normalized to *Gapdh* by the comparative C_T ($2^{-\Delta\Delta CT}$) method (Livak and Schmittgen, 2001). Data are presented as Mean ± SEM of biological triplicates. Statistical significance was calculated between the normalized expression values ($2^{-\Delta CT}$) from the IP and input biological replicates for each gene by Student's *t*-test in Microsoft Excel.

Results

Serotonin robustly inhibits L6 pyramidal neurons of mPFC

Here, we investigated the electrophysiological consequences of 5-HT on pyramidal neurons in L6 of mPFC. Experiments in voltage clamp showed robust and replicable outward currents (58.3 ± 6.4 pA, n = 28, **Figure 1A**, **1B**) in response to bath application of 5-HT (10 µM, 30 s). These 5-HT-elicited currents were dose-dependent, with an EC_{50} of 5.7 ± 0.1 µM (n = 7, r^2 = 0.9). The lack of significant change in these responses to blocking voltage-gated sodium channels with TTX (2 µM, 10 minutes in bath; $t_3 = 1.2$, $P = 0.3$, n = 4, paired *t*-test), to blocking AMPA, NMDA, and GABA-A receptors (CNQX [20 µM], APV [50 µM], picrotoxin [100 µM], $t_6 = 0.5$, $P = 0.6$, n = 7, paired *t*-test, **Figure 1A**, **D**), as well as to blocking these synaptic

receptors together with GABA-B blockade with CGP52432 (10 µM) (t_3 = 0.27, P = 0.8, n = 4, paired t-test) suggest direct mediation by 5-HT receptors on L6 pyramidal neurons themselves. We found that the specific 5-HT_{1A} antagonist WAY100635 (30 nM, 10 minutes in bath) significantly reduced the 5-HT mediated current in L6 pyramidal neurons (~ 70% reduction to 18.3 ± 2.6 pA, t_{18} = 4.9, P < 0.0001, n = 19, unpaired t-test, **Figure 1B**, **C**, **D**). The 5-HT_{1A} agonist 8-OH-DPAT (10 µM) elicited outward currents of similar magnitude to the 5-HT current in L6 pyramidal neurons (5-HT: 61.2 ± 12.8 pA; 8-OH-DPAT: 48.5 ± 11.1 pA, t_5 = 1.6, P = 0.2, n = 6, paired t-test).

To investigate the functional effects of 5-HT on L6 pyramidal neurons during excitation, we used current-clamp and bath applied 5-HT in the presence of injected positive depolarizing current sufficient to elicit action potential firing (2-3 Hz). Under these conditions, 5-HT hyperpolarized L6 neurons (-16.3 ± 1.6 mV, n = 17) and fully and significantly inhibited action potential firing in every recorded neuron (t_7 = 13.5, P < 0.0001, n = 8, paired t-test) (**Figure 1E**, **H**, **I**). This suppression was repeatable in the same neuron after washout, and was not affected by the presence of synaptic blockers (**Figure 1E**, **H**, **I**). Antagonism of 5-HT_{1A} receptors by WAY100635 significantly reduced the 5-HT mediated hyperpolarization (-7.8 ± 0.7 mV, t_{18} = 5.0, P < 0.0001, n = 19, unpaired t-test; **Figure 1F-I**). Unexpectedly, however, 5-HT still robustly and significantly inhibited action potential firing in every neuron (t_{13} = 12, P < 0.0001, n = 14, paired t-test) (**Figure 1F-I**). This strong and significant suppression of L6 spiking by 5-HT was also observed in the presence of synaptic blockers (t_3 = 4.8, P = 0.02, n = 4, paired t-test). These data show a robust and repeatable 5-HT inhibition of L6 neurons by 5-HT with a component mediated by 5-HT_{1A} receptors. However, the continued suppression of action

potential firing by 5-HT after blockade of 5-HT_{1A} receptors suggests the involvement of an additional 5-HT-mediated mechanism for inhibition of mPFC L6 pyramidal neurons.

Serotonergic 5-HT_{1A} and 5-HT_{2A} receptors co-operate in inhibiting L6 pyramidal neurons

To interrogate this unidentified component of 5-HT inhibition of L6 pyramidal neurons, action potentials were elicited by a series of incremental square depolarizing pulses before and during 5-HT application. Despite the stronger activation, 5-HT still significantly reduced spike frequency at each injected current step, as shown by the significant right-shift in the input-output curve (inhibitory effect of 5-HT: $F_{(1, 168)} = 31$, $P < 0.0001$, n = 22, repeated measures two-way ANOVA, **Figure 2A**). *Post hoc* tests show that significantly fewer action potentials were elicited by input current in 5-HT compared to baseline at every step ($P < 0.05$, Sidak's multiple comparisons test). Of note, the 25 pA step was supra-threshold for 17/22 neurons at baseline and only for 3/22 neurons in 5-HT ($P < 0.0001$, Fisher's exact test). Spiking was significantly restored following a 5 minute washout of 5-HT ($F_{(1, 168)} = 28$, $P < 0.0001$, repeated measures two-way ANOVA). Activation of 5-HT_{1A} receptors by 8-OH-DPAT also significantly suppressed firing of L6 neurons (50 pA current injection, baseline: 4.0 ± 1.1 Hz; 8-OH-DPAT: 0.8 ± 0.6 Hz; $t_5 = 5.3$, $P = 0.003$, n = 6, paired *t*-test), as did 8-OH-DPAT across a range of depolarizing steps, ($F_{(5,12)} = 35.7$, $P < 0.0001$, two-way repeated measures ANOVA, data not shown).

Yet, further experiments suggest that 5-HT recruits an additional receptor beyond 5-HT_{1A} to inhibit the excitability of L6 pyramidal neurons. Significant 5-HT suppression of L6 neuronal excitability continued after antagonism of 5-HT_{1A} receptors by WAY100635, with a significantly right-shifted input-output (inhibitory effect of 5-HT in WAY100635: $F_{(1, 72)} = 72$, $P < 0.0001$, n

= 10, repeated measures two-way ANOVA, **Figure 2B**). Consistent with our above data, this result suggests the participation of at least one additional subtype of 5-HT receptor in inhibiting L6 pyramidal neurons. The 5-HT_{2A} receptors that are co-expressed with 5-HT_{1A} receptors in 48% of L6 pyramidal neurons in mouse mPFC (Table 3 in Amargós-Bosch et al., 2004) are an unusual candidate to underlie the 5-HT-mediated supra-threshold suppression of spiking. These receptors typically recruit excitatory effectors (Lambe and Aghajanian, 2001; Zhang and Arsenault, 2005; Weisstaub et al., 2006; Benekareddy et al., 2010; Weber and Andrade, 2010; Avesar and Gulledge, 2012); although previous work has demonstrated the capacity of serotonin and 5-HT_{2A} agonists to exert direct inhibitory effects through 5-HT_{2A} receptors or heteromers (Carr et al., 2002; Kurrasch-Orbaugh et al., 2003; González-Maeso et al., 2007; Moreno et al., 2011). We found that adding the selective 5-HT_{2A} antagonist MDL100907 abolished the remaining inhibitory effects elicited by 5-HT on the input-output of L6 neurons (no significant effects of 5-HT in WAY100635 and MDL100907: $F_{(1,32)} = 0.8$, $P = 0.4$, n = 5, repeated measures two-way ANOVA, **Figure 2C**). A similar blockade of the inhibitory effects of 5-HT on L6 neurons was also seen when other 5-HT_{2A} antagonists were applied together with WAY100635, such as ketanserin (2 µM) or ritanserin (300 nM -1 µM) ($F_{(1,48)} = 0.3$, $P = 0.6$, n = 7, repeated measures two-way ANOVA).

To probe further the power of 5-HT_{2A} receptors to inhibit L6 pyramidal neurons in mPFC, we applied a potent 5-HT_{2A} agonist, TCB-2 (300 nM - 1µM). Here, we observed a strong inhibition of L6 neuronal excitability, with a significant right-shift of the input-output relationship (inhibitory effect of TCB-2: $F_{(1,80)} = 24$, $P < 0.0001$, n = 11, repeated measures two-way ANOVA, **Figure 2D**). Pre-treatment with MDL100907 abolished the inhibitory effect of TCB-2 ($F_{(1,16)} = 1.2$, $P = 0.3$, n = 3, repeated measure two-way ANOVA). Taken together, our

results suggest that 5-HT inhibition of mPFC L6 pyramidal neurons is mediated by a combination of 5-HT_{1A} and 5-HT_{2A} receptors acting in concert. However, substantial future work will be needed to elucidate the mechanisms by which these receptors individually and together work to suppress the excitability of L6 pyramidal neurons.

Transgenic mouse for examining the effect of L6 activation on L5 interneurons

It has been shown that L6 pyramidal neurons in primary sensory cortex exert robust gain modulation over superficial layers of the cortical column (Olsen et al., 2012) through strong connections to FS interneurons (Vélez-Fort and Margrie, 2012). In mPFC, recent work has demonstrated the importance of fast-spiking interneurons in L5 for performance on attention tasks (Kim et al., 2016). To investigate the effects of prefrontal L6 activation on its targets in the cortical column, we utilized the Epyc-Cre BAC transgenic mice that target Cre recombinase to L6 cells in mPFC, then generated Epyc-cre;Ai:32 mice (Epyc-ChR2) to obtain expression of channelrhodopsin in those cells. **Figures 3A** and **3B** show a similar distribution of L6 cells labeled by Syt6-eGFP fluorescence, used in the initial electrophysiology experiments, and by Epyc-cre, used for the optogenetic experiments. Due to the lack of reliable histological markers for L6 pyramidal neurons in mPFC, the translating ribosome affinity purification (TRAP) technique (Heiman et al., 2008; Doyle et al., 2008) was used to interrogate the identity of the Epyc-Cre cells. An adeno-associated virus (AAV) vector (AAV-FLEX-EGFPL10a) to express EGFP-tagged ribosomal protein L10a (EGFPL10a) in a Cre-dependent manner was injected into the mPFC of Epyc-Cre mice and anti-EGFP immunoprecipitations (IPs) were performed to isolate tagged polysomes. Bound mRNAs were then purified and analyzed by quantitative RT-PCR (qPCR). These data are plotted in **Figure 3C**. There was a significant enrichment for the

356 excitatory neuron marker, *Slc17a7* (VGluT1), in the Epyc TRAP IP when compared to whole
357 PFC input. Two genes known to be expressed in L6 corticothalamic cells, *Ntsr1* (Gong et al.,
358 2007; Olsen et al., 2012; Mease et al., 2014) and *Foxp2* (Ferland et al., 2003), were also
359 significantly enriched in IP samples. In contrast, genes that label inhibitory interneurons (*Gad1*),
360 astrocytes (*Aldh1l1*), or oligodendrocytes (*Cnp*) were significantly depleted from the IPs. Taken
361 together, these data suggest that Epyc-Cre labels a population of L6 corticothalamic pyramidal
362 cells. Importantly, the qPCR also revealed that *Syt6* was highly enriched in the Epyc cells,
363 demonstrating that the Syt6-eGFP and Epyc-Cre mice label an overlapping population of
364 neurons. By contrast, levels of the housekeeping gene *Gapdh* were found to be similar between
365 the TRAP IPs and whole PFC input. Similar results were obtained for the 5-HT receptors, *Htr1a*
366 and *Htr2a,* suggesting these genes are expressed but not enriched in the Epyc cells, which was
367 not surprising given the expression of 5-HT_{1A} and 5-HT_{2A} in other populations of neurons in
368 mouse mPFC beyond L6 (Chalmers and Watson, 1991; Pompeiano et al., 1992, 1994; Cornea-
369 Hébert et al., 1999; Amargós-Bosch et al., 2004).
370
371 **Optogenetic activation of L6 pyramidal neurons is sensitive to serotonin**
372 In electrophysiological experiments from Epyc-ChR2, we found that L6 pyramidal
373 neurons, but not non-pyramidal neurons, were strongly depolarized upon light stimulation (473
374 nm, train of 2-ms duration pulses at 20 Hz for 1 s), which was targeted to L6 mPFC with optic
375 fiber (**Figure 4A, B**). In contrast, prefrontal L6 neurons of littermate controls lacking
376 channelrhodopsin did not respond to light stimulation. To verify that L6 pyramidal neurons were
377 directly activated by light stimulation, we measured the kinetics of their light-evoked excitation.
378 L6 pyramidal neurons rapidly responded to light (< 1 ms latency to onset of excitation),

consistent with direct activation through expressed channelrhodopsin (Ernst et al., 2008). This response rises to threshold, giving an action potential peak at 4.7 ± 0.7 ms (time-to-L6-spike; n = 5).

The channelrhodopsin-expressing L6 neurons from Epyc-ChR2 mice showed similar sensitivity to 5-HT as the Syt6-eGFP cells in the above experiments. Light-mediated excitation of Epyc-ChR2 L6 neurons was significantly suppressed in the presence of 5-HT ($F_{(1,24)} = 10.3$, $P < 0.004$, repeated measures two-way ANOVA).

Optogenetic activation of L6 drives excitation of L5 interneurons

Since fast-spiking GABAergic neurons in mPFC are important to normal performance in attention tasks (Kim et al., 2016), we patched mPFC L5 interneurons in Epyc-ChR2 mice as potential downstream projection targets of L6 pyramidal neurons. We anticipated that light-mediated activation of L6 pyramidal neurons by targeted optic fiber would elicit postsynaptic responses in patched L5 interneurons. GABAergic interneurons were visually identified by their morphology and intrinsic properties, and their spiking patterns in response to depolarizing current steps were documented. This experimental protocol yielded two distinct populations of interneurons: FS cells with characteristic action potential firing >40 Hz and nFS cells which displayed low-threshold firing characteristics. The intrinsic properties of these neurons are illustrated in **Table 1**. A subset of patched interneurons (n = 6 FS interneurons, n = 5 nFS interneurons) was filled with Alexa-594 (20 μM) or Texas red dextran (0.15%) in the patch solution to verify their morphology. Filled FS (6/6) and nFS (5/5) interneurons were morphologically characteristic of the respective subtypes of interneurons in cortex (Markram et al., 2004; Ascoli et al., 2008).

All of the L5 FS (n = 19) and nFS (n = 22) interneurons recorded responded to light stimulation positioned over L6 (20 Hz, 2 – 5 ms pulse duration, 20 pulse train). Light stimulation over other cortical layers did not produce a response. Latency between time-to-L6-spike and response onset in L5 interneurons was 1.1 ± 0.3 ms, consistent with a monosynaptic connection (Markram et al., 1997; Feldmeyer et al., 2005; Frick et al., 2008) from L6 (**Figure 4C**). Activation of both FS and nFS interneurons by optogenetic stimulation of L6 were substantially and significantly reduced by TTX ($F_{(1,80)} = 19$, $P < 0.0001$, two-way ANOVA). Together with the need for light activation over L6, it appears that channelrhodopsin is predominantly localized in the L6 pyramidal cell bodies and not in axon terminals impinging on the L5 interneurons.

Light stimulation over L6 elicited action potential firing in 100% of FS cells (**Figure 4D**) and 70% of nFS cells. The firing pattern elicited in these two types of interneurons was different, with a greater number of spikes seen at the start of L6 stimulation in FS neurons and a more evenly-distributed firing pattern observed in the nFS neurons (**Figure 5**). The minimal L6 light to elicit a suprathreshold excitatory response did not differ significantly between FS and nFS L5 interneurons ($t_{14} = 0.2$, $P = 0.8$, unpaired *t*-test), despite a significant difference in input resistance ($t_{14} = 4.3$, $P = 0.0006$, unpaired *t*-test; **Table 1**). In response to maximal L6 light stimulation of L6, FS interneurons fired more action potentials than nFS interneurons ($t_{14} = 4.4$, $P = 0.0007$, unpaired *t*-test).

Serotonin suppresses L6 activation of L5 interneurons

Since optogenetic stimulation in L6 resulted in robust and highly stable excitation of interneurons in L5 that did not decrease over time at baseline conditions ($t_{11} = 0.8$, $P = 0.4$, paired *t*-test; **Figure 6A**), it was straightforward to test the effect of 5-HT on this local circuit.

We found that 5-HT strongly and significantly suppressed the number of action potentials elicited in L5 interneurons by optogenetic activation of L6 (FS cells: $t_8 = 3.8$, $P = 0.005$, n = 9, paired *t*-test; nFS cells: $t_6 = 5.7$, $P = 0.001$, n = 7, paired *t*-test; **Figure 6B**). Of note, this suppression appeared to arise from 5-HT effects in L6 since interneurons in L5 showed minimal direct responses to 5-HT at -75 mV (2.7 ± 6.0 pA, $P = 0.9$, n = 29, one sample *t*-test). Furthermore, these interneurons showed no change to spiking elicited by depolarizing steps of current amplitudes similar to those elicited by optogenetic stimulation of L6 (FS interneurons: $F_{(1,9)} = 3.1$, $P = 0.1$, repeated measures two-way ANOVA; nFS interneurons: $F_{(1,18)} = 3.4$, $P = 0.1$, repeated measures two-way ANOVA, data not shown). The excitation of L5 interneurons by L6 optogenetic activation was no longer sensitive to 5-HT upon blockade of 5-HT_{1A} and 5-HT_{2A} receptors ($t_{15} = 0.9$, $P = 0.4$, paired *t*-test, n = 10 FS interneurons, n = 6 nFS interneurons; **Figure 6C**). Overall, these results demonstrate the ability of L6 pyramidal neurons to excite a diverse group of inhibitory interneurons in L5 and the sensitivity of this effect to suppression by serotonergic 5-HT_{1A} and 5-HT_{2A} receptors.

Discussion

In this study, we show robust serotonergic inhibition of L6 pyramidal neurons and their output to L5 interneurons. This suppression of L6 activity by 5-HT is driven by the combined effects of 5-HT_{1A} and 5-HT_{2A} receptors. Using transgenic mice and optogenetic techniques, we illustrate a functional link between L6 pyramidal neurons and L5 interneurons potentially important to performance on attention tasks. Light stimulation in L6 strongly excited L5 interneurons. This excitatory connection was inhibited by 5-HT and was restored in the presence of 5-HT_{1A} and 5-HT_{2A} antagonists. Taken together, these results suggest that 5-HT_{1A} and 5-HT_{2A}

448 receptors mediate a strong inhibitory drive in L6 that can suppress its local activation of cortical
449 targets in L5, which others have shown to be critical to attention (Kim et al., 2016).
450
451 **Prefrontal L6 pyramidal neurons excite a diverse group of interneurons in L5**
452 We found that L6 pyramidal neurons excited both FS and nFS interneurons in L5 of
453 mPFC. These groups of interneurons likely represent the parvalbumin- (PV) and/or somatostatin-
454 expressing (SOM) groups of interneurons, which together form the majority of interneurons in
455 cortical L5 (Kawaguchi and Kubota, 1997; Rudy et al., 2011). Both perisomatic PV and
456 dendrite-targeting SOM interneurons are strong mediators of activity on downstream cortical
457 pyramidal output (Kawaguchi and Kubota, 1997; Glickfeld et al., 2009; Kvitsiani et al., 2013;
458 Hangya et al., 2014), with mPFC PV interneuron activity particularly important to normal
459 performance on attention tasks (Kim et al., 2016). Our finding of a functional connection
460 between L6 and L5 interneurons suggests a means by which L6 could influence mPFC cortical
461 gain modulation, as has been observed in primary sensory cortices (Olsen et al., 2012; Bortone et
462 al., 2014). These findings are in agreement with previous work in primary sensory cortex that
463 examined anatomical and functional connections within the cortical column (Zhang and
464 Deschênes, 1997; Thomson et al., 2002; Mercer et al., 2005; Watts and Thomson, 2005; West et
465 al., 2006; Kim et al., 2014). This research suggests that activation of L6 is a driver of
466 intracortical inhibition leading to a widespread suppression of cortical targets, observed *in vivo* in
467 visual cortex (Beierlein et al., 2003; Olsen et al., 2012; Bortone et al., 2014). The L6 excitation
468 of nFS in addition to FS interneurons in mPFC suggests additional complexity in association
469 cortex. Our data show for the first time that excitatory output from L6 can drive interneuron
470 activity in L5 in mPFC, a region critical for attention and other executive functions.

Serotonergic inhibition of this L6 to L5 intracortical circuit

We found that 5-HT, via stimulation of 5-HT_{1A} and 5-HT_{2A} receptors, inhibited L6 pyramidal neurons and their activation of L5 interneurons. These two receptors show substantial co-localization in L6 pyramidal neurons in mPFC of mouse (Table 3 in Amargós-Bosch et al., 2004), yet typically appear to exert opposing electrophysiological effects in other cortical layers (Benekareddy et al., 2010; Avesar and Gulledge, 2012; Stephens et al., 2014). While 5-HT_{1A} receptors are known inhibitory receptors acting via Kir3 channels (Goodfellow et al., 2014; Johnston et al., 2014), 5-HT_{2A} receptors act through a less well-characterized set of channels to excite certain populations of neurons, including a subset of L5 pyramidal neurons in mPFC (Willins et al., 1999; Benekareddy et al., 2010; Weber and Andrade, 2010; Avesar and Gulledge, 2012). Direct inhibition of L6 pyramidal neurons by 5-HT_{2A} receptors could arise from a number of possible mechanisms: including through the suppression of sodium channels (Carr et al., 2002) or via 5-HT_{2A} heteromers with inhibitory signaling (González-Maeso et al., 2007; Moreno et al., 2011; Viñals et al., 2015). Pyramidal neurons in L6 also have prominent afterhyperpolarizations (Proulx et al., 2015), known to affect excitability. Accordingly, the $G\alpha_q$-coupled 5-HT_{2A} receptors may affect the excitability of these neurons by modulating channels contributing to different phases of the afterhyperpolarization (Gulledge et al., 2005; Gulledge et al., 2009; but see also: Villalobos et al., 2005; Villalobos et al., 2011). Complex and carefully controlled future work will be necessary to identify the mechanisms underlying the 5-HT_{2A} receptor mediated inhibition of L6 pyramidal neuron excitability.

In investigating FS and nFS interneurons in L5, we found that the majority of these cells did not respond strongly to 5-HT. A minority showed electrophysiological responses (FS: 4/13;

494 nFS: 2/16), predominantly inward currents (< -20 pA) that were insufficient to elicit spiking.
495 These proportions are consistent with the literature on the expression of 5-HT receptors only in a
496 small proportion of L5 interneurons (Abi-Saab et al., 1999; Santana et al., 2004; Rudy et al.,
497 2011; Celada et al., 2013). Our findings were not significantly altered by the inclusion or
498 exclusion of these neurons. Control experiments with GABA-A and GABA-B blockers suggest
499 that 5-HT receptors on interneurons are not significantly involved in the 5-HT suppression of L6
500 pyramidal neurons. Taken together, our data support the hypothesis that the combined activation
501 of both 5-HT_{1A} and 5-HT_{2A} receptors can inhibit neuronal excitability in L6 neurons of
502 prefrontal cortex. However, further pharmacological work is required to examine the specific
503 downstream mechanisms underlying this inhibition of L6 pyramidal neurons. Furthermore,
504 additional investigations into the consequences of serotonergic inhibition of L6 on local network
505 dynamics will provide more insight into the nature of these important associative circuits and
506 how they control attention.
507
508 **Serotonin, prefrontal attention circuitry, and attention deficits in psychiatric illness**
509 Prefrontal attention circuitry is complex and attentional performance can be perturbed by
510 extremes of mPFC activity in either direction (Pezze et al., 2014). Serotonin shapes and biases
511 attention in humans and rodents: with low levels of 5-HT enhancing attention (Schmitt et al.,
512 2000; Gallagher et al., 2003; Booij et al., 2005) and higher levels of 5-HT disrupting attention
513 (Riedel et al., 2005; Wingen et al., 2007; Watson et al., 2015) through 5-HT_{1A} and 5-HT_{2A}
514 receptors (Wingen et al., 2007). Stress is well known to raise prefrontal serotonin levels (Adell et
515 al., 1997; Fujino et al., 2002; Bland et al., 2004) and similar behavioral manipulations disrupt
516 attention (Minor et al., 1984; Sänger et al., 2014). Intriguingly, elevation of intra-cortical 5-HT

517 has been strongly associated with deficits in attention (Puumala and Sirviö, 1998) and increases
518 in impulsivity (Dalley et al., 2002). Specific activation of 5-HT_{1A} and 5-HT_{2A} receptors result in
519 similar attention deficits (Carli and Samanin, 2000; Koskinen et al., 2000). Conversely, infusion
520 of antagonists to 5-HT_{2A} receptors into mPFC improved performance on attention tasks in
521 rodents and reduced impulsivity (Passetti et al., 2003; Winstanley et al., 2003).

522 While manipulation of 5-HT receptors specifically in mPFC can manipulate attention,
523 certain key experiments (i.e. attention under stress or in models of dysregulated 5-HT signaling)
524 remain to be done. Based on our findings, rising levels of cortical 5-HT may increase cortical
525 noise due to suppression of L6 mediated cortical inhibition. Increasing the signal-to-noise ratio is
526 strongly correlated with attentional focus (Briggs et al., 2013; Pratte et al., 2013), whereas
527 deficits can arise from increasing cortical noise in prefrontal cortex (Pezze et al., 2014),
528 Similarly, disruptions to normal excitation of L6 neurons of mPFC can lead to attention deficits
529 in rodents, although previous studies have focused on cholinergic stimulation of L6 (Bailey et
530 al., 2010; Guillem et al., 2011). Our study is the first to demonstrate the strong inhibitory
531 modulation exerted on L6 by 5-HT, and the resultant decrease in its ability to stimulate
532 interneuron activity in L5. Taken together, we provide evidence that L6 of mPFC is a candidate
533 locus of action for the modulatory effects of 5-HT on attention. Based on recent work in
534 nonhuman primates (Watson et al., 2015), it is tempting to speculate that 5-HT levels in deep
535 mPFC may modulate the balance between social vigilance and attentional task performance, a
536 phenomenon that is impaired in several neuropsychiatric illnesses.
537
538

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Figure Legends

Figure 1. Serotonin (5-HT) inhibits L6 pyramidal neurons of medial prefrontal cortex. Responses to 5-HT were probed in voltage-clamp and current-clamp by bath application of 5-HT. Representative voltage-clamp traces of the 5-HT response in L6 pyramidal neurons show that responses to 5-HT are: (A) stable and persist in the presence of synaptic blockers, (B) significantly suppressed by WAY100635, and (C) similarly suppressed by a combination of WAY100635 and synaptic blockers. (D) 5-HT elicits strong outward currents on L6 pyramidal neurons of mPFC (n = 28) that can be pharmacologically modulated ($F_{(2, 53)} = 11.8$, $P < 0.0001$, one-way ANOVA). *Post hoc* analyses show that these currents persist in the presence of synaptic blockers ($q = 0.2$, $P > 0.05$, n = 7, Dunnett's Multiple Comparison Test), but are significantly suppressed by WAY100635 ($q = 4.6$, $P < 0.0001$, n = 19). Responses to 5-HT were probed in current-clamp in L6 pyramidal neurons of medial prefrontal cortex with current injection to elicit steady firing (~2-3 Hz) at baseline. Representative current-clamp traces show that responses to 5-HT are: (E) inhibitory, repeatable, and unaffected by synaptic blockers, (F) not fully blocked by WAY100635, nor with (G) WAY100635 and synaptic blockers. (H) L6 pyramidal neurons of mPFC are strongly hyperpolarized by 5-HT (n = 26). *Post hoc* analyses of pharmacological effects on this hyperpolarization ($F_{(2, 50)} = 17$, $P < 0.0001$, one-way ANOVA) show that this inhibition is unaffected by synaptic blockers ($q = 0.8$, $P > 0.5$, n = 6, Dunnett's Multiple Comparison Test) , but greatly reduced by WAY100635 ($q = 5.4$, $P < 0.0001$, n = 21). (I) Action potential firing was significantly affected by 5-HT ($F_{(4,103)} = 37$, $P < 0.0001$, one-way ANOVA). *Post hoc* analyses reveal that baseline firing was strongly suppressed by 5-HT ($q = 9$, $P < 0.0001$, n = 17, Dunnett's Multiple Comparison Test), remain suppressed by 5-HT in synaptic blockers ($q = 6.1$, $P < 0.0001$, n = 6). The suppression is not blocked by WAY100635

(q = 8.8, P < 0.0001, n = 16), and returns to baseline levels following washout of 5-HT (q = 2.3, P > 0.05, n = 36).

Figure 2. Combined activation of serotonergic 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors mediate inhibition of L6 neuronal excitability at suprathreshold potentials. Incremental current steps were injected into patched L6 pyramidal neurons and their output in firing frequency was measured. Shown are representative recordings of the response to a 150 pA current step in single L6 pyramidal neurons (left), and the response to the same 150 pA current step in the presence of 5-HT (middle). The input-output relationship for each group is plotted (right). (A) The input-output relationship of L6 pyramidal neurons is significantly right-shifted by 5-HT ($F_{(1, 168)}$ = 31, P < 0.0001, repeated measures two-way ANOVA). *Post hoc* analysis showed significantly fewer elicited action potentials at every input step (P < 0.05, Sidak's multiple comparisons test). (B) L6 excitability is significantly suppressed by 5-HT in the presence of WAY100635 (P < 0.0001, $F_{(1, 72)}$ = 72, repeated measures two-way ANOVA), an effect especially prominent at higher input steps (125 – 200 pA steps, P < 0.05, Sidak's multiple comparisons test). (C) L6 suppression by 5-HT is fully blocked by simultaneous blockade of both 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors by specific antagonists WAY100635 and MDL100907 ($F_{(1,32)}$ = 0.8, P = 0.4, repeated measures two-way ANOVA). (D) TCB-2, a selective 5-HT$_{2A}$ receptor agonist, inhibits L6 neuronal firing ($F_{(1,80)}$ = 24, P < 0.0001, repeated measures two-way ANOVA), also more prominently at higher input steps (125 – 200 pA steps, P < 0.05, Sidak's multiple comparisons test).

Figure 3. Characterization of L6 neurons in medial prefrontal cortex (mPFC) expressing synaptotagmin 6 and epiphycan. (A) Neurons expressing eGFP driven by the synaptotagmin-6 (Syt6) BAC promoter are localized to L6 pyramidal neurons in the prelimbic region of mPFC. (B) EGFP is also seen in L6 pyramidal neurons in prelimbic mPFC by anti-EGFP immunohistochemistry in Epyc-Cre mice crossed to a Cre-dependent eGFP reporter. Image is adapted from www.gensat.org. (C) Quantification (mean+SEM) by qRT-PCR of the expression of selected genes in mPFC Epyc-vTRAP IP samples compared to whole PFC input. Positive values indicate enrichment in the IP and negative values indicate depletion. Dotted lines indicate a 2-fold difference in either direction. Green bars represent genes that are >2-fold enriched in the Epyc cells, red bars represent genes that are >2-fold depleted, and gray bars are genes expressed at similar levels to the rest of PFC. Of note, mRNA for serotonin receptors *Htr1a* and *Htr2a* were expressed, but not enriched in Epyc cells, a not unexpected finding given the expression of 5-HT_{1A} and 5-HT_{2A} in other populations of neurons in mouse mPFC beyond L6. **P* < 0.05; ***P* < 0.01 by Student's *t*-test.

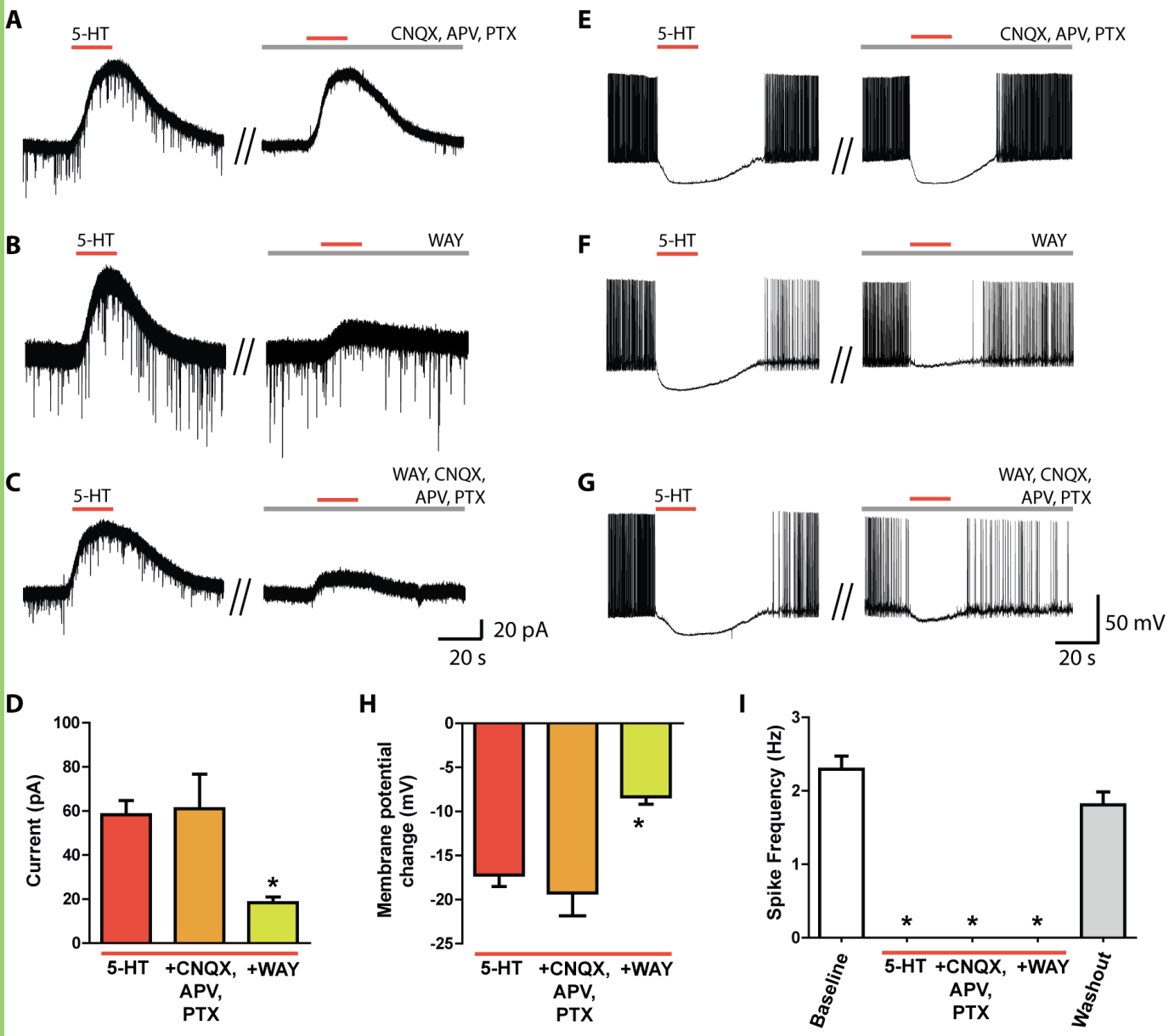
Figure 4. Optogenetic activation of L6 pyramidal neurons of medial prefrontal cortex excites L5 interneurons. (A) Schematic representation of light activation of L6 pyramidal neurons of medial prefrontal cortex in Epyc-ChR2 mice with axons projecting to L5 interneurons. (B) Channelrhodopsin-expressing pyramidal L6 neurons were robustly excited by targeted light stimulation over L6. The effects of increasing L6 light power are shown for one example L6 pyramidal neuron. (C) Light activation of L6 robustly excited L5 interneurons. The effects of increasing L6 light power are shown for three different L5 interneurons. (D) Top, close-up of the initial light-evoked action potential in L6 to show the timing from onset of light

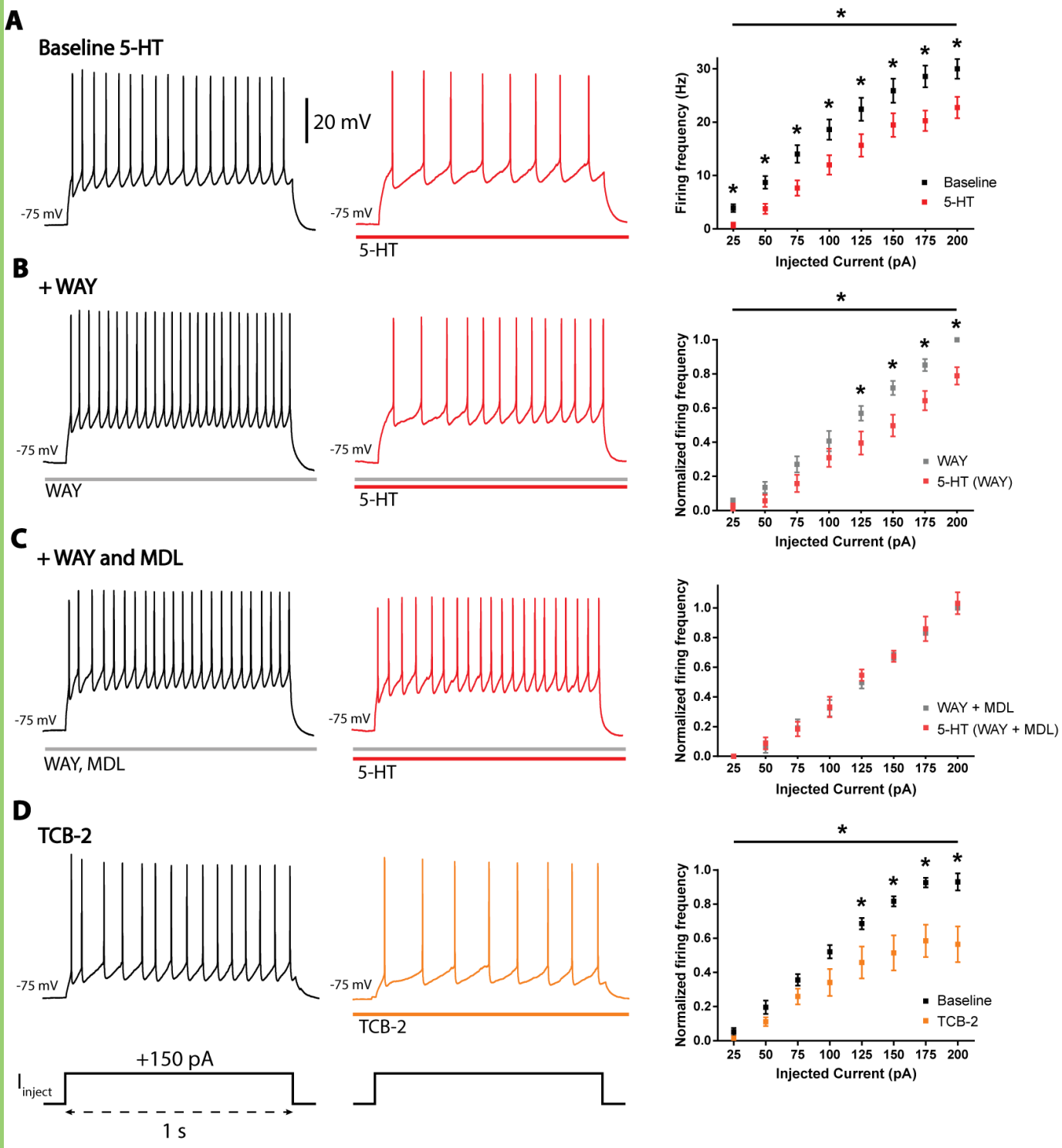
(blue dotted line) to peak of the spike (black dotted line). Scale bar: 20 mV, 1 ms. Note: in L6 the onset of depolarization from light is < 1 ms. Bottom, voltage-clamp recording showing the light-evoked postsynaptic response in a L5 interneuron to demonstrate response latency. Scale bar: 40 pA, 1 ms. Post-synaptic responses in L5 interneurons were initiated 1.1 ± 0.3 ms following initial spike of L6 pyramidal neurons, as indicated by an arrow.

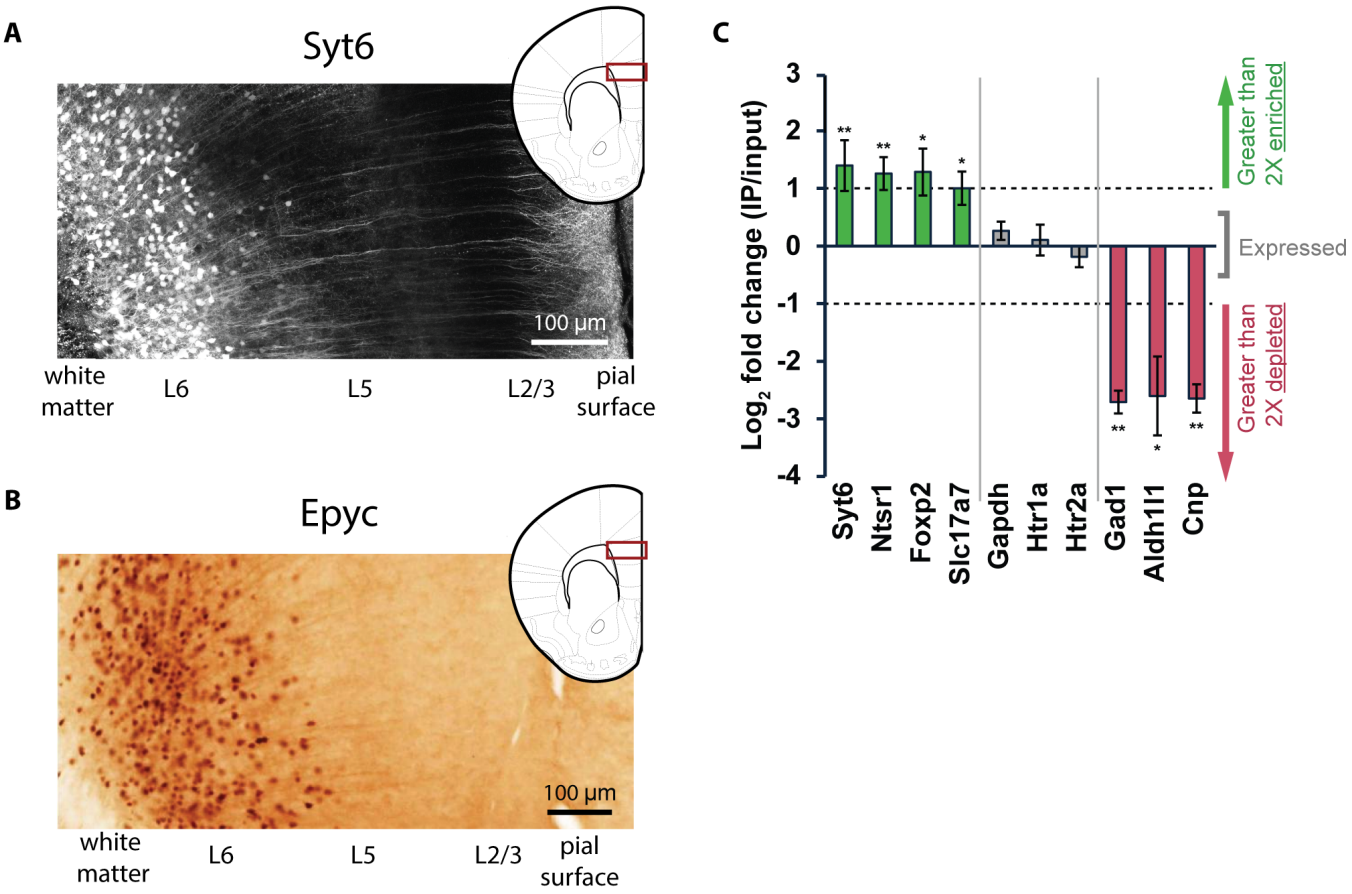
Figure 5. Two distinct groups of interneurons are found in L5 and are activated by light stimulation of L6 pyramidal neurons. (A) Fast-spiking (FS) interneurons of L5 characterized by injection of current steps. (B) Representative trace of a L5 FS interneuron activated by L6. Note the rapidly depressing response to L6 activation. (C) Activation of FS interneurons by L6 elicited action potential firing primarily during the initial phase of activation that rapidly depressed over the duration of the stimulation (# of elicited action potentials in first half of stimulation vs. second half: $t_9 = 7.2$, $P < 0.0001$, unpaired *t*-test). (D) Non-fast-spiking (nFS) interneurons of L5 characterized by injection of current steps. (E) Representative trace of a L5 nFS interneuron activated by L6, demonstrating a more regular firing pattern. (F) L5 nFS interneurons were activated by L6, and fired in a regular pattern over the course of the stimulation (# of elicited action potentials in first half of stimulation vs. second half: $t_7 = 1.9$, $P = 0.1$, unpaired *t*-test).

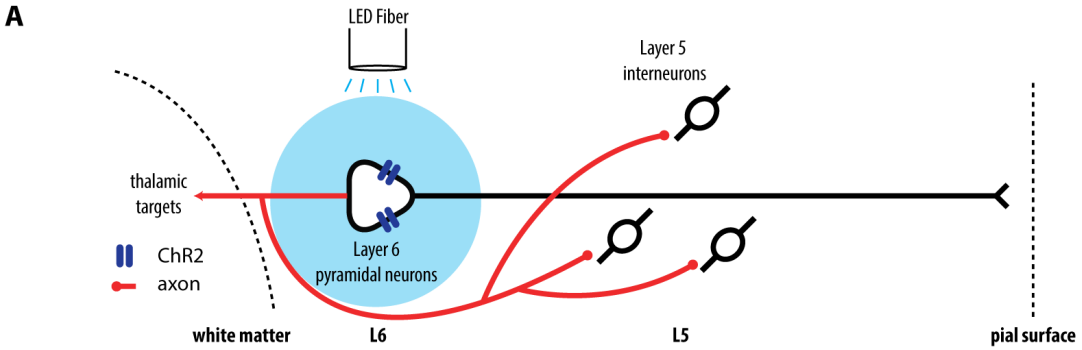
Figure 6. L6 activation of L5 interneurons in medial prefrontal cortex is stable over time, but suppressed by 5-HT. (A) Excitatory effects on L5 interneurons by optogenetic activation of L6 were stable and repeatable over time. Shown here are the postsynaptic responses in a L5

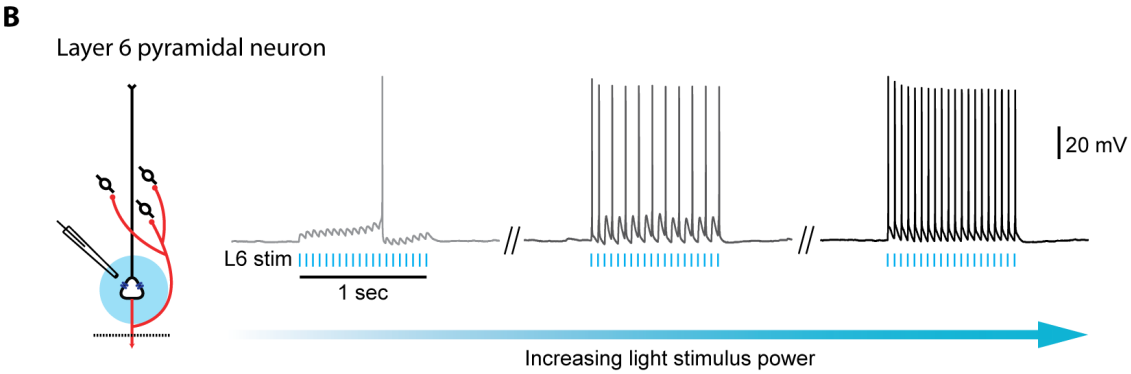
interneuron to L6 light-stimulation repeated over 15 minutes. The number of spikes elicited initially and upon repetition in L5 interneurons are plotted on the bar graph at the right (mean ± SEM). There was no significant difference (t_{11} = 0.8, P = 0.4, paired t-test), showing that the postsynaptic effect in L5 interneurons does not decrease over time under baseline conditions. (B) L6 activation of L5 FS interneurons were significantly suppressed by 5-HT (t_8 = 3.8, P = 0.005, paired t-test, n = 9). Shown here are repeated recordings (baseline, 5-HT, washout) from 3 different L5 interneurons. The number of spikes elicited at baseline and in the presence of 5-HT in L5 interneurons are illustrated in the bar graph on the right (mean ± SEM). (C) Antagonists of serotonergic 5-HT_{1A} and 5-HT_{2A} receptors blocked the inhibitory effects of 5-HT on L6 activation of L5 interneurons (t_{15} = 0.9, P = 0.4, paired t-test, n = 16). Shown here is one representative L5 interneuron excited by L6 stimulation, which is suppressed by 5-HT applied alone, and no longer suppressed by 5-HT in the presence of WAY100635 and MDL100907. The results are plotted on the bar graph at the right (mean ± SEM).

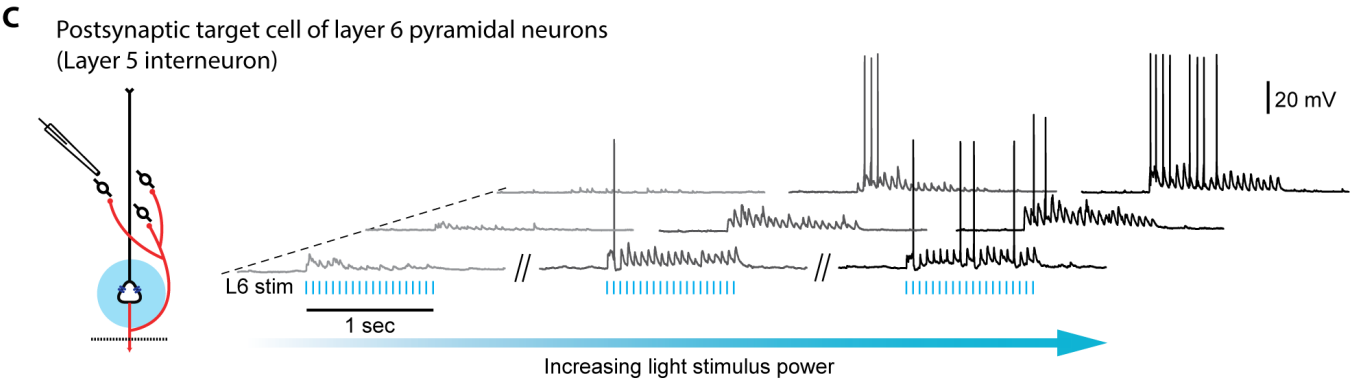


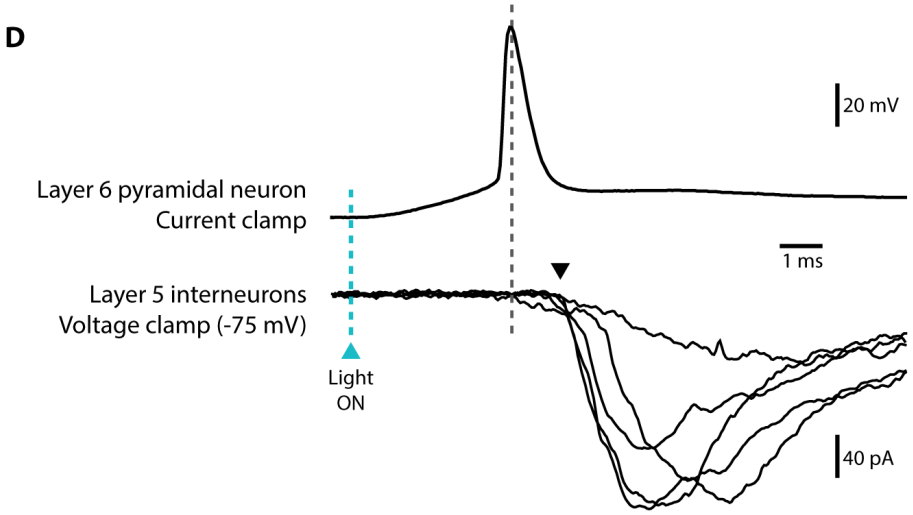


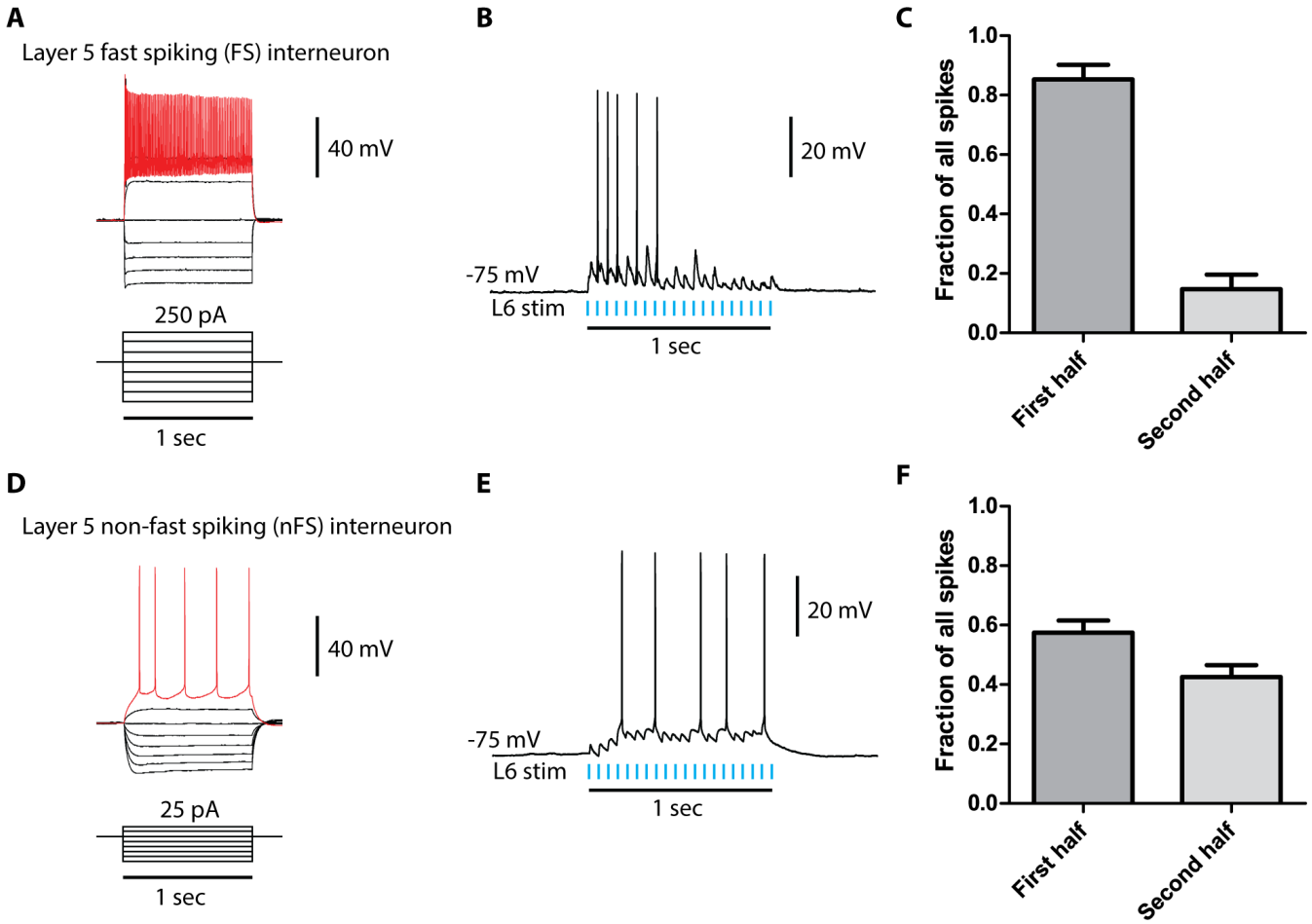


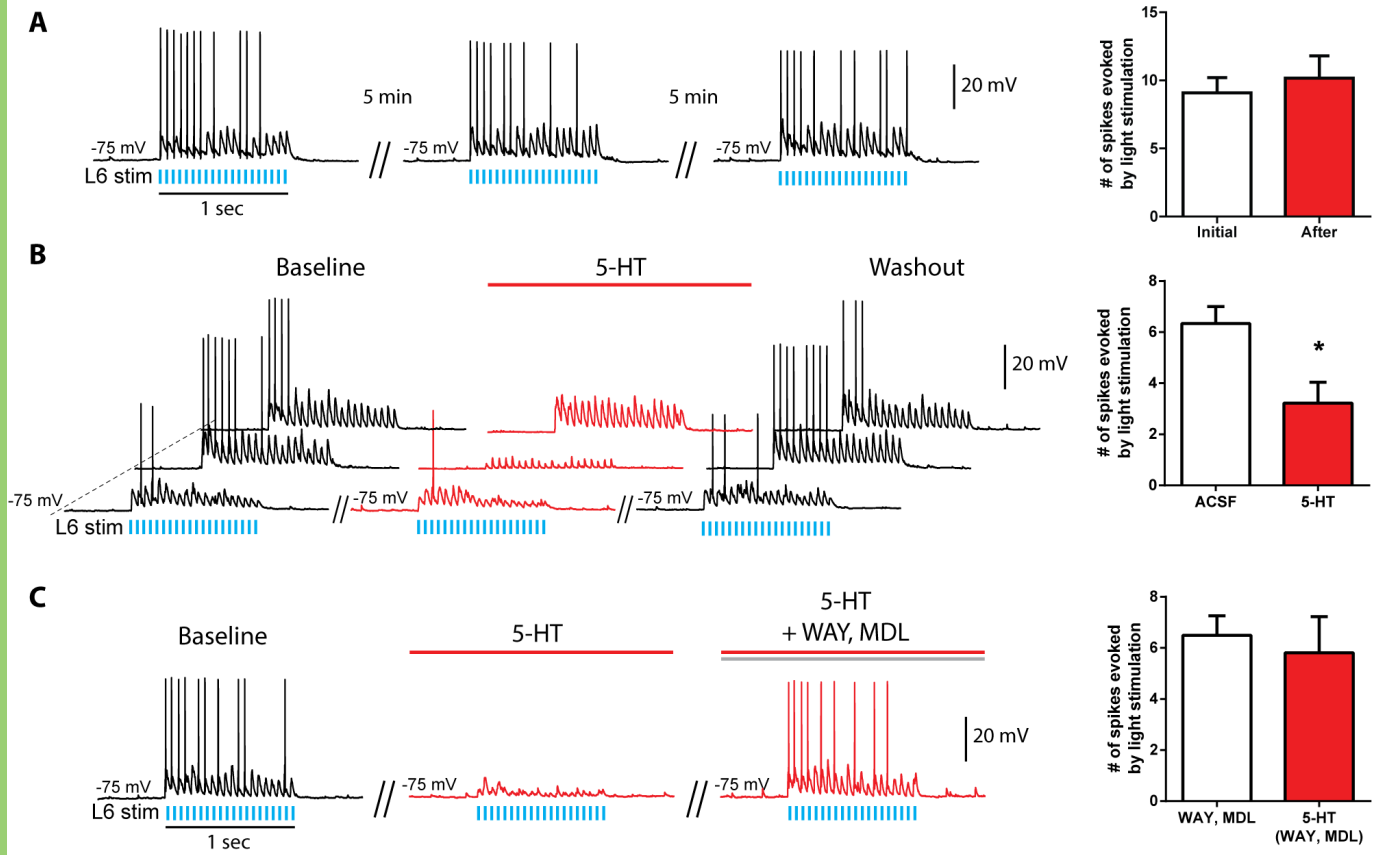












	Neuron type	RMP (mV)	Input Resistance (MΩ)	Spike Amplitude (mV)	Peak firing frequency (Hz)	N
Layer 6	**Pyramidal neurons**	-90.2 ± 0.6	128.5 ± 3.9	83.1 ± 0.6	22.4 ± 0.8	122
Layer 5	**FS interneurons**	-85.2 ± 1.2	114.3 ± 9.8	59.5 ± 2.2	109.8 ± 23.3	19
	nFS interneurons	-82.7 ± 1.6	235.9 ± 30.0*	79.7 ± 1.9**	34.1 ± 3.0**	22

Table 1. Intrinsic electrophysiological properties of three groups of neurons recorded: pyramidal neurons in L6, fast-spiking (FS) interneurons in L5, and non-fast-spiking (nFS) interneurons in L5. Neuronal properties shown are: resting membrane potential (RMP), input resistance, spike amplitude, and peak firing frequency upon injection of a maximal suprathreshold current. Data are shown as mean ± SEM. Comparisons between L5 FS and nFS interneurons: $*P < 0.05$, $*P < 0.001$ by unpaired *t*-tests.

Symbol	Gene Name	Source	Assay	Dye
Aldh1l1	aldehyde dehydrogenase 1 family, member L1	Life Technol.	Mm03048957_m1	FAM
Cnp	cyclic nucleotide phosphodiesterase 1	Life Technol.	Mm01306640_m1	FAM
Foxp2	forkhead box P2	Life Technol.	Mm00475030_m1	FAM
Gad1	glutamic acid decarboxylase 1	Life Technol.	Mm00725661_s1	FAM
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	Life Technol.	Mm99999915_g1	FAM
Htr1a	5-hydroxytryptamine (serotonin) receptor 1A	Life Technol.	Mm00434106_s1	FAM
Htr2a	5-hydroxytryptamine (serotonin) receptor 2A	Life Technol.	Mm00555764_m1	FAM
Ntsr1	neurotensin receptor 1	Life Technol.	Mm00444459_m1	FAM
Slc17a7	vesicular glutamate transporter 1	Life Technol.	Mm00812886_m1	FAM
Syt6	synaptotagmin VI	Life Technol.	Mm01308768_m1	FAM

Table 2. TaqMan Gene Expression Assay