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Development of VIP-SST Interneuron Associations in Mouse Neocortex and Entorhinal Cortex

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Abstract

Cortical networks depend upon inhibition through the neurotransmitter GABA to control and coordinate specific spatiotemporal circuit patterns, underlying the exquisite complexity of neural signaling. Disinhibition, a form of inhibition where inhibitory neurons inhibit other inhibitory cells, further aids in amplifying local neural processing in a selective, organized manner. A subset of GABAergic interneurons, vasoactive intestinal peptide-expressing (VIP) cells, preferentially inhibit somatostatin interneurons, which provide inhibitory input onto pyramidal cells, thus creating an archetypal circuit illustrating disinhibition in the cortex. The aim of this anatomical study was to investigate variations in GABAergic VIP synapses onto somatostatin-expressing inhibitory interneurons in mice at different developmental stages. Through a series of age-based and regional quantifications of somatic terminals and terminal distances, we sought to determine the inception of these associations and their distances from the nuclei. Our data show that although VIP terminal distances remained within a similar range as the animal developed, there was a significant change in the number of VIP terminal associations onto SST cells. Pups at the age of one week had far fewer terminals than older juvenile or adult mice. Among brain regions, neocortical cells had a greater number of terminals than entorhinal cells, but terminal distances generally overlapped in range. The results support previous research about the critical period of VIP interneuron genesis, while regional diversity in terminals may highlight how this crucial disinhibitory circuit functions differently in the neocortex and entorhinal cortex. These findings have broad implications for understanding the temporal patterns of inhibitory circuit development, which can act as

an important indicator of the differences between control animals and those expressing pathological conditions associated with dysfunctional inhibitory networks.

Introduction

Inhibition in the cortex plays a profound role in coordinating the activity that stimulates and constructs complex cognitive functions. Neocortical inhibition is promoted through a heterogeneous grouping of GABAergic interneurons that orchestrate signal processing. Precise, regulated interconnections between interneurons allow for modulating computations of the inhibitory circuits they participate in. (Pfeffer et al., 2013; Rossignol, 2011). Cortical inhibitory interneurons are integral components in altering the dynamic ranges of excitatory cells and the information processing capacity of neural circuits. Computations in the neocortex rely upon these interneurons to preserve the equilibrium between excitation and inhibition by countering uncontrolled excitation (Burkhalter, 2008; Pronneke et al., 2015; Rossignol, 2011; Rudy et al., 2011).

Most cortical neurons, approximately 80%, are excitatory principal cells, and use glutamate, an excitatory neurotransmitter. With pyramidal morphology, they respond to sensory stimuli and use extensive axonal projections to target both remote and nearby cells. These neurons reside across various cortical layers and are composed of many diverse subcategories (Harris and Mrsic-Flogel, 2013). GABAergic interneurons account for about 20% of all neurons in the cortex. These cells primarily establish associations between other neurons. Distinct from motor, sensory, or principal neurons, they send more local projections (Harris and Mrsic-Flogel, 2013; Kelsom and Lu, 2013). GABA, or γ-aminobutyric acid, from presynaptic cells acts upon postsynaptic membranes to

hyperpolarize them. Despite being a minority population of neurons, interneurons have considerable branching of their axons, allowing them to modulate all other neocortical neurons. Their inhibition dampens and regulates the effects of target cells when they respond to changes in excitation (Druga, 2009; Rossignol, 2011; Rudy et al., 2011).

Cortical networks depend upon inhibition through GABA to control neural signaling and create specific spatiotemporal patterns. While playing an important role in the control of plasticity and sensory receptive fields, they also synchronize firing within networks to generate particular rhythms in the cortex (Huang, Di Cristo, and Ango, 2007; Rossignol, 2011; Rudy et al., 2011). Through state-dependent gating, interneurons have a critical duty to regulate the flow of information across neocortical areas (Francavilla et al., 2015; Kelsom and Lu, 2013; Pfeffer, 2014). Dysfunction among interneurons and unreliable inhibitory networks can lead to serious pathologies. Evidence has linked imbalances between excitation and inhibition with autism, epilepsy, anxiety-based disorders, and schizophrenia (Rudy et al., 2011; Silberberg and Markram, 2007).

A particular form of neuronal control through inhibition, disinhibition, entails inhibitory neurons that inhibit other inhibitory cells. The suppression of these neurons allows for greater control over cortical processing, and heightens the gain of principal excitatory cells by releasing them from inhibition. Importantly, disinhibition provides for gain modulation and gating in the nervous system (Pi et al., 2013; Pronneke et al., 2015). In gain modulation, input information from more than one neuron is incorporated, and the sensitivity of the target neuron to one input neuron varies based upon the activity of the other.

With synaptic gating, gatekeeper neurons send gating signals that may disable the firing of a target neuron by activating inhibitory interneurons that synapse onto it, even though it may be receiving excitatory inputs from other cells. To close the gate, the inhibitory interneurons diminish any excitatory signals by altering the target axon's membrane conductance, and thereby selectively preventing the transmission of specific information. A significant example of such strengthening of local processing and selective inhibition through gating are pain pathways.

The mechanism of disinhibition guarantees the temporal precision of firing in principal cells. Excitatory input is relayed to interneurons and projection cells, where the interneurons facilitate feed-forward inhibition on the excitatory neurons after a brief delay. This gives the principal neurons a short opportunity to fire before they become inhibited. Controlled disinhibition assists in complex neural computations, but unspecific and maladaptive disinhibition is damaging and can cause various disorders. Some possible cognitive impairments include epilepsy, schizophrenia, autism, and intellectual disabilities (Letzkus, Wolff, and Luthi, 2015).

Disinhibition acts as intermediary in the balance between excitation and inhibition, but can also aid in learning, memory acquisition, and memory expression. Research on behaving animals shows that specific events promote the disinhibition of principal neurons to induce and enhance excitatory activity, leading to a memory trace. Work on spatial navigation and auditory fear learning demonstrate links to disinhibition as a prominent and maintained circuit mechanism in diverse brain regions, timescales, and types of projection neurons (Letzkus, Wolff, and Luthi, 2015).

Inhibitory interneurons in the cortex are diverse both physiologically and morphologically, and include cells that express parvalbumin, somatostatin, and 5HT3aR, with these three subgroups making up almost all of them. Research has shown that all three neurons types co-localize the inhibitory neurotransmitter GABA, resultant of their umbrella grouping as interneurons (Bayratkar et al., 1997; Bayratkar et al., 2000; David et al., 2007; Kawaguchi and Kubota, 1998; Kubota, Hattori, and Yui, 1994; Porter et al., 1998).

One of the largest subtypes of interneurons is the somatostatin positive population (SST), which also makes up about 30% of all neocortical interneurons. Most, but not all, are Martinotti cells and many reside in layer V, but are also found in layers II-VI. Their axons extend up to layer I and synapse onto principal neurons' tuft dendrites in that region, and axon collaterals project into nearby cortical columns as well (Rossignol, 2011). Another subset of somatostatin-expressing interneurons specifically target layer IV neurons (Kelsom and Lu, 2013; Pfeffer et al., 2013; Rudy et al., 2011).

As GABAergic cells, somatostatin neurons provide inhibitory inputs to the principal cells through that connection. This makes them ideal candidates to assist in regulating dendritic integration in pyramidal neurons, based on behavior. For example, in the visual cortex, somatostatin cells are activated by locomotion, which drives inhibition of pyramidal cells and allows for the control of top-down excitatory effects from those pyramidal cells. As a result, somatostatin neurons suppress exorbitant and repetitive excitation throughout networks, especially during states of high activity within the cortex. Somatostatin interneurons strongly inhibit many populations of cells, but never other somatostatin cells (Harris and Mrsic-Flogel, 2013; Rossignol, 2011).

Parvalbumin-positive neurons make up about 40% and include chandelier cells and fast spiking basket cells. The 5HT3aR group accounts for 30% of GABAergic interneurons and encompasses VIP-positive cells, a heterogeneous grouping of VIP-negative cells, of which a major group is Reelin-expressing neurons, and neurogliaform cells (Rudy et al., 2011). Among these populations of neurons, studies have proven that parvalbumin, somatostatin, and VIP-expressing interneurons are three non-overlapping, disparate groups. Comprehensively, inhibitory cells with these markers make up about 60% of all GABAergic neurons (Kawaguchi and Kubota, 1998; Xu, Roby, and Callaway, 2010).

Most interneurons arise from a structure in the ventral telencephalon called the ganglionic eminence, or GE, during embryonic brain development. The ganglionic eminence is comprised of lateral, medial, and caudal regions, termed LGE, MGE, and CGE, respectively, where different regions generate distinct subtypes of cortical interneurons (Druga, 2009; Kelsom and Lu, 2013). Interneurons; however, mainly develop in the MGE and CGE, as opposed to the LGE. The MGE generates about 80%, and therefore the majority, of all other interneuron types, including somatostatin-positive Martinotti cells and parvalbumin-positive basket cells (Fishell, 2007).

Approximately 20% of interneurons arise from the CGE, a number of which are bipolar, VIP-expressing cells. In addition, other CGE-derived interneurons contain bipolar calretinin neurons, and reelin-positive cells (Rudy et al., 2011). Around E12.5, rodent GABAergic interneurons begin migrating tangentially from the MGE and CGE to their eventual cortical layer destinations (Druga, 2009; Kelsom and Lu, 2013). As cells migrate, the primary source of interneurons in superficial cortical layers is the CGE, in contrast to the MGE (Rossignol, 2011).

Out of all caudal ganglionic eminence interneurons, 40% are VIP interneurons, which are found mostly in layers II and III. (Druga, 2009; Francavilla et al., 2015; Rudy et al., 2011). In the cortex, those VIP cells in layers II and III are about 60% of all VIP neurons, and the other 40% are distributed across varying layers. In terms of their electrophysiology and morphology, layer II/III VIP neurons are distinct from those resident in layers IV to VI, especially when considering axonal and dendritic spread. Dendrite arborization in layer II/III interneurons is limited to layers I, II, and III, while VIP cells in layers IV-VI extend dendrites through all cortical layers. Axonal branching deviates from this pattern with layer II/III axons reaching all layers, but axons from deeper layer neurons (IV-VI) restricted to only layers V and VI (Pronneke et al., 2015; Rossignol, 2011).

Although as interneurons, vasoactive intestinal peptide-expressing (VIP) cells associate with a variety of neurons, their primary inputs are on other interneurons, making them optimal candidates to specialize in the disinhibition of pyramidal cells (Francavilla et al., 2015; Kelsom and Lu, 2013; Pi et al., 2013; Rossignol, 2011; Rudy et al., 2011). Innumerable studies have shown that among those interneurons, VIP cells preferentially inhibit somatostatin interneurons. VIP to SST inhibition is transmitted to a much greater degree than the inhibition mediated by VIP neurons that synapse onto principal cells. These somatostatin interneurons provide inhibitory input onto principal cells in layer II/III and V (Francavilla et al., 2015; Pfeffer et al., 2013; Pronneke et al., 2015).

It has long been known that the neuropeptide VIP is expressed predominantly in the cerebral cortex and the hypothalamus. It is a crucial factor in cortical metabolic control, and adjusts cerebral blood flow, as well as promoting glycogenolysis. In addition, VIP interneurons are bipolar, and vertically oriented in the cortex (Druga, 2009; Ferron,

Siggins, and Bloom, 1985; Magistretti, 1990; Porter 1998). One of the more excitable populations of interneurons, they also have an irregular-spiking pattern and a high input resistance. Bitufted, calretinin positive interneurons are the most abundant subtype of VIP-labeled cells and send their axons down to deeper layers of the cortex. Calretinin negative VIP neurons are the second most common, but in contrast, their projections are far more widely arborized in nearby layers and in the deeper cortex (Kelsom and Lu, 2013; Kubota, Hattori, and Yui, 1994; Rossignol, 2011). Although they do not co-express parvalbumin or somatostatin, VIP neurons do also express 5HTa3 receptors, making up about 40% of all 5HTa3 neurons (Francavilla et al., 2015; Kelsom and Lu, 2013; Rossignol, 2011; Rudy et al., 2011).

Overall, the radial dendrites perpendicular to the pial surface, and strong vertical descent of VIP axons through many layers is excellent in helping to collect and integrate input both from different laminae and afferent networks (Francavilla et al., 2015). The bipolar morphology of the interneurons is possibly related to the columnar layout of the cortex as well, and might help to construct them in the developing brain. Cells in each vertical column stretching from the pia to the white matter share response patterns specific to a given stimulus and participate in inter- and intralaminar inhibition. This implies that they interact and relate to each other in a functional manner, in conjunction with residing in a similar locality (Bayratkar et al., 1997; Bayratkar et al., 2000; Druga, 2009; Staiger et al., 2004).

VIP interneurons facilitate neural signaling through disinhibitory circuits in all notable neocortical regions. In the visual cortex, VIP-labeled terminal boutons cluster around the somas of vertically oriented principal cells to inhibit them (Peters, 1990,

Pronneke et al., 2015). VIP cells also target parvalbumin-positive interneurons in the somatosensory cortex to a high degree. Parvalbumin neurons innervate pyramidal cells, so this circuit also influences and controls excitatory output. As a result of this disinhibition, the circuit allows for more precise responses to tactile stimuli by the principal neurons, a mechanism aided by the neruomodulatory impacts of VIP (David et al., 2007; Druga, 2009).

Another target of VIP cells in the somatosensory cortex are calbindin-immunoreactive interneurons found in layers II-VI. Studies describe functional synaptic connections between calbindin dendrites and somas and VIP-expressing axon terminals using electron and light microscopy. All interneurons containing the calcium binding protein calbindin are acted upon by VIP neurons, and calbindin cells provide inhibitory input onto pyramidal cells as their primary targets, specifically those in layers II, III, and V. This leads to a circuit that entails the disinhibition of the principle cells in an indirect manner. VIP neurons that co-localize with calretinin, a distinct calcium binding protein, provide abundant innervation to other VIP-expressing cells, and also to calbindin interneurons, just as they do without calretinin (Druga, 2009; Francavilla et al., 2015; Staiger et al., 2004).

Research using whole-cell patch-clamp recordings has established that within the cortex, pyramidal cells directly excite VIP-positive interneurons through glutamatergic inputs. Bolstered by its various synaptic targets, VIP modulates the activity and excitability of other cortical neurons, especially other interneurons, so this pathway affects critical functions in the brain. A wider perspective shows that principal cells from diverse cortical regions direct input onto excitatory cells in layers II and III; these pyramidal neurons further target VIP cells. As a result, VIP interneurons are ideally located in the neocortical

network to consolidate, modulate and regulate communication across the cortex. Despite this crucial role, they only constitute 1-3% of all neocortical cells (Druga, 2009; Francavilla et al., 2015; Porter et al., 1998; Rossignol, 2011).

In various studies, VIP neurons' disinhibition has been described as active during certain behavioral states, in many areas of the cortex. In the primary sensory cortex and visual cortex, VIP-positive cells had heightened activation during locomotion. Additionally, Pi *et al*. found that during discrimination tasks, VIP cells were recruited in the auditory cortex by both positive and negative reinforcement signals. Further, whisking increased the activity of VIP interneurons in the somatosensory cortex. Such observations show that the recruitment of VIP cells during distinct behavioral states further indicates their role in modulating the gating of information across the cortex. VIP activity only within specific conditions implies how such regulation allows for the control of neural disinhibition (Francavilla et al., 2015; Pronneke et al., 2015).

Whisking-related activity was studied by Lee *et al.* through an extensive excitatory protection transmitting from vM1, the motor cortex, to vS1, the somatosensory cortex. They recorded depolarization in diverse inhibitory neurons and principal cells, finding that VIP interneurons received the most intense excitation. Although parvalbumin and somatostatin neurons account for most GABAergic cortical cells, they had far smaller depolarizations, as compared to the recruitment of VIP. Following this circuit, they showed that only somatostatin interneurons received inhibition from VIP cells, and that the long-range projections from the motor cortex hyperpolarized vS1 somatostatin neurons. *In vivo*, mouse whisking lead to active VIP cells and inactive SST cells, while non-whisking periods

meant that VIP cells were silent and SST cells were active (Harris and Mrsic-Flogel, 2013; Lee et al., 2013; Pffeffer, 2014).

Pi *et al.*'s auditory cortex experiments also showed a circuit where VIP interneurons inhibit somatostatin and parvalbumin neurons, which in turn affect pyramidal cells. They found that both reward and punishment activated VIP interneurons, which went on to activate principal neurons, within an auditory discrimination task. Their work also exemplifies a neocortical microcircuit that proceeds through disinhibition when in certain behavioral states (Letzkus, Wolff, and Luthi, 2015; Pi et al., 2013). Further observations in the processing of incoming sensory afferents by VIP interneurons have been detailed by Staiger et al. Thalmocortical afferents associate onto VIP-immunoreactive cells in the primary somatosensory cortex, assisting in the regulation of this input through VIP circuits (Bayraktar et al., 2000).

Recent research in the rodent visual cortex revealed the role of VIP interneurons in relation to locomotion, in a form of sensory-motor integration. When running, mouse VIP cells were activated and somatostatin neuron activity was decreased, providing disinhibition onto pyramidal cells. Directly, locomotion inhibited somatostatin cells, and increased the responses of excitatory cells. As another example of behavioral state-dependent gain control, they showed that locomotion strengthens visual response through this disinhibitory circuit by activating nearby excitatory pyramidal neurons (Fu et al., 2014).

Another study by Letzkus *et al.* also focused on the auditory cortex, and how disinhibition relates to memory. During fear conditioning, principal neurons were disinhibited by foot-shocks, which increased their response to a tone that sounded

concurrently. Disinhibition here gated the plasticity at the cells affected by the tone, and helped in creating a memory trace that was preserved. They believe that both somatostatin and parvalbumin interneurons in this circuit were inhibited by VIP neurons, which allowed them to disinhibit pyramidal, projection neurons perisomatically (Letzkus, Wolff, and Luthi, 2015).

It is an undeniably accurate prediction to expect that interneurons that inhibit other interneurons, particularly as opposed to principal cells, will be disinhibitory towards principal cells (Pfeffer et al., 2013). As noted above, since VIP interneurons have an inclination to inhibit somatostatin cells over others, and as a result of somatostatin cells targeting the distal dendrites of pyramidal neurons, this creates an archetypal circuit illustrating disinhibition in the cortex. Therefore, by releasing pyramidal cells of their inhibition, this neural pathway based upon the feed-forward inhibition of SST interneurons by VIP interneurons, leads to a heightened gain of function in the realm of sensory information processing. Experimental reports show that axonal boutons of VIP cells are mostly found in layers II/III, and that a significant population of inhibitory interneurons in those layers are somatostatin positive, providing further proof of the legitimacy and operational merit of this circuit motif (Lee et al., 2013; Pronneke et al., 2015).

VIP neurons also form this circuit in the entorhinal cortex of the brain. Situated in caudal region of the temporal lobe, the entorhinal cortex acts as the principal intermediary between the neocortex and the hippocampus. It is organized into two major components called the lateral and medial entorhinal areas, LEA and MEA, respectively (van Groen, Miettinen, and Kadish, 2003). It works as a relay center for the hippocampus, allowing information in and out through circuits that extend between the cortex and the

hippocampus. As an overview, input from the cortex enters superficial layers of the entorhinal cortex. It is then projected to regions of the hippocampus, and the hippocampal output is received in deep layers of the entorhinal cortex. From there, it is transmitted back to cortical structures. Horizontal neurons in the entorhinal cortex are inhibitory, and VIP-expressing interneurons are found in the lateral entorhinal area. They traverse layers I and II, remaining mostly superficial, but have also been occasionally found in deeper layers (Canto, Wouterlood, and Witter, 2008).

The aim of this anatomical study was to investigate variations in GABAergic VIP synapses onto somatostatin-expressing inhibitory interneurons. Through a series of age-based and regional quantifications of somatic terminals and terminal distances, we sought to determine the inception of these associations and their distances from the nuclei. A cross between VIP-Cre mice and a tdTomato reporter line allowed for the expression and visualization of genetically labeled GABAergic VIP target neurons in the neocortex and the entorhinal cortex of mice aged 1 week, 1 month, and 3 months. Our results indicate the age around which synaptic connections between VIP and SST interneurons approach the expression pattern of the adult animal, confirming previous research about the critical period of interneuron genesis. Regional diversity in terminals may highlight how this crucial disinhibitory circuit functions differently in the neocortex and entorhinal cortex. Understanding interneuron development processes and timelines may help researchers design more effective drugs to combat the various neural pathologies that stem from impairments in the control of cortical processing through disinhibition.

Methods

Immunocytochemistry

This experiment utilized 7 VIP-Cre/tdTomato mice at varying ages. Three-month old adult mice (2), four-week old juvenile mice (2), and one-week old pups (3) were anesthetized with isofluorane. Following cervical dislocation, mice were transcardially perfused with PBS containing heparin (50µg/ml) followed by 4% paraformaldehyde in PBS. Brains were excised out and fixed in 4% paraformaldehyde overnight at 4°C. Brains were then washed three times in PBS for 10 minutes each at room temperature. Brains were sectioned using a vibratome (VT-1000S; Leica) into 80 µm coronal slices. 3 pup brains were embedded in agarose before similarly sectioning. Sections were separated into wells with PBS while maintaining their serial order. Blocking solution for the dilution of antibodies was prepared with 3% goat serum or 6% horse serum in PBS/0.1% Triton-X-100. Immunostaining of the sections was performed by incubation in rat anti-somatostatin (1:1000**,** Millipore) primary antibody for 48 hours at 4°C on a rocker. Tissue was washed with blocking solution twice for 10 minutes each, followed by a 1-hour wash at room temperature. Sections were then incubated in goat anti-488 (1:200**,** Invitrogen), an Alexa Fluor dye-conjugated secondary antibody, for 48 hours at 4°C on a rocker. Tissue was again washed with blocking solution twice for 10 minutes each, followed by a 1-hour wash at room temperature. Nuclear counterstaining was performed by adding Hoechst 33342 (1:1000, Invitrogen) to the 1-hour wash for the final 20 minutes. Additionally, to determine GABA immunoreactivity in cells, some sections were incubated in mouse anti-gephyrin (1:200**,** Synaptic Systems) primary antibody, followed by goat anti-mouse (1:400, Invitrogen) secondary antibody using the same time frames and blocking procedures

detailed above. Sections were then mounted onto glass slides, coverslipped using ProLong Gold Antifade reagent (Life Technologies) and allowed to dry.

Imaging

Fluorescent imaging was done on a Leica TCS SP8 confocal laser scan microscope. Sections were imaged at 10x for regional determination, then at 63x for identifying target neurons through tdTomato fluorescence and for capturing z-stack images at 0.4 μm. Additionally, some sections were further imaged using structured illumination microscopy.

Quantification

Terminal identification and quantification analysis was performed manually, and the co-localization of GABA with VIP terminals was determined visually. Distances in microns were measured from a centrally located point in the nucleus of a cell to each identified terminal. Maximal intensity projections of z-stacked images were used for analysis throughout.

Results

25 cells were analyzed at each developmental age (adults, juveniles, and pups) and for each brain region (neocortex and entorhinal cortex) at that age. The number of somatic terminals per each target cell was determined, and their varying distances from the nucleus were measured. Gephyrin staining was performed to confirm inhibitory synapses on target somatostatin cells and the terminals were seen to be co-localized with GABA (Figure 1). Distance value determinations are aggregates of all measured terminals within the range of

a single integer; for example, a value of 3 microns encompasses any terminal distances from 3.000 to 3.999 microns. Structured illumination microscopy was also used to examine the volume of adult terminals in the neocortex (Figure 2)

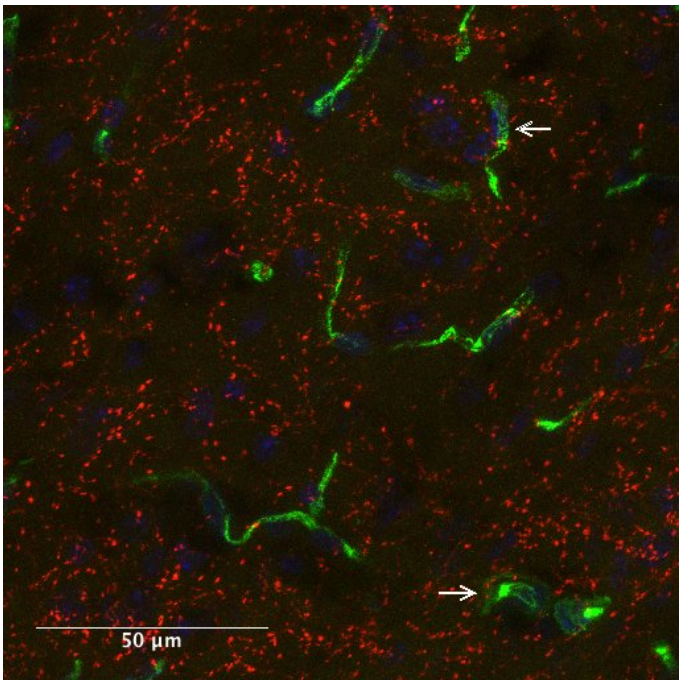


Figure 1: Co-localization of GABA staining with anti-gephyrin antibody (green) and VIP terminals (red) on adult somatostatin interneurons. Example target GABA-immunoreactive cells on upper and lower right in the confocal image.

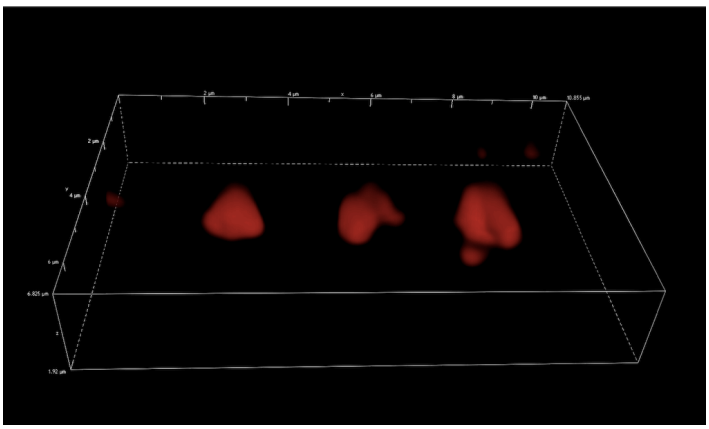
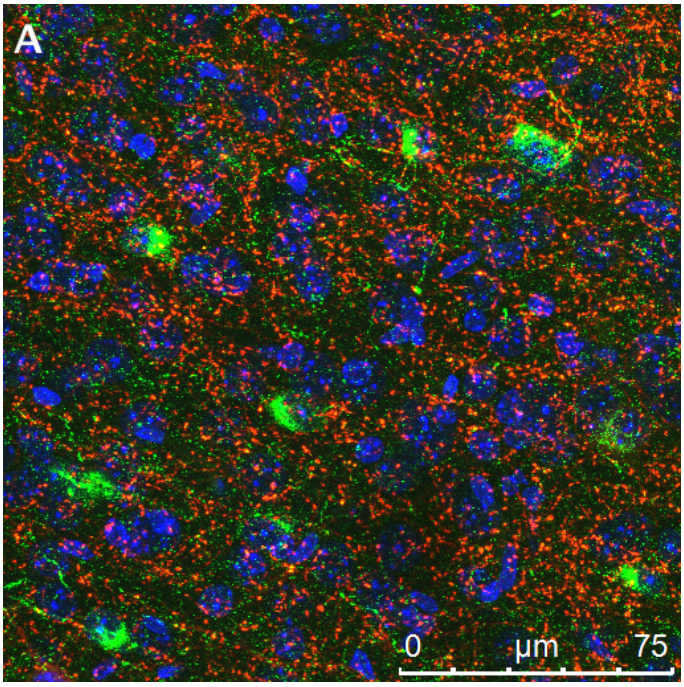


Figure 2: Structured illumination microscopy image of volume of VIP terminals in adult neocortex.

Neocortex

The adult and juvenile animals (Figure 3) shared a similar number of terminals in the neocortex. Their ranges, from 5 to 16 terminals, overlapped completely with each other. Most adult cells; however, had far fewer terminals, with a mode of 6, as compared to juveniles. The juvenile mode was 10 terminals, though 4 adult cells also had 10 terminals, the second highest terminal count for adult cells after the 5 cells that had 6. The pups had very few terminals (Figure 4) and their range just barely overlapped with the older animals, with the majority of cells not having any terminals at all. One pup cell had 5 terminals, but their terminal density was strongly skewed towards the lower end, with 13 cells that had none, within a range of 0 to 5 (Figure 5).



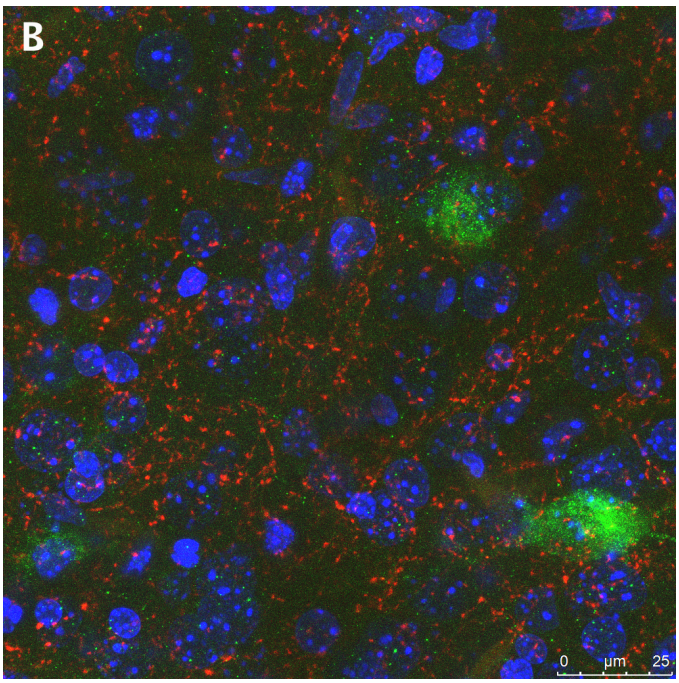


Figure 3: Adult neocortical cells (A), with VIP terminals (red) associating onto somatostatin neurons (green). 2 juvenile neocortical somatostatin cells (B) with VIP synapses.

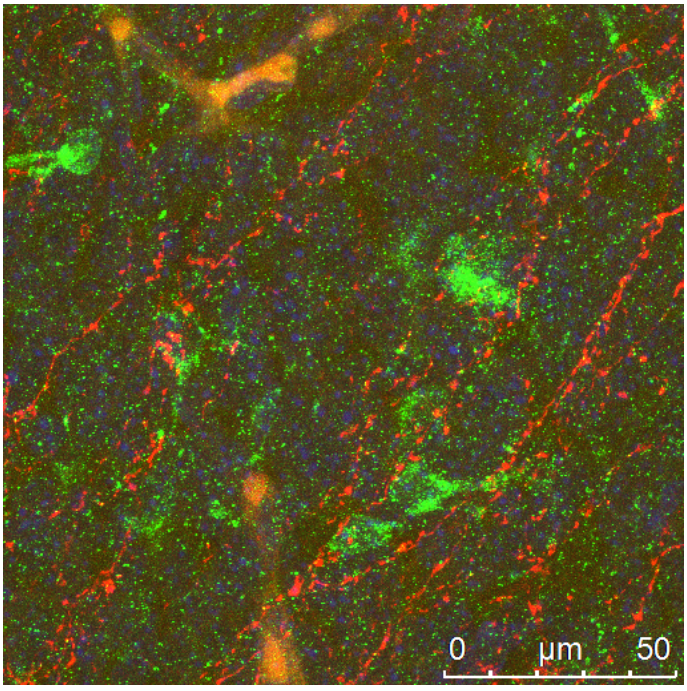


Figure 4: Pup neocortical cells. Note several somatostatin cells have no VIP innervation.

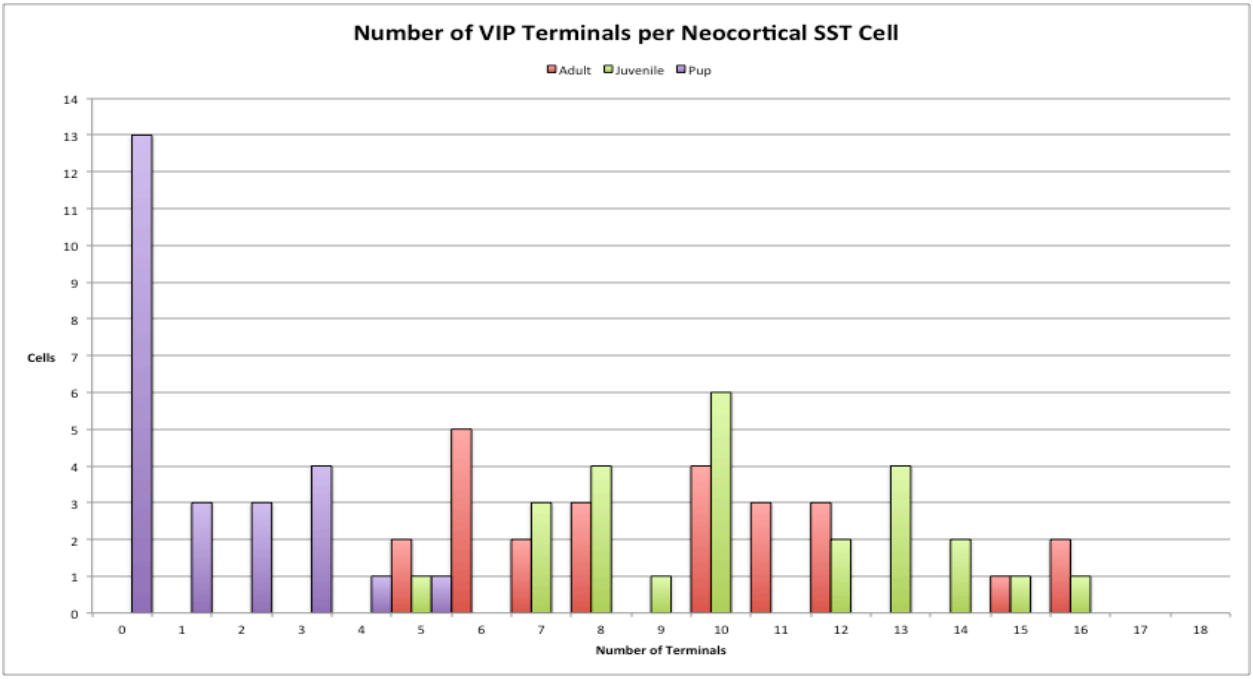


Figure 5: Number of VIP terminals per neocortical SST cell. Older animals show overlapped ranges, although juveniles had a higher mode value. Pups' cells had fewer terminals, and many had none.

Neocortical terminal distances had less variation across development in terms of both ranges and mode values. Adult terminals ranged from 0 to a high of 19 microns, but most terminals (45) were 5 microns away from the nucleus. Juvenile cells had terminals ranging from 1 to 21 microns, and 41 of them were 6 microns in distance. The pups' cells had fewer terminals overall, but among the cells that did, 9 of them were 7 microns away. The pups also had a narrower range of 3 to 12 microns (Figure 6).

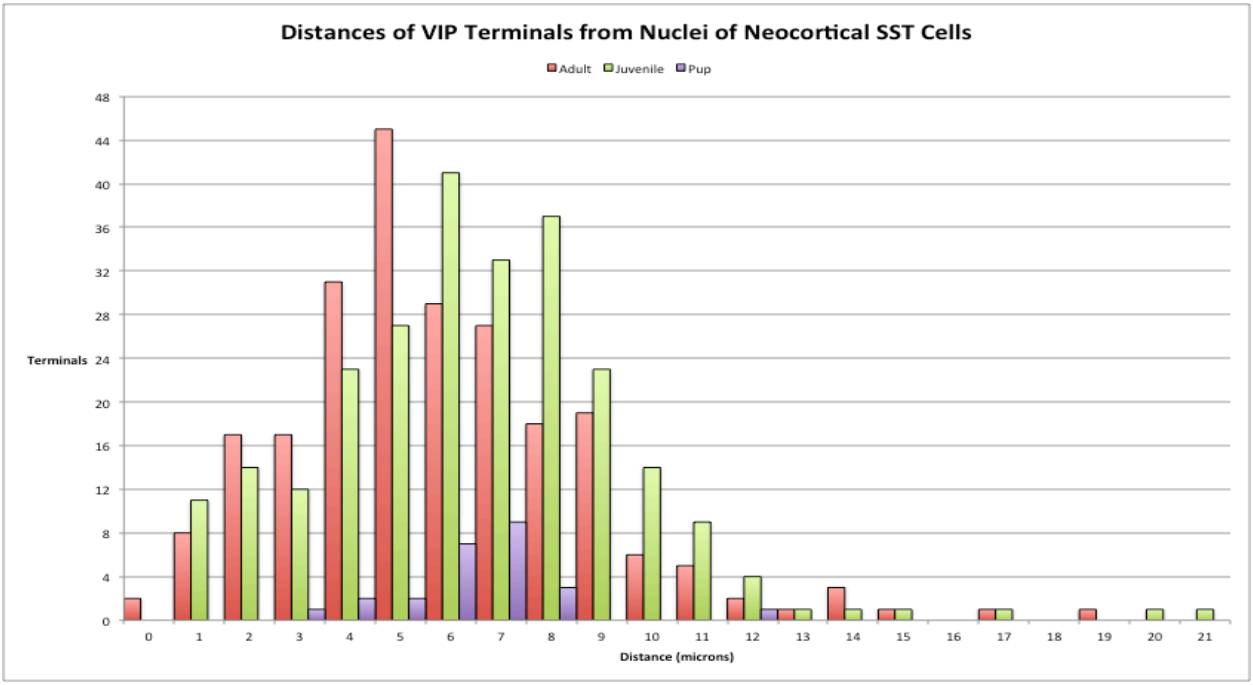


Figure 6: Distances of VIP terminals from nuclei of neocortical SST cells. Modes among all 3 developmental ages were similar, ranging from 5 to 7 microns.

Entorhinal Cortex

Adult mice had a much narrower range of terminal numbers in the entorhinal cortex, as compared to juveniles (Figure 7). Juvenile cells had 0 to 13, while adults had 2 to 9 terminals. The majority of juvenile cells did overlap with adult cells, despite the broader range. Most adult cells (6) had 5 terminals, and 5 juvenile cells each shared the modes of 6 and 8 terminals. Juvenile mice generally had cells with more terminals than adult cells. Pups again had far fewer terminals (Figure 8), with 19 cells having 0. They ranged from 0 to 3 terminals, so they did overlap with the ranges of adult and juvenile animals (Figure 9).

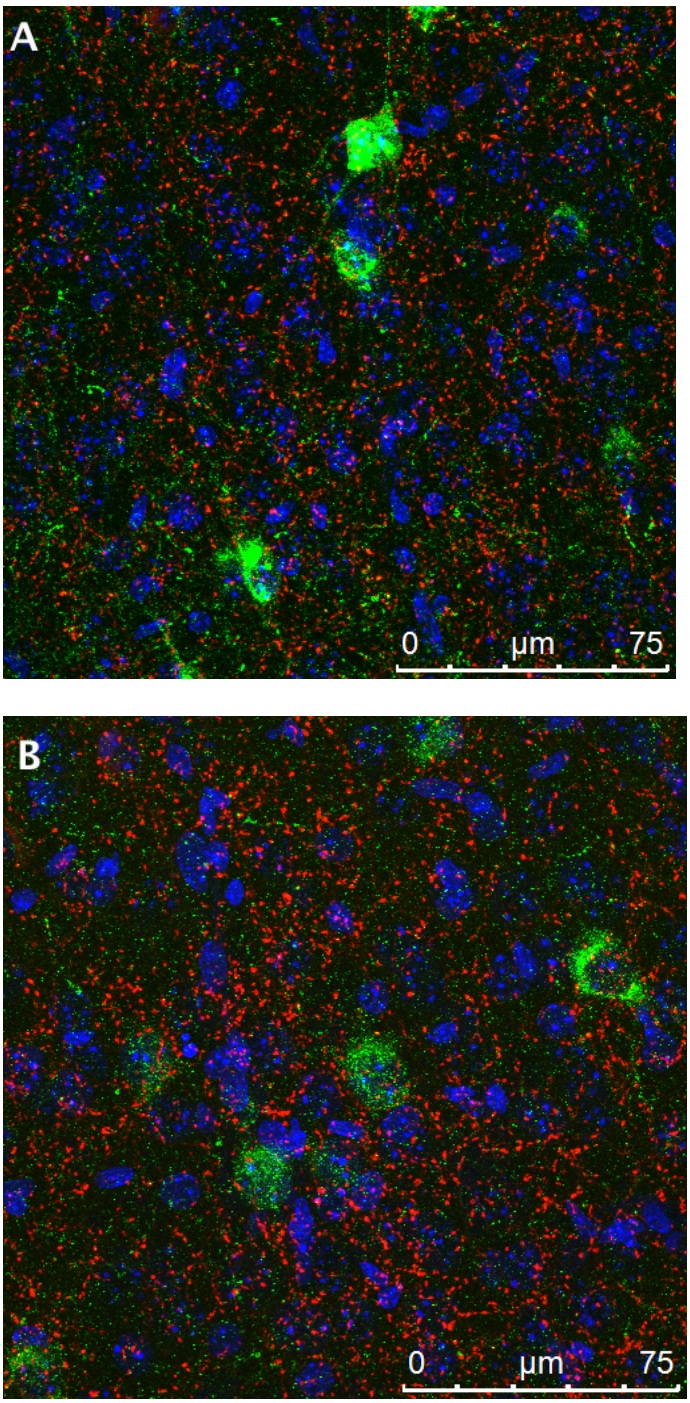


Figure 7: 3 Adult entorhinal cells (A), with VIP terminals (red) associating onto somatostatin neurons (green). Juvenile entorhinal somatostatin cells (B) with VIP synapses.

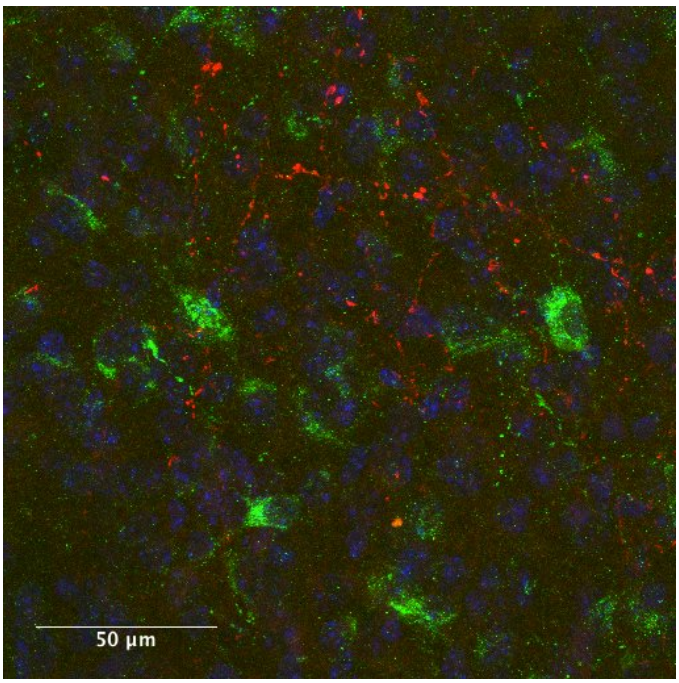


Figure 8: Pup entorhinal cells. Note several somatostatin neurons have no VIP innervation.

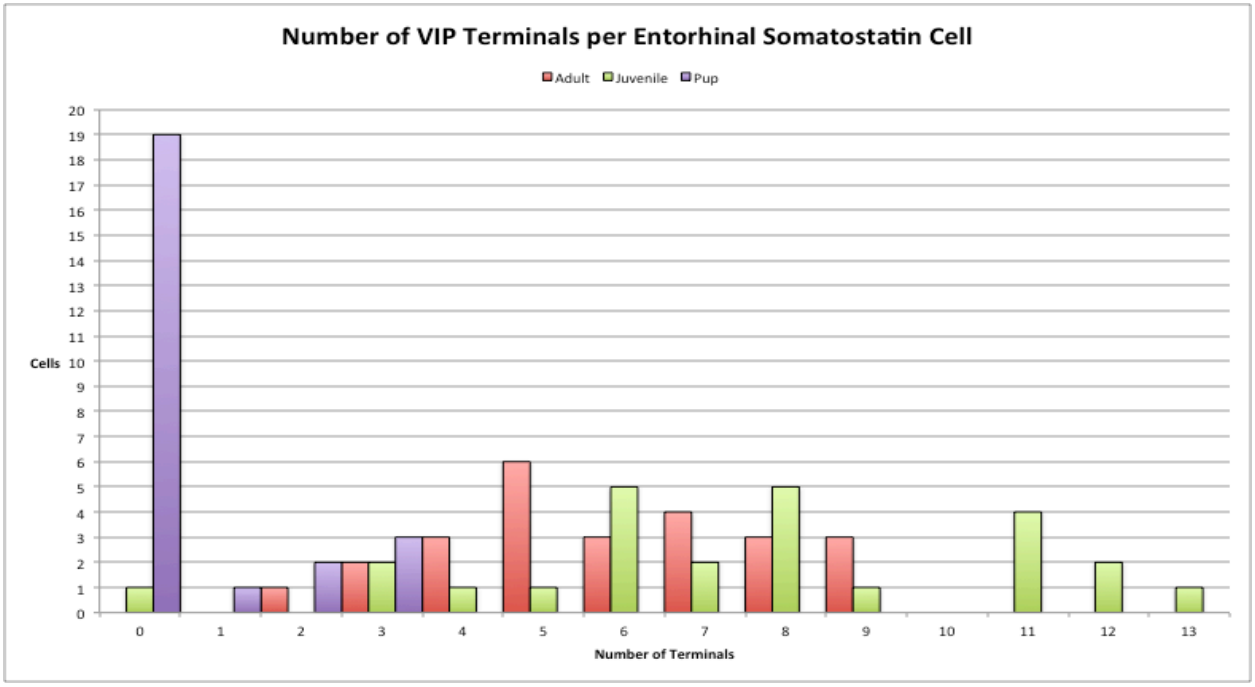


Figure 9: Number of VIP terminals per entorhinal SST cell. Adult animals had a narrower range, and juveniles had higher numbers of terminals comparatively. Most pup neurons had 0 terminals.

Across ages, VIP terminal distances had similar mode values. 30 adult cells each shared the modes of 4 and 5 microns, with a range between 1 and 11. Juvenile terminal distances were skewed much higher, with a broad range of 1 to 24, however, most cells had distances between 1 and 11, so there was a clear overlap between those two developmental stages. Most juvenile cells (30) had terminals 6 microns away from the nucleus. Pups had a range between 3 and 10 microns, and shared their mode of 6 microns with juvenile animals, with 5 cells (Figure 10).

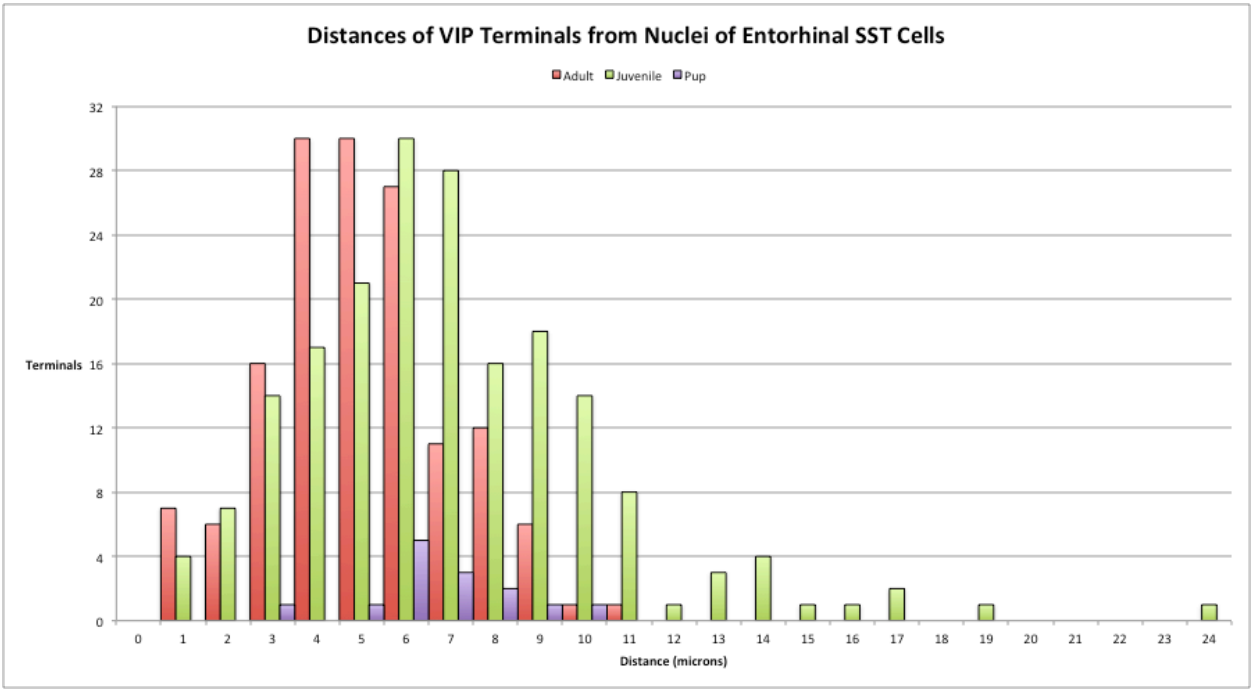
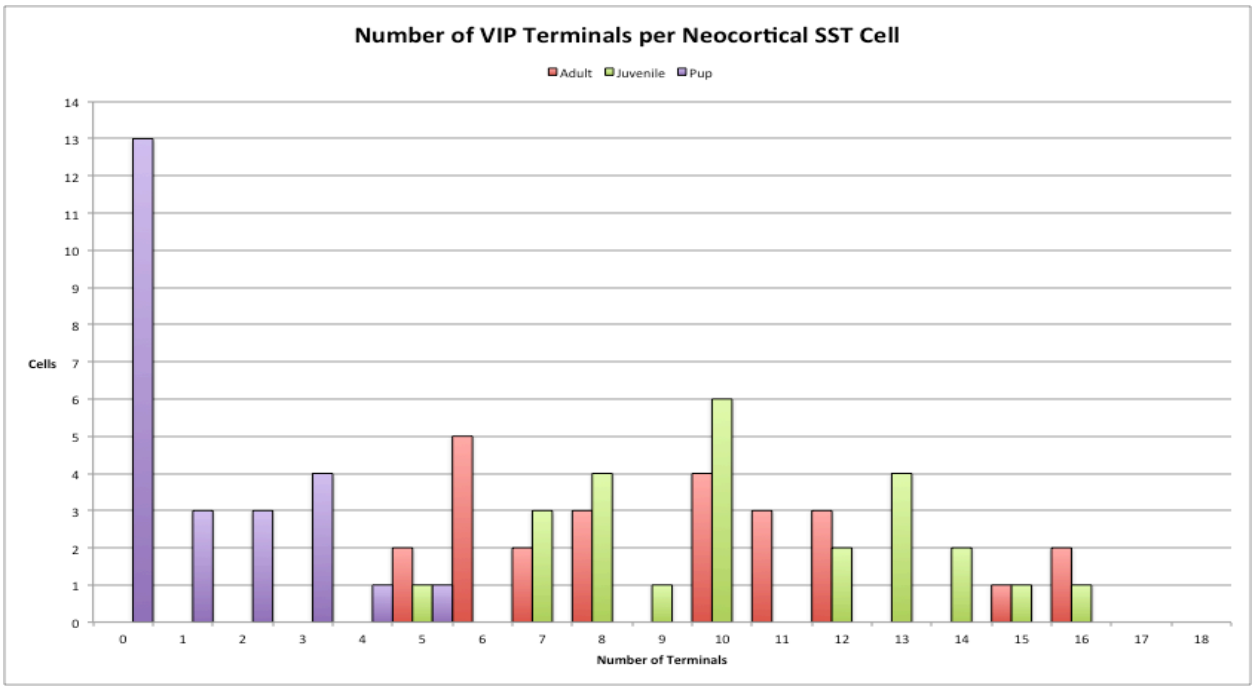


Figure 10: Distances of VIP terminals from nuclei of entorhinal SST cells. Terminal distances generally overlapped between adult and juvenile mice, and both juvenile and pup cells shared a mode of 6 microns.

Terminals

There were fewer terminals in the entorhinal cortex than in the neocortex across all ages (Figure 11). Both regions in the adult animals had similar numbers of terminals as their modes; 5 in the entorhinal cortex, and 6 in the neocortex. Overall, the neocortical

terminal numbers were skewed higher. Similarly, the juvenile animals had fewer terminals overall in the entorhinal cortex as compared to the neocortex, with one of the entorhinal cells having none. The mode in the neocortex was much higher at 10, than the shared modes of 6 and 8 in the entorhinal cortex. However, the ranges overlapped to a greater degree than they did in adults. Although pups had fewer terminals in both regions versus the older animals, the entorhinal cortex had 6 extra cells at zero terminals. The pups' ranges overlapped with the adult and juvenile mice in both brain areas, but continuing the trend seen in their cells, the neocortical cells had a higher number of terminals, with a maximum of 5 in comparison to a maximum of 3 in the entorhinal cortex.



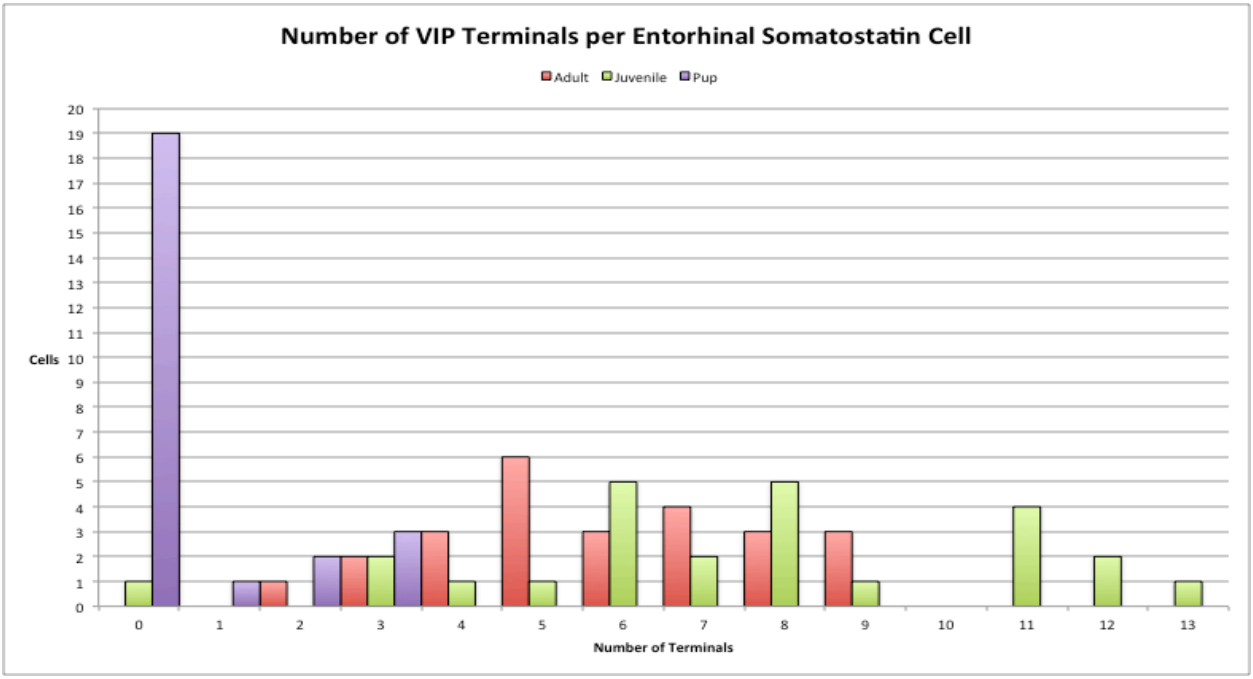


Figure 11: Terminals by age in the neocortex and entorhinal cortex. Fewer entorhinal terminals were seen at all developmental stages, in terms of modes and the overall skew of ranges.

Terminal Distances

The distances between regions didn't vary as dramatically as terminal numbers did and mostly overlapped (Figure 12). The adult mode in the neocortex was 5, and in the entorhinal cortex, it was shared mode of 4 and 5. There was a larger variation in neocortical distances, with the maximum extending up to 9 microns. However, the majority of distances clustered between 0 and 12, where the distances from both regions overlapped. The juvenile animals' brain areas showed a similar clustering and overlap between 1 and 14 microns, although the entorhinal cortex maximum was 24 microns. Both regions had a mode of 6 microns. The pups had terminals farther away as compared to adult and juvenile animals in both areas, with both minimums starting at 3 as opposed to 0 or 1. The two areas overlapped with each other, and the neocortical mode was 7, just

slightly higher than 6 in the entorhinal cortex. The modal terminal distances decreased by about 1 micron through increasing developmental stages.

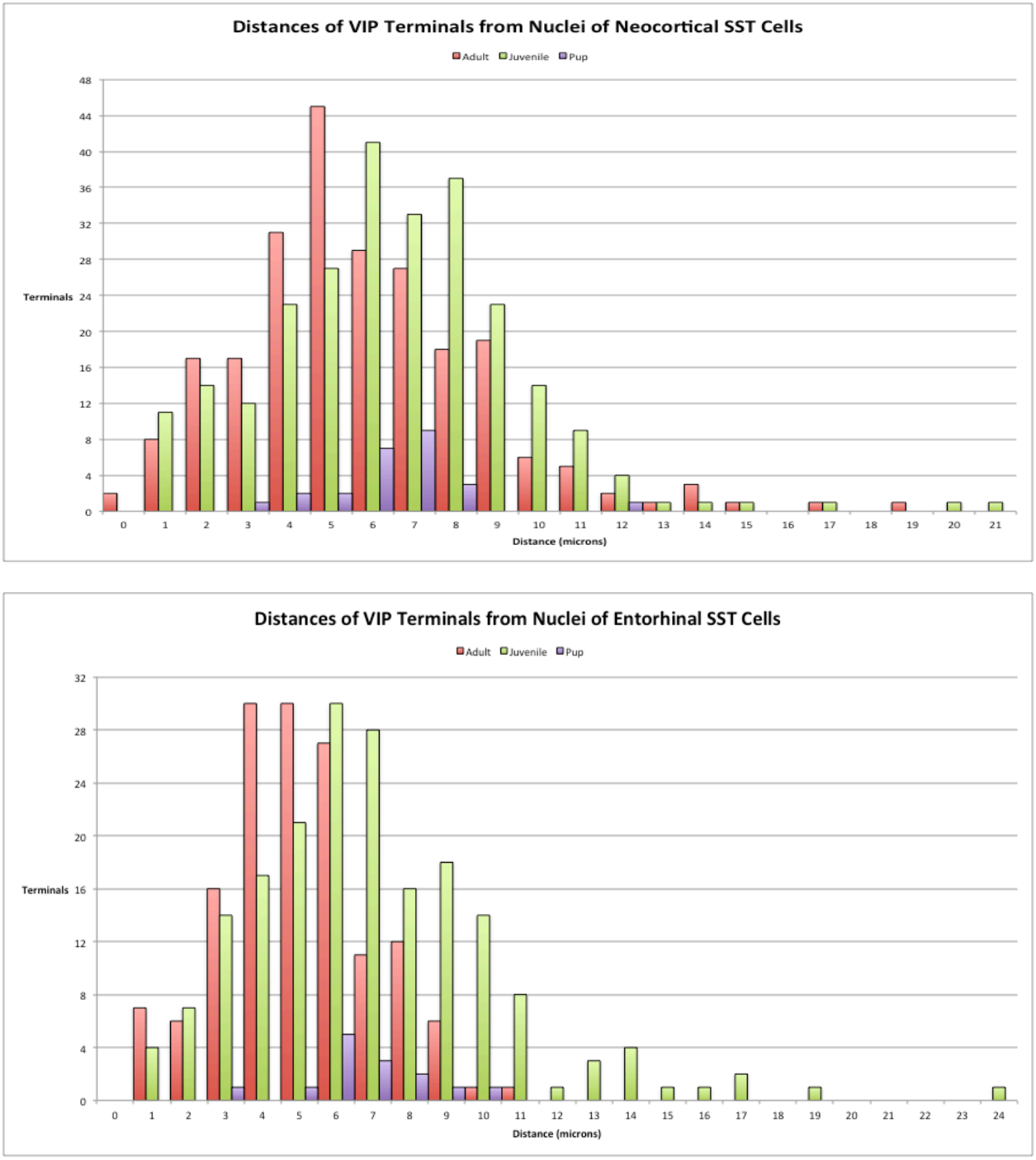


Figure 12: Neocortical and entorhinal terminal distances by age. Distances from nuclei were similar across ages and overlapped in range to a greater degree. Adults had the

lowest mode at 5 microns, then juveniles at 6, followed by pups at 7 microns from the nucleus.

Discussion

Neural inhibitory network construction is initiated during gestation, when GABAergic signaling begins. Inhibitory transmission assists in neuronal development, facilitating the morphogenesis of further GABAergic synapses by refining their growth and activity. This coupling between neurotransmitter activity and morphological maturation allows for precise temporal and spatial associations in a newly developing circuit. Often, there can be a gap between the birth of an interneuron and its maturation into forming working inhibitory circuits, usually extending into the juvenile period; activity-dependent maturation continues throughout the postnatal life of the animal into adolescence (Huang, Di Cristo, and Ango, 2007; Taniguchi et al., 2011).

The data in the present study show that although VIP terminal distances remained within a similar range as the animal developed, there was a significant change in the number of VIP terminal associations onto SST cells. Pups at the age of one week had far fewer terminals than older juvenile or adult mice. Among brain regions, neocortical cells had a greater number of terminals than entorhinal cells, but terminal distances generally overlapped in range.

Our experiments coincide with previous research regarding the growth of VIP interneurons. Although both neurons begin expression during the neonatal period, VIP cells are born and develop after neurons that express somatostatin. This difference in temporal origin may explain the presence of somatostatin interneurons in young pups, but very few VIP-labeled terminals, as well as the constant terminal distances based on mature SST cell bodies (Xu, de la Cruz, and Anderson, 2003). Our results also complement a study

by Taniguchi *et al.* who have observed that VIP interneuron generation occurs around P0 and after radial migration, their expression is widespread by P21 (Taniguchi et al., 2011). Our pups at P7 did not have VIP connections that matched the fully developed networks of juvenile (1 month) and adult (3 months) mice, suggesting that they were still migrating and hadn't formed lasting associations with SST interneurons.

Other concurrent observations have found that VIP interneurons develop into adult-like mature neurons by P12 in the occipital cortex (Hajos, Zilles, and Gallatz, 1990). Separate studies of VIP cells in the visual cortex show correlating temporal expression patterns. Evidence suggests that VIP cells can be seen in layers V and VI by P4-5, and migrate to more superficial layers like II and III as the animal develops. The researchers found that the neurons increased in number and displayed morphological maturation, such as growing arborization of processes through the second and third weeks of life. They attained a mature state by the time the individuals were 1 month old, with no ongoing extension in dendrites or growth in size past the fourth week (Gotz and Bolz, 1989; McDonald et al., 1982). This further corroborates our results, which show juvenile mice having reached the terminal association density of adults, as well as a reasonable implication that while pups at P7 may have somatic VIP expression, the neurons may not have migrated far or have extensive enough projections to form associations yet. In addition, diversity among the number of terminals between the neocortex and entorhinal cortex may be relevant to the differing functions of this circuit by region.

Understanding the critical period of circuit development can act as an important indicator of the differences between control animals and those with pathological conditions through comparisons. As previously described, numerous neurodevelopmental disorders

such as autism, anxiety, epilepsy, hypersensitivity, schizophrenia, and mental retardation have been associated with interruptions of inhibitory interneuron activity (Rossignol, 2011; Silberberg and Markram, 2007). It is possible that the proposed mechanism and timeline of VIP interneuron development and its associations with SST cells is malformed in such diseased states, illustrating the significance of distinguishing such networks. This may be particularly relevant to promoting the design of drugs that can more effectively resist the effects of neural impairments. To fully characterize and interpret the nuances of interneuron development, additional experiments are recommended as the future direction of this work.

References

Bayraktar, T., Staiger, J., Acsady, L., Cozzari, C., Freund, T., & Zilles, K. (1997). Co-localization of vasoactive intestinal polypeptide, γ-aminobutyric acid and choline acetyltransferase in neocortical interneurons of the adult rat. *Brain Research, 757*(2), 209-217.

Bayraktar, T., Welker, E., Freund, T. F., Zilles, K., & Staiger, J. F. (2000). Neurons immunoreactive for vasoactive intestinal polypeptide in the rat primary somatosensory cortex: Morphology and spatial relationship to barrel-related columns. *The Journal of Comparative Neurology J. Comp. Neurol., 420*(3), 291-304.

Burkhalter, A. H. (2008). Many specialists for suppressing cortical excitation. *Frontiers in Neuroscience, 2*(2), 155-167.

Canto, C. B., Wouterlood, F. G., & Witter, M. P. (2008). What Does the Anatomical Organization of the Entorhinal Cortex Tell Us? *Neural Plasticity,* 1-18.

Dávid, C., Schleicher, A., Zuschratter, W., & Staiger, J. F. (2007). The innervation of parvalbumin-containing interneurons by VIP-immunopositive interneurons in the primary somatosensory cortex of the adult rat. *European Journal of Neuroscience, 25*(8), 2329-2340.

Druga, R. (2009). Neocortical Inhibitory System. *Folia Biologica, 55*(6), 201-217.

Ferron, A., Siggins, G. R., & Bloom, F. E. (1985). Vasoactive intestinal polypeptide acts synergistically with norepinephrine to depress spontaneous discharge rate in cerebral cortical neurons. *Proceedings of the National Academy of Sciences, 82*(24), 8810-8812.

Francavilla, R., Luo, X., Magnin, E., Tyan, L., & Topolnik, L. (2015). Coordination of dendritic inhibition through local disinhibitory circuits. *Frontiers in Synaptic Neuroscience, 7*(5).

Fishell, G. (2007). Perspectives on the Developmental Origins of Cortical Interneuron Diversity. *Cortical Development: Genes and Genetic Abnormalities Novartis Foundation Symposia,* 21-44.

Fu, Y., Tucciarone, J., Espinosa, J., Sheng, N., Darcy, D., Nicoll, R., . . . Stryker, M. (2014). A Cortical Circuit for Gain Control by Behavioral State. *Cell, 156*(6), 1 139-1152.

Götz, M., & Bolz, J. (1989). Development of vasoactive intestinal polypeptide (VIP)-containing neurons in organotypic slice cultures from rat visual cortex. *Neuroscience Letters, 107*(1-3), 6-11.

Groen, T. V., Miettinen, P., & Kadish, I. (2003). The entorhinal cortex of the mouse:

Organization of the projection to the hippocampal formation. *Hippocampus, 13*(1), 133-149.

Hajos, F., Zilles, K., & Gallatz, K. (1990). Vasoactive intestinal polypeptide (VIP) containing cells in the developing rat occipital hemisphere. *Anat Embryol Anatomy and Embryology, 182*(1).

Harris, K. D., & Mrsic-Flogel, T. D. (2013). Cortical connectivity and sensory coding. *Nature, 503*(7474), 51-58.

Huang, Z. J., Cristo, G. D., & Ango, F. (2007). Development of GABA innervation in the cerebral and cerebellar cortices. *Nature Reviews Neuroscience, 8*(9), 673-686.

Kawaguchi, Y. (1997). GABAergic cell subtypes and their synaptic connections in rat frontal cortex. *Cerebral Cortex, 7*(6), 476-486.

Kelsom, C., & Lu, W. (2013). Development and specification of GABAergic cortical interneuron. *Cell Bioscience, 3*(19).

Kubota, Y., Hattori, R., & Yui, Y. (1994). Three distinct subpopulations of GABAergic neurons in rat frontal agranular cortex. *Brain Research, 649*(1-2), 159-173.

Lee, S., Kruglikov, I., Huang, Z. J., Fishell, G., & Rudy, B. (2013). A disinhibitory circuit mediates motor integration in the somatosensory cortex. *Nature Neuroscience, 16*, 1662-1670.

Letzkus, J., Wolff, S., & Lüthi, A. (2015). Disinhibition, a Circuit Mechanism for Associative Learning and Memory. *Neuron, 88*(2), 264-276.

Mcdonald, J. K., Parnavelas, J. G., Karamanlidis, A. N., & Brecha, N. (1982). The morphology and distribution of peptide-containing neurons in the adult and developing visual cortex of the rat. II. Vasoactive intestinal polypeptide. *J Neurocytol Journal of Neurocytology, 11*(5), 825-837.

Magistretti, P. J. (1990). VIP neurons in the cerebral cortex. *Trends in Pharmacological Sciences, 11*(6), 250-254.

Peters, A. (1990). The axon terminals of vasoactive intestinal polypeptide (VIP)-containing bipolar cells in rat visual cortex. *Journal of Neurocytology, 19*(5), 672-685.

Pfeffer, C. K., Xue, M., He, M., Huang, Z. J., & Scanziani, M. (2013). Inhibition of inhibition in visual cortex: The logic of connections between molecularly distinct interneurons. *Nature Neuroscience, 16*(8), 1068-1076.

Pfeffer, C. (2014). Inhibitory Neurons: Vip Cells Hit the Brake on Inhibition. *Current Biology, 24*(1).

Pi, H., Hangya, B., Kvitsiani, D., Sanders, J. I., Huang, Z. J., & Kepecs, A. (2013). Cortical interneurons that specialize in disinhibitory control. *Nature, 503*(7477), 521-524.

Porter, J. T., Cauli, B., Staiger, J. F., Lambolez, B., Rossier, J., & Audinat, E. (1998). Properties of bipolar VIPergic interneurons and their excitation by pyramidal neurons in the rat neocortex. *European Journal of Neuroscience, 10*(12), 3617-3628.

Prönneke, A., Scheuer, B., Wagener, R. J., Möck, M., Witte, M., & Staiger, J. F. (2015). Characterizing VIP Neurons in the Barrel Cortex of VIPcre/tdTomato Mice Reveals Layer-Specific Differences. *Cerebral Cortex, 25*(12), 4854-4868.

Rudy, B., Fishell, G., Lee, S., & Hjerling-Leffler, J. (2010). Three groups of interneurons account for nearly 100% of neocortical GABAergic neurons. *Developmental Neurobiology, 71*(1), 45-61.

Rossignol, E. (2011). Genetics and Function of Neocortical GABAergic Interneurons in Neurodevelopmental Disorders. *Neural Plasticity,* 1-25.

Silberberg, G., & Markram, H. (2007). Disynaptic Inhibition between Neocortical Pyramidal Cells Mediated by Martinotti Cells. *Neuron, 53*(5), 735-746.

Staiger, J. F., Masanneck, C., Schleicher, A., & Zuschratter, W. (2003). Calbindin-containing interneurons are a target for VIP-immunoreactive synapses in rat primary somatosensory cortex. *The Journal of Comparative Neurology J. Comp. Neurol., 468*(2), 179-189.

Taniguchi, H., He, M., Wu, P., Kim, S., Paik, R., Sugino, K., . . . Huang, Z. (2011). A Resource of Cre Driver Lines for Genetic Targeting of GABAergic Neurons in Cerebral Cortex. *Neuron, 71*(6), 995-1013.

Xu, Q. (2003). Cortical Interneuron Fate Determination: Diverse Sources for Distinct Subtypes? *Cerebral Cortex, 13*(6), 670-676.

Xu, X., Roby, K. D., & Callaway, E. M. (2010). Immunochemical characterization of inhibitory mouse cortical neurons: Three chemically distinct classes of inhibitory cells. *The Journal of Comparative Neurology J. Comp. Neurol., 518*(3), 389-404.