Mediodorsal and Ventromedial Thalamus Engage Distinct L1 Circuits in the Prefrontal Cortex

Highlights
- Prefrontal cortex L1 has sublayers distinguished by thalamic axons from VM and MD
- VM and MD drive parallel L1 micro-circuits composed of NDNF and VIP interneurons
- NDNF and VM synapses are highly enriched at the apical dendrites of L5 PT cells
- NDNF-mediated dendritic inhibition controls VM-evoked Ca^{2+} signals at L5 PT cells

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In Brief
Anastasiades et al. show how VM and MD thalamus differentially engage NDNF and VIP interneurons located in L1 of the mouse prefrontal cortex. These interneurons participate in distinct inhibitory and disinhibitory micro-circuits, with feedforward inhibition via NDNF interneurons regulating VM-evoked Ca^{2+} signals in the apical dendrites of L5 pyramidal cells.
Mediodorsal and Ventromedial Thalamus Engage Distinct L1 Circuits in the Prefrontal Cortex

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SUMMARY

Interactions between the thalamus and prefrontal cortex (PFC) play a critical role in cognitive function and arousal. Here, we use anatomical tracing, electrophysiology, optogenetics, and 2-photon Ca2+ imaging to determine how ventromedial (VM) and mediodorsal (MD) thalamus target specific cell types and subcellular compartments in layer 1 (L1) of mouse PFC. We find thalamic inputs make distinct connections in L1, where VM engages neuron-derived neurotrophic factor (NDNF+) cells in L1a and MD drives vasoactive intestinal peptide (VIP+) cells in L1b. These separate populations of L1 interneurons participate in different inhibitory networks in superficial layers by targeting either parvalbumin (PV+) or somatostatin (SOM+) interneurons. NDNF+ cells also inhibit the apical dendrites of L5 pyramidal tract (PT) cells to suppress action potential (AP)-evoked Ca2+ signals. Lastly, NDNF+ cells mediate a unique form of thalamus-evoked inhibition at PT cells, selectively blocking VM-evoked dendritic Ca2+ spikes. Together, our findings reveal how two thalamic nuclei differentially communicate with the PFC through distinct L1 micro-circuits.

INTRODUCTION

Communication between the thalamus and prefrontal cortex (PFC) is critical for cognition and disrupted in mental health disorders (Huang et al., 2019; Ouhaz et al., 2018; Pergola et al., 2018). The PFC engages several higher order nuclei, including reciprocal interactions with mediodorsal (MD) and ventromedial (VM) thalamus (Collins et al., 2018; Gabbott et al., 2005). Although the behavioral and computational roles of these nuclei have not been fully elucidated, connections with MD sustain delay period activity during working memory (Bolkan et al., 2017; Schmitt et al., 2017), whereas those with VM regulate arousal (Honjoh et al., 2018). The behavioral roles of these nuclei likely reflect their impact on different circuits within the PFC, with MD inputs driving layer (L)2/3 pyramidal cells (PYRs) and VM synapsing onto, but not firing, L2/3 and L5 pyramidal cells (Collins et al., 2018). Interestingly, both MD and VM inputs also project to L1, but it remains unclear how these connections contribute to their functional influence on the PFC.

Both MD and VM elicit robust inhibition in the PFC (Collins et al., 2018), which can be mediated by a variety of GABAergic interneurons. MD targets parvalbumin-positive (PV+) cells in L2/3 (Delevich et al., 2015), but targeting of L1 interneurons is unknown. In contrast, VM engages L1 interneurons (Cruikshank et al., 2012), which have diverse properties (Chu et al., 2003; Jiang et al., 2013; Lee et al., 2013; Schuman et al., 2019; Wozny and Williams, 2011; Zhou and Hablitz, 1996). L1 interneurons express 5HT3a receptors (5HT3aR+) (Rudy et al., 2011) and are further classified by the expression of either vasoactive intestinal peptide (VIP+) or neuron-derived neurotrophic factor (NDNF+) (Schuman et al., 2019). Throughout the cortex, VIP+ cells typically target somatostatin (SOM+) cells to mediate disinhibition (Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013). In contrast, NDNF+ cells are less understood but appear to be neuroglialiform cells (Schuman et al., 2019) that can inhibit pyramidal cell dendrites (Abs et al., 2018). By targeting either VIP+ or NDNF+ cells, MD and VM could either inhibit or disinhibit the PFC, with important implications for network activity (Letzkus et al., 2011; Palmer et al., 2012; Pi et al., 2013).

Thalamic inputs to L1 are also well positioned to contact the apical dendrites of pyramidal cells residing in deeper layers (Rubio-Garrido et al., 2009). Recent studies from frontal cortex show VM innervation is localized to the apical dendrites of pyramidal tract (PT) cells in L5 (Collins et al., 2018; Guo et al., 2018). Although these inputs do not appear to drive somatic firing (Collins et al., 2018), they may generate dendritic Ca2+ spikes (Zhu and Connors, 1999). Interestingly, dendritic Ca2+ signals are tightly regulated by GABAergic inhibition via SOM+ and 5HT3aR+ interneurons (Chalifoux and Carter, 2011; Chiu et al., 2013; Marlin and Carter, 2014; Palmer et al., 2012; Pérez-Garcia et al., 2006). If VM inputs engage a subset of 5HT3aR+ cells, they could also mediate a local feedforward inhibitory circuit.
Figure 1. Thalamic Axon and Cortical Interneurons Define Two L1 Sublayers

(A) Left: schematic of AAV-XFP (either AAV-EGFP or AAV-mCherry) injections into the ventromedial (VM) and mediiodorsal (MD) thalamus. Right: Representative images of superficial layers of PFC show VM axon (blue), MD axon (green), and merge with DAPI (grayscale). Scale bars represent 25 μm.

(B) Left: summary of axon density for VM (blue) and MD (green) as a function of L1 depth (0% is pial surface). Lines are normalized axon density plots from individual slices. Right: summary of L1 depth at which MD and VM axon density peaks.

(C) Left: schematic of AAV-FLEX-XFP injection into the PFC of NDNF-Cre, VIP-Cre, or 5HT3aR-Cre mice. Right: representative images of superficial layers of PFC show labeled NDNF+, VIP+, and 5HT3aR+ interneurons. Scale bars represent 50 μm.

(D) Left: summary of cumulative frequency of interneurons as a function of L1 depth. Right: relative cell density for NDNF+, VIP+, and 5HT3aR+ interneurons in L1a and L1b.

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within L1. Because PT cells project back to thalamus (Harris and Shepherd, 2015), this local inhibition could influence cortico-thalamic-cortical loops that are critical for PFC-dependent behaviors (Bolkan et al., 2017; Guo et al., 2017; Schmitt et al., 2017).

Here, we combine anatomical tracing, electrophysiology, optogenetics, 2-photon Ca\(^{2+}\) imaging, and pharmacology to dissect how higher order thalamic inputs from MD and VM engage multiple circuits in the superficial layers of the prelimbic PFC. We first show that L1 consists of two sublayers, with VM selectively driving NDNF+ cells in L1a and MD engaging VIP+ cells in L1b. We then show that these interneurons participate in distinct inhibitory networks, with NDNF+ cells inhibiting PV+ cells and VIP+ cells inhibiting SOM+ cells in L2/3. We also show that NDNF+ cells inhibit the apical dendrites of pyramidal cells, suppressing action potential (AP)-evoked Ca\(^{2+}\) signals in the distal dendrites of PT cells in L5. Lastly, we show that NDNF+ cells mediate thalamus-evoked inhibition, blocking VM-evoked Ca\(^{2+}\) signals in the apical dendrites of PT cells. Together, our findings reveal key differences between two higher order thalamic inputs to PFC, highlighting how they target distinct networks of L1 interneurons and suggesting novel roles for both VM inputs and NDNF+ cells in gating communication between the cortex and thalamus.

**RESULTS**

**Thalamic Inputs and GABAergic Interneurons**

**Distinguish Sublayers of L1**

Both VM and MD thalamus innervate L1 of the prelimbic PFC, but how they influence local micro-circuits remains unclear. To explore these connections, we co-injected AAVs expressing EGFP or mCherry into VM and MD (n = 3 mice each; Figure 1A). We found both afferents densely innervated L1 of the prelimbic cortex, with VM axon prominent in the outer half (L1a) and MD axon greater in the inner half (L1b; peak axon density [% L1 depth]: MD = 45.7% ± 1.3%; VM = 9.3% ± 1.6%; Figures 1B and S1) and a second band of MD axon in L3 (Figure S1; Collins et al., 2018). The spatial separation of these axon arborizations suggested that VM and MD may have different postsynaptic targets within L1. Several classes of GABAergic interneurons are found in L1, with the main subtypes being NDNF+ and VIP+ cells (Schuman et al., 2019). We examined the distributions of these cells by injecting AAV-FLEX-EGFP or AAV-FLEX-tdTomato into the PFC of SHT3AR-Cre, NDNF-Cre, or VIP-Cre mice (n = 3 mice each; Figure 1C). We found that SHT3AR+ cells spanned L1, whereas NDNF+ cells were biased toward L1a and VIP+ cells were restricted to L1b (Figure 1D), indicating that these cell types are also spatially segregated.

To characterize the properties of L1 interneurons in the prelimbic PFC, we next made whole-cell recordings from ex vivo slices. We found VIP+ cells in L1b had bitufted or bipolar morphologies, along with non-fast-spiking (NFS), irregular-spiking (IS), or fast-adapting (fAD) firing properties (n = 17 VIP+ cells, 8 mice; NFS = 9/17; IS = 5/17; fAD = 3/17; Figures 1E–1G and S1). In contrast, NDNF+ cells in L1a had horizontal morphologies and late-spiking (LS) firing properties (n = 12 NDNF+ cells, 4 mice; LS = 11/12; non-LS = 1/12; Figures 1E–1G and S1), with a slow ramp to spike at near-threshold potentials (Chu et al., 2003; Schuman et al., 2019). VIP– cells in L1a of VIP-Cre x Ai14 mice had indistinguishable properties to NDNF+ cells (n = 17 cells, 8 mice; Figures 1E–1G and S1). These results show that NDNF+ and VIP+ cells have distinct morphological and physiological properties and segregate into separate sublayers, indicating the two main classes of L1 interneurons may differentially receive and process MD and VM inputs, suggesting complementary activation of superficial networks.

**Brain-wide Retrograde Tracing Indicates Differential Interneuron Innervation**

The distributions of VIP+ and NDNF+ cells in L1 indicated they may receive and process different local and long-range inputs. To map their brain-wide innervation, we performed monosynaptic input tracing using a conditional rabies virus approach (Wall et al., 2010). We first injected AAV-FLEX-TVA-mCherry and AAV-FLEX-oG into the PFC of either NDNF-Cre or VIP-Cre mice (Figure 2A). After allowing for expression, we then injected SAD\(\Delta\)GFP (EnvA) pseudotyped rabies virus to infect Cre+ interneurons. Starter cells were primarily located in prelimbic PFC (Figures 2A and S2), with GFP+ monosynaptic input neurons found in all layers of the local circuit (Figure 2B). We also observed GFP+ cells across the rostral-caudal axis of the brain (VIP-Cre: n = 23,483 cells, 5 mice; NDNF-Cre: n = 21,448 cells, 3 mice; Figures 2C and S2). The main extra-cortical input was thalamus, in addition to striatum and pallidum, hippocampus, amygdala, castrum, olfactory areas, hypothalamus, and various midbrain and hindbrain structures (Figures 2D, S2, and S3; Tables S1 and S2). VIP+ cells received a greater proportion of input from prefrontal regions, although no specific subregion accounted for this bias (Figure S3). In contrast, NDNF+ cells received greater subcortical input from cholinergic areas, suggesting they may be differentially modulated (Figure S2). Thalamic input to both interneuron populations came mostly from higher order/secondary nuclei (VIP+ = 81.7% ± 1.2%; NDNF+ = 79.5% ± 2.2%; Figure S3; Philips et al., 2019). However, we observed major differences in the relative composition of this input, with VIP+ cells receiving a greater proportion of input from MD (VIP+ = 36.0% ± 1.2%; NDNF+ = 17.3% ± 0.6%; p < 0.0001) and NDNF+ cells from VM (VIP+ = 14.3% ± 1.6%; NDNF+ = 23.8% ± 4.5%; p = 0.0004; Figures 2E and 2F). This difference was highlighted by the MD/VM ratio for input to VIP+ and NDNF+ starter cells (VIP+ = 2.6, confidence interval [CI] = 1.8–
3.8; NDNF+ = 0.8, CI = 0.4−1.5; VIP+ versus NDNF+, p = 0.036; Figure 2G). Together, these findings indicate both interneuron subtypes receive considerable brain-wide input but suggest differential thalamic targeting by MD and VM.

**MD and VM Innervate and Activate Different Subtypes of L1 Interneurons**

Although rabies tracing provides clues about differences in connectivity, it cannot reveal the strength or dynamics of synapses. To understand how VM and MD engage L1 micro-circuits, we next injected AAV-ChR2-EYFP into VM or MD of VIP-Cre × Ai14 mice and recorded whole-cell responses at VIP+ and L1 cells in VIP+ mice (Figure 3A). Because VIP+ cells in L1a are physiologically and morphologically indistinguishable from NDNF+ cells, this approach allows us to compare the activation of the two main types of L1 interneurons in the same slice. Trains of VM inputs (5 pulses @ 10 Hz with a 473-nm LED) elicited robust excitatory postsynaptic currents (EPSCs) at VIP+ cells, which were much stronger than those at VIP+ cells in L1b (VIP+ = −146 ± 41 pA, VIP+ = −6 ± 2 pA, p = 0.016; n = 7 pairs, 5 mice; Figures 3A and 3B). In contrast, we found comparable MD-evoked EPSC amplitudes at each cell type (VIP+ = −57 ± 22 pA, VIP+ = −41 ± 13 pA, p = 0.68; n = 7 pairs, 5 mice; Figures 3A and 3B). This differential thalamic targeting of L1 interneurons was highlighted by VIP+/−VIP+ input ratios, which were close to unity for MD but an order of magnitude higher for VM (MD = 0.94, CI = −0.03–3.4, p = 0.58; VM = 25.3, CI = 8.4–92.6, p = 0.016; MD versus VM, p = 0.001; Figure 3C). Interestingly, both MD and VM inputs to VIP+ cells showed facilitation, although VIP− cells displayed initial facilitation followed by depression (Figure 3D). These results indicate that thalamic inputs differentially engage L1 interneurons, with VM stronger onto VIP− cells and MD showing no bias. They also show how differences in synaptic strength depend on the source of thalamic input, whereas short-term dynamics depend on the postsynaptic cell type.

To determine how differences in connectivity impact action potential (AP) firing, we performed separate current-clamp experiments. VM inputs drove robust AP firing of VIP− cells in L1a, with minimal activation of VIP+ cells in L1b (Figures 3E−3G; VIP− p-spike = 0.88 ± 0.07, VIP+ p-spike = 0.14 ± 0.14, p = 0.03; n = 7 pairs, 4 mice). In contrast, MD input activated VIP+ cells in L1b more strongly than VIP− cells in L1a (Figures 3E−3G; VIP− p-spike = 0.33 ± 0.13, VIP+ p-spike = 0.81 ± 0.10, p = 0.04; n = 8 pairs, 5 mice). This reflects the intrinsic properties of VIP+ cells, which are much more excitable than NDNF+/VIP− cells (Figure 1G). Together, these findings indicate that L1 contains two distinct inhibitory micro-circuits, one mediated by NDNF+ interneurons in L1a and strongly activated by VM and the other by VIP+ interneurons in L1b and driven by MD.

**VIP+ and NDNF+ Interneurons Participate in Distinct Output Pathways**

Having determined how MD and VM engage L1 interneurons, we next examined how these cells in turn engage the local network. The axonal projections of VIP+ and NDNF+ interneurons suggested that they may target distinct postsynaptic cells. To test this idea, we crossed VIP-Cre or NDNF-Cre mice with either G42 or GIN reporter mice, which label subsets of PV+ and SOM+ interneurons, respectively (Chattopadhyaya et al., 2004; Oliva et al., 2000), allowing us to distinguish them from unlabeled L2/3 pyramidal cells (PYRs) (Figure S4). We then injected AAV-DIO-ChR2-mCherry into the PFC and recorded light-evoked inhibitory postsynaptic currents (IPSCs) from L2/3 interneurons or PYRs in the presence of TTX and 4-AP (Anastasiades et al., 2018; Petreanu et al., 2009; Figures 4A and 4B). For VIP-evoked IPSCs, we found large responses at SOM+ interneurons but neither PYR nor PV+ cells (PYR = 21 ± 7 pA, n = 10 cells, 7 mice; PV+ = 28 ± 9 pA, n = 9 cells, 6 mice; SOM+ = 342 ± 77 pA, n = 10 cells, 5 mice; SOM+ versus PYR, p < 0.0001; SOM+ versus PV+, p = 0.0003; Figures 4A and 4C). For NDNF−evoked IPSCs, we found the opposite relationship, with large responses at PYR and PV+ cells, but not SOM+ cells (PYR = 88.5 ± 21 pA, n = 14 cells, 5 mice; PV+ = 56 ± 16 pA, n = 10 cells, 5 mice; SOM+ = 10 ± 6 pA, n = 8 cells, 4 mice; SOM+ versus PYR, p = 0.0004; SOM+ versus PV+, p = 0.0085; Figures 4B and 4C). These findings indicate that NDNF+ and VIP+ interneurons participate in distinct micro-circuits in superficial layers of the PFC, potentially engaging different disinhibitory pathways via SOM+ and PV+ interneurons, respectively.

Figure 2. Brain-wide Input to NDNF+ and VIP+ Cells via Transsynaptic Rabies Tracing

(A) Left: schematic of AAV-Flex-oG and AAV-Flex-TVA-mCherry injection into PFC of NDNF-Cre or VIP-Cre mice, followed 5 weeks later by EnvA-RV-GFP. Right: AAV-helper virus–infected cells in L1 of prelimbic PFC are labeled in red. Presynaptic cells are labeled in green. NDNF+ and VIP+ starter cells are labeled in yellow and indicated by arrows. Scale bar represents 50 μm.

(B) Left: representative images of GFP+ presynaptic cells across layers of prelimbic PFC in NDNF-Cre or VIP-Cre mice. Right: quantification of percentage of GFP+ presynaptic cells in different layers. Dashed lines indicate layer boundaries. Scale bar represents 100 μm.

(C) Left: example images of GFP+ presynaptic cells in coronal sections relative to bregma. Right: summary distribution of GFP+ presynaptic cells along the rostro-caudal axis, with plots for individual mice (light traces) and averages (dark trace).

(D) Summary of percentage of GFP+ presynaptic cells in different brain regions. Data points from individual mice are shown as colored circles. Amyg, amygdala; Clau, claustrum; Ctx, remainder of cortex (excluding IPC); Hipp, hippocampus; iPFC, ipsilateral PFC (prelimbic, infralimbic, and rostral component of dorsal anterior cingulate cortex); Thal, thalamus; S & P, striatum and pallidum.

(E) Summary of percentage of thalamic GFP+ presynaptic cells in different nuclei, including MD and VM. Data points from individual mice are shown as colored circles.

(F) Left: example images of GFP+ presynaptic cells in the thalamus of NDNF-Cre (top) and VIP-Cre (bottom) mice. Middle: location of individual cells from NDNF-Cre mice mapped onto thalamic nuclei using the Allen brain atlas (slice 65, https://portal.brain-map.org/), where MD (green) and VM (blue) are highlighted. Right: similar for VIP-Cre mice. Scale bar represents 200 μm.

(G) Ratio of GFP+ presynaptic neurons found in MD and VM for NDNF-Cre and VIP-Cre mice. Averages are mean ± SEM (B, D, and E) or geometric mean ± 95% CI (G). *p < 0.05. See also Figures S2 and S3 and Tables S1 and S2.
Figure 3. MD and VM Differentially Recruit Interneuron Populations in L1a and L1b

(A) (Left) Schematic for recordings of VM (top) and MD (bottom) inputs onto pairs of VIP- (equivalent to NDNF+) cells in L1a (orange) and VIP+ cells in L1b (red). (Right) Average voltage-clamp recordings at -60 mV show VM-evoked (blue triangles) and MD-evoked (green triangles) EPSCs at pairs of L1 interneurons.

(B) Summary of VM-evoked (top) and MD-evoked (bottom) EPSC amplitudes versus pulse number at pairs of L1 interneurons.

(C) Summary of (VIP-/VIP+) input ratio for first VM-evoked (blue) and MD-evoked (green) EPSC amplitude at pairs of L1 interneurons. Note the logarithmic axis.

(D) Summary of paired-pulse ratio (PPR) for VM-evoked (solid lines) and MD-evoked (dashed lines) EPSCs at VIP- and VIP+ cells.

(E) Similar to (A) for VM-evoked (top) and MD-evoked (bottom) EPSPs and APs at pairs of VIP- (left) and VIP+ (right) cells in response to stimulus trains (triangles), evoked from resting membrane potential. Traces are shown from representative pairs of neurons, where each panel shows five traces recorded from the same cell.

(F) Similar to (B) for AP probability (p-spike) for VM (top) and MD (bottom) stimulation.

(G) Summary of maximum VM-evoked (top) and MD-evoked (bottom) p-spike at pairs of L1 interneurons across a stimulus train. Lines represent individual pairs. Averages are mean ± SEM (B, F, and G) or geometric mean ± 95% CI (C). *p < 0.05.
In other cortices, L1 neurogliaform cells, which are a subset of NDNF+ interneurons, evoke inhibitory responses via slow GABAA and GABAB receptors (Olah et al., 2007, 2009). Consistent with this idea, we found NDNF-evoked IPSCs were markedly slower than VIP-evoked IPSCs (Figures 4D and S4). To further examine connections onto PV+ and PYR cells in the PFC, we also performed a separate set of current-clamp recordings, using selective pharmacology to assay the involvement of ionotropic and metabotropic receptors. Both PYR and PV+ cells had biphasic inhibitory postsynaptic potentials (IPSPs) (Figures 4E and S4; NDNF+ → PYR: n = 5 cells, 2 mice; NDNF+ → PV+: n = 5 cells, 4 mice), with a slow component sensitive to the GABAB-R antagonist CGP (2 μM) and fast component blocked by the GABAA-R antagonist gabazine (GZ) (10 μM; Figures 4E, 4F, and S4). In contrast, VIP+ → SOM+ connections did not reveal any GABAB-mediated IPSP (Figures 4E and 4F; VIP+ → SOM+: n = 7 cells, 3 mice). These findings indicate that NDNF+ cells engage slow inhibition via GABAB receptors and fast inhibition via GABAA receptors. The latter component suggests a role for NDNF+ cells in rapid, thalamus-evoked feedforward inhibition at PFC pyramidal neurons.
Differential Innervation of PT and IT Cells by NDNF+ Interneurons

Our results show that NDNF+ cells synapse onto PYRs, suggesting they play a role in thalamus-evoked feedforward inhibition. Because their axons extend across L1, NDNF+ cells may also target the dendrites of pyramidal cells residing in deeper layers (Abs et al., 2018; Jiang et al., 2013; Zhou and Hablitz, 1996). L5 pyramidal cells segregate into PT and intratelencephalic (IT) cells, which possess distinct properties (Harris and Shepherd, 2015). For example, the strength of excitatory inputs from thalamus and inhibitory inputs from PV+ and SOM+ cells differ at PT and IT cells (Anastasiades et al., 2018a; Collins et al., 2018; Hilscher et al., 2017). To test whether this is also true for connections from NDNF+ cells, we applied widefield optogenetic stimulation of NDNF+ cells and recorded from triplets of retrogradely labeled PT and IT cells, as well as unlabeled PYR cells in L2/3 (Figure 5A). Calculating the pairwise ratio of IPSC amplitudes for PT/PYR and PT/IT cells, we found that NDNF-evoked IPSCs were larger at PT cells than IT cells, with no difference between PT and PYR cells (PT/PYR ratio = 1.5, CI = 0.5–2.5, p = 0.64; PT/IT ratio = 2.4, CI = 1.5–3.3, p = 0.016; n = 8 triplets, 3 mice; Figures 5A, 5B, and S5). These findings indicate that NDNF+ cells synapse onto a variety of pyramidal cells, making particularly strong connections onto PT cells.

Although these experiments indicate connection strength, they do not reveal the location of presynaptic cells. To confirm that presynaptic neurons were found in L1, we also performed local circuit mapping with soma-restricted optogenetics (Baker et al., 2016; Mardinly et al., 2018). In control experiments, we first confirmed that our stimulation parameters allowed reliable, spatially restricted firing in st-ChroME-expressing interneurons (Figures 5C and S5C). We then assessed connections from NDNF+ cells to PT cells, which represent their strongest outputs in the local micro-circuit. We found that PT cells received pronounced inputs from NDNF+ cells located in L1, with markedly less input from L2/3 and L5 (n = 10 cells, 3 mice; Figures 5D and S5). In contrast, L2/3 SOM+ cells received VIP+ input from both L1b and superficial L2/3 (n = 12 cells, 6 mice; Figures S5A, S5B, and S5C). These findings indicate that NDNF+ cells synapse onto a variety of pyramidal cells, making particularly strong connections onto PT cells.

Figure 5. NDNF+ Cell Targeting of Cortical Pyramidal Neuron Subtypes

(A) Left: schematic for studying outputs from NDNF+ cells (orange) onto PYR (black), IT (blue), and PT (red) cells. Right: voltage-clamp recordings at +10 mV show NDNF-evoked IPSCs at the three different cell types. Light traces are individual cells, and dark traces are averages. Triangles show light stimulation.

(B) Summary of IPSC amplitude ratios from recorded triplets, calculated by dividing the IPSC amplitude recorded in PT cells by the IPSC amplitude recorded in either PYR or IT cells. Note the logarithmic axis.

(C) Top left: recording schematic, showing grid of light spots. Top right: representative examples of light-evoked action potentials (APs) recorded in cell-attached mode from an NDNF+ st-ChroME+ cell stimulating over the soma. Blue bar shows light stimulation. Bottom: summary of the number of light-evoked APs per pixel as a function of distance from the soma for all NDNF+ st-ChroME+ cells recorded in cell-attached mode.

(D) Left: recording schematic, showing grid of light spots. Middle: normalized maps of NDNF-evoked IPSCs at PT cells are shown, indicating location of presynaptic cells. Triangles show soma depth of recorded cells. Individual pixels are 75 × 75 μm. Right: representative examples of NDNF-evoked IPSCs recorded in voltage-clamp at +10 mV across different layers. Light traces are individual trials, and dark traces are averages. Blue bar shows light stimulation.

(E) Left: summary of IPSC amplitude per pixel calculated by dividing the total current (in pA) per layer by the number of pixels, showing relative input strength across different layers for maps of NDNF+ connections onto PT cells. Right: similar for VIP+ connections onto L2/3 SOM+ cells. Values are mean ± SEM (C and E) or geometric mean ± 95% CI (B and F). *p < 0.05. See also Figure S5.
5E and S5). Consistent with the sublaminar distribution of NDNF+ and VIP+ cells in L1a and L1b, the ratio of L1a/L1b input was also higher for NDNF-evoked IPSCs at PT cells, recorded at +10 mV in the presence of TTX and 4-AP, indicating synapse location. Triangles show soma depth of recorded cells. Individual pixels are 75 × 75 μm. Right: representative examples of NDNF-evoked IPSCs at the different layers. Light traces are individual trials, and dark traces are averages. Blue bar shows light stimulation.

NDNF+ Cells Mediate Inhibitory Control over Pyramidal Cell Apical Dendrites

NDNF+ axons are particularly dense in L1, suggesting they may selectively innervate the distal apical dendrites of L5 pyramidal cells. The dendrites of PT and IT cells differ in complexity, with the more extensive arbors of the former potentially enabling more connections (Figures 6A and S6). To assess the dendritic location of NDNF+ synapses at pyramidal cells, we performed subcellular ChR2-assisted circuit mapping (sCRACM) in the presence of TTX (1 μM) and 4-AP (0.1 mM; Petreanu et al., 2009). We found NDNF-evoked IPSCs were biased onto the distal apical dendrites of PT cells (n = 7 cells, 3 mice; Figures 6B and 6C). Similar targeting was also observed at IT cells (n = 8 cells, 3 mice; Figures 6C and S6), suggesting that NDNF+ cells may play an important role in inhibiting apical dendrites.

To assess the influence of this targeting, we used high-frequency bursts of APs, which back-propagate into the dendrites to engage voltage-gated ion channels and generate dendritic Ca²⁺ signals (Larkum et al., 1999; Stuart and Sakmann, 1994; Stuart et al., 1997). Interneurons can strongly inhibit these Ca²⁺ signals (Chiu et al., 2013; Marlin and Carter, 2014; Palmer et al., 2012; Pérez-García et al., 2006), suggesting NDNF+ cells may also perform this role. To test this idea, we recorded in current-clamp from identified PT cells, filling them with the Ca²⁺ indicator Fluo-5F (0.5 mM; Figure 6D; Chalifoux and Carter, 2011). High-frequency trains of APs (3 APs @ 100 Hz) evoked an afterdepolarization (ADP) measured at the soma, which we confirmed by recording simultaneous Ca²⁺ signals in the distal apical and basal dendrites (Figures 6D and 6E). In interleaved trials, we activated NDNF+ inputs with 1-photon optogenetics while imaging Ca²⁺ with 2-photon microscopy. We found
NDNF+ inputs to the apical dendrites significantly reduced the ADP (fraction of baseline: apical = 0.69 ± 0.05, p = 0.0156; basal = 0.89 ± 0.05, p = 0.0625; n = 6 cells, 5 mice; Figure 6D) and completely blocked Ca$^{2+}$ signals (fraction of baseline: apical = 0.09 ± 0.04, p = 0.0156; basal = 0.98 ± 0.05, p = 0.8438; Figure 6E). However, NDNF inputs to the basal dendrites or apical obliques had minimal influence on either the ADP or Ca$^{2+}$ signals (Figures 6D, 6E, and S6). Similar results were observed in IT cells (n = 8 cells, 6 mice; Figures 6D, 6E, and S6), consistent with similarly prominent NDNF+ inputs to their apical dendrites. These experiments indicate that NDNF+ synapses are enriched at the apical dendrites of PT cells, where they strongly inhibit electrogensis and Ca$^{2+}$ signals.

**Selective Thalamic Input onto the Apical Dendrites of L5 Pyramidal Cells**

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By targeting the distal apical dendrites of PT cells, VM inputs may generate local electrogenic and \( \text{Ca}^{2+} \) signals. To test this idea, we next combined whole-cell recordings, optogenetics, and 2-photon \( \text{Ca}^{2+} \) imaging (Figure 7D). We found that VM stimulation over the apical dendrites of PT cells evoked both sub-threshold somatic excitatory postsynaptic potentials (EPSPs) (5.82 ± 1.09 mV; \( n = 12 \) cells, 10 mice) and \( \text{Ca}^{2+} \) signals in apical dendrites and spines (0.043 ± 0.01 \( \Delta \text{G}/\text{G}_{\text{sat}} \); Figures 7D and 7F). Consistent with the enrichment of VM input to the apical dendrites, \( \text{Ca}^{2+} \) signals were absent from the basal dendrites (Figures 7D and 7F). Furthermore, \( \text{Ca}^{2+} \) signals were much smaller in both the apical and basal dendrites of IT cells (\( n = 9 \) cells, 7 mice; Figures 7E and 7F). Importantly, simultaneous recordings from PYRs remained subthreshold, indicating these findings are not due to recurrent network activity, which would otherwise complicate our interpretation (Figure S7). Moreover, responses in PYRs were similar across recordings sessions for PT and IT cells, indicating results are not due to differences in the strength of stimulation between experiments (Figure S7). Instead, these findings indicate a unique functional role for VM input onto the distal apical dendrites of PT cells.

**Dendritic Excitation-Inhibition Balance Shapes PT Cell Activation**

Our results indicate that VM activates the apical dendrites of PT cells but also engages NDNF+ cells that inhibit the same dendrites. To assess this feedforward inhibition, we used dual-color optogenetics (Delevich et al., 2015), activating VM axons with or without suppression of NDNF+ cells. We injected AAV-ChR2 into VM, the fluorescently labeled retrograde tracer cholera toxin subunit B (CTB) into the periaqueductal gray (PAG), and AAV-FLEX-ArchT (Chow et al., 2010; Han et al., 2011) into PFC of NDNF-Cre mice (Figure 8A). Recording from NDNF+ cells, we found yellow light (590 nm for 200 ms) activated ArchT to cause a hyperpolarization that silenced APs evoked by either current injection or activation of VM inputs with blue light (473 nm; Figure 8B). Recording from PT cells, we observed VM-evoked EPSCs and IPSCs (\( n = 9 \) cells, 4 mice), with a strong bias toward inhibition (\( E/I \) ratio = 0.36; \( C_l = 0.17–0.73 \); Figures 8C and 8D). However, suppression of NDNF+ cells reduced VM-evoked IPSCs (fraction of control = 0.57 ± 0.05; \( p = 0.004 \); Figures 8C and 8E) without impacting EPSCs (fraction of control = 1.02 ± 0.06; \( p = 0.91 \); Figures 8D and 8E). These findings confirm that the VM-evoked inhibition of PT cells involves the activation of NDNF+ cells.

Lastly, to determine the functional impact of VM-evoked inhibition, we compared synaptic responses before and after bath application of GABA-R antagonists. Blocking inhibition significantly increased VM-evoked EPSPs (before drug = 118 ± 20 mV*m; after drug = 275 ± 54 mV*m; \( p = 0.0078 \); \( n = 8 \) cells, 7 mice) and dendritic \( \text{Ca}^{2+} \) signals (before drug = 2.5 ± 0.5 \( \Delta \text{G}/\text{G}_{\text{sat}} \)*ms; after drug = 4.6 ± 0.9 \( \Delta \text{G}/\text{G}_{\text{sat}} \)*ms; \( p = 0.016 \); Figures 8E and 8F). To approximate bursts of thalamic activity, we also examined the impact of inhibition on responses to stimulus trains of VM inputs (3 LED pulses @ 50 Hz). With intact inhibition, PT cells and PYRs displayed summatng but subthreshold EPSPs (Figure 8G); however, when inhibition was blocked, PT cells fired a barrage of APs (p-spike: before drug = 0.0 ± 0.0; after drug = 0.77 ± 0.15; \( p = 0.016 \); Figures 8G and 8H), with greatly increased dendritic \( \text{Ca}^{2+} \) signals (before drug = 5.0 ± 1.6 \( \Delta \text{G}/\text{G}_{\text{sat}} \)*ms; after drug = 28.5 ± 6.3 \( \Delta \text{G}/\text{G}_{\text{sat}} \)*ms; \( p = 0.0078 \); Figures 8G, 8H, and 8I). Importantly, these changes were not due to local network activity, as simultaneous recordings from PYRs remained subthreshold in all cases (Figures 8G, 8H, and 8I). These results indicate that inhibition both prevents VM inputs from firing PT cells and suppresses dendritic \( \text{Ca}^{2+} \) signals. Together, our findings characterize a monosynaptic input-output circuit between VM and PT cells, which is tightly regulated by strong thalamus-evoked inhibition mediated by NDNF+ cells in L1a.

**DISCUSSION**

Our findings demonstrate that two higher order thalamic nuclei innervate distinct populations of L1 interneurons in the PFC. MD primarily drives VIP+ cells in L1b, which in turn inhibit L2/3 SOM+ cells, engaging a local disinhibitory circuit. In contrast, VM engages NDNF+ cells in L1a, which then inhibit L2/3 PV+ cells, along with L2/3 and L5 pyramidal cells. By targeting the apical dendrites of PT cells, NDNF+ cells robustly inhibit VM-mediated activation of dendritic \( \text{Ca}^{2+} \) signals. NDNF+ cells therefore participate in both a thalamus-evoked inhibitory circuit and a separate, distinct disinhibitory circuit. Together, these results indicate that higher order thalamic inputs engage different inhibitory networks to have distinct functional influences on the PFC.

Thalamic nuclei are categorized in multiple ways, based on their cytology, inputs, and outputs (Jones, 1998; Kuramoto et al., 2017; Sherman and Guillery, 1999). In sensory cortex, first-order thalamic drives cells in L4 (Agnon and Connors, 1991; Cruikshank et al., 2010), whereas higher order thalamic influences networks in other layers (Audette et al., 2018; Crandall et al., 2017; Williams and Holtmaat, 2019). In the PFC, thalamic inputs exclusively arrive from a variety of higher order nuclei (Collins et al., 2018), with both MD and VM considered “secondary” based on their transcriptomic profiles (Phillips et al., 2019). However, these inputs have different axonal projections, with MD innervating L1b and L3 and VM arborizing in L1a. We previously found MD drives L2/3 pyramidal cells (Collins et al., 2018), and our new data indicate MD also activates L1b VIP+ cells. In contrast, VM engages NDNF+ cells in L1a, which in turn play a critical role in regulating the activity of L5 PT cells. These results highlight how synaptic connectivity provides an important additional metric for understanding the diversity of thalamic nuclei.

Recent whole-brain, rabies-tracing studies have not revealed major differences in thalamic innervation of interneurons in the PFC (Åhrlund-Richter et al., 2019; Sun et al., 2019), which differs from our results. One explanation for this discrepancy is that we focused on L1 interneurons, motivated by dense thalamic axon to this layer. In contrast, rabies tracing pools inputs to cells across layers and cannot account for the strength and dynamics of presynaptic inputs or the intrinsic physiology of postsynaptic cells. Our data show that the mechanisms behind differences in thalamic activation of L1 interneuron subtypes only emerge after voltage-clamp recordings to measure synaptic connections and current-clamp recordings to account for intrinsic membrane properties. For example, our rabies tracing data suggested both
Figure 8. NDNF+ Cells Control PT Dendritic Electrogenesis and Firing
(A) Schematic of injections of AAV-ChR2 into VM, AAV-FLEX-ArchT into PFC, and CTB-647 into PAG of NDNF-Cre mice.
(B) Left: recording schematic. Middle: NDNF+ cell firing evoked by current step in the absence (orange trace) and presence (gray trace) of yellow light to activate ArchT (590 nm, 200 ms) and hyperpolarize the NDNF+ cell. Right: similar for VM-evoked firing with blue light to activate ChR2 (473 nm).
(C) Left: recording schematic. Middle: VM-evoked IPSCs measured at +10 mV from PT cells, evoked with blue light to activate ChR2 in the absence (red) or presence (gray) of yellow light to activate ArchT, with black trace showing ArchT-only control. Right: summary of normalized VM-evoked IPSC amplitudes, without (red) and with (gray) the activation of ArchT.
(D) Similar to (C) for VM-evoked EPSCs at −60 mV, showing no effect.
(E) Left: schematic of recordings from PYR (gray) and PT cells (red), 1-photon stimulation of VM inputs (blue circles), and 2-photon line scans (dashed lines). Middle: VM-evoked EPSPs at PT cells before (red) and after (green) wash in of the GABA-R antagonists GZ and CGP. Right: corresponding VM-evoked Ca²⁺ signals evoked in the apical dendrites before and after GABA-R antagonists.
(F) Left: summary of VM-evoked EPSP integral before and after GABA-R antagonists. Right: similar for VM-evoked Ca²⁺ signals.
(G) Similar to (E) for trains of VM inputs (3 × 50 Hz), also showing PYR cells (gray).
(H) Left: summary of VM-evoked firing probability in PYR (gray) and PT (red) cells before and after GABA-R antagonists. Right: similar for VM-evoked Ca²⁺ signals at PT cells.
Values are mean ± SEM (C, D, F, and H). *p < 0.05. See also Figure S8.
MD and VM connect with VIP+ and NDNF+ interneurons and our voltage-clamp experiments show MD inputs are similar in strength at the two cells, but current-clamp recordings show MD preferentially activates VIP+ cells due to their higher intrinsic excitability. In contrast, VM inputs are much stronger at NDNF+ cells, which they selectively activate despite their lower intrinsic excitability. Together, our combined experimental approach demonstrates the importance of detailed circuit analysis considering the location of cells, postsynaptic physiology, and presynaptic dynamics.

Our results indicate that VM and MD engage two distinct classes of L1 interneurons in the PFC, which are segregated between L1a and L1b. Evidence from sensory cortices suggests that this sublaminar organization may be a general principle, with VIP+ cells consistently located further from the cortical surface (Abs et al., 2018; Schuman et al., 2019). Interestingly, MD targeting of VIP+ cells in PFC resembles activation of somatosensory VIP+ cells by inputs from the motor cortex and higher order thalamic nuclei (Lee et al., 2013; Williams and Holtmaat, 2019). VIP+ cells in turn inhibit SOM+ cells, a motif observed across cortex (Lee et al., 2013; Williams and Holtmaat, 2019), including the PFC (Pi et al., 2013). Thus, our results suggest that MD activates a conserved disinhibitory circuit, with the inhibition of SOM+ cells suppressing dendritic inhibition of pyramidal cells, potentially enabling dendritic electrogenesis and synaptic plasticity (Williams and Holtmaat, 2019). In contrast, VM strongly activates L1a NDNF+ cells, which in turn inhibit PV+ cells that synapse at the soma of pyramidal cells. A similar form of disinhibition via PV+ cells has been reported to drive a form of non-VIP-dependent plasticity during auditory learning (Lietzkes et al., 2011). Therefore, our results indicate that thalamic nuclei engage distinct inhibitory micro-circuits that can influence dendritic and somatic activity, with important implications for PFC function.

Although cortical interneurons are often divided into cardinal groups, there is substantial heterogeneity in each of these populations (Markram et al., 2004; Tremblay et al., 2016). For example, SOM+ cells are among the most diverse neurons in the cortex (Tasic et al., 2018), with different subtypes mediating distinct functions (Hilscher et al., 2017; Naka et al., 2019; Tremblay et al., 2016; Xu et al., 2013), and the GIN line we used labels dendrite-targeting Martinotti cells (Oliva et al., 2000). Consistent with our findings, there is also a high probability of connection from VIP+ cells to SOM+ cells in superficial layers of visual cortex (Pfeffer et al., 2013), although targeting in deep layers may be more diverse (Zhou et al., 2017). Similarly, the G42 line used here only labels a subset of PV+ cells (Chattopadhyaya et al., 2004), although the functional diversity of the PV+ population is currently poorly understood. VIP+ cells also consist of different subtypes based on expression of calretinin (CR) and cholecystokinin (CCK) (Acsády et al., 1996; Tremblay et al., 2016), which have distinct morphology and physiology (Acsády et al., 1996; He et al., 2016). Many of the L1b VIP+ cells we recorded matched the VIP+/CR+ subtype known to target other interneurons (Anastasiades et al., 2019), consistent with our output mapping of strong VIP+ → SOM+ connectivity. Lastly, NDNF+ cells comprise at least two subgroups, distinguished by expression of neuropeptide Y (NPY), with late-spiking cells enriched in the NPY+ population (Schuman et al., 2019). In the future, it will be important to further refine our understanding of how these different interneuron subtypes respond to thalamic inputs and communicate with each other and pyramidal cells in superficial layers of the PFC.

In addition to synapsing on interneurons, NDNF+ cells also strongly contact both L2/3 and L5 pyramidal cells, with particularly strong connections onto L5 PT cells. Interestingly, this biased inhibition has recently been observed for PV+, SOM+, and CCK+ inputs onto L5 pyramidal cells (Anastasiades et al., 2018b; Liu et al., 2020). The axons of NDNF+ cells primarily arborize within L1, making most of their connections onto the distal apical dendrites of PT cells. Optogenetic activation of NDNF+ cells strongly inhibits PT cell apical dendrites and suppresses the somatic ADP, as recently reported in sensory cortex (Abs et al., 2018). By measuring the spatial influence of this inhibition using 2-photon imaging, we further observed a complete block of local Ca2+ signals, which was highly restricted to the distal apical tuft. These findings complement our previous work on local GABAAR-mediated dendritic inhibition, identifying NDNF+ cells as a subpopulation of 5HT3AR+ cells that strongly inhibit AP-evoked Ca2+ signals in unlabeled pyramidal cells (Marlin and Carter, 2014). SOM+ cells also inhibit dendritic Ca2+ signals in pyramidal cells (Chiu et al., 2018b; Marlin and Carter, 2014) but are primarily activated by local connections (Kapfer et al., 2007). Interestingly, our results suggest that MD may relieve dendritic inhibition at pyramidal cells by activating VIP+ cells, suggesting contrasting roles for VM and MD in inhibitory control of dendritic electrogenesis.

In addition to activating interneurons, both MD and VM directly innervate the apical dendrites of PT cells, with weaker connections onto IT dendrites (Collins et al., 2018; Guo et al., 2018). This targeting appears specific to thalamic circuits, as it is not observed for callosal, hippocampal, or amygdala inputs (Anastasiades et al., 2018; Liu and Carter, 2018; A. Manouchehi and A.G.C., unpublished data). Consistent with this connectivity, VM readily triggers dendritic Ca2+ signals but only in PT cells and not IT cells, which reflects thalamic input rather than local network activity, because L2/3 pyramidal cells are quiescent. These dendritic Ca2+ signals are regulated by VM-evoked inhibition mediated by NDNF+ cells acting on GABA receptors. Although our ArchT experiments did not completely abolish VM-evoked IPSCs, the reduction is similar to related studies (Delevich et al., 2015; Lee et al., 2013). Incomplete suppression could be due to lateral inhibition between adjacent NDNF+ cells (Fan et al., 2020) or variable ArchT expression. Blocking GABA receptors enhances dendritic Ca2+ signals and somatic EPSPs, allowing bursts of VM inputs to elicit action potentials. These dendritic non-linearities may play an important role in amplifying VM input to selectively recruit PT cells (Larkum and Zhu, 2002; Stuart et al., 1997).

Our results indicate that VM activation of PT cells is tightly controlled by NDNF+ cells, suggesting these L1 interneurons can gate long-range feedback loops between thalamus and PFC. In principle, a variety of mechanisms could release this inhibition by regulating the activity of NDNF+ cells: first, depressing VM input or NDNF+ output, similar to feedforward inhibition mediated via PV+ cells in sensory cortex (Cruikshank et al., 2010); second, neuromodulation, including via selectively
expressed acetylcholine or noradrenergic receptors (Brombas et al., 2014; Salgado et al., 2011); third, local inhibition via SOM+ cells (Abs et al., 2018) or lateral inhibition via other NDNF+ cells (Fan et al., 2020); and fourth, long-range inputs from inhibitory regions, including the basal ganglia and basal forebrain (Saunders et al., 2015a, 2015b), which our rabies data show project to NDNF+ cells. In the future, it will be important to uncover which mechanisms are engaged and how they contribute to VM-evoked firing and dendritic Ca2+ spikes in PT cells.

Our experiments suggest multiple ways in which MD and VM differentially impact specific micro-circuits to shape activity in the PFC (Bolkan et al., 2017; Schmitt et al., 2017). MD input to L1b VIP+ cells may combine with input to L3 pyramidal cells (Collins et al., 2018) to maintain local excitation and support delay period activity (Bolkan et al., 2017; Schmitt et al., 2017). Similarly, MD input may also amplify cortical or basolateral amygdala inputs to superficial layers (Little and Carter, 2013; Schmitt et al., 2017), as suggested for higher order thalamic inputs to sensory cortex (Williams and Holtmaat, 2019; Zhang and Bruno, 2019). Activation of superficial networks also influences deep layers, including connections onto L5 PT cells that “close the loop” between PFC and thalamus (Collins et al., 2018). By targeting their dendrites, VM can also directly and selectively engage PT cells, which innervate numerous downstream targets (Collins et al., 2018; Economo et al., 2018). Consistent with our findings, a recent study from sensory cortex showed how activity within PT dendrites plays an essential role in regulating cortical function via increasing outputs to thalamus and other regions (Takahashi et al., 2020). Therefore, VM-evoked activation of PT cell dendrites has important implications for cognitive behaviors that depend upon communication between PFC, thalamus, and subcortical structures (Bolkan et al., 2017; Guo et al., 2017; Schmitt et al., 2017; Warden et al., 2012).

In summary, we have established that MD and VM have different influence on the PFC, depending on the presynaptic input, cortical sublayer, postsynaptic cell type, and subcellular compartment. Our findings add to a growing literature about how long-range inputs from the thalamus, cortex, amygdala, hippocampus, and claustrostrum differentially engage PFC micro-circuits (Anastasiades et al., 2018a; Cruikshank et al., 2012; Delevich et al., 2015; Jackson et al., 2018; Little and Carter, 2013; Liu and Carter, 2018; McGarry and Carter, 2016). Our observation that multiple thalamic nuclei engage L1 interneuron subtypes, which subsequently inhibit L2/3 interneurons, suggests an important role for thalamus in gating superficial layer pyramidal neurons. This includes a myriad of projection neurons, including cortico-cortical, cortico-amgydala, and cortico-striatal neurons, in addition to PT cells. Therefore, our findings suggest thalamus plays a central role in orchestrating activity across PFC networks that engage many downstream targets, albeit through distinct mechanisms to studies of thalamo-cortical networks in sensory cortices (Audette et al., 2018; Crandall et al., 2017; Cruikshank et al., 2010). In the future, it will be important to study how other thalamic inputs influence the PFC, including from the intralaminar nuclei (Mandelbaum et al., 2019) and nucleus reuniens (Banks et al., 2020). Similarly, it will be important to test whether the thalamic connectivity motifs observed in the PFC are present in other frontal cortices, including anterior lateral motor cortex (ALM) (Guo et al., 2018). Our advancing understanding of these networks will help understand how they contribute to complex cognitive tasks associated with higher order thalamo-cortical loops (Bolkan et al., 2017; Gao et al., 2018; Guo et al., 2017; Schmitt et al., 2017).

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead Contact
  - Materials Availability
  - Data and Code Availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Stereotaxic injections
  - Slice preparation
  - Electrophysiology
  - Optogenetics
  - Soma-restricted optogenetics
  - Two-photon microscopy
  - Rabies virus tracing
  - Histology
  - Data analysis
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.neuron.2020.10.031.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

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### KEY RESOURCES TABLE

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**Experimental Models: Organisms/Strains**

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| Mouse/NDNF-Cre   | Jackson Lab | RRID: IMSR_JAX:028536 |
| Mouse/Ai14       | Jackson Lab | RRID: IMSR_JAX:007908 |
| Mouse/VIP-Cre    | Jackson Lab | RRID: IMSR_JAX:010908 |
| Mouse/G42        | Jackson Lab | RRID: IMSR_JAX:007677 |
| Mouse/GIN        | Jackson Lab | RRID: IMSR_JAX:003718 |
| Mouse/5HT3aR-Cre | GENSAT | RRID: MMRRRC_036680-UCD |

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RESOURCES AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Adam Carter (adam.carter@nyu.edu).

Materials Availability
This study did not generate new unique reagents.

Data and Code Availability
All data and codes generated during this study are available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Acute slices were prepared from healthy, immune-competent P42-P90 wild-type mice, NDNF-Cre mice (Tasic et al., 2016), VIP-Cre mice (Taniguchi et al., 2011), SHT3a-R-Cre (Gong et al., 2003), VIP-Cre crossed to Ai14 reporter mice (Madisen et al., 2010), and NDNF-Cre or VIP-Cre crossed to G42 (Chattopadhyaya et al., 2004) or GIN (Oliva et al., 2000) interneuron reporter mice. Mice were bred on mixed background. No animals had been involved in previous procedures. Animals were group-housed with same-sex littersmates in a dedicated animal care facility and were on a 12-h light/dark cycle at 18-23°C. Food and water were available ad libitum. All physiology and anatomy experiments used male and female mice, and no significant differences were found between groups. All procedures followed guidelines approved by the New York University animal welfare committee.

METHOD DETAILS

No formal method for randomization was used and experimenters were not blind to experimental groups. No pre-test analyses were used to estimate sample sizes. No data were excluded from final analyses.

Stereotaxic injections
P28–P50 mice were anesthetized with a mixture of ketamine and xylazine or isoflurane and head fixed in a stereotax (Kopf Instruments). A small craniotomy was made over the injection site, through which retrograde tracers and viruses were injected. Injection site coordinates were relative to bregma (mediolateral, dorsoventral, and rostrocaudal axes): PFC = ± 0.3, −2.1, +2.2 mm (Allen mouse common coordinate framework (CCF): ± 0.3, 2.35, 2.03 mm); anterior MD thalamus = −0.4, −3.5, −0.4 mm (Allen mouse CCF: −0.7, 1.2, −1.17 mm); anterior VM thalamus = −2.9, −3.4, −0.4 mm, at an angle of 30° from upright (Allen mouse CCF: −1.0, 0.15, −1.17 mm), PAG = −0.5, −3.0, −4.0 mm (Allen mouse CCF: −0.5, 2.01, −4.07 mm). For examples of thalamus injection sites, see Figures S1A–S1C. Borosilicate pipettes with 5-10 μm tip diameters were backfilled and 100-500 nL was pressure-injected using a Nanoject II (Drummond) with 30–45 s inter-injection intervals. For retrograde labeling, pipettes were filled with red retrobeads (Lumafluor) or cholera toxin subunit B (CTB) conjugated to Alexa 647 (Thermo Fisher). Axon labeling was achieved using AAV1-CB7-mCherry (UPenn Vector Core) or AAV1-hSyn-EGFP (UPenn Vector Core). Interneuron labeling was achieved using AAV1-CAG-FLEX-tdTomato (UPenn Vector Core) or AAV9-CAG-FLEX-EFGP (UPenn Vector Core). Optogenetic stimulation was achieved using AAV1-hSyn-hChR2-EYFP (UPenn Vector Core), AAV1-CAMKIIa-hChR2-mCherry (UPenn Vector Core), AAV1-EF1a-DIO-hChR2-EYFP (UPenn Vector Core), AAV1-EF1a-dfox-hChR2-mCherry (Addgene), or AAV expressing soma-tagged ChroME AAV9-CAG-DIO-ChronosM140E-ST-p2A-H2B-mRuby (Addgene) (Mardinly et al., 2018). Optogenetic inhibition was achieved using AAV9-FLEX-ArchT-GFP (UNC Vector Core). For ArchT experiments two separate injections were performed. AAV1-CAMKIIa-hChR2-mCherry was injected into VM then 14-20 days later AAV9-FLEX-ArchT-GFP was injected and allowed to express for an additional 8-10 days. This was necessary to avoid blue light mediated activation of ArchT, which was found to occur at longer expression durations. Following injections, the pipette was left in place for an additional 10 min before being slowly withdrawn. After all injections, animals were returned to their home cages for 1-4 weeks before being used for experiments.

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Neuron 109, 314–330.e1–e4, January 20, 2021
Slice preparation
Adult mice (P42–P90) were anesthetized with an intraperitoneal injection of a lethal dose of ketamine/xylazine and perfused intracardially with an ice-cold cutting solution containing (in mM): 65 sucrose, 76 NaCl, 25 NaHCO3, 1.4 NaH2PO4, 25 glucose, 2.5 KCl, 7 MgCl2, 0.4 Na-aspartate, and 2 Na-pyruvate (bubbled with 95% O2/5% CO2). 300 μM coronal sections were cut in this solution and transferred to ACSF containing (in mM): 120 NaCl, 25 NaHCO3, 1.4 NaH2PO4, 21 glucose, 2.5 KCl, 2 CaCl2, 1 MgCl2, 0.4 Na-aspartate, and 2 Na-pyruvate (bubbled with 95% O2/5% CO2). Slices were recovered for 30 min at 35°C and stored for at least 30 min at 24°C. For thalamocortical optogenetics, the thalamus was also sectioned and inspected under a fluorescent dissection scope to confirm that ChR2 was targeted to MD or VM. If injections missed the target nucleus, slices were not recorded. In some cases, injection in VM led to ChR2 expression in adjacent nuclei such as VPL and VPM, but this was unlikely to influence our results, as labeling was often weak in these areas and our rabies tracing data suggest that input from these nuclei is at least an order of magnitude less than from VM (Table S2). With the exception of sCRACM and st-ChroME mapping experiments, which were performed at room temperature, recordings were conducted at 30-32°C.

Electrophysiology
Targeted whole-cell recordings were made from projection neurons or interneurons in the prelimbic PFC using infrared-differential interference contrast. Layers were defined by distance from the pial surface, as described previously (Anastasiades et al., 2019). L1a and L1b were distinguished by subdividing the depth of L1 at its midpoint, with the inner half L1b and outer half L1a. IT and PT neurons were identified by the presence of retrobeads or CTB. Targeted recordings from L1 interneurons were performed using Cre-dependent fluorophore after injection with AAVs or crossing to Ai14 reporter mice.

Optogenetics
Glutamate release was triggered by activating channelrhodopsin-2 (ChR2) present in presynaptic terminals of either thalamic inputs to the PFC, or local circuit interneurons as previously described (Anastasiades et al., 2018a; Little and Carter, 2012). ChR2 was activated with 1–8 ms pulses of 473 nm light from a blue light-emitting diode (LED; 473 nm; Thorlabs) through a 10X 0.3 NA objective (Olympus) with a power range of 0.1-20 mW. For widefield recordings in the PFC, the objective was always centered 200 μm from the pial surface of the cortex. For focused optogenetic stimulation over the apical or basal dendrites blue (473 nm) LED light was focused through a 60X 1.0NA objective (Olympus). For ArchT mediated suppression of activity this was interleaved with light from a yellow (590 nm) light focused through the same 60X 1.0NA objective. Subcellular targeting (sCRACM) experiments were performed using a Polygon DMD device (Mightex) focused through a 60X 1.0NA objective (Olympus) with pixel size calibrated to 75 μm. Pulses were delivered at 1 Hz using a pseudo-random 10 × 10 grid pattern, yielding an effective mapping area of 750 μm × 750 μm. Experiments used a 4 ms LED pulse yielding an effective power of 0.17 mW per pixel.

Soma-restricted optogenetics
To map the outputs of st-ChroME+ interneurons, stimulation parameters were developed to produce robust, spatially restricted AP firing of these cells. Expression time was tightly controlled (14–16 days post injection) to ensure reliable, yet spatially restricted, firing across the presynaptic population. Calibration recordings were performed in cell-attached mode to avoid perturbing the intracellular environment of the cell. A blue (473 nm) LED was passed through a Polygon DMD device (Mightex) and focused through a 10X 0.3 NA objective (Olympus) with pixel size calibrated to 75 μm. Pulses were delivered at 1 Hz using a pseudo-random 10 × 10 grid pattern, yielding an effective mapping area of 750 μm × 750 μm. Experiments used a 1 ms LED pulse yielding an effective power of 0.02 mW per pixel. Presynaptic APs occurred within 30 ms of LED onset and a postsynaptic IPSCs detection window was set to include responses 100 ms after LED onset to calculate the IPSC peak amplitude per pixel, as described previously (Anastasiades et al., 2018b).
Two-photon microscopy

Two-photon imaging was performed on a custom microscope, as previously described (Chalifoux and Carter, 2010; Marlin and Carter, 2014). Briefly, a Titanium:Sapphire laser (Coherent Ultra II) tuned to 810 nm was used to excite Alexa Fluor-594 or 488 and Fluo-5F to image dendrite morphology and monitor Ca²⁺ signals, respectively. Line scans were acquired across dendrites or dendrite-spine pairs at 500 Hz. Reference frame scans were routinely taken to correct for any spatial drifts over time at the imaging location. Ca²⁺ signals were quantified as the change in Fluo-5F fluorescence [green (G)] normalized to the Alexa Fluor 594 fluorescence [red (R)], giving units of ΔG/ΔR. These signals were then normalized to the Gsat/R value measured with a saturating concentration of Ca²⁺ added to the internal solution in a thin-walled pipette, giving final measurements in units of ΔG/Gsat. Recordings were discarded if an increase in baseline fluorescence was detected, which could indicate photo damage. Experiments testing the impact of optogenetic activation of NDNF+ interneurons on AP Ca²⁺ signals involved four interleaved trials: 1 = no APs + no LED, 2 = 3 x APs + no LED, 3 = no APs + LED, 4 = 3 x APs + LED. ChR2 activation (2ms light pulse) was followed after 10ms by 3 x 1ms, 1.5-3 nA somatic current steps delivered at 100 Hz to drive AP generation (Marlin and Carter, 2014). For experiments testing the dendritic Ca²⁺ response to VM axon stimulation, NBQX, CCP, GZ and CGP were added to the bath at the end of the experiment, and line scans were repeated to test for LED artifacts. All Imaging was performed with a 60X 1.0NA objective (Olympus). A fast shutter was closed for 25ms after light delivery to protect the PMTs during any experiments involving imaging during LED stimulation. Morphological reconstruction and analysis of 2-photon image stacks were by manually tracing over max projections of the reconstructed neurons.

Rabies virus tracing

For monosynaptic rabies virus tracing, AAV1-EF1α-FLEX-TVA-Cherry (130 nL) (UNC Vector Core) and AAV9-CAG-FLEX-oG (450 nL) (Salk) were injected into a single hemisphere of the PFC of either NDNF-Cre or VIP-Cre mice. After allowing 4-5 weeks for expression and analysis of 2-photon image stacks were by manually tracing over max projections of the reconstructed neurons.

Histology

Mice were anesthetized and perfused intracardially with 0.01 M PBS followed by 4% PFA. Brains were stored in 4% PFA for 12-18 hours at 4 °C before being washed three times in 0.01 M PBS. Slices were cut on a VT-1000S vibratome (Leica) at 75 μm thickness and placed on gel-coated glass slides. ProLong Gold anti-fade reagent with DAPI (Invitrogen) or VectaShield with DAPI (Vector Labs) was applied to the surface of the slices, which were then covered with a glass coverslip. Fluorescent images were taken on an Olympus VS120 microscope, using a 10X 0.25NA objective (Olympus) or on a Leica TCS SP8 confocal microscope, using either a 10X 0.4NA or 20X 0.75NA objective (Olympus).

Data analysis

Offline analysis was performed using Igor Pro (WaveMetrics). For current-clamp recordings, input resistance was measured using the steady-state response to a 500 ms, –10, –20 or –50 pA current injection. The membrane time constant (tau) was measured using exponential fits to these hyperpolarizations. Action potential latencies were measured as the time between onset of current injection and membrane voltage crossing 0 mV for spikes at rheobase. The ADP was calculated as the area under the curve of the voltage trace in the 25 ms following termination of the third AP in a burst of 3 APs delivered at 100 Hz. For voltage-clamp recordings, EPSC and IPSC amplitudes were measured as the average value across 1 ms around the peak response.

Cell counting was performed in ImageJ using the Cell Counter plugin. For cumulative frequency plots of L1 interneurons the depth of each cell was determined and normalized to the depth of L1, with the L1/2 border determined for each slice based on DAPI labeling. Axon distributions in PFC and thalamus were quantified using un-binned fluorescence. The average fluorescence profile for each slice was peak-normalized and multiple individual traces are shown. For rabies tracing labeled cell bodies were manually counted for every slice between rostro-caudal co-ordinates +2.2 to −5.0 relative to bregma. Presynaptic input regions were determined relative to the Allen Brain Atlas at the appropriate rostro-caudal co-ordinate. For cross laminar analysis of rabies labeled cells in the local circuit, the distance from the pial surface was used to sort cells into 25 μm bins for those cells assigned to the prelimbic subdivision of the mPFC. The number of cells per bin was averaged across 3 slices from each animal.

**Quantification and Statistical Analysis**

N values are reported within the Results and Supplemental Figure legends as number of recorded cells (for physiology) or number of animals (for anatomy). Summary data are reported in the text and figures as arithmetic mean ± SEM unless otherwise stated. Ratio data are displayed in figures on logarithmic axes and reported as geometric mean ± 95% confidence interval (CI). In some graphs with three or more traces, SEM waves are omitted for clarity. Statistical analyses were performed using Prism 7.0 (GraphPad Software). Hierarchical cluster analysis was performed in MATLAB (MathWorks). Comparisons between unpaired data were performed using two-tailed Mann-Whitney tests. Comparisons between data recorded in pairs were performed using two-tailed Wilcoxon matched-pairs signed rank tests. Ratio data were compared to a theoretical median of 1 using Wilcoxon signed rank tests. For rabies tracing, comparisons were performed using an unpaired t test correcting for multiple comparisons using the Holm-Sidak method. Significance was defined as p < 0.05.