Synaptic Specificity and Plasticity in Parvalbumin-Basket Cell Circuits

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Synaptic specificity and plasticity in parvalbumin-basket cell circuits

A dissertation presented

by

Luke Joseph Bogart

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

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Synaptic specificity and plasticity in parvalbumin-basket cell circuits

Abstract

Inhibitory interneurons regulate experience-dependent plasticity across brain regions. Perisomatic inhibition by fast-spiking, parvalbumin-positive basket cells (PV-cells) is central to these processes, but which synapses are key remains unknown.

Here we show using immunohistochemistry that PV-input to pyramidal cells in layer 5 of primary visual cortex (V1) differs on a cell type-specific basis, with subcortically-projecting pyramidal cells more highly-innervated by PV-cells than callosally-projecting pyramidal cells. Surprisingly, the density of PV-inputs to either pyramidal cell-type was not changed by dark-rearing mice to adulthood, a classical manipulation that delays V1 plasticity. Instead, dark-rearing selectively reduced PV-inputs onto other PV-cells, which normally form highly-recurrent networks. To investigate the circuit-level basis of this plasticity, PV-cells in both normal and dark-reared mice were labeled by Brainbow. In both conditions, individual innervations of PV-cell bodies by PV-axons were mediated on average by just 2 boutons/axon, revealing that loss of inputs to dark-reared PV-cells results from decreased convergence within the PV-network.

On a molecular level, PV-cells sorted from dark-reared mice exhibited a reduction in GABA\textsubscript{A} receptor \(\alpha1\)-subunit mRNA, a marker of functionally-mature inhibition. In whole-cell recordings of dark-reared PV-cells \textit{in vitro}, spontaneous inhibitory postsynaptic currents (sIPSCs) were broader than in light-reared controls, an effect not seen in pyramidal cells in which \(\alpha1\)-level was unchanged. Optogenetics experiments revealed that PV-cell-mediated synaptic events exhibited broader currents selectively in PV-cells. Also, consistent with their loss
of PV-inputs, the frequency of sIPSCs received by dark-reared PV-cells was markedly lower than in light-reared controls, with sIPSC amplitude decreased as well.

We modeled the loss of fast, PV-inhibition onto PV-cells through conditional gene deletion of the α1-subunit from PV-cells. This did not physically disconnect PV-PV connections, but rather decreased the amplitude and broadened the decay of individual sIPSCs. α1-deletion alone was sufficient to extend plasticity in light-reared adult V1, implicating recurrent PV-inhibition in critical period regulation. Together, our results suggest that reorganization of this recurrent PV-cell network by early experience or gene mutation may contribute to aberrant plasticity and associated cognitive disorders more broadly.
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CHAPTER 1

Introduction
Critical periods of development

Development of the nervous system occurs along a protracted timeline that extends into early postnatal life (Hensch, 2004). As the nervous system undergoes periods of growth and maturation, an organism gains sensitivity to new stimuli in the environment as well as the ability to process these stimuli in novel ways. Interestingly, this enhanced sensitivity is the result of environmental influence on the nervous system’s own development, which adapts to respond optimally to the environmental features that are present. For example, neurons in the visual cortex of kittens are sensitive to the direction of motion of visual stimuli, a response property that matures over the first few weeks of life. In cats reared under a strobe light, however, this sensitivity fails to develop as motion stimuli are absent from the environment (Cynader & Chernenko, 1976). Strikingly, transfer of strobe-reared cats to a normal visual environment does not promote recovery of direction selectivity, suggesting that development of this response property is confined to early life.

These windows in time during which environmental experience has a heightened ability to impact an organism’s nervous system are called critical periods. Development is comprised of numerous critical periods, each of which corresponds to the structural and/or functional maturation of underlying brain systems (Hensch, 2004). Critical periods occur in a sequential fashion, with “lower” brain areas (i.e., those proximal to stimulus detection) maturing before “higher” areas (i.e., those further downstream). For example, the emergence of tonotopy in primary auditory cortex precedes language acquisition in humans, as maturation of upstream areas involved in the basic perception of sounds is needed before these sounds can be comprehended as language (Werker & Hensch, 2015). It is during these windows that synaptic connections important for proper function throughout life are fine-tuned. After the close of the
critical period these connections become resistant to change, even following changes in the environment (Hensch, 2004). As such, while critical periods are a time of progress in an organism’s life, they are also a time of vulnerability. For instance, natural experiments have shown that the loss of sensory input by chronic ear infections during the critical period can impair language acquisition in children (Werker & Hensch, 2015). Similarly, failure to correct either congenital cataracts or a “lazy” eye early in life can lead to amblyopia, permanent cortical blindness of visual input to an eye despite the eye itself being healthy (Hensch, 2004). These outcomes highlight the finite nature of the critical period, as well as the importance of receiving quality sensory experience during these key windows of brain development. However, the mechanisms controlling both the onset and closure of the critical period are incompletely understood. Elucidating these mechanisms will inform our understanding of how these processes can lead to negative outcomes, as well as generate insights into how these outcomes can be treated clinically.

While critical periods for both sensory and non-sensory systems have been documented (e.g., development of stress/anxiety; Meaney, 2001), many critical periods are modality-specific, reflecting the segregated flow of information from the periphery (Hensch, 2004). Due to the ease with which an organism’s environmental experience can be manipulated, sensory-based critical periods have emerged as tractable model systems in which to study the mechanisms of critical period regulation. Among these, the critical period for ocular dominance plasticity in primary visual cortex (V1) has been studied most intensively. As V1 was our system of choice for the experiments in this thesis, it is described below in detail.
Primary visual cortex and the critical period for ocular dominance plasticity

In a largely sequential fashion, structures early in the visual pathway like the retina and lateral geniculate nucleus of the thalamus form and refine their connections in response to spontaneously generated activity (Katz & Shatz, 1996). In contrast, the hallmark structural and functional organization of downstream V1 requires patterned visual experience in order to mature properly. In carnivores, eye-specific thalamocortical projections terminating in layer 4 (L4) of V1 initially generate overlapping axonal arborizations, giving rise to a preponderance of binocularly-driven cells early in life (LeVay et al., 1978). As development proceeds, competition between projections serving the two eyes leads to their segregation into alternating eye-specific patches, a process involving further axonal growth in some areas and pruning in others (Antonini & Stryker, 1993). This results in the formation of stereotyped ocular dominance (OD) columns, as well as more cells being monocularly-driven (LeVay et al., 1978). Notably, withholding visual experience by “dark-rearing” animals to adulthood delays plasticity until exposure to light, and results in incomplete segregation of OD columns (Mower et al., 1985). These and other findings identify a role specifically for patterned visual experience in driving these processes, as spontaneous activity, which persists in the dark, is insufficient for complete maturation.

V1 develops as described above when visual experience is “normal,” meaning that light-evoked activity from both eyes reaches cortex. On the functional level, this balanced input is exhibited across the population of V1 cells as roughly equal levels of responsiveness to stimulus presentations to the two eyes (i.e., neutral ocular dominance; Hubel & Wiesel, 1962). However, when one eye is deprived of vision from shortly after birth (“monocular deprivation,” MD) and the activity levels of these competing inputs becomes imbalanced, V1 undergoes a dramatic rearrangement both functionally and structurally (Hensch, 2004). Specifically, when the eye is
reopened and cellular responses to visual stimulation of both the open and previously-closed eye are compared, the ocular dominance profile of the recorded cells exhibits a marked shift in favor of the open eye (Wiesel & Hubel, 1963). This shift has anatomical correlates, as OD columns formed by open-eye inputs to V1 expand while those belonging to deprived-eye inputs shrink (Shatz & Stryker, 1978). Strikingly, these effects, termed ocular dominance plasticity, wane in magnitude when MD is begun later in life (Wiesel & Hubel, 1963). While virtually all cells shift to being driven exclusively by the open eye when cats are deprived from one week of age, the OD shift is less pronounced when deprivation is started at 9 weeks of age, and is absent following MD in adult cats. This lessening of plasticity with age and its disappearance after early postnatal life is the essence of the critical period.

Role of local cortical circuits in V1 plasticity

Interestingly, plasticity of thalamocortical projections does not account for the full range of OD plasticity-related phenomena observed in V1. For example, even after dark-rearing has frozen in place incompletely-segregated OD columns (Mower et al., 1985), subsequent monocular visual experience can bring about functional plasticity outside of L4, suggesting that targets downstream of thalamocortical terminals may mediate this plasticity. As such, interest in the role of local cortical circuits in critical period plasticity (Hensch, 2005) has grown immensely over the past 10-15 years, and is now the main focus of research into critical period mechanism (Maffei & Turrigiano, 2008; Takesian & Hensch, 2013). This shift towards investigating V1-intrinsic bases of plasticity has paralleled the field’s migration from cats to mice, which are more tractable for genetic manipulation. Crucially, although mice lack ocular dominance columns and show only limited structural plasticity of thalamocortical arbors upon MD (Antonini et al., 1999),
mice still exhibit functional plasticity following deprivation and do so in the context of a critical period (Gordon & Stryker, 1996). This critical period opens around postnatal day 21 (P21) and peaks at P28, then largely recedes by ~P35 (although residual plasticity remains until ~P60; Hensch, 2004).

Rising interest in the contribution of local V1 circuits to OD plasticity has meant reexamining the role of intracortical inhibition, which came to be thought of mainly as a plasticity-limiting factor (e.g., Kirkwood et al., 1995). However, earlier reports suggested that inhibitory signaling may sculpt OD as a receptive field property of V1 neurons. Recording in cats monocularly-deprived during the critical period, Duffy et al. (1976) found that blockade of inhibition via intravenous administration of bicuculline unmasks binocular responses in the majority of V1 neurons, suggesting that OD plasticity may involve the strengthening of inhibition selectively at sites of deprived-eye input. This result was confirmed using iontophoresis of γ-Aminobutyric acid (GABA) antagonist directly into V1, from which Sillito et al. (1981) concluded that the receptive field-altering inhibition may be mediated by circuits local to V1.

More recently, genetic disruption of GABAergic neurotransmission has provided strong evidence that inhibition is not only involved in critical period plasticity, but actively gates its expression (Hensch, 2005). Specifically, targeted gene deletion of the synaptically-localized isoform of the GABA synthetic enzyme glutamic acid decarboxylase 65 (GAD65-knockout (KO) mice) abolishes critical plasticity (Hensch et al., 1998). In the highly-decussated visual system of the mouse, cells in the binocular zone normally exhibit a strong response bias towards stimuli presented to the contralateral eye (Gordon & Stryker, 1996). While cells in critical period-aged wildtype (WT) mice show a shift towards greater ipsilateral eye responsiveness after
closure of the contralateral eye for 4 days (“brief MD”), GAD65-KO mice fail to demonstrate this shift. This finding suggests that without the capability for local, fast GABA synthesis by GAD65 to meet the demands of synaptic activity, the critical period fails to open due to compromised inhibitory transmission (Hensch et al., 1998). Indeed, augmenting inhibition by infusing diazepam – a positive allosteric modulator of type A GABA receptors (GABA$_A$Rs) – into V1 restores plasticity, allowing the OD profile in GAD65-KO mice to shift in response to MD. Diazepam infusion was later shown to initiate a critical period much like the one that occurs endogenously in WT mice, in that it is a singular event of finite length (Fagiolini & Hensch, 2000). Remarkably, diazepam infusion in P15 WT mice initiates an early critical period just days after eye-opening (~P12), as revealed by MD from P16-P20 (“early MD”; ibid). Together, these findings suggest that the critical period for OD plasticity is gated by the maturation of inhibition. Further, as pharmacological enhancement of inhibition permits precocious plasticity, critical period opening in V1 may simply be “waiting” for inhibition to mature. Indeed, overexpression of brain-derived neurotrophic factor (BDNF), a molecule regulating inhibitory maturation (Rutherford et al., 1997), leads to both precocious strengthening of inhibition and earlier onset of OD plasticity (Huang et al., 1999).
Figure 1.1 = The neocortical inhibitory system is characterized by subcellular domain-specific innervation of target cells by interneurons. Postsynaptic cells, such as the central pyramidal cell (green), express in a synapse-specific manner GABA_ARs with different alpha subunits (e.g., α1, α2), which are recruited on the basis of presynaptic cell-type. ais, axon initial segment; CRC, Cajal-Retzius cell; CCK, cholecystokinin-positive cell; Ch, chandelier cell; DB, double bouquet cell; PV, parvalbumin-positive cell; M, Martinotti cell; Ng, neurogliaform cell. (adapted from Hensch, 2005)
Following the discovery that inhibitory maturation gates critical period onset, the next step was to investigate this phenomenon on the circuit-level. While overall inhibitory tone was potentially the trigger, the high level of diversity within the population of GABAergic interneurons (Markram et al., 2004) suggested that certain inhibitory circuits may be key. Specifically, if synapses containing particular $\text{GABA}_A$-R-subunits (Figure 1.1) could be identified as mediating critical period onset, then the cells forming these synapses could be identified as the source of the key inhibition. Taking advantage of this organization, subunit-specific transgenic lines containing mutated benzodiazepine binding-sites were employed (Fagiolini et al., 2004), with the idea that mutation of the key subunit would block diazepam-mediated precocious plasticity. Indeed, through elimination it was found that diazepam was able to facilitate an OD shift with early MD in all mutants but the $\alpha_1$-subunit-specific mutant line, suggesting that inhibitory transmission at $\alpha_1$-subunit-containing synapses is the trigger for critical period onset.

Knowledge of the cellular localization of various $\alpha$-subunits was then able to suggest which subtype of inhibitory interneuron is associated with $\alpha_1$-subunit-containing $\text{GABA}_A$Rs (Figure 1.1). Specifically, it had been shown in the hippocampus via immuno-EM that pyramidal cell somata enrich their $\text{GABA}_A$Rs with certain alpha subunits depending on the type of cell forming the synapse (Klausberger et al., 2002). Specifically, somatic synapses formed by boutons expressing the calcium-binding protein parvalbumin (PV) show a greater than 3-fold higher level of $\alpha_1$-subunit than those formed by PV-negative boutons, even though $\text{GABA}_A$R number and synapse size are equivalent in each case. The $\alpha_2$-subunit, in contrast, is enriched 5-fold at PV-negative somatic synapses and is rarely found at nearby PV+ synapses (Nyiri et al., 2001). Given that pyramidal cells only receive somatic inhibitory contacts from cells that express either PV or the neuropeptides cholecystokinin (CCK) and vasoactive intestinal polypeptide
(VIP), these PV-negative synapses containing the α2-subunit are highly likely to be formed by CCK+/VIP+ cells (ibid). Paired recordings between interneurons and pyramidal cells have since extended this finding to rat somatosensory cortex (S1; Ali & Thomson, 2008). Specifically, application of the GABA\(_A\)R allosteric modulator zolpidem, which has a higher affinity for α1-subunit-containing GABA\(_A\)R than those containing α2/3-subunits (Smith et al., 2001), boosts PV+ cell-mediated IPSPs substantially more than those from CCK+ cells.

Overall, these findings suggest that PV+ interneurons, through their inhibitory transmission via α1-subunit-containing GABA\(_A\)Rs, are responsible for gating the critical period. The PV+ class of interneurons is comprised largely of basket cells, a morphological subtype that powerfully inhibits the somata of their target cells (Markram et al., 2004). Reviewing what is known about these cells and their location in cortical microcircuits will provide context for understanding their putative role in plasticity.
**Figure 1.2** = (a) Cajal’s original drawing of the pericellular basket surrounding a pyramidal cell body, as seen in a Golgi-stained sample of human infant cerebral cortex. (b) Reconstruction of a basket cell in superficial cat V1, as viewed from the cortical surface. The soma and dendrites are shown in green and the axon is shown in black, with the cell bodies of PV+ basket cells postsynaptic to the reconstructed axon labeled in red. Scale bar, 500µm. (a, adapted from Cajal, 1899, via Marin-Padilla, 1974; b, adapted from Kisvárday et al., 1993)
Basket cells, a classical cell-type

Basket cells were first identified in the cerebellum by Ramón y Cajal (1888), and were then characterized extensively in the neocortex by Marin-Padilla (1969) using Golgi-stained tissue from human infants. Baskets, for which basket cells are named, are dense meshworks around cell bodies that are formed by the convergence of bouton-rich axonal segments originating from numerous distinct basket cells (Figure 1.2.a). These stellate cells are found throughout layers 2/3 to 6, and have dendrites that are long, straight, and which branch close to the soma if at all. Basket cells are distinguished by their axon, which branches upon leaving the cell body to give rise to multiple collaterals that project horizontally for distances of up to 1mm or more (Somogyi et al., 1983). Finer, vertically-oriented segments arise from these, which either terminate as part of a basket or contribute en passant boutons to baskets as they continue through the neuropil and across layers to contact multiple cells. Overall, an individual basket cell is thought to contact hundreds of pyramidal cells and dozens of other basket cells (Wang et al., 2002), forming a highly-interconnected recurrent inhibitory network in V1 (Figure 1.2.b; Kisvárday et al., 1993).

Rodent basket cells – parvalbumin-positive, fast-spiking interneurons

As visual neuroscience has shifted from cats to mice, the identity of basket cells has been established with greater precision. In cat, cells were often characterized in vivo for their receptive field properties while they were filled intracellularly with horseradish peroxidase, permitting subsequent visualization of gross axonal morphology and identified synaptic contacts by correlated light & electron microscopy (e.g., Martin et al., 1983). These anatomical observations, including arbor shape and the targeting of boutons to cell bodies, were then used to classify
neurons as basket cells. Antibodies against GABA and other markers like parvalbumin and cholecystokinin were later applied, allowing molecularly-identifiable subtypes of basket cell to begin to be defined (Celio, 1986; Naegele & Barnstable, 1989). While this more recent approach was used relatively sparingly in cat (e.g., Kiszvárdy et al., 1993), converging evidence from immunohistochemistry and \textit{in vitro} physiology studies across species has identified one subtype, the PV+ basket cell, as the predominant variety in cortex (Hu et al., 2014).

Recent, intensive efforts to identify inhibitory cell-types by immunostaining for various markers has revealed their laminar distribution in mouse V1 (Gonchar et al., 2008). These markers, while functionally important in their own right, are useful for studying interneuron diversity in that they segregate cells by their place and time of birth, effectively defining cell-types (Wonders & Anderson, 2006; Rudy et al., 2011). PV is a marker of late-born cells originating from the medial ganglionic eminence, a transient, developmental structure from which PV+ interneurons emigrate to reach V1 around P11 in mice (del Rio et al., 1994; Wonders & Anderson, 2006). PV+ cells are the main inhibitory cell-type overall (Celio, 1986), accounting for nearly half of all interneurons in layers 4, 5, & 6, and over one-third of those in L2/3 (Gonchar et al., 2008; for similar results in rat V1, see Uematsu et al., 2008). While some diversity exists within the PV+ population and is discussed in later chapters, PV+ cells are generally soma-targeting basket cells (Uematsu et al., 2008). (Briefly, two other classes of PV+ interneuron, chandelier cells & multipolar bursting cells, are few in number in V1 and mostly restricted to superficial layers (Rudy et al., 2011), where they form non-α1-subunit-containing synapses onto non-somatic targets (Hensch, 2005)). PV-basket cells are also distinguished by their fast-spiking (FS) behavior (Kawaguchi et al., 1987). In response to current injection, FS-cells produce a high frequency, non-adapting train of action potentials, each of which is narrow
in time and followed by a deep afterhyperpolarization. Together, these hallmarks of parvalbumin expression and fast-spiking behavior define PV-basket cells.

Basket cell axonal arbors show variability in the extent of their projections, which anatomists have used as a basis for defining subtypes (Markram et al., 2004). The classical cells described above, which have long, horizontally-projecting axons that reach at least three times as far as the dendrites (Somogyi et al., 1983), are called large basket cells. Small basket cells, by contrast, have highly local arbors confined to a small region around the parent soma. There is also a hybrid type, the nest basket cell (Wang et al., 2002), whose arbor has both local and semi-long distance elements. Although the precise neurochemical identity of each of these subtypes was controversial for some time (Markram et al., 2004), it is now generally accepted that the PV+ population includes most large & nest basket cells, with small basket cells instead expressing the neuropeptides VIP and/or CCK (Wang et al., 2002; Rudy et al., 2011). While large basket cells expressing CCK and other markers have been reported (e.g., Wang et al., 2002), it is unclear how prevalent these subtypes may be, especially in deeper layers where CCK+ cells are rare in V1 of both cats (Demeulemeester et al., 1988) and mice (Gonchar et al., 2008). That half of deep layer GABAergic interneurons are PV+ (Gonchar et al., 2008) and that the vast majority of these are basket cells suggests that whatever their precise morphological subtype, PV-basket cells play a dominant role in mediating neocortical inhibition (Rudy et al., 2011).

**Location of PV-cells in cortical microcircuits**

A common theme across neocortical areas is that certain interneurons receive especially strong thalamic activation. Early degeneration studies in mouse S1 showed that while non-
pyramidal cell somata are densely innervated by thalamocortical terminals (White et al., 1984),
the somata of spiny stellate cells are largely devoid of these terminals (White & Rock, 1980),
with their dendrites receiving only relatively sparse innervation (Benshalom & White, 1986).
This shorter electrotonic distance contributes to non-pyramidal cell responses to thalamocortical
activation that are faster and stronger than those of pyramidal or stellate cells (Cruikshank et al.,
2007).

The synaptic mechanisms of this intense thalamocortical innervation have recently been
dissected in mouse S1 (Cruikshank et al., 2007). By stimulating thalamus and recording evoked,
monosynaptic currents in L4 cells, it was found that FS-cells exhibit an excitatory conductance
nearly 8-fold larger than regular-spiking (RS) cells. Interestingly, thalamic minimal stimulation
revealed that not only are unitary conductances stronger in FS-cells, but that FS-cells receive
inputs from greater numbers of thalamic relay neurons than RS-cells do. Further,
thalamorecipient glutamate receptors on FS-cells are optimized for extreme speed (Angulo et al.,
1999), enabling the excitatory conductance in these cells to nearly completely elapse before it
even peaks in RS-cells (Cruikshank et al., 2007). Together, these specializations provide for
feedforward inhibition onto both FS and RS-cells that is fast and strong, with the IPSC peaking
roughly 3ms after the onset of excitation. However, while in FS-cells the effect of inhibition is to
curtail an excitatory conductance that has already largely elapsed, the slower timecourse of
excitation in RS-cells confers inhibition the ability to override much of this excitation. Indeed,
cell-attached recordings show that while FS-cells usually spike in response to thalamic
stimulation, this seldom happens in RS-cells. Overall, this differential connectivity of afferents
onto FS- and RS-cells sheds light on the functional architecture of the thalamocortical gate
(Hensch, 2005; Wang et al., 2010), and suggests the importance of both FS-RS and FS-FS connections in controlling information flow to cortex.

Additionally, PV-cells extensively form both electrical and chemical synapses with one another (Galarreta & Hestrin, 2001a). Typically formed at the juxtaposition of two dendrites, gap junctions are low-resistance structures allowing the passage of charged ions between cells. By doing so, gap junctions “couple” cells electrically, resulting in the flow of current from one cell into another. As such, paired whole-cell recordings of coupled cells reveal that depolarization of one cell causes simultaneous depolarization of the other, with the “coupling coefficient” quantifying the level of signal attenuation (Gibson et al., 1999). While both sub- and supra-threshold responses to junctional current are possible, coupling acts as a low-pass filter, more strongly attenuating high-frequency signals like action potentials (Galarreta & Hestrin, 1999). This results in a spike from one cell appearing as a low-amplitude “spikelet” in the other, while the slower post-spike afterhyperpolarization is able to infiltrate the coupled cell to a greater degree. Together, the combination of electrical and GABAergic connections between PV-cells endows these networks with the ability to detect the synchrony of inputs (Galarreta & Hestrin, 2001b), a property discussed at length in Chapter 7. Briefly, while inputs with high temporal coherence produce maximal activation of the PV-cell-network due to the spread of excitation by gap junctions, asynchronous inputs dampen network activity due to the presence of reciprocal inhibitory synapses.

**Role of PV-cells in mediating ocular dominance plasticity**

Beyond the large-scale structural plasticity of thalamocortical arbors in response to MD (Antonini et al., 1999), plasticity of thalamocortical synapses onto V1 neurons has also been
observed. Specifically, brief MD weakens functional thalamocortical input to both L4 star pyramidal (Wang et al., 2013) and PV+ cells (Quast & Hensch, 2012), as well as causes physical shrinkage of thalamocortical inputs to PV+ cell somata (ibid). In vivo, this experience-dependent plasticity of thalamocortical input to L4 cells would lead to changes in the spike rate of recorded cells following presentations of visual stimuli. Indeed, Yazaki-Sugiyama et al. (2009) found that in the binocular zone, brief MD shifts spiking of pyramidal cells from contralaterally biased to neutral, an effect potentially mediated by a weakening of thalamocortical input from the deprived/contralateral eye. Interestingly, the spike bias of FS-cells is also acutely regulated by visual experience. While FS-cells normally exhibit no spike bias, brief MD shifts this bias toward the deprived-eye (ibid), a counterintuitive result considering classical Hebbian plasticity mechanisms (Hebb, 1949). By gaining greater influence over FS-cell spiking via a spike-timing-dependent plasticity rule (Yazaki-Sugiyama et al., 2009), deprived-eye inputs can drive inhibition in target pyramidal cells, reducing spiking to the deprived-eye stimuli concurrently trying to excite these cells. The precise contribution of inhibitory transmission to this process was evident after the GABA_A R blocker picrotoxin contained within the recording pipette diffused into pyramidal cells. In mice given brief MD, picrotoxin remarkably unmasked ipsilateral-biased pyramidal cells as preferring contralateral input (i.e. an “inversion” of bias), revealing that inhibitory transmission was mediating the shift in OD preference from pre-MD values. In non-deprived mice, however, picrotoxin only slightly altered spike bias on a per cell basis and produced markedly fewer inversions, indicating that inhibition has a more subtle effect on OD receptive field sculpting in the absence of imbalanced input.

A growing number of studies have addressed the plasticity of PV-cells’ connections to their target cells following monocular deprivation (Takesian & Hensch, 2013). Together, their
results suggest that this plasticity may be a causal mechanism underlying changes in pyramidal cells. For instance, the evoked spike rate of L2/3 pyramidal cells recorded in vivo increases after just 1 day of MD (Kuhlman et al., 2013), plasticity attributed to reduced responsiveness of PV-cells at this time-point. As assayed in vitro, this decrease in PV-cell spiking is mediated by a reduction in feedforward excitatory drive to these cells (ibid), an effect previously mentioned as also having a thalamocortical basis (Quast & Hensch, 2012). Overall, this decrease in PV-cell-mediated inhibition is thought to be an early step permitting initiation of subsequent plasticity mechanisms (Kuhlman et al., 2013). When paired recordings were used to directly assay feedback inhibition between FS and pyramidal (PYR) cells after a full 3 days of MD (Maffei et al., 2006), it was found that both PYR-FS and FS-PYR connections more than tripled in strength in the deprived hemisphere, potently suppressing pyramidal cell firing. Interestingly, long term potentiation (LTP) of the inhibitory connection between FS & PYR cells (LTPi) could be induced by pairing FS-cell spiking with subthreshold depolarization of PYR cells (ibid). This plasticity was occluded by prior MD, however, suggesting that LTPi is a likely mechanism by which deprivation-induced loss of deprived-eye responsiveness is mediated. Taken together, these studies suggest that PV-pyramidal connections are involved in mediating critical period plasticity, either by depressing pyramidal cell responses (Maffei et al., 2006) or sculpting their receptive field properties (Yazaki-Sugiyama et al., 2009), or permitting transient relief from inhibition (Kuhlman et al., 2013).

Despite all we have learned about the effects of deprivation on PV-pyramidal connections (Takesian & Hensch, 2013), pyramidal cells are only one of the two major cell-types targeted by PV-cells (Markram et al., 2004; Pfeffer et al., 2013). Additionally, PV-cells heavily innervate other PV-cells (Kisvárday et al., 1993; Tamás et al., 1998; Galaretta & Hestrin, 2001a), as well
as form substantial numbers of autapses onto themselves (Tamás et al., 1997a). These PV-PV connections are also enriched in the α1-subunit-containing GABA<sub>A</sub>Rs (Klausberger et al., 2002) shown to regulate critical period onset (Fagiolini et al., 2004), and actually contain >3-fold higher levels of α1-subunit than do PV-pyramidal synapses (Klausberger et al., 2002). Despite the strength and prevalence of these connections (Galaretta & Hestrin, 2002) and their role in mediating the thalamocortical gate (Cruikshank et al., 2007), the role of PV-PV connectivity in critical period regulation remains relatively unexplored. To better understand the experience-dependent plasticity of PV-cell synapses with a fuller appreciation of their target-cell diversity, we set out to investigate PV-cell connectivity to both PV-cells and different sub-types of pyramidal cell (Leyva-Díaz & López-Bendito, 2013).
CHAPTER 2

Methods
Immunohistochemistry

PV-Cre mice (Hippenmeyer et al., 2005) were either light-reared until P60 (12hr light/dark cycle, LR, n=3), dark-reared until P60 (DR, n=4), dark-reared until P60 then re-exposed to light for 5 weeks (DR+light, n=3), or light-reared until P15 (P15, n=3), then anesthetized and perfused intracardially with ice-cold saline followed by 4% PFA in 0.1M PB, postfixing overnight at 4°C in the same solution. Coronal vibratome sections were cut at 60µm, blocked for 1hr at room temperature (RT) in 95% StartingBlock (Thermo Scientific), 1% Triton-X-100 (Sigma), & 4% PBS, washed in PBS-T (0.25% TX-100), then incubated for 4 nights in primary antibody at 4°C: a base of 99% [0.02% NaN₃ PBS] + 1% TX-100, with 1:500 rabbit anti-PV (Swant, ‘PV27’), 1:250 mouse-IgG2A anti-Synaptotagmin-2 (DSHB, ‘znp-1’), & 1:500 biotinylated Wisteria Floribunda Agglutinin (WFA, Sigma, ‘L1516’), with 1:50 mouse-IgG1 anti-Satb2 (Abcam, ‘ab51502’) & 1:200 rat anti-Ctip2 (Abcam, ‘ab18465’) present only for the 4th night. Sections were then washed in PBS-T, incubated for 2 nights in secondary antibody at 4°C (Alexa Fluor conjugates from Life Technologies, all goat anti-primary species and used at 1:500: rabbit-405, rat-488, mouse-IgG2a-546, mouse-IgG1-594; Streptavidin-647), washed, incubated for 30mins at RT in 1:300 NeuroTrace 435/455 (in PBS-T, Life Technologies), washed, and mounted on slides using Vectashield (Vector Labs, ‘H-1000’).

To perform immunostaining of PV and the GABAₐR α1-subunit, mice were anesthetized and perfused as above, then postfixied for 2hrs. Coronal cryostat sections were cut at 35µm, then blocked for 1hr at RT in 10% normal goat serum & 0.1% Tween-20 in PBS, incubated overnight in primary antibodies (1:500 rabbit anti-PV, Swant; 1:250 mouse anti-GABAₐR α1, NeuroMab, ‘75-136’) at 4°C, washed in PBS, incubated for 4hrs at RT in secondary antibodies (mouse-488
& rabbit-594), washed in PBS, and mounted with DAPI-Fluoromount-G medium (Southern Biotech).

Imaging and analysis

Images for Synaptotagmin-2 bouton analysis were taken at the Nyquist resolution (voxel size = 94nm XY & 250nm Z-step, with optical sections of ~0.6µm / channel; 12-bits; 16x line-averaging) on a Zeiss LSM 710 confocal using a 63x, 1.4NA oil immersion PlanAPO objective (1.1x zoom). Z-stacks centered on the bright Syt2+/Ctip2+ band in layer 5 of monocular V1 were acquired through the thickness of the section using multi-track mode and depth-correction settings. Acquisition parameters were determined first for DR samples, which had slightly dimmer Syt2 signals (Sommeijer & Levelt, 2012), to ensure that dim boutons could reliably be identified above background. Then, detector gain was decreased very slightly for light-reared samples, in order to prevent saturation of pixels.

Images were corrected for chromatic aberration in three dimensions using a custom ImageJ/Fiji plugin (D. Cai, U Michigan) and global pixel-shift values determined from a set of single antibody, multi-label images acquired using the same settings. While photobleaching was minimal, Fiji’s bleach correction plugin was run using the histogram-matching method in order to render planar intensity uniform throughout the stack for a given image channel, aiding identification of weakly-labeled structures. Images were convolved with a Gaussian kernel (1px radius) to reduce the appearance of super-resolution shot noise for the following reason, which is also why we did not deconvolve our images (Ichinohe et al., 2008): deconvolution resulted in poor blending of colors in brainbow-labeled axons, but a minimal convolution greatly improved
color blending and discriminability. For consistency, we performed Syt2-bouton analysis on images processed the same way.

To quantify PV-innervation, Syt2+ boutons apposed to NeuroTrace-labeled cell bodies were manually identified by morphology and counted using ObjectJ, an annotation plugin for ImageJ (http://simon.bio.uva.nl/objectj/). Boutons were identified by their aspect ratio and characteristic donut-like structure, with a central Syt2-exclusion zone. Soma-apposed boutons identified using light microscopy have been shown to “invariably” make synaptic contact when re-examined on the electron microscopic level (Somogyi et al., 1983). Only appositions lacking dark pixels between the bouton and the cell body profile were considered (Ichinohe et al., 2008). All boutons apposed to the cell body throughout its 3D extent were counted, and only cells fully contained within the stack were analyzed. Bouton density was calculated using the total surface area of the cell body, which was measured from perimeters drawn manually on each z-plane. Surface area was computed using the trapezoidal rule, and was corrected using the thickness of the section as measured optically. Cells were chosen for analysis blind to the Syt2 channel, with only cell-type markers being visualized. To measure Syt2+ bouton volume, the perimeter of each identified bouton was outlined on each z-plane on which it was present to obtain the area, with the volume computed using the trapezoidal rule. All data reported as mean±SEM, with 15-18 cells imaged across multiple mice analyzed per condition.

For validation of PV:GABA\textsubscript{A}-\textalpha{1}\textsuperscript{-/-} mice via immunostaining, images were acquired using an Olympus FV1000 confocal at a resolution of 124nm XY under a 20x objective with 2x digital zoom. PV and GABA\textsubscript{A}-\textalpha{1} immunostains were imaged at the mid-plane of identified cells, with L4 PV+ cells identified by their intense somatic expression of PV, and non-PV cells identified by DAPI. Cells of similar size were chosen for comparison and analysis. To quantify
α1-signal fluorescence in ImageJ, intensity inside a manually-drawn cell perimeter was averaged. Total somatic α1 intensity was normalized to that of non-PV cells in control mice, with 18 cells of each type being analyzed per genotype across multiple mice.

**Whole-cell recordings**

To evaluate synaptic inhibition, voltage-clamp recordings (Axopatch 1D; Axon Instruments) were obtained from L5 cells using mice expressing channelrhodopsin-2 (ChR2) in PV-cells (Ai32ChR2-YFP^{f/f} mice (JAX) crossed to PV-Cre, either LR (n=4) or DR (n=5) to P55-66). 300µm coronal slices were prepared using an NMDG-based cutting solution after Zhao et al. (2011), containing (in MM): 93 NMDG, 93 HCl, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 10 MgSO4, & 0.5 CaCl2, with 5 sodium ascorbate, 2 Thiourea, and 3 sodium pyruvate to promote slice longevity. Slices were cut at RT, recovered in a 33°C bath for 12mins, and then transferred to the following RT solution for at least 1hr before recording: (in mM) 92 NaCl, 2.5 KCl, 1.2 NaH2PO4, 30 NaHCO3, 20 HEPES, 25 glucose, 2 MgSO4, & 2 CaCl2, with 5 sodium ascorbate, 2 Thiourea, and 3 sodium pyruvate.

PV-cells were targeted via YFP expression and pyramidal (PYR) cells were targeted by soma shape using IR-DIC. The internal solution contained (in mM): 100 KCl, 40 K-glucconate, 8 NaCl, 10 HEPES, 2 MgCl2, 0.1 EGTA, 2 ATP, 0.3 GTP, and 5 QX-314 (pH=7.2 with KOH), with a high chloride concentration being used to obtain inward inhibitory postsynaptic currents (IPSCs) at a holding potential of -60mV. The tip resistance of the patch electrode filled with internal solution was ~2-3 MΩ. Spontaneous IPSCs (sIPSCs) were recorded in the presence of ionotropic glutamate receptor blockers DNQX (20mM, Sigma) and (RS)-CPP (20mM,
Tocris), added to the superfusing ACSF (in mM: 125 NaCl, 2.5 KCl, 1.2 NaH2PO4, 25 NaHCO3, 25 Glucose, 1 MgCl2, 4 SrCl2), at RT.

After recording sIPSCs (2.5mins), a 200µm diameter fiber optic coupled to a 473nm laser (IkeCool) was used for ChR2 excitation of PV-cells in the cortical area around the patched cell (2ms pulses, 2Hz train of 5 pulses every 20s, 2mW on sample). Replacement of extracellular calcium with strontium desynchronizes presynaptic release, permitting analysis of “evoked quantal” events following decay of bulk IPSC (and channelrhodopsin current, in PV-cells) in the patched cell to within 10% of baseline amplitude. Only those cells whose rate of evoked IPSCs upon light-activation showed a ≥3x increase over that of the earlier-recorded sIPSC-rate were taken as exhibiting sufficient enrichment of “PV-IPSCs” (Nahmani & Turrigiano, 2014). Data were acquired at a sampling rate of 10 kHz using an IGOR (WaveMetrics) program. IPSCs were analyzed offline using MiniAnalysis (Synaptosoft Inc.) with the following thresholds: amplitude ≥6pA, 10-90% rise time ≤3ms. All data reported as mean±SEM.

**Brainbow labeling and analysis**

Adult PV-Cre mice (n=4) were anesthetized with vaporized isoflurane prior to undergoing aseptic surgery, during which the scalp was cleaned, hair and skin were cut and moved aside, and a small hole was drilled over V1m at Bregma -3.0mm and ±2.0mm from the midline. A 32 gauge syringe (Hamilton, #80014) containing a titer-balanced cocktail of Brainbow-AAVs (Cai et al., 2013; Penn Vector Core #s p2453 & p2454) diluted to 3x10^{12} GC/mL in saline was lowered 400µm below the cortical surface, where 1µL of virus solution was injected at 0.1µL/min. After settling time, the needle was slowly retracted and the scalp incision sutured, with post-operative pain management achieved via Meloxicam (Norbrook
Laboratories). After at least 4 weeks survival time, mice were perfused and brains were sectioned as described above. To keep light exposure to a minimum during surgeries on DR mice (n=4), animals were transported from the facility in a darkened anesthetization induction box and then maintained under isoflurane at a level sufficient to abolish tail-pincho reflexes, as per standard procedure. Mice were then returned to the dark-room for the survival period. All procedures were approved by the Harvard University Institutional Animal Care and Use Committee.

Immunohistochemistry was performed as above on sections of V1 containing Brainbow-labeled cells and axons in order to yield high-quality images for quantitative analysis. Custom-made primary antibodies against each of the 4 fluorescent proteins (XFPs) in the Brainbow-AAV cocktail were used (chicken-GFP (labels EYFP), guinea pig-mKate2 (labels TagBFP), rabbit-mCherry, & rat-mTFP1.0; Covance), which were then visualized with the following secondary antibodies: 1:500 donkey anti-rabbit-405 (Jackson ImmunoResearch), and 1:500 each of goat anti-chicken-488, rat-546, & guinea pig-647 (Life Technologies). Stacks of L5 PV-cells were acquired as above, with the sections then demounted, washed, and incubated overnight in biotinylated-WFA followed by 1:500 Streptavidin-594. Sections were then re-mounted, and the previously imaged cells were relocated to check the PNN-status of each one, with only PNN-bearing PV-cells analyzed further.

For analysis, image stacks were corrected individually for chromatic aberration, the level of which varied mildly across slides. This correction was critically important for accurate assessment of each axon’s true color, as Brainbow relies on the combinatorial nature of component colors and proper blending is essential, especially in the axial dimension. ObjectJ was used to mark the boutons of those Brainbow-labeled axons contacting a given PV-cell body. Like-colored axonal segments were tracked back to a branch point to check if they were part of
the same axon (i.e., a terminal fork, Chattopadhyaya et al. 2004), and were further disambiguated by toggling display of the 4th Brainbow-channel – usually left un-visualized on 3-color RGB displays – to further enhance color diversity. These strategies permitted straightforward assignment of virtually all axonal segments and boutons as being either uniquely-colored among a target PV-cell’s complement of inputs or belonging to a previously identified axon.

**Fluorescence-activated cell (FAC) sorting and quantitative Polymerase Chain Reaction (qPCR)**

Visual cortices from PV-Cre x Ai32ChR2-YFP<sup>ef</sup> mice that were light-reared (n=4) or dark-reared (n=4) until adulthood were freshly dissected and dissociated using a Papain dissociation system (Worthington Biochemical) and trehalose as described previously (Saxena et al., 2012). Dissociated neurons were then subject to FAC-sorting (MoFlo Legacy Cell Sorter; Beckman Coulter) at the Harvard Center for Systems Biology Bauer Core Facility to isolate YFP-enriched and non-YFP control cell fractions. Cells were then lysed in Trizol reagent, and RNA extraction was carried out using RNeasy Micro extraction kit (Qiagen). RNA from YFP+ and YFP- fractions was subject to reverse transcription using a high-capacity RNA-to-cDNA kit (Life Technologies, LT), and qPCR reactions were setup using Taqman Universal Master Mix (LT). Taqman probes (FAM/MGB) were as follows: gabra1 (LT Mm00439045_m1), gabra2 (LT Mm00433435_m1), gabra3 (LT Mm01294271_m1), gabrag2 (LT Mm00433489_m1), and endogenous GAPDH control (LT 4352932E). All results were normalized to GAPDH expression and then to light-reared level per gene, with differences in expression level assayed across conditions on a per gene basis using t-tests.
Monocular deprivation (MD) and visual evoked potential (VEP) recordings

For MD, the eyelids of one eye were sutured shut with the mouse under isoflurane anesthesia, with sutures monitored daily thereafter to ensure complete closure for 4d. On the fifth day, the sutured eye was opened and VEP recordings carried out. Mice were anesthetized using a mixture of nembutal/chlorprothixene, and a tracheotomy was performed to provide constant O₂ supply. After exposure of V1 via craniotomy, a high-resistance tungsten electrode was inserted down 350-400µm beneath the cortical surface to L4. Transient VEPs in response to abrupt contrast reversal (100%, 1Hz) of spatial frequency gratings ranging from 0.05-0.5 cycles/deg were band-pass filtered (0.1-100Hz), amplified, and fed to custom computer software where ≥20 events were averaged in synchrony with the stimulus contrast reversal (Fagiolini & Hensch, 2000). A linear regression between VEP amplitude and spatial frequency was plotted, and visual acuity was defined as the spatial frequency at which the regression line reaches zero. Mice not receiving MD were used as control. Animal numbers were as follows: WT (strain C57BL/6J, JAX; P60 no MD control = 7; P60 MD = 3; P150 no MD = 5; P150 MD = 5) and PV:GABA₅R-α1(-/-) (P60 no MD = 3; P60 MD = 6; P150 no MD = 6; P150 MD = 6).

Voltage-sensitive dye imaging and analysis

The voltage-sensitive dye Di-4-ANEPPS (Life Technologies), dissolved at 10 mg/mL in DMSO stock solution, was diluted (1.3µL/mL) in ACSF to a final concentration of 10 µg/L. Coronal slices through V1 were incubated in dye (>90 min) before transfer to an ACSF recording chamber (20–22 °C), then imaged using an Olympus MVX10 microscope with a 1x, 0.25NA objective. Stimulation (0.01-1mA, 1ms pulse) of white matter with an ACSF-filled glass pipette was controlled by a programmable pulse generator (MiCam Ultima) linked to a constant...
current stimulus isolation unit (Iso-Flex, A.M.P.I.). Excitation light from a shuttered 150-W halogen lamp (MHF-G150LR, Moritex) was band-pass filtered (515-535nm) and reflected toward the sample by a 570-nm dichroic mirror. Emitted fluorescence, reflecting a change in potential across neuronal membranes (Grinvald & Hildesheim, 2004), was long-pass filtered (590 nm) and imaged using a MiCam Ultima CMOS-based camera (SciMedia). Fluorescence changes (recorded for 512 periods at 1000Hz) were averaged across 10 trials and integrated across user-determined regions of interest (ROIs; 5x5 pixels covering 125x125ȝm) by spatial averaging and across different trials using MiCam Ultima analysis software. Fluorescence change was normalized to resting fluorescence (∆F/F₀).

For each individual slice, a horizontal array of 9 ROIs in both L2/3 and L5 was placed, with the maximum single-pixel intensity value in the first ROI in L5 – centered tangential to the stimulation site – being used to normalize all ∆F/F values for the entire slice for a given trial, controlling for slice-to-slice variability in staining intensity and electrode depth. Individual time course traces were subsequently exported to MATLAB (MathWorks) to combine data from a number of slices (Barkat et al., 2011). Maximum fluorescence change (∆F/F) and integrated sums of ∆F/F over the first 100ms following stimulation were calculated at each region of interest for each trial. The following number of animals (aged P55-60) and slices were used per condition: LR PV-Cre = 3 mice / 13 slices; DR PV-Cre = 4/20; PV:GABAₐR-α1(+/+) = 4/17; PV:GABAₐR-α1(−/−) = 6/25.

Statistics

Most statistical tests were performed using Prism (GraphPad), with Kolmogorov-Smirnov (KS) tests performed using MATLAB’s ‘kstest2’ function and a random sampling of
n=50 events per group compared. For comparisons that were significant, the test used and the significance level (P<0.05, *; P<0.01, **; P<0.001, ***) are reported in the figure and corresponding legend. For relevant comparisons that were insignificant, the test and P-value are reported in the main text.
CHAPTER 3

Cell type-specificity of PV-cell

afferent connectivity
ABSTRACT

Neurons of the cerebral cortex can be divided into two main classes: excitatory pyramidal cells whose axons project outside of the cortex, and inhibitory interneurons whose axons remain within the cortex. Each of these classes is comprised of numerous distinct cell-types that differ in both form and function. Pyramidal cell-types send their axons to different regions of the brain (Leyva-Díaz & López-Bendito, 2013), while different types of interneurons contact their target cells along stereotyped subcellular domains (Markram et al., 2004). PV-cells, which innervate the perisomatic domain of their target cells, are known to contact both pyramidal cells (e.g., Somogyi et al., 1983) as well as other PV-cells (e.g., Kisvárday et al., 1993). However, the relative strength of each of these circuits is unknown. Here, we used anatomical techniques to investigate the level of PV-input to both other PV-cells and genetically-identifiable subtypes of pyramidal cell in V1.
INTRODUCTION

Our understanding of the wiring diagram of cortical inhibition has evolved considerably over time. This progression has paralleled what we have learned about the intracortical connectivity of pyramidal cells: previously thought to synapse on whatever somatodendritic structures their sparse axons cross (Szentágothai, 1975), we now know that their selection of postsynaptic partners is guided by target cell-type identity (Brown & Hestrin, 2009). Similarly, due to its local, dense nature and high degree of redundancy in connections made, GABAergic inhibition was also thought to be fairly non-specific (Peters & Regidor, 1981). However, the advent of modern cell-types, for interneurons (Ascoli et al., 2008) as well as for their target cells (Leyva-Díaz & López-Bendito, 2013), has provided new insight into the architecture of inhibitory circuits.

Domain-specific innervation, the ability of cells to contact their targets within a stereotyped subcellular region (Figure 1.1), has emerged as a major organizing principle in our understanding of the structure-function relationship of interneurons (Markram et al., 2004). Chandelier cells, a class of PV-expressing interneurons scarcely found in mouse sensory cortex (ibid), represent the ultimate case of domain-selective targeting, only innervating pyramidal cells and only along their axon initial segments (Somogyi, 1977). While other inhibitory cell-types do not show such absolute specificity in their connections, most do exhibit a bias towards contacting cells either proximally or distally with respect to their cell bodies (Markram et al., 2004). Synapses made by PV-expressing basket cells (“PV-cells”) are proximally-biased and typically fall within the “perisomatic” domain of the target cell, which includes the soma and proximal dendrites. Although contacts onto more distal dendrites have been observed (Somogyi et al., 1983), the soma-targeting nature of PV-cells is highlighted most often given the rarity of this
preference among cortical cell-types (Peters & Proskauer, 1980; DeFelipe & Fairén, 1982; Somogyi et al., 1983; Martin et al., 1983), as well as for the powerful functional control such innervation confers (Hensch, 2005). Through inhibitory control of target somata, PV-cells are positioned to regulate spike-timing dependent plasticity by gating the back-propagation of action potentials into dendrites, mediating refinement of synaptic strength in an input-specific manner.

Synaptic specificity, cells’ ability to select targets for innervation on the basis of cell-type identity (Sanes & Yamagata, 2009), has until recently been studied largely in the context of projection neurons. However, both excitatory (Brown & Hestrin, 2009) and inhibitory cells (Krook-Magnuson et al., 2012) are now appreciated to take target-cell identity into account when forming local connections. This is perhaps best illustrated by cholecystokinin-expressing basket cells (“CCK-cells”) in the medial entorhinal cortex (MEC; Varga et al., 2010). In this region, two distinct populations of pyramidal cell (PYR) comingle in layer 2, with one expressing reelin and projecting to the ipsilateral dentate gyrus, and the other expressing calbindin and projecting to the contralateral entorhinal cortex. CCK-cell axons were found to heavily innervate the somata of calbindin-PYRs while seldom contacting reelin-PYRs, a result confirmed physiologically. Interestingly, PV-cells in the MEC were found to contact the somata of both types of PYR equally, suggesting that different populations of interneuron regulate the specificity of their connections independently. However, these interneuron type-specific differences in degree of selectivity appear to be region-specific, as PV-cells in hippocampal CA1 preferentially innervate deep rather than superficial PYRs in stratum pyramidale, a distinction not observed in CCK-cell innervation patterns (Lee, Marchionni et al., 2014). Within CA1, PV-cells also exhibit projection target-specific innervation of PYRs, inhibiting PYRs that project to the amygdala more strongly & via more terminals than those that project to the prefrontal cortex.
While connections from PV-cells to pyramidal cells have long been studied in neocortex, the question of whether pyramidal cell subtypes there receive different amounts of perisomatic inhibition has remained relatively unexplored. Indeed, after an early study in which immunohistochemistry for the GABA-synthesizing enzyme glutamic acid decarboxylase was used to label gross inhibitory structure, the observation that the largest pyramidal cells in layers 3 & 5 were surrounded by a “continuous sheet” of labeled terminals has received little follow-up attention (Ribak, 1978; also see Marin-Padilla, 1974). Recently, however, Lee et al. (2014) have shown in medial prefrontal cortex (mPFC) that two types of layer 5 (L5) pyramidal cell – thick-tufted type A neurons which project subcortically, and thin-tufted type B neurons which project callosally – are differentially innervated by PV-cells. Type A neurons not only receive stronger PV-mediated inhibition, but have much higher rates of connectivity with PV-cells as well. In a parallel to the MEC result of Varga et al. (2010), these PYR subtype-specific differences in innervation are not common among all inhibitory cell-types, but apply only to PV-cell inputs and not to those from somatostatin-expressing interneurons. While a previous study quantified the number of GABA+ terminals synapsing onto the somata of PYR-subtypes in primary visual cortex (V1), different cell-types were compared to one another and not in layer 5 (Fariñas & DeFelipe, 1991), leaving unanswered the question of whether PV-cell inputs to type A & B neurons in V1 differ.

To better understand how the circuits through which PV-cells mediate ocular dominance plasticity are organized, we investigated the prevalence of PV-cell inputs to various cell-types in V1 of the mouse. We focused on L5 as it is the primary output layer of the cortex (Leyva-Díaz & López-Bendito, 2013), and processing there is likely to be behaviorally-relevant. Like in the mPFC, L5 of V1 contains two subtypes of pyramidal cell that comingle with one another, the
characteristics of which map onto the type A & B neurons of Lee et al. (2014). These subtypes can be distinguished by their expression of transcription factors involved in determining their genetic fate: Ctip2+ neurons project subcortically, are thick-tufted, and correspond to type A neurons; Satb2+ neurons project callosally, are thin-tufted, and correspond to type B neurons (Leyva-Díaz & López-Bendito, 2013). We reasoned that by comparing the level of PV-cell input to these two subtypes, we would learn something about how the two processing streams mediated by their projections may be involved in the expression of ocular dominance.

We also studied a third type of connection, that of PV-cell inputs to other PV-cells. Largely unappreciated until recent decades, Kisvárday et al. (1993) showed that not only do PV-cells form axosomatic synapses onto other PV-cells, but that basket cells seem to preferentially contact other basket cells, establishing a highly-recurrent network in cat V1 (Figure 1.2.b). Subsequent work has confirmed the existence of robust, proximally-targeted interconnections between basket cells, which are often reciprocal and accompanied by substantial numbers of autapses, effective regulators of spiking precision (Tamás et al, 1997a; Tamás et al, 2000; Bacci & Huguenard, 2006). In order to study such homotypic connections using fluorescence immunohistochemistry, we took advantage of an antibody that labels an isoform of the presynaptic release protein synaptotagmin, which was recently characterized as a high-fidelity marker of PV+ boutons in mouse V1 (‘Syt2’; Sommeijer & Levelt, 2012). By visualizing PV-cell gross anatomy in one channel and PV-cell boutons specifically in another, we were able to discriminate individual PV-PV contacts onto intensely-stained cell bodies, permitting complete reconstructions of basket cells’ primary domain of innervation to be obtained.
RESULTS

Synaptotagmin-2 as a tool for studying PV-PV innervation

We immunostained sections of adult mouse brain with antibodies against PV and Syt2, finding that a mouse monoclonal antibody raised against the zebrafish protein (‘znp-1’, Trevarrow et al., 1990) replicates the high-fidelity staining pattern reported by Sommeijer & Levelt (2012), who used the original rabbit polyclonal antibody (‘i735’) produced by Dr. Thomas Sudhof’s lab (who confirmed the quality of the znp-1 antibody; personal communication to T.K. Hensch). As expected, Syt2 labels PV+ boutons and is not present in either PV+ cell bodies or dendrites, allowing ready identification of Syt2+ boutons making putative contacts onto PV+ / NeuroTrace-labeled cell bodies (Figure 3.1.a-b).
Figure 3.1 = (a) (Left) PV+ structures surrounding a pyramidal cell body in L5 of V1. (Right) Colocalization with Syt2 confirms most elements in contact with the cell body as boutons. Scale bars, 5µm. (b) (Left) Syt2 is restricted to axons and does not label either somata or dendrites of PV+ cells, permitting visualization of axosomatic PV-PV connections (Right). Arrows in (a) and (b) indicate dendrites of PV+ cells, unlabeled by Syt2.
**Cell-type-specific regulation of PV-innervation in L5 of V1**

We next assessed the pattern of Syt2 staining across layers (Figure 3.2). Syt2+ perisomatic puncta rings were observed throughout layers 2-6, with the most intense staining found in L5 (Figure 3.2.b). To investigate whether the level of Syt2+ input to individual cells in L5 may be organized in a cell type-specific manner, we next stained for markers of various cell-types. We stained for PV (Figure 3.2.c) and *Wisteria Floribunda* agglutinin (WFA, Figure 3.2.d), a marker of the perineuronal nets that surround the perisomatic region of a subset of PV+ cells. We also stained for two markers of pyramidal cell (PYR) subtypes, the transcription factors Satb2 & Ctip2 (Figure 3.3). These markers identify callosally-projecting and subcortically-projecting PYRs, respectively, and are organized in a laminar pattern across cortical areas (Figure 3.3.b; Leyva-Díaz & López-Bendito, 2013). As expected, we found that Ctip2+ nuclei were concentrated in L5, with less intensely-stained nuclei also present in L6 (Figure 3.3.c). In contrast, Satb2+ nuclei were found throughout layers 2-6, with the most intensely-stained nuclei located in the lower part of L2/3 (Figure 3.3.d). Within L5, Ctip2+ nuclei and Satb2+ nuclei comingled with one another in a salt-and-pepper pattern (Figure 3.3.e), permitting the subtype-identity of individual L5 PYRs to be determined (Figure 3.3.f).

PV+ and pyramidal cells in L5 of monocular V1 (V1m) were identified, and confocal image stacks were acquired through the thickness of the tissue section. During image analysis, stacks were first visualized blind to the Syt2 channel, with only cell-type marker (Satb2, Ctip2, & WFA) channels turned on in order to identify PYR-subtypes and perineuronal net-bearing PV-cells, respectively (Figure 3.4.a). To ensure that Ctip2+ & Satb2+ cells chosen for analysis were indeed pyramidal cells, somatodendritic morphology was examined via the NeuroTrace channel for the presence of an apical dendrite. After selecting a cell that was fully contained within the
bounds of the stack, each bouton apposed to the NeuroTrace-labeled cell body throughout its 3D extent (Figure 3.4.b) was marked and counted, and the cell body perimeter was measured on each section to compute total surface area. The density of Syt2+ boutons per \( \mu m^2 \) soma was then calculated (Kameda et al., 2012; Hioki et al., 2013), with the results shown in Figure 3.4.c. Syt2+ bouton density differed significantly by cell-type, with both Ctip2+ PYRs and PV-cells having significantly higher somatic Syt2-input density than Satb2+ cells. In each case, this result was a function of bouton number differing significantly by cell-type (Figure 3.4.d), and was not due to unexpected differences in surface area between cell-types (Figure 3.4.e).
Figure 3.2 = (a) A coronal section (Bregma -3.8mm) through monocular V1 stained with NeuroTrace, a fluorescent Nissl stain, shows the cortical cell layers and underlying white matter. Scale bars, 200µm. Orientation bar indicates dorsal (D) and medial (M) neuroanatomical directions. The remaining panels show antibody labeling of the same section for: Syt2 (b), PV (c), and Wisteria Floribunda agglutinin, WFA (d). In this figure, brightness & contrast were manually set for each channel to aid visualization.
Figure 3.3 = (a) A coronal V1 section stained with NeuroTrace. Scale bars, 200µm. Orientation bar indicates dorsal (D) and medial (M) neuroanatomical directions. (b) Co-visualization of antibody labeling for Ctip2 & Satb2, which are shown individually in (c) & (d), respectively. Ctip2 & Satb2 are transcription factors involved in determining the genetic fate of subtypes of pyramidal cells, which are intermingled in cortical layer 5 (e). (f) Ctip2+ pyramidal cells (blue asterisks) and an adjacent Satb2+ pyramidal cell (red asterisk) in L5 of monocular V1. Scale bar, 10µm. In this figure, brightness & contrast were manually set for each channel to aid visualization.
Figure 3.4 = (a) (Left) Satb2+ pyramidal (PYR) cell, callosally-projecting, typically smaller; (Middle) Ctip2+ PYR cell, corticofugally-projecting, typically larger; (Right) WFA-labeled perineuronal net that surrounds a PV+ cell. Scale bars, 5µm. (b) An example cell of each type shown in (a) is shown via a maximum intensity projection through the thickness of its soma. Syt2+ boutons can be seen outlining the perimeter of Ctip2+ PYR and PV+ cells, but are less common around Satb2+ PYR cells. (c) Density of soma-apposed Syt2+ boutons by cell-type (Kruskal-Wallis test, P<0.0001; brackets, Dunn’s multiple comparison tests). (d) Nominal number of Syt2+ boutons per soma by cell-type (Kruskal-Wallis test, P<0.0001; brackets, Dunn’s multiple comparison tests). (e) Surface area of the cell bodies analyzed by cell-type (Kruskal-Wallis test, P<0.0001; brackets, Dunn’s multiple comparison tests).
DISCUSSION

Experimental considerations

In this study, we assessed the abundance of PV-cell inputs to various cell-types in L5 of V1, as a means of investigating the architecture of circuits involved in visual cortical function and ocular dominance plasticity. We took advantage of an antibody recently characterized as a specific label of PV+ boutons in mouse V1 (Sommeijer & Levelt, 2012), and used it to quantify the level of PV-innervation to both pyramidal and PV-cell somata. While PV-cells also provide substantial innervation to the proximal (Di Cristo et al., 2004, Hioki et al, 2013) and occasionally more distal dendrites (Somogyi et al., 1983) of their target cells, we focused on somatic inputs for a number of reasons. First, many studies report that a substantial fraction of basket cell synapses – typically 30% of the total – are formed on somata (typically ~20-50%: Somogyi et al., 1983; Martin et al., 1983; Kisvárday et al, 1985; Tamás et al., 1997a; Kawaguchi & Kubota, 1998). Second, quantifying only somatic inputs provided a basis for normalization and comparison across cells and cell-types. Specifically, while PV-cells are often multipolar in shape, their exact somatodendritic morphology can differ from cell-to-cell, with the number of primary dendrites being variable (Markram et al., 2004). Somata and dendrites can receive different amounts of innervation (Hioki et al, 2013), making normalization between PV-cells more difficult if dendritic innervation were to be considered (let alone normalization to pyramidal cells, whose somatodendritic morphology is markedly different from that of PV-cells; Parnavelas et al., 1977). Third, while mapping inputs to dendrites is fraught with uncertainty over whether an immunostained punctum putatively synapses onto the labeled dendritic element of interest or another, unlabeled structure (Ichinohe et al., 2008), Somogyi et al. (1983) reports that somatic contacts by basket cells viewed at the light level “invariably” show a synapse when
verified at the EM-level, a relationship that is more unreliable for dendrites. Finally, somatic synapses have the strongest impact on action potential generation (Rudolph et al., 2007), important for plasticity processes thought to mediate ocular dominance plasticity (Hensch, 2004, 2005).

Despite their common origins and general non-expression of other markers (Gonchar et al., 2008), parvalbumin-expressing cells do show some diversity as a class (Rudy et al., 2011). Fortunately, each non-basket-forming PV+ cell-type has characteristics that make it unlikely to have been considered here. The best known non-basket-forming PV+ cell-type is the chandelier cell, a GABAergic interneuron that only contacts pyramidal cells and only on the axon-initial segment (Somogyi, 1977; Markram et al., 2004). Chandelier cells are not abundant in caudal mouse cortex, and when present are typically restricted to the L1/L2 border region (Peters et al., 1982). A second PV+ cell-type, the so-called multipolar bursting cell, is also restricted to this superficial border region and differs from PV-basket cells in that it innervates more distal dendrites and lacks fast-spiking behavior (Blatow et al., 2003). Together, these cell-types are unlikely to have been examined here, given both their non-soma-targeting behavior and superficial location within cortex (Freund et al., 1983).

Relationship to previous studies – inhibitory innervation of pyramidal cells

Axosomatic synapses onto pyramidal cells in sensory cortical areas have previously been studied by other groups. An early study combining GABA-labeling of cells with EM-validation and typing of synapses by Gray’s criteria (Gray, 1959) observed that across the whole of layers 2-6 in rat V1, both GABA(-) and GABA(+) cell bodies receive large numbers of inhibitory synapses, estimated to be on the order of at least 0.055 synapses/μm² (Wolff & Chronwall,
Another study, in cat V1, compared the number of GABA+ inputs to callosally-projecting PYRs in deep layer 3 with that of corticothalamic PYRs in upper layer 6, finding that callosal cells receive heavier innervation (Fariñas & DeFelipe, 1991). While these subtypes do not align exactly to those examined in the present study (i.e., L3 and L5 callosal cells are different; Leyva-Díaz & López-Bendito, 2013), our results are consistent on the basis of morphological subtype: L3 callosal cells, which are thick-tufted, receive more GABA+ inputs than L6 corticothalamic cells, which are thin-tufted. In this light, the reported densities of ~0.2 synapses/µm² for thick-tufted cells and ~0.1 synapses/µm² for thin-tufted cells approximate the numbers and ratio observed in our study (thick-tufted = ~0.13, thin-tufted = ~0.08; Figure 3.4.c), considering that inputs from PV-cells are a subset of those that are symmetric. Taken together, these results suggest that perisomatic inhibition may be regulated not only on the basis of PYR subtype-identity as a function of long-range projection target, but according to morphological and other characteristics as well (Marin-Padilla, 1974).

Beyond the principle of domain-specific innervation (Markram et al., 2004), the specificity with which inhibitory neurons wire-up to their target cells remains contentious. The classical view of inhibition has been that connections are unspecific, perhaps owing to early ideas of inhibition as mainly serving to prevent runaway excitation of circuits. This idea has contemporary support, with recent glutamate-uncaging, circuit-mapping studies reporting that both somatostatin-positive (Fino & Yuste, 2011) and PV-positive (Packer & Yuste, 2011) interneurons connect promiscuously to nearby pyramidal cells, forming a dense, “homogenous matrix” of inhibition. However, substantial regional- and laminar-variability was observed for PV-cells, with overall connection probabilities ranging from 0.18 in L2/3 to 0.35 in L5 of mouse somatosensory cortex (S1). While connectivity between pairs with inter-somatic distances of
≤200µm was twice as likely, overall rates were also 50% lower at young adult ages (<0.1 in L2/3 at P31-45), suggesting that connections from PV-cells onto pyramidal cells are actually fairly sparse. Interestingly, the soma morphology of L5 cells was not described, suggesting that both thick- & thin-tufted pyramidal cells were included in the dataset (Hattox & Nelson, 2007). As any subtype-specific differences in connectivity that may have existed would have been averaged-out, it remains unclear whether PV-cells show sub-laminar, cell type-specific differences in connectivity onto L5 pyramids in S1. Thus, our results do not conflict with those of Packer & Yuste (2011), but highlight the need for cell type-specific analyses whenever possible.

In contrast, our data supports an emerging view positing that similar to how pyramidal cells choose their intracortical targets on the basis of their long-range projection patterns (Brown & Hestrin, 2009), interneurons may regulate their innervation of putative target cells on the basis of cell-type identity as well (Krook-Magnuson et al., 2012). Our results agree with recent observations made by Lee et al. (2014) in L5 of mPFC, where two subtypes of pyramidal cell were found to be differentially innervated by PV-cells. Our Satb2+ PYRs, which correspond to their type B neurons, received many fewer Syt2+ boutons than Ctip2+ PYRs, which correspond to their type A neurons, consistent with PV-cells having a dramatically lower connection probability with type B neurons (Lee et al., 2014). Taken together, these results argue for interneurons’ ability to form specific connections on the basis of target-cell identity, as well as provide evidence of a canonical inhibitory circuit motif in L5 that may subserve a common function in both sensory and higher-order cortical areas.
Relationship to previous studies – inhibitory innervation of PV-cells

Previously unexplored outside of the hippocampus (Gulyás et al., 1999), the distribution of synapses on the somatodendritic domain of PV-cells has recently been described in mouse S1, where inhibitory inputs were found to concentrate on the soma and proximal dendrites (Kameda et al., 2012). Starting at a density of 0.41 VGAT+ puncta/µm² on the soma (VGAT = vesicular GABA transporter, a pan-GABA+ presynaptic marker), the density of inhibitory inputs decreases linearly out to the distal dendrites, to 0.26 VGAT+ puncta/µm² (for L5 PV-cells; Hioki et al., 2013). Surprisingly, it was reported that PV+ inputs to PV-cell bodies comprised only a modest fraction of these inputs (0.043 puncta/µm²), with vasoactive intestinal polypeptide (VIP)+ inputs making up two-thirds of the total at 0.27 puncta/µm² (and with minimal somatostatin-positive input density, 0.006 puncta/µm²). Hioki et al. (2013) note that the density of PV+ inputs to PV-cell somata that they observed was lower than expected from previous studies, many of which examined visual cortex (Kisvárday et al., 1993; Ahmed et al., 1994; Tamás et al, 1998; Tamás et al, 2000; Sommeijer & Levelt, 2012). Further work will be needed to determine whether these regional differences in the density of PV-inputs to PV-somata between S1 (0.043 puncta/µm², Hioki et al., 2013) and V1 (0.136 puncta/µm², the present study) are real, or arise from methodological differences.

Interestingly, Hioki et al. (2013) report that the density of PV-PV inputs is highest on the dendrites (0.23 puncta/µm² for proximal locations), suggesting that detection of PV-inputs was reliable. However, the extent to which visualizing homotypic connections onto intensely-stained somata presented difficulties is unclear (Sommeijer & Levelt, 2012; the present study), despite PV-cells being labeled with membrane-targeted GFP. Considering the possibility that the density of PV-inputs to PV-somata in S1 could actually be 0.136 puncta/µm² as we have found in V1,
this would bring the total density of somatic inputs contributed by identified inhibitory subtypes to 0.412 puncta/µm², equivalent to the reported VGAT+ puncta density of 0.41/µm², and accounting for a 22% shortfall in the observed total density of inhibitory subtype inputs (0.319 puncta/µm²). Overall, our results and those of Hioki et al. (2013) provide evidence of strong, proximally-targeted PV-innervation of PV-cells, which may influence circuit function (Galaretta & Hestrin, 2001b; Bartos et al., 2007) and plasticity (Hensch, 2005) in numerous ways.

**Functional impact of cell type-specific regulation of PV-innervation**

Consideration of further results from Lee et al. (2014) in mPFC may aid in our understanding of the expression of ocular dominance plasticity in V1. In their study, they found that activation of callosal inputs from the contralateral mPFC can drive spiking in Ctip2+ (Type A) cells. Further, the potent inhibition that Ctip2+ cells receive is recruited by callosal inputs, and is suggested to be PV-cell-mediated. That callosal inputs, which mediate a substantial portion of the ipsilateral-eye response recorded in V1 (Restani et al., 2009; Cerri et al., 2010), provide both excitation and disynaptic inhibition to Ctip2+ cells raises an interesting possibility. Following monocular deprivation (MD), silencing of callosal inputs via injection of muscimol into V1 contralateral to the recording site (i.e., into the non-deprived hemisphere, which is driven most strongly by the open eye) unmasks deprived-eye inputs in the deprived-hemisphere, resulting in stronger responses to stimulation of that eye (Restani et al., 2009). Further, that deprived-eye strengthening occurs immediately upon callosal silencing suggests that this effect may be mediated by relief from callosally-driven inhibition (ibid) that is functioning to mask deprived-eye afferents (Duffy et al., 1976; Sillito et al., 1981; Yazaki-Sugiyama et al., 2009). Interestingly, callosal-silencing during MD blocks the normal weakening of the deprived eye.
(Restani et al., 2009), suggesting that the strengthening of ipsilateral input onto PV-cells with long-term MD (Yazaki-Sugiyama et al., 2009) may involve callosal inputs from the non-deprived hemisphere. Driving PV-cells by the open eye via callosal inputs could also potentiate their connections onto deprived-hemisphere pyramidal cells (Maffei et al., 2006), facilitating the masking of deprived-eye inputs to these cells (Restani et al., 2009).

Interestingly, cortical UP states, slow oscillations of spontaneous depolarization and spiking in ensembles of cells, are thought to originate in L5 (Beltramo et al., 2013) and rely on inhibitory modulation from PV-cells for their initiation (Neske et al., 2015). UP states can be evoked by synchronous afferent input, such as is transmitted thalamocortically (MacLean et al., 2005) during phasic responses to viewing of natural scenes (Hensch, 2004, 2005). As callosal cells are concentrated in superficial layers (Mizuno et al., 2007) and would receive feedforward excitation following thalamocortical excitation of L4 (Feldman & Brecht, 2005), it is possible that UP states may arise from interhemispheric activation of L5. Further, callosal inputs to Ctip2+ cells do not depress and actually weakly facilitate (Lee et al., 2014), suggesting that callosally-mediated spiking of these cells may be sufficient to reliably drive UP states. Triggering of UP states, particularly in the deprived-hemisphere by callosal inputs mediating open-eye activity (Restani et al., 2009), may help create the conditions appropriate for spike-timing-dependent plasticity (Hensch, 2004, 2005) to facilitate strengthening of open-eye responses, as part of the late-stage response to monocular deprivation.
CHAPTER 4

Plasticity of PV-cell circuits

following dark-rearing
ABSTRACT

The maturation of PV-cell synapses onto V1 neurons has been implicated in gating the critical period for ocular dominance plasticity (Huang et al., 1999; Fagiolini et al., 2004). However, the exact location of the operant synapses within the cortical microcircuit has not been determined. In Chapter 3, we found that PV-cells in primary visual cortex innervate their targets in a cell type-specific manner, with both PV-cells and certain pyramidal cells receiving high levels of PV-input. To examine whether PV-pyramidal or PV-PV connections may be selectively important for critical period regulation, we raised mice in the dark to delay visual cortical plasticity until adulthood. By analyzing brains that were developmentally-mature but that had not yet undergone experience-dependent maturation, we reasoned that observing either of these connections in a weakened state might signal that that connection regulates the status of the critical period. We addressed this question of dark-rearing’s effects on V1 PV-circuitry using both anatomical and electrophysiological techniques.
INTRODUCTION

Beyond uncovering the targeting-preferences of cortical cell-types, a key goal of developmental neuroscience is to understand the forces that regulate the strength of various circuits. Many connections develop in an activity-dependent manner, with much of the activity in primary sensory areas originating from powerful thalamic inputs (Hensch, 2004, 2005). The activity level of these inputs is governed by an animal’s sensory experience, and the changes that occur in response to altered sensory drive are referred to as experience-dependent plasticity. This phenomenon has been studied using various paradigms, one of which involves depriving an animal of sensory experience during the critical period. By removing the influence of experience during the time when its ability to sculpt circuits is strongest, the circuitry can be captured in an impoverished state and the missing, experience-dependent component of development can be identified. In the visual cortex (V1), sensory deprivation is achieved by rearing an animal in complete darkness, such that spontaneous (Katz & Shatz, 1996) but not light-evoked activity is transmitted along the visual pathway. Dark-rearing prevents experience-dependent maturation and keeps V1 in a juvenile state, with numerous cortical response properties failing to develop properly (Fagiolini et al., 1994). Crucially, the critical period for ocular dominance (OD) plasticity remains open in the dark, with monocular deprivation (MD) able to induce an OD shift in adult animals upon exposure to light. While the effects of dark-rearing on circuit maturation have previously been investigated (e.g. Desai et al., 2002; Tropea et al., 2006), most studies have focused on changes in excitation. Of the studies addressing inhibition, most have considered either global alterations to the V1 GABA system (e.g., Mower et al., 1986; Chen et al., 2001), or cellular changes in inhibition exclusively onto pyramidal cells (e.g., Morales et al., 2002; Katagiri et al., 2007). Given the robust innervation of PV-cells by other PV-cells described in
Chapter 3, we decided to investigate how PV-input to both pyramidal and PV-cells is affected by dark-rearing. After providing an overview of what is already known about the effects of dark-rearing on V1, our results on PV-specific plasticity will be described.

**Cortical plasticity in response to dark-rearing**

Throughout many decades of dark-rearing’s use as a tool to study experience-dependent plasticity, a rich picture of its impact on V1 development has emerged. As mentioned above, its most striking effect is its ability to maintain the critical period for ocular dominance plasticity in an open state for as long as an animal remains in the dark (Fagiolini et al., 1994). This outcome is directly dependent on the animal’s sensory history, as interruption of dark-rearing (DR) with as little as 6hrs of light is sufficient to trigger subsequent critical period closure in cats (Mower et al., 1983; in mice, several days of light are required, Iwai et al., 2003). Further, it is the complete lack of sensory-evoked activity that maintains critical period openness, as binocular lid suture (BS) – a form of visual deprivation in which only dim, un-patterned light reaches the retina – fails to prevent critical period closure (Mower et al., 1981). Interestingly, these manipulations have similar anatomical outcomes in that segregation of thalamocortical axons serving the two eyes into ocular dominance columns remains incomplete, with even subsequent MD unable to refine their highly overlapping distributions (Mower et al., 1985). However, DR and BS affect the receptive field properties of V1 neurons in dramatically different ways. While after DR most cells remain binocularly-driven, BS produces cells whose responses are largely restricted to one eye or the other, with many cells showing no light-evoked responses at all (Mower et al., 1981).

These results highlight two important aspects of using dark-rearing to study experience-dependent plasticity. First, as mentioned above, sensory-evoked activity needs to be completely
eliminated in order to maintain V1 in a truly immature state. Further, dark-rearing removes the experience-dependent component of V1 development specifically, without disrupting spontaneous activity (e.g., through intraocular injection of TTX, Chattopadhyaya et al., 2004) or producing axonal degeneration (e.g., through enucleation, Nys et al., 2015). Second, dark-rearing does not arrest V1 development completely, but instead slows down the maturational process of certain receptive field properties (Kang et al., 2013). For instance, cats 6 weeks old and raised in the light are more plastic than dark-reared cats of the same age (Beaver et al., 2001), indicating that the entire critical period for OD plasticity is shifted later in the absence of experience. Similarly, while in light-reared (LR) mice visual acuity matures rapidly after eye-opening to reach adult levels by ~P21, this process is delayed but not fully prevented by dark-rearing, which yields acuity that is substantially reduced at P21 but which fully matures even in the dark by P55 (Kang et al., 2013). Notably, reduction of acuity by MD (i.e., amblyopia) remains possible only during the critical period even in DR mice, with adult MD failing to alter acuity (ibid) despite producing a robust ocular dominance shift (Fagiolini et al., 1994). As acuity is measured in L4, its lack of change in adult DR mice draws a parallel to the static OD columns described by Mower et al. (1985), highlighting the notion that dark-rearing’s greatest impacts are on intracortical circuits (Beaver et al., 2001).

**Synaptic plasticity following dark-rearing**

Dark-rearing has a multifaceted set of effects on neurons and synapses in V1. Early studies were focused on excitation, with the goal of elucidating a role for N-Methyl-D-aspartate (NMDA) glutamate receptor-dependent plasticity mechanisms in the experience-dependent plasticity of V1. As DR delays an experience-dependent loss of NMDA-receptor function in cat
V1 (Fox et al., 1991), it was hypothesized that critical period status may be associated with the ability to induce long-term potentiation (LTP). Consistent with this, Kirkwood et al. (1995) found that while white matter stimulation during the third postnatal week produced a robust LTP onto targets in layer 3, at five weeks of age LTP could no longer be induced. Intriguingly, dark-rearing until this later age preserved the potential for plasticity, with P35 DR slices exhibiting LTP as robust as P15 LR slices.

On the level of individual neurons, V1 can be characterized as responding to the reduction in sensory drive that results from dark-rearing by increasing the strength of excitation (Desai et al., 2002). For example, miniature excitatory postsynaptic currents (mEPSCs) in layer 4 star pyramidal cells normally increase in frequency and decrease in amplitude over the course of the third postnatal week, but with dark-rearing during this time amplitude remains high. The delay of such experience-dependent weakening of excitation is paralleled by increases in the expression of both α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)- & NMDA-receptor genes (Tropea et al., 2006) and NMDA-receptor protein (Gordon et al., 1997; Chen et al., 2000). Additionally, this excitatory plasticity may be an attempt by the brain to restore activity levels in cortical circuits to a baseline level, yielding firing rates that are constant regardless of afferent input (Desai et al., 2002).

Similar to how dark-rearing delays an experience-dependent decline in NMDA-receptor function, it also prevents an experience-dependent maturation of inhibition (Morales et al., 2002). Normally, feedforward inhibition onto L2/3 pyramidal cells matures with a time-course that parallels the critical period, with the amplitude of the maximal inhibitory postsynaptic current (IPSC) increasing over the first several weeks postnatal. Shortly after the peak of the critical period the maximal IPSC reaches adult levels (by ~P35; ibid), a milestone thought to
mark the end of V1 plasticity (Kirkwood et al., 1995). DR to five weeks of age prevents this maturation as maximal IPSC size becomes arrested at the three week level, with two days of light-exposure after DR leading to only partial recovery (Morales et al., 2002). This delay in inhibitory maturation following DR is also apparent on the level of postsynaptic GABA<sub>A</sub>R clusters, as uncaging GABA on the soma of patched pyramidal cells was found to produce IPSCs with sharply lower amplitude in DR slices (Katagiri et al., 2007). Further, it was revealed that this decrease is mediated by the presence of fewer open channels overall, suggesting that postsynaptic GABA<sub>A</sub>R number is regulated by visual experience. While GABA<sub>A</sub>Rs will be discussed in greater detail in Chapter 6, the site-specific regulation of connection strength uncovered by this study (i.e., amplitude following uncaging onto the axon initial segment did not change with experience) provided further evidence that inhibition mediated by soma-targeting PV-cells may itself be plastic, suggesting its involvement in critical period mechanism. Indeed, recent studies of connections between fast-spiking cells and pyramidal cells have documented experience-dependent plasticity at this synapse, finding that DR leads to smaller unitary IPSCs in L2/3 but not in L4 (Jiang et al., 2010).

**Structural plasticity arising from dark-rearing**

Compared to our emerging understanding of how dark-rearing impacts the physiological maturation of V1 circuits, relatively little is known about the structural correlates of this plasticity. On a gross level, dark-rearing causes a dramatic shrinkage of primary visual cortex, with cortical thickness and volume declining, and cellular density increasing as a result (Takács et al., 1992). Interestingly, these changes are not accompanied by a reduction in cell number. This holds true for GABA-expressing cells, which, when differences in cellular density are
corrected for, are no less prevalent in DR cat V1 (Mower et al., 1988; but also see Benevento et al., 1995, where lower GABA-cell density was observed in DR rat V1). Although DR leads to a decrease in the size of individual cell bodies, loss of neuropil structure is thought to underlie the bulk of V1 volume reduction (Takács et al., 1992). However, early studies examining synaptic structure reported relatively little change following DR, either in terms of fine-scale morphometry (in rabbits DR to 7 months; Vrensen & de Groot, 1974) or overall synapse number (in cats DR to 7 months; O’Kusky, 1985). More recently, studies in rats have found that while synaptic morphometry is indeed unchanged by DR, a V1-specific drop in synaptic density results, from which auditory cortex is spared (occurs between P28-56; Bakkum et al., 1991). While this study found no evidence of a selective loss of asymmetric vs. symmetric synapses, Gabbot & Stewart (1987) observed a specific decrease in symmetric-synapse density in rats DR to P52. Consistent with this, Valverde (1976) observed that in mice DR to P19, extragranular cells with intracortical axons (i.e., interneurons) showed a dramatic 70% reduction in total axonal length (although different cell-types may not have been equally represented between groups in this small study). Taken together, these anatomical findings identify the neuropil as the major site of dark-rearing’s action in V1, suggesting that altered synaptic connectivity, possibly involving inhibitory interneurons, may result from loss of visual experience.

While most studies agree that the number/density of inhibitory cells is not significantly altered in dark-reared V1 (Mower et al., 1988; Mower & Guo, 2001), reports of a change in PV-cell number specifically are varied, with some studies finding no change (Grabert & Wahle, 2009; Kind et al., 2013) and others a mild (Ye & Miao, 2013) to moderate (Tropea et al., 2006) decrease. Although the sensitivity of some studies’ detection capabilities are unclear, experiments involving over-expression of brain-derived neurotrophic factor (BDNF) – which is
normally expressed in an experience-dependent manner – make clear the link between experience and PV-cell development (Huang et al., 1999). In mice whose expression of BDNF is uncoupled from visual experience, both the density of PV-cell bodies and the exuberance of perisomatic puncta-rings were found to develop precociously. This, along with a sharp downregulation of parvalbumin gene expression following DR (Tropea et al., 2006), suggested to us that investigating the structural plasticity of PV-circuits specifically may be fruitful. While previous studies have found no difference in the density of GAD65-labeled axon terminals following DR (Mower & Guo, 2001), we reasoned that a cell type-specific analysis of PV-input to various neuronal populations might inform our understanding of the persistent plasticity observed in DR mice.
RESULTS

Cell type-specific regulation of PV-innervation by experience

To investigate how experience regulates the level of PV-innervation to various cell-types, we dark-reared (DR) mice to P60 and then performed immunostaining for synaptotagmin-2 (Syt2) and markers of various L5 cell-types (Satb2, Ctip2, WFA) as described in Chapter 3. We confirmed that there was robust expression of Syt2 in PV+ boutons even in dark-reared tissue (Sommeijer & Levelt, 2012), as expected for an activity-independent marker. Initially, we were struck by the appearance of robust Syt2+ perisomatic input to pyramidal cells in dark-reared mice (Figure 4.1.a, DR Ctip2+ PYR cell), which was similar to that observed in light-reared (LR) mice. Upon quantifying the density of Syt2+ inputs to pyramidal cell somata, we found that there was indeed no difference between rearing conditions for either cell-type (Figure 4.1.b). We then considered whether Syt2+ input to PV-cells may change with experience. Unexpectedly, we found that the density of Syt2+ inputs to PV-cell bodies was nearly 40% lower in DR mice (Figure 4.1.b), indicating that the plasticity of PV-innervation is regulated in a cell type-specific manner.
Figure 4.1 = (a) Example Satb2+ PYR, Ctip2+ PYR, and PV+ cells from light-reared (LR, top) and dark-reared (DR, bottom) V1. Note the decreased innervation of PV+ cells by Syt2+ boutons in DR V1. All scale bars, 5µm. (b) Density of Syt2+ boutons by cell-type for various rearing conditions. In 2way ANOVA of all LR & DR cells, significant effect of rearing condition for PV+ cells (Bonferroni posttest, P<0.001). Brackets represent Dunn’s multiple comparison test results from 1way ANOVA of all PV+ cells. (c) Example PV+ cells from V1 of mice dark-reared until adulthood and then exposed to light for several weeks (DR+light), and pre-critical period-aged mice (P15). (d) Volume of Syt2+ boutons contacting PV+ cells in LR & DR V1 (Mann Whitney test).
We next asked whether the level of Syt2-input to dark-reared PV-cells could be rescued by subsequent visual experience. Following dark-rearing from birth, re-exposure to light sets in motion the critical period for ocular dominance plasticity (Mower et al., 1983), suggesting that if PV-PV connections are important in this process they should mature in turn. In mice that were DR until P60 and then placed in a normal light/dark cycle for several weeks, Syt2+ input density to PV-cell somata was found to have recovered to near LR levels (DR+light, Figure 4.1.b).

Having obtained further evidence that PV-PV connectivity matures in an experience-dependent manner, we next looked at cells in mice reared normally to P15, an age when expression of PV in V1 cell bodies is first becoming detectable (Figure 4.1.c ; del Rio et al., 1994). We found that Syt2-input to pre-critical period-aged PV-cells was similar to that received by DR PV-cells (Figure 4.1.b), suggesting that the low level of PV-PV connectivity observed in DR mice reflects a failure of experience-dependent maturation.

As the size of presynaptic boutons has been observed to increase following strengthening of inhibitory synapses (Nusser et al., 1998), we also measured the 3D volume of the Syt2-inputs contacting both light-reared and DR PV-cells. Following loss of visual experience in DR mice, the volume of Syt2+ boutons onto PV-cells was sharply decreased vs. light-reared controls (Figure 4.1.d).

To gain further insight into this cell type-specific regulation of PV-innervation by experience, we turned next to slice physiology. By recording spontaneous inhibitory postsynaptic currents (sIPSCs) in both fluorescently-labeled PV-cells and non-fluorescing pyramidal cells in layer 5 of adult V1, we hoped to find a functional correlate to our Syt2 results. Specifically, sIPSCs are thought to originate predominantly from synapses close to the soma (Soltesz et al., 1995), as removal of much of the dendritic tree from patched hippocampal pyramidal cells leaves...
the distribution of sIPSCs largely unaltered. As such, we aimed to use the frequency of sIPSCs recorded at the soma as a proxy for the number of inhibitory axon terminals contacting the perisomatic region of a given neuron. Although additional classes of interneuron other than PV-cells target the perisomatic domain of both PV+ (Hioki et al., 2013) and pyramidal (Markram et al., 2004) cells, PV-cells are considered to be the dominant source of perisomatic inhibition in the neocortex (Rudy et al., 2011). In order to focus on perisomatically-located inputs and thereby enrich our sample of sIPSCs with those arising from cell-types whose innervation is proximally-biased (Soltesz et al., 1995), we restricted our analyses to those events with a 10-90% rise-time of <3ms (Nahmani & Turrigiano, 2014).
Figure 4.2 = (a) Pharmacologically-isolated spontaneous inhibitory postsynaptic currents (sIPSCs) recorded from YFP-expressing PV-cells in LR (left) and DR (right) acute V1 slices. Scale bar, 15pA, 1s. (b) sIPSC frequency by cell-type. In 2way ANOVA, P<0.05 effect of rearing for PV-cells, via Bonferroni posttest. Asterisks are from Mann Whitney test of PV+ cells. (c) 10-90% rise time by cell-type. In 2way ANOVA, P<0.01 effect of rearing for PV-cells, via Bonferroni posttest. Asterisks are from Mann Whitney test of PV+ cells.
In the presence of DNQX & (RS)-CPP (20mM each, to block AMPA & NMDA receptors, respectively) and using a high-chloride internal solution, we recorded sIPSCs as robust inward events in both PV+ and pyramidal cells voltage-clamped at -60mV (Figure 4.2.a). When we compared the frequency of sIPSCs recorded in PV-cells between LR and DR slices, we found a striking 55% reduction in the DR cells, consistent with their receiving fewer Syt2/PV-inputs (Fig 4.2.b). In contrast, the sIPSC rate recorded in pyramidal cells was not significantly different between rearing conditions, though it trended lower in agreement with previous reports (Morales et al., 2002, ~40% lower in both studies). That the average rise-times of our events were ~1.15ms for PV-cells and ~1.4ms for pyramidal cells (Figure 4.2.c) suggests that these events originated close to the soma (Soltesz et al., 1995), consistent with their being mediated by perisomatic-targeting inhibitory cell-types such as PV-cells. Interestingly, rise-times were longer selectively in DR PV-cells (Figure 4.2.c), suggesting that the fraction of sIPSCs arising from highly-proximal inputs may be smaller in these cells. Thus, these results are compatible with the notion that there is cell type-specific regulation of PV-innervation by experience.

To examine whether inhibitory synaptic strength may be altered as a function of experience, we next analyzed the amplitude of our recorded sIPSCs. Although previous studies have found that the maximal evoked-IPSC recorded in pyramidal cells is lower in amplitude in DR tissue (Morales et al., 2002), the amplitude of mIPSCs shows a counterintuitive increase in size (ibid). We confirmed this result, finding that our sIPSCs recorded in pyramidal cells were significantly larger in amplitude in DR slices (Figure 4.3.a). In contrast, sIPSCs recorded in PV-cells were smaller in DR slices, indicating bidirectional plasticity of sIPSC amplitude on the basis of cell-type.
**Figure 4.3** = (a) Amplitude of sIPSCs by cell-type. In 2way ANOVA, P<0.05 effect of rearing for PV-cells, via Bonferroni posttest. Asterisks are from Mann Whitney tests of PV+ cells and PYR cells.
In order to investigate a potential role for PV-cells in these sIPSC amplitude changes, we used a recently-developed optogenetic technique to isolate PV-cell-mediated IPSCs specifically (Nahmani & Turrigiano, 2014; C.G. Lau & V.N. Murthy, unpublished). Recording in ACSF in which calcium has been replaced with strontium ($\text{Sr}^{++}$) causes desynchronization of presynaptic release, such that bindings of individual quanta can be visualized after decay of the bulk current. In addition to expressing EYFP, the PV-cells in the mice that we recorded from also expressed the light-gated ion channel channelrhodopsin-2 (Nagel et al., 2003; Boyden et al., 2005). Light-activation of the PV-cell network in the presence of strontium-ACSF enriches the population of IPSCs with activation-mediated events (Figure 4.4.a), increasing the IPSC rate recorded in patched cells above the sIPSC rate (Nahmani & Turrigiano, 2014; Lau & Murthy, unpublished).

In cells in which the evoked-IPSC rate is substantially higher than the sIPSC rate (>3x), the IPSC population can be considered to be highly-enriched with activation-mediated events (“PV-IPSCs”), the properties of which can then be compared to the broader sIPSC population.

First, we extended this technique to study PV-PV connections. In previous reports (Nahmani & Turrigiano, 2014), this technique was used to study connections made by a channelrhodopsin-expressing cell-type (PV-cells) onto non-channelrhodopsin-expressing target cells (L4 star pyramidal cells). When we patched fluorescing, channelrhodopsin-expressing PV-cells and light-activated the local PV-cell network – including the patched cell – with 2ms pulses of blue light, we observed a large, inward bulk current followed by desynchronized, putatively quantal events (Figure 4.4.a). As PV-cells exhibited an average 5.4-fold increase in IPSC rate following light pulses (Figure 4.4.b), we were convinced that we were recording PV-IPSCs in PV-cells. Thus, we functionally confirmed our anatomical findings indicating robust PV-PV innervation in L5 of V1 (Chapter 3), a layer previously shown in somatosensory cortex to
contain extensive interconnections between PV-cells (Gibson et al., 1999; Galaretta & Hestrin, 2002). We obtained a similar enhancement of IPSC rate in patched pyramidal cells upon light-activation of the PV-cell network (6.2-fold increase, Figure 4.4.b), suggesting that we generated PV-IPSCs with similar efficiency in both cell-types.

We then compared the amplitudes of PV-IPSCs between cells recorded in LR and DR slices. Interestingly, PV-IPSC amplitude did not change as a function of experience in either cell-type (Figure 4.4.c). However, upon comparing the average PV-IPSC amplitudes of all PV-cells with those of all pyramidal cells, we found that PV-cells exhibited PV-cell-mediated currents that were significantly larger than those seen in pyramidal cells (P<0.05, Mann-Whitney test), not unexpected given previously reported specializations at synapses between FS-cells in the hippocampus (Bartos et al., 2007).

We next sought to explore the relationship between the PV-IPSCs that we recorded and the broader sIPSC population. In the original report of this technique, the properties of sIPSCs and PV-IPSCs recorded in L4 star pyramidal cells were found to be very similar, with the rise time and amplitude of the two populations being nearly identical (Nahmani & Turrigiano, 2014). This similarity supported the notion that the PV-IPSCs were indeed quantal, and further suggested that the majority of sIPSCs recorded in L4 star pyramidal cells are in fact PV-cell-mediated. In our L5 recordings, the PV-IPSCs that we recorded differed from spontaneous events in important ways (Figure 4.5).
Figure 4.4 = (a) Light-activation (vertical blue bar) of local PV-cells in PV-ChR2-YFP slices generates inward channelrhodopsin current and bulk IPSC in PV-cells voltage-clamped at -60mV (initial negative peak clipped for clarity). Desynchronization of presynaptic release with extracellular strontium (Sr$^{++}$) isolates PV-cell-driven synaptic events after decay of bulk IPSC (“PV-IPSCs”, asterisks). Scale bar, 10pA, 25ms. (b) (Left) IPSC frequency of PV+ cells prior to (sIPSC) and following (PV-IPSC) light-activation of the PV-cell network. Only cells exhibiting at least a 3-fold increase in IPSC frequency were included (average ~5.4-fold; Wilcoxon signed rank test). (Right) In PYR cells, average ~6.2-fold increase in IPSC frequency following light-activation of the PV-cell network; Wilcoxon signed rank test. (c) Amplitude of PV-IPSCs by cell-type.
Figure 4.5 = (a) (Left) Cumulative distribution of 10-90% rise times of sIPSCs (black lines) & PV-IPSCs (green lines) recorded in light-reared (dashed lines) & dark-reared (solid lines) PV+ cells. Asterisk, results from KS tests comparing events sampled from their broader populations (LR sIPSC vs. DR sIPSC; LR PV-IPSC vs. DR PV-IPSC; DR sIPSC vs. DR PV-IPSC). (Inset) Cell-averaged representation of the same data shown in the cumulative distributions. Red symbols, Mann Whitney test (#, P=0.0519). Blue symbols, paired t-tests comparing sIPSCs and PV-IPSCs recorded in the same cells. (Right) 10-90% rise times for PYR cells. Asterisk, KS test (DR sIPSC vs. DR PV-IPSC). (Inset) Cell-averaged data for PYR cells. (b) (Left) Cumulative distribution of event amplitudes recorded in PV+ cells. Asterisk, KS tests (LR sIPSC vs. LR PV-IPSC; DR sIPSC vs. DR PV-IPSC, **). (Inset) Cell-averaged data. Red symbol, Mann Whitney test. Blue symbols, paired t-tests. (Right) Amplitudes for PYR cells. Asterisk, KS tests (LR sIPSC vs. LR PV-IPSC, **; DR sIPSC vs. DR PV-IPSC). (Inset) Cell-averaged data for PYR cells. Red symbol, Mann Whitney test.
Across cell-types and conditions, PV-IPSCs exhibited 10-90% rise times that were faster than sIPSCs recorded in the same cells (Figure 4.5.a, left & right, insets; blue symbols signify results from paired t-tests). Further, PV-IPSCs recorded in LR PV-cells were nearly significantly faster on a cell-averaged basis (P=0.0519, Mann Whitney test) than those recorded in DR PV-cells, a difference that was manifest when events sampled from the entire PV-IPSC population were compared using a KS test (Figure 4.5.a, left, LR PV-IPSC vs. DR PV-IPSC, P=0.012). Interestingly, of all events recorded in PV-cells, the rise times of DR sIPSCs (solid black line, Figure 4.5.a) were significantly slower than those of all other groups (P<0.05, KS tests), and by a considerable margin. This suggests that fast, PV-cell-mediated events may make up a smaller fraction of the sIPSC population in DR PV-cells than they do in the LR sIPSC population, the curve for which (dashed black line) is closer to its PV-IPSC counterpart (dashed green line). As mentioned above, pyramidal cells from both light-reared and DR slices received PV-IPSCs with 10-90% rise times that were faster than sIPSCs recorded in the same cells (Figure 4.5.a, right). However, while both sIPSCs and PV-IPSCs trended towards showing faster rise times in DR pyramidal cells, this failed to reach significance (P=0.16 & P=0.11, respectively; Mann Whitney test), likely due in part to small sample size.

Finally, we considered how the amplitude of PV-cell-mediated IPSCs may differ from that of sIPSCs. In both LR & dark-reared PV-cells, PV-IPSCs were larger in amplitude than sIPSCs recorded in the same cells (Figure 4.5.b, left, inset). Notably, although sIPSC amplitude was significantly reduced in DR PV-cells as reported above (Figure 4.3), PV-IPSC amplitude was nearly identical across rearing conditions (Figure 4.5.b, left, inset). That PV-IPSC amplitude was not lower in DR PV-cells suggests that the decreased sIPSC amplitude exhibited by these cells may arise through indirect means (discussed below). Analysis of the amplitude of PV-
IPSCs recorded in pyramidal cells was not as fruitful due to higher variability in this cell-type as well as small sample size (Figure 4.5.b, right, inset), although PV-IPSCs trended towards larger amplitudes in both LR (P=0.09) and DR (P=0.1) pyramidal cells (paired t-tests). However, results from KS tests comparing events sampled from the entire PV-IPSC population suggest that the amplitude of PV-IPSCs is indeed larger than that of sIPSCs (LR, P=0.006; DR, P=0.019).
DISCUSSION

In this Chapter, we studied how PV-cell input to various L5 cell-types is affected by dark-rearing, a classical manipulation that delays V1 plasticity until adulthood (Hensch, 2005). As development of V1 continues over the first few weeks of postnatal life (Maffei & Turrigiano, 2008), we studied dark-reared rather than juvenile V1 in order to focus our analyses specifically on how loss of visual experience affects the maturation of PV-cell-mediated inhibition. Surprisingly, we found that while PV-input to excitatory pyramidal cells was unchanged in the dark, PV-input to PV-cells was dramatically reduced, suggesting that experience acts in a synapse-specific manner to mature PV-PV circuits and regulate plasticity. This specificity is the central finding of this Chapter, and its precedence, potential mechanisms, and impact on V1 physiology will be discussed.

Our study of the effects of dark-rearing on inhibitory maturation in V1 differs from past studies in important ways. While previous studies have found that loss of visual experience reduces inhibitory input to pyramidal cells, these studies either used different deprivation paradigms (Chattopadhyaya et al., 2004), analyzed different layers (Morales et al., 2002), or considered changes to inhibition only globally and without respect to presynaptic cell-type (Kreczko et al., 2009). For instance, using a transgenic line in which a subset of PV-cells expresses GFP, Chattopadhyaya et al. (2004) looked at their input to L5 pyramidal cells after intraocular injection of TTX during the critical period. Following complete blockade of one eye’s activity, pyramidal cells in the binocular zone of the deprived hemisphere received fewer PV-boutons, suggesting that a severe imbalance in afferent input can drive rewiring of downstream PV-axons. In contrast, our selective removal of visually-evoked activity from both eyes failed to alter PV-input to pyramidal cell somata (Figure 4.1.a-b), suggesting that such
innervation may, at least on the anatomical level, develop in a largely experience-independent manner.

By using the PV-specific marker synaptotagmin-2 (Sommeijer & Levelt, 2012), we focused our analyses on a single class of interneuron implicated in regulating plasticity (Sugiyama et al., 2008). In doing so, we avoided other, non-PV+ inhibitory cell-types whose inputs would be labeled by broad markers such as GAD65 (Kreczko et al., 2009), which may exhibit counter-directional changes (e.g., Papadopoulos et al., 1993) that mask an overall effect. This limitation necessarily affects many physiology studies, as recordings of either spontaneous or evoked IPSCs include responses mediated by a broad population of presynaptic cell-types. At this resolution it is difficult to pinpoint which circuits may be undergoing plasticity, even when L4-to-L2/3 feedforward inhibition is probed specifically (Morales et al., 2002). Nevertheless, maturation of PV-cells and their connectivity has been shown to occur in an experience-dependent manner (in mouse S1: Jiao et al., 2006, 2011; in mouse V1: Huang et al., 1999; Sugiyama et al., 2008), including in L4 (Quast & Hensch, 2012) where PV-cells are highly abundant (Gonchar et al., 2008) and form many feedforward connections (Cruikshank et al., 2007). While the results we obtained in L5 regarding the plasticity of sIPSC frequency & amplitude are similar to those of Morales et al. (2002) in L2/3 (i.e., sIPSC frequency in our L5 pyramidal cells was not as definitively reduced as it was in their L2/3 cells), there are reasons to think that the responses of these two layers to dark-rearing may not be identical.

For instance, the upper and lower layers of V1 differentially express polysialic acid (PSA; Di Cristo et al., 2007), a cell-surface molecule shown to negatively regulate inhibitory maturation and critical period onset by its attenuation of the function of neural cell adhesion molecule (NCAM; Gascon et al., 2007). Before eye-opening, expression of PSA is initially high
throughout all layers, then declines in an experience-dependent manner in the lead up to the critical period (Di Cristo et al., 2007). This decline permits PV-cells’ perisomatic innervation of pyramidal cells to mature, as evidenced by enzymatic digestion of PSA facilitating precocious maturation. However, experience-dependent clearance of PSA from lower layers is incomplete even in normal, light-reared mice, with L5 displaying persistent expression of PSA throughout the critical period. That PV-innervation still matures in the face of locally-higher PSA levels – which are even higher in V1 of DR mice (ibid) – suggests that PV-cells in particular may possess mechanisms (e.g., Okaty et al., 2009; Rossier et al., 2015) of overcoming this barrier to synaptogenesis (Gascon et al., 2007).

Indeed, at least one previous study of inhibitory maturation has found that sensory deprivation fails to reduce somatic innervation. Specifically, Micheva & Beaulieu (1995) report that in barrel cortex of rats deprived of whisker experience from birth until 2 months of age, somatic innervation by GABA(+) terminals actually nearly doubles in L2/3 & L5, with only L4 & L6 showing mild decreases. Surprisingly, most of the synaptic loss was concentrated on dendrites and particularly on dendritic spines (ibid), which are also synaptic targets of PV-cell terminals (Kawaguchi & Kubota, 1998). As such, it is likely that previous studies analyzing bulk inhibitory innervation of pyramidal cells (e.g., Morales et al., 2002) would have detected this loss of inhibition to dendrites, which we did not address in our study of PV-cell-mediated somatic inputs specifically.
Mechanisms governing inhibitory development

Compared to our wealth of knowledge on the formation and plasticity of excitatory synapses, we know considerably less about how these processes are regulated with respect to inhibition (Huang et al., 2007). However, insights into the diverse roles of adhesion molecules, the extracellular matrix, and other factors in inhibitory synaptogenesis and maturation have recently started to emerge (Huang & Scheiffele, 2008; Flores et al., 2014). While the mechanisms permitting target cell-specific regulation of plasticity are not yet fully known, there are several possibilities that may explain what we have observed to occur for PV-PV vs. PV-pyramidal connections following dark-rearing.

Cell adhesion molecules

Neuroligin 2 (NL2), a cell adhesion molecule found exclusively at inhibitory synapses (Varoqueaux et al., 2004), is one such candidate by which the synaptic specificity of plasticity may be achieved. Through its activation of another key inhibitory postsynaptic molecule, collybistin, NL2 recruits gephyrin and can drive self-organization of GABAergic postsynapses complete with GABAA receptors (GABAARs; Poulopoulos et al., 2009). Interestingly, NL2−/− animals show deficits in synaptic assembly at perisomatic but not dendritic sites (ibid; also, Jedlicka et al., 2011), which leads to a loss of inhibitory events with fast-onset times (Poulopoulos et al., 2009). Strikingly, in acute slices of NL2−/− brains, paired whole-cell recordings of PV-cells & postsynaptic pyramidal cells reveal strong deficits in the amplitude of unitary IPSCs, an effect not seen in connections between somatostatin-positive cells & pyramidal cells (Gibson et al., 2009). As the expression level of NL2 impacts inhibitory synapse number (Fu & Vinici, 2009), experience-dependent regulation of NL2 expression in PV-cells may be one
mechanism by which perisomatic inputs are controlled \textit{in vivo}. Further, NL2 overexpression speeds a developmental shift in the switch from slow-decaying $\alpha_2/\alpha_3$-subunit-containing GABA$_A$Rs to fast-decaying $\alpha_1$-receptors (ibid), suggesting that elucidation of such a shift in PV-cells with experience may offer further support to this hypothesis.

However, there are at least two caveats to experience-dependent regulation of NL2 expression as a candidate mechanism for reduced PV-PV innervation with dark-rearing. First, the frequency of connections between FS & PYR cells is unaltered in NL2$^{-/-}$ mice, suggesting that NL2 may regulate the function of PV-cell synapses more so than their formation (Gibson et al., 2009), and not necessarily explain the reduced PV-PV innervation that we observed. However, it is important to note that synaptotagmin-2 staining alone does not reveal the number of presynaptic partners contacting a given cell body, only their total number of boutons. It is possible, for example, that dark-reared PV-cells may possess the same number of presynaptic partners, and that the presence of fewer boutons overall is mediated by each partner contributing fewer boutons. Connection probability would be static in this scenario, and the reduction in unitary IPSC amplitude that results from NL2 deletion might then be due to a reduction in the number of release sites. Different anatomical techniques, such as Brainbow-labeling of the population of presynaptic PV-cell axons, could potentially clarify the circuit-level basis of the reduced PV-PV innervation observed in dark-reared mice.

A second caveat is that NL2$^{-/-}$ mice exhibit an overall decrease in mIPSC amplitude, an effect suggested to arise from an observed decrease in the amplitude of PV-cell-mediated events on the quantal level (Gibson et al., 2009). While we did observe a decrease in sIPSC amplitude in dark-reared PV-cells, our strontium recordings indicated that PV-IPSCs were not reduced in size. Nevertheless, that our reduction in sIPSC amplitude may be due to large, PV-cell-mediated
events comprising a smaller fraction of the sIPSC population is still compatible with the notion that downregulation of NL2, rather than total knockout, can modulate inhibitory synapse number downward (Fu & Vinici, 2009).

IgSF9b, an NL2-linked cell adhesion molecule, may provide another level of control (Woo et al., 2013). IgSF9b is highly expressed in GABAergic interneurons and less so in hippocampal pyramidal cells, where it is mainly present at inhibitory synapses both pre- & postsynaptically and may mediate homophilic trans-synaptic adhesion. Crucially, Woo et al. (2013) show that postsynaptic knockdown of IgSF9b reduces inhibitory synapse number specifically onto interneurons, as pyramidal cells fail to exhibit a significant reduction (~18% decrease vs. ~12%, with no effect on excitatory synapses for either cell-type). While for interneurons this was confirmed functionally as a ~50% reduction in mIPSC frequency, no data is shown for pyramidal cells, leaving the specificity with which IgSF9b regulates inhibitory-inhibitory connections somewhat uncertain. Woo et al. (2013) suggest that the breadth of IgSF9b expression in the inhibitory population (~80% of cells) indicates a lack of cell type-specificity, a conclusion further supported by an overlapping distribution of the labeling of known splice variants. Even so, as PV-cells do not extensively innervate other inhibitory cell-types (Pfeffer et al., 2013), the possibility that experience may regulate IgSF9b expression in PV-cells and function to scale up network interconnectivity is intriguing.

**Extracellular matrix**

The extracellular matrix (ECM) provides another avenue by which inputs to PV-cells may be regulated. In V1 and other areas of the nervous system, ECM components have long been observed to aggregate around the soma and proximal dendrites of a sparse neuronal
population, later revealed to be PV+ basket cells (Lüth et al., 1992). The resulting structures are called perineuronal nets (PNNs; Wang & Fawcett, 2012), and they are comprised of chondroitin sulfate proteoglycan (CSPG) and tenascin-R molecules attached via link proteins to hyaluronan, a cell surface-expressed polysaccharide. PNN components like the CSPG aggrecan accumulate around PV-cells in an experience-dependent manner across brain regions (Guimaraes et al., 1990; Lander et al., 1997; McRae et al., 2007; Balmer et al., 2009; Ye & Miao, 2013), a process that is retarded by sensory deprivation. PNNs are thought to stabilize synaptic connections and prevent further plasticity (Wang & Fawcett, 2012), and their removal via enzymatic digestion of CSPGs reopens V1 plasticity in adult animals (Pizzorusso et al., 2002). Despite this, the link between PNNs and plasticity remains incompletely understood.

A number of studies identify deficits in net formation and in perisomatic inhibition following genetic deletion of ECM/PNN components, suggesting that these molecules may play a role in the recruitment and stabilization of PV-inputs. For example, knockout of major ECM component tenasin-R causes a 35% reduction in the number of inhibitory active zones apposed to CA1 pyramidal cell somata (Nikonenko et al., 2003). While experience-dependent expression of tenascin-R on a V1-wide level (Ye & Miao, 2013) would appear not to affect the maturation of perisomatic inhibition onto L5 pyramidal cells (Figure 4.1.a-b), it is possible that additional molecules found exclusively in net-bearing cells (Carulli et al., 2006) may interact with ECM components such as tenascin-R to provide more local regulation of inhibitory innervation. One such molecule is cartilage link protein (Crtl1), which shows a peak in expression shortly after eye opening and is thought to function as a key trigger in PNN formation via its linkage of ECM components to cell surface-expressed hyaluronan (Carulli et al., 2010). Knockout of Crtl1 does not affect the ECM at large, but does attenuate its aggregation into PNNs. Interestingly, the
number of Crtl1-expressing cells is sharply lower in dark-reared V1, suggesting a mechanistic link between sensory deprivation and a resulting loss of PNNs. If Crtl1-induced aggregation of ECM components like tenascin-R is curtailed in the dark, this may lead to a loss of inhibitory inputs specifically onto Crtl1-expressing PV-cells. Strikingly, Crtl1-knockout mice also show adult plasticity (ibid), raising the possibility that dark-rearing-induced loss of PV-PV connectivity may be a common mechanism owing to diminished PNNs.
4.6. (a) A light-reared PV-cell enwrapped by a WFA+ perineuronal net, with interdigitating Syt2+ boutons. The cell is shown at its largest cross-section (top row) and closer to one pole (bottom row). All scale bars, 5µm. (b) WFA+ perineuronal nets found around L5 PV-cells in light-reared (top row) and dark-reared (bottom row) V1. Note that nets around LR cells are crisper and somewhat higher contrast than those around DR cells.
However, the connection between the level of perisomatic innervation that a cell receives and the exuberance of its PNN is not entirely clear. Indeed, despite Syt2+ puncta being localized to holes in the WFA+ lattice (Figure 4.6.a), Syt2-input density appears to correlate only weakly with PNN complexity on a within-rearing-condition basis. Nevertheless, in agreement with previous reports (e.g., Carulli et al., 2010), PNNs surrounding LR PV-cells appear more well-formed than do those of DR PV-cells (Figure 4.6.b), suggesting that there may be some basis to the notion that PNN complexity and PV-innervation level are linked. Even so, it remains unclear how PNN removal via chondroitinase-induced digestion of CSPGs restores plasticity (Pizzorusso et al., 2002), and what effect this has on the afferent synapses located within the net.

Interestingly, some aspects of this latter point are starting to be addressed. Treating mature dissociated hippocampal cultures with chondroitinase produces no change in the density of inhibitory puncta apposed to basket cell somata (Dityatev et al., 2007), suggesting that PNNs may not be involved in actively maintaining these inputs. However, a study in which chondroitinase was delivered to rat V1 in vivo found that inhibitory events onto unspecified L4 cells recorded in acute slices were weaker (Liu et al., 2013), suggesting that maintenance of inhibitory inputs whose development is guided by visual experience and not spontaneous activity may respond differently to PNN removal. Further, as we have not examined the prevalence and potential for plasticity of other, non-PV+ inputs to either PV+ or pyramidal cell bodies, such as those contributed by VIP+ small basket cells (Hioki et al., 2013), it is unknown whether any compensation in somatic input by other interneuron classes, which may be more or less reliant on PNNs for their formation and stabilization, may occur with DR. Overall, more work is needed to determine exactly how the ECM and PNNs may regulate experience-dependent perisomatic innervation of various cell-types.
Finally, emerging evidence suggests that PV-cells may be able to facilitate their own structural plasticity via expression of matrix metalloproteinases (MMPs; Okaty et al., 2009; Rossier et al., 2015). MMPs are a class of proteases that are either targeted to the surface of developing neurites or secreted extracellularly, processes that are thought to occur in an activity-dependent manner (Rivera et al., 2010; Huntley, 2012). Once activated, they are capable of degrading various ECM components including CSPGs and tenasin-R, can regulate the function of cell adhesion molecules, and can activate neurotrophins such as BDNF as well as their receptors. The role of MMPs in synaptic plasticity has been most studied at excitatory synapses, where following NMDA receptor activation they may digest CSPGs in the vicinity of dendritic spines and facilitate synaptic remodeling. It is possible to imagine similar receptor-linked activation of MMP release at excitatory synapses onto PV-cells. In PV-cells of light-reared mice experiencing higher levels of thalamocortical activation via powerful, proximally-biased inputs (White et al., 1984; Cruikshank et al., 2007), MMP-mediated digestion of CSPGs at these sites may permit increased innervation by perisomatically-targeting PV-cell axons. As pyramidal cells seldom receive excitatory inputs onto their somata (White & Rock, 1980) but PV-cells often do (White et al., 1984; Quast & Hensch, 2012), glutamate-receptor-linked activation of local MMP release (Rivera et al., 2010) may mediate cell type-specific regulation of PV-innervation as a function of experience. Tissue inhibitors of metalloproteinases (TIMPs; Huntley, 2012), the primary endogenous inhibitors of MMPs, could further regulate cell type-specific inhibitory maturation if their clearance from PV-cell somata occurs in an experience-dependent manner.
Impact of reduced reciprocal PV-inhibition on V1 physiology

Using immunolabeling of Syt2 as a proxy for PV+ boutons, we found that PV-input to PV-cell bodies was reduced in dark-reared mice. In our slice physiology experiments, we found that the frequency of sIPSCs received by DR PV-cells was reduced, consistent with their receiving fewer Syt2+/PV-cell-mediated inputs. However, as any spontaneously active inhibitory input that is located electrotonically close enough to the recording pipette (within ~30um; Kubota, 2014) would contribute to the measured frequency, we cannot conclude that the decrease in frequency we observed was mediated solely by PV-cell-mediated events, as other inhibitory cell-types contact the perisomatic region as well (Markram et al., 2004). Nevertheless, other data that we obtained provide indirect evidence suggesting that loss of PV-cell-mediated inputs/events did indeed occur in DR PV-cells. Specifically, in dark-reared PV-cells, strontium-desynchronized PV-IPSCs were static in amplitude vs. light-reared controls, but sIPSCs did show an amplitude decrease. That the frequency of sIPSCs, along with the density of Syt2+ boutons, was decreased in dark-reared mice, offers indirect evidence that the decrease in sIPSC amplitude was mediated by the presence of fewer large, PV-cell-mediated events. Thus, overall, these results suggest that PV-cells in dark-reared V1 receive less functional inhibition from other PV-cells.

That dark-rearing, which keeps V1 plasticity open (Fagiolini et al., 1994), selectively maintains PV-PV and not PV-pyramidal connections in an immature state suggests that the former circuit may be a site of critical period regulation. In other words, from an initially weaker state that permits plasticity, the experience-dependent strengthening that PV-PV connections undergo may in turn mark the end of the critical period. In published work, the PV-PV connection has received relatively little attention in the context of experience-dependent
plasticity. In one of the only reports, Maffei et al. (2006) show that 3 days of monocular deprivation (MD) at the beginning of the critical period (P18-21) does not alter the connection strength of FS-FS pairs in L4 of rat V1b. However, as perisomatic innervation of pyramidal cells in organotypic slices nearly doubles between P18 & P24 (Chattopadhyaya et al., 2004) as well as undergoes further experience-dependent maturation until the peak of the critical period (ibid) and beyond (Huang et al., 1999; Morales et al., 2002), our theory is not incompatible with the results of Maffei et al. (2006). Specifically, structural and functional maturation of the PV-cell network may simply occur with a time-course that parallels the critical period, without a requirement for MD-induced plasticity at this synapse to be present. Alternatively, other properties of FS-cell networks, such as the connection probability or kinetics of FS-FS connections (Bartos et al., 2007), could exhibit MD-induced plasticity that reflects what we have observed here in response to dark-rearing. Further, individual layers may respond to deprivation in different ways (Maffei & Turrigiano, 2008; Jiang et al., 2010), with L5 potentially more plastic than L4 (Beaver et al., 2001).
CHAPTER 5

Circuit-level impact of dark-rearing

on PV-PV connections
ABSTRACT

In Chapter 4, we found that the density of PV-inputs to PV-cells is reduced by nearly 40% in primary visual cortex of dark-reared mice. However, the circuit-level basis of this plasticity was not evident from the techniques used. Specifically, this loss of boutons could have resulted from two distinct structural mechanisms: a reduction in the number of presynaptic partners contacting each dark-reared PV-cell, or a reduction in bouton number per partner. To investigate whether one or both of these mechanisms – which would alter network architecture and function in distinct ways – may underlie the dark-reared phenotype, we used the Brainbow technique to study this circuit (Livet et al., 2007; Cai et al., 2013). By labeling members of the PV-cell population with semi-unique colors via combinatorial expression of fluorescent proteins, we were able to analyze the contribution of individual PV-cells’ axons to perisomatic baskets, and identify which mechanism accounts for the loss of boutons onto dark-reared PV-cells.
INTRODUCTION

For over one hundred years, the morphology of interneurons has been studied on various scales. Initially, anatomists took a macroscopic approach and focused on uncovering the “parts list” of the brain, documenting the gross anatomy of interneurons and their prevalence across regions and layers. From this emerged a more microscopic perspective and a focus on domain-targeting, interneurons’ tendency to innervate their target cells within a stereotyped subcellular region. An intermediate level of morphological analysis has followed, focused on addressing the precise circuit-level organization of interneurons and their synaptic partners. By examining which cells provide input to and/or receive output from a given cell or cell-type, a greater understanding of interneurons’ function in microcircuits will emerge. While basket cells have been studied extensively on both the macro- & microscopic scales (Markram et al., 2004), circuit-level analysis has received considerably less treatment, due in part to technological challenges inherent to its study. After reviewing what is known on each of these three scales, our efforts to address the circuit-level organization of basket cells will be described.

Basket cells on the macroscopic-scale

As described in Chapter 1, classical basket cells are characterized by their long, horizontally-projecting axons that form finer, vertical collaterals as they innervate cells in a “patchy fashion” throughout the arbor (Somogyi et al., 1983). While in cat these arbors can stretch for millimeters and typically have a substantial translaminar component, rodent arbors usually span several hundred microns across and are often confined to the layer of origin (Dumitriu et al., 2007). Most arbors are locally dense, but some have sparse horizontal segments extending a short distance from the arbor edge (Kawaguchi & Kubota, 1998). Parvalbumin-
expressing (PV+), fast-spiking (FS) basket cells (“PV-cells”) are the predominant variety in cortex (Rudy et al., 2011), where they mediate the bulk of both local and lateral inhibition (Helmstaedter et al., 2008). PV-cells are most abundant in the middle layers of sensory cortex, with 40% of the total PV+ population in V1 located in L5 (Gonchar et al., 2008). In this layer, the high density of their axon terminals forms a bright band in images of synaptotagmin-2 immunolabeling (Figure 3.2.b), indicating a high degree of connectivity to L5 cells.

**Domain-specific targeting by PV-cells: perisomatic baskets**

As introduced in Chapter 3, most inhibitory cell-types exhibit domain-specific innervation, the tendency to contact their target cells within a stereotyped subcellular region (Markram et al., 2004). Basket cell axons show a preference for the “perisomatic” domain, a region comprised of the soma and proximal dendrites. Although contacts onto more distal dendrites have been reported (e.g., Somogyi et al., 1983), the soma-targeting nature of basket cells is highlighted most often given the rarity of this preference among cortical cell-types (Peters & Proskauer, 1980; DeFelipe & Fairén, 1982; Somogyi et al., 1983; Martin et al., 1983; Wang et al., 2002). Indeed, 90-95% of synapses onto pyramidal cell somata in cat V1 are symmetric (Freund et al., 1983; Fariñas & DeFelipe, 1991), indicating that the overwhelming majority of input to this domain originates from the ~25% of cortical neurons that are inhibitory (Markram et al., 2004), roughly half of which are PV-basket cells (Uematsu et al., 2008). Together, these perisomatic synapses comprise a “basket,” the meta-structure formed around cell bodies by the convergence of bouton-rich axonal segments originating from numerous distinct basket cells (Ramón y Cajal, 1899; Marin-Padilla, 1969). Although some fraction of somatic synapses – especially onto interneurons (Ahmed et al., 1994; Kameda et al., 2012) – are
excitatory, “baskets” typically refer to the inhibitory inputs received by both pyramidal and non-pyramidal somata.

Basket cell axons innervate somata via both *en passant* and terminal boutons (Somogyi et al., 1983). In both cases, the axon is often myelinated right up to the boutons (ibid), which are large at 1-1.5µm across (Marin-Padilla, 1969). These two modes of innervation may also appear together in the form of “terminal forks” (Chattopadhyaya et al., 2004), short collaterals that extend off of a main branch and allow a cell to be contacted repeatedly on multiple sides. While terminal forks do exist *in vivo* (e.g., Martin et al., 1983), their prevalence may have been exaggerated by studies using long-term organotypic slice culture *in vitro* (Chattopadhyaya et al., 2004), a preparation that leads to excessive growth of neurites and a higher frequency of miniature synaptic currents (Cho et al., 2007). Rather, *en passant* contacts may be most common in samples prepared from intact animals, with many axons failing to show “basked-like formations” as they innervate cell bodies (DeFelipe & Fairén, 1982; also, Somogyi et al., 1983).

Unlike chandelier cell axons, which always innervate axon initial segments in the distal-to-proximal direction (Somogyi et al., 1977), basket cells contact their targets without respect to orientation (Marin-Padilla, 1969), placing boutons along any trajectory of approach. As only ~30% of basket cell boutons are somatic (Somogyi et al., 1983), innervation of the soma by any individual axon is not obligatory, with some innervations being purely dendritic (Wang et al., 2002). However, many innervations falling within the perisomatic domain do include some number of somatic contacts.
Circuit-level analyses of PV-cells: inputs and outputs

While uncovering the gross anatomy and synaptic preferences of basket cells has identified their location within cortical microcircuits, a better understanding of their functional role can be obtained by analyzing their inputs and outputs on a cell-wide basis (Bezaire & Soltesz, 2013). A number of different types of studies have been performed to this end, each of which takes a slightly different perspective. For instance, the distribution of synaptic inputs along the somatodendritic domain of PV-cells has recently been documented in mouse S1 (Kameda et al., 2012). This type of data is useful as it reveals the balance of excitatory vs. inhibitory inputs that a cell receives, and can further indicate the source of these inputs. Intracortical excitatory connections express vesicular glutamate transporter (VGlut) 1 and outnumber both VGlut2+ thalamic inputs and GABAergic inputs to L4 PV-cells by 2.4-fold and 3.1-fold, respectively. This suggests substantial influence of corticocortical connections on PV-cell physiology, even at the soma where both VGlut2+ & GABA+ inputs are found at relatively high density, yet even together do not equal the density of VGlut1+ inputs. Studies like this (see also Hioki et al., 2013) shed light on the fuller range of inputs governing PV-cell function in vivo and provide context in which to interpret data on specific connections, such as those examined using whole-cell recordings.

However, as useful as cell-wide input-mapping studies are, a level of abstraction is created by not knowing how many presynaptic neurons contribute the sum of the inputs to a given cell. While this may be less important for intracortical excitatory connections, which are often mediated by a single input (Gulyás et al., 1993; but also see Bezaire & Soltesz, 2013, for other cases), GABAergic innervations often involve the targeting of multiple boutons to a postsynaptic cell (Buhl et al., 1994; Tamás et al., 1998). As a result, the number of presynaptic
partners converging onto a given cell is difficult to predict with high precision, leaving a key edge in the cortical connectivity graph unknown. This so-called convergence ratio is functionally relevant (Bezaire & Soltesz, 2013), contributing a level of redundancy to cortical circuits and ensuring strong and reliable transmission (Wang et al., 2002).

The number of basket cells participating in the formation of a typical perisomatic basket has previously been studied in both hippocampus and neocortex. To estimate convergence, most studies take the following approach (Buhl et al., 1994). First, the total number of somatic synapses/boutons received by a cell-type of interest is determined. Second, the average number of boutons that a single basket cell contributes as it innervates the soma is determined. Third, the total number of somatic boutons is divided by the average single-cell bouton number, yielding an estimate of the total number of basket cells that are needed to account for all of the boutons. This is a valid approach, but the precision of the estimate can be limited by the sample size of the study. For example, Buhl et al. (1994) serially-sectioned a CA1 pyramidal cell for electron microscopy and found that of the 124 somatic synapses, 5 were contributed by a biocytin-filled basket cell, yielding a convergence estimate of ~25 presynaptic basket cells per pyramidal cell body. While this was a heroic early attempt at estimating convergence, its accuracy relies upon both the cell and innervation chosen being representative of the broader population, which can exhibit substantial variability (Bezaire & Soltesz, 2013).

**Dissecting PV-cell circuit architecture and its plasticity using Brainbow**

Our finding that dark-rearing results in the loss of PV-inputs to PV-cells was presented in Chapter 4. For reasons described in the above section, however, the circuit-level basis of this plasticity could not be discerned from a simple input-mapping study. Specifically, it remained
unclear whether the loss of PV-inputs was mediated by a reduction in *bouton number per innervating axon*, or whether a reduction in the *number of actual presynaptic partners* had occurred. To distinguish between these two possibilities, we needed to assess the level of convergence in the PV-PV circuit.

To study convergence using the approach described above (Buhl et al., 1994), cells of interest need to be labeled so that their contacts to target cells can be visualized. Due to the high density of cortical neuropil, strategies that label the neuronal population sparsely are needed in order to discriminate individual processes and contacts with high confidence. Sparse labeling has historically been achieved using the Golgi stain, a technique that labels a seemingly random 2% of cells (Spacek, 1992). However, this random nature as well as the technique’s unsatisfactory labeling of myelinated axons make it unsuitable for our purpose. More recently, researchers have developed techniques that better label axons and that are even sparser, such as bulk injections of biocytin (e.g., Kisvárday et al., 1993) and intracellular filling of cells with horseradish peroxidase *in vivo* (e.g., Somogyi et al., 1983). While much of what we know about basket cells has come from these invaluable techniques, they are technically challenging and labor intensive, and do not necessarily produce labeling patterns in which individual target cells are multiply-innervated by distinct basket cells. Reconstructions of cells filled with biocytin during whole-cell recording experiments *in vitro* present a similar problem, although this type of data could be used for estimating convergence in other ways (see Discussion, below). Further, these classical techniques label all structures the same color, limiting their use when more than a small number of cells are labeled. Specifically, single-color labeling makes it difficult, if not impossible, to ascertain whether contacts onto multiply-innervated cells arise from different presynaptic partners or merely from different branches of a single cell’s axon.
One approach to enhance the resolution of presynaptic-cell identity is to label cells with different colors, using either biolistic delivery of dyes (Gan et al., 2000) or genetic expression of fluorescent proteins (“XFPs”; Feng et al., 2000; Kasthuri & Lichtman, 2003). By combining just three primary colors in different ratios, dozens of distinct secondary colors are possible, affording sufficient resolution to disambiguate target-cell identity in brain circuits characterized by dense convergence of many axons. To address the question of convergence from the perspective of the postsynaptic cell we used Brainbow (Livet et al., 2007), a neuroanatomical technique in which cells are labeled with a semi-unique color via combinatorial expression of fluorescent proteins. By using color to discriminate each of the axons making up a basket, the number of boutons contributed by distinct presynaptic partners can be counted, permitting highly precise estimates of convergence to be obtained due to increased sample size. To measure the level of convergence in PV-PV circuits, we used a version of Brainbow that was adapted for expression via adeno-associated viruses (AAVs; Cai et al., 2013).
RESULTS

Brainbow-labeling of PV-cells

At the heart of the Brainbow technique lies the Brainbow cassette, a stretch of recombinant DNA that includes inverted, transcription-inaccessible genes for a number XFPs (Livet et al., 2007). These XFP genes are separated by alternating, non-compatible pairs of \textit{lox} sites, which the bacterial recombinase Cre acts on to recombine the intervening DNA, permitting subsequent transcription. Due to the low efficiency of recombination this process occurs stochastically, meaning that for any given binding of Cre to DNA the outcome as to which sites are recombined is random. For each copy of the cassette, this process results in the transcription-ready presentation of a single XFP gene to the cellular machinery, allowing expression and accumulation of the XFP throughout the cell. Additional copies of the cassette, delivered to the cell through infection by multiple AAVs, enhance color diversity (Cai et al., 2013).

The Brainbow-AAV system incorporates a total of 4 XFPs, which are distributed across two AAVs that contain two XFPs each (Penn Vector Core #s p2453 (TagBFP + EYFP) & p2454 (mCherry + mTFP1.0)). With assistance from Dr. Dawen Cai at University of Michigan, we produced custom-made antibodies against each of these XFPs. The XFPs, which were chosen for this version of Brainbow because they come from different species and have low sequence homology, generated highly-specific antibodies that labeled only the XFP used to raise the antibody (data not shown). These antibodies allowed us to prepare samples that were more easily imaged than those exhibiting only native fluorescence, as the brighter & crisper signals in immuno-enhanced samples made small morphological features easier to identify (compare Figure 5.1.a & Figure 5.1.b). Further, as native mTFP is challenging to image (data not shown),
the antibodies also allowed us to use all 4 XFPs by “moving” mTFP to another location within the visible spectrum by choosing an appropriate secondary antibody.
**Figure 5.1** = (a) Expression of fluorescent proteins (XFPs) in PV-cell neurites following injection of Brainbow-AAVs into V1 of PV-Cre mice. XFP mixtures differing in their relative concentrations of mCherry (red), EYFP (green), and TagBFP (blue) gave individual PV-cells semi-unique colors. Native fluorescence of XFPs is shown, including in axonal boutons (bottom). Scale bars: top image (63x), 10µm; bottom images, 2µm. (b). Enhancement of Brainbow labeling with antibodies specific to each XFP in (a). Observe that antibody-enhanced images are higher contrast and show greater detail than images of native fluorescence, including in axonal boutons (bottom). Scale bars: top image (40x), 10µm; bottom images, 2µm.
To label members of the PV-cell population an array of different colors, we injected a titer-balanced cocktail of the two Brainbow-AAVs into V1 of both light-reared (LR) and dark-reared (DR) adult PV-Cre mice. Since we observed a mild “whiting-out” of colors at the injection site due to too many XFPs being present (Figure 5.1.b), we refined our approach in two ways. First, we diluted the cocktail to one-fifth its original concentration (i.e., $3 \times 10^{12}$ genome-copies/mL) in order to reduce multiple infections of the same cell, which can result in the number of Brainbow cassettes being too high. Second, we relocated the injection site from the center of V1 to its medial edge, and moved it further rostral as well. We did this in order to further reduce copy number within V1, as well as reduce damage to the central & caudal regions of V1 that we intended to analyze (Bregma -3.5mm to -4.0mm). We injected 1µL of virus solution at a depth of 400µm from the cortical surface, which yielded a consistent pattern of labeling across animals (Figure 5.2.a). This pattern was characterized by: (1) a several hundred micron-wide core region in which most of the cortical thickness contained densely-labeled structure, and (2) a region that stretched for a few hundred microns medially and for a couple of millimeters laterally, in which layers 5 & 6 were densely labeled but where more superficial layers mainly contained sparse, ascending collaterals originating from infragranular cells (Figure 5.2.b). As dorsal hippocampus was also labeled out to a similar lateral distance (Figure 5.2.a), we think that virus likely settled in the white matter tract below medial V1 and then diffused laterally, ultimately seeping out both dorsally (into cortex) and ventrally (into hippocampus) to create the characteristic labeling pattern observed. Since the axons of rodent basket cells are largely contained to the layer of origin (Dumitriu et al., 2007; Tremblay & Rudy, 2012), we were satisfied with this pattern of labeling (Figure 5.2.b) as we reasoned that axons of most of the PV-cells targeting L5 PV-cell bodies were being visualized. Overall, these refinements greatly
enhanced the color diversity of our samples, allowing us to acquire images in which PV-cell axons of many different colors were evident (Figure 5.2.c-d).
Figure 5.2 = (a) Brainbow labeling in a coronal section proximal to the injection site (asterisk) in rostral V1. Observe two different regions of labeling: (1) a core region proximal to the injection site in which most layers are densely labeled, and (2), a more distal region where labeling is mainly confined to L5/6. Scale bar, 500µm. Orientation bar indicates dorsal (D) and lateral (L) neuroanatomical directions. WM, white matter; Hipp, hippocampus. (b) Larger view of labeling in the distal lateral region, which extends caudally to cover much of monocular V1 (where this image was taken). Observe labeling of numerous PV-cell bodies in L5 & L6. Scale bar, 100µm. (c) Optimal color diversity in an area of visual cortex located within the distal region (compare to Figure 5.1.b). Brainbow-labeled PV-cell axons form baskets around putative neuronal somata (asterisks). Maximum projection of a few microns of tissue, also in (d). Scale bar, 10µm. (d) Larger view of the rightmost basket in (c). Axons originating from different PV-cells express distinct mixtures of XFPs, permitting single-process analysis of somatic innervation. Scale bar, 5µm. Brightness of all images increased by 10% to aid visualization.
PV-PV convergence in L5 of light-reared & dark-reared V1

We examined connectivity in L5 of V1m by acquiring confocal image stacks of Brainbow-labeled PV-cell bodies and their axons. Similar to classic reports of robust perisomatic innervation by either bulk axonal structure (Ramón y Cajal, 1899; Marin-Padilla, 1969) or GAD+ or PV+ axon terminals (Ribak, 1978; Celio, 1986), we found that Brainbow-labeled PV-cell axons heavily innervate the somata of both pyramidal and non-pyramidal cells (Figure 5.3.a). Further, as Brainbow-labeled axons originating from different cells possess their own semi-unique color, it was readily apparent that large numbers of PV-cells converge onto each target cell, confirming definitively impressions gleaned from Golgi-stained tissue (i.e., Marin-Padilla, 1969). AAV-mediated expression of Brainbow worked equally well in dark-reared PV-Cre mice, indicating that expression of Cre from the PV promoter – which exhibits experience-dependent changes in strength (Tropea et al., 2006; Sugiyama et al., 2008) – remained sufficiently strong to drive recombination of the Brainbow cassette.

To compare the level of PV-PV convergence between LR & DR mice, we examined Brainbow-labeled PV-cell bodies contacted by Brainbow-labeled PV-cell axons (Figure 5.3.b). Boutons mediating these contacts were present in both conditions, and, similar to synaptotagmin-2 (Syt2) puncta, were large in size and easily identified by their characteristic dark center (Figure 5.3.c). This protein exclusion zone was even more pronounced in Brainbow-labeled axons, likely due to the XFPs being membrane-targeted. As with Syt2 puncta, contacts made by labeled axons onto labeled somata were readily discriminated, with boutons often exhibiting a flat edge where they abut cell bodies (Figure 5.3.c, upper-left). Interestingly, whereas innervations of pyramidal cells more often involved “terminal forks” (Martin et al., 1983; Chattopadhyaya et al., 2004) in which larger numbers of boutons were present (Figure 5.3.a), PV-PV connections were typically
mediated by a smaller number of boutons. This pattern has been reported previously in hippocampus, where CA1 PV-basket cells were observed to contact their PV+ targets with only one or a few boutons per connection, reserving more boutons for pyramidal cells (Sik et al., 1995). Indeed, the largest group of connections was mediated by a single bouton (Figure 5.3.d), with both LR & DR PV-cell bodies receiving on average just ~2 boutons per incoming axon (Figure 5.3.d, inset; P=0.88, Mann Whitney test). This indicated that bouton number per axon is not regulated by visual experience, suggesting that the decrease in total Syt2+ input number to DR PV-cells may be due to a reduction in the number of presynaptic partners. Therefore, we estimated the convergence ratio of the PV-PV circuit by taking the average number of Syt2+ boutons received by both LR and DR PV-cell bodies (mean ± SEM: LR, 82.7 ± 7.4; DR, 44.8 ± 2.6; P<0.0001, Mann Whitney test) and dividing this by the corresponding average per-axon bouton number (1.94 and 2.0, respectively; Figure 5.3.d, inset). This revealed that while LR PV-cells receive somatic input from ~43 other PV-cells, PV-PV convergence is dramatically reduced following DR, with PV-cell bodies receiving input from only ~23 other PV-cells (Figure 5.3.e). Thus, that DR PV-cells receive fewer PV-inputs is not because each presynaptic partner contributes fewer boutons to the somatic basket, but because each basket is comprised of fewer presynaptic PV-cell partners.
Figure 5.3 = (a) AAV-Brainbow-labeled PV-cell bodies (arrows) and axons in L5 of V1 (max projection of 3µm of tissue). Observe somatic innervation of NeuroTrace-labeled cell bodies (gray). Scale bar, 10µm. (b) Single-plane images of Brainbow axons & boutons converging onto both light-reared (LR, left) and DR (right) PV-cell bodies. Scale bars, 5µm. (c) Brainbow axons forming putative contacts (arrows) onto Brainbow cell bodies. The complete extent of each axon’s innervation is shown. Scale bars, 2µm. (d) Cumulative distribution of the number of soma-targeted boutons per axon. (Inset) Mean somatic bouton number per innervating axon. (e) PV-PV convergence, the quotient of average Syt2+ bouton number for LR & DR PV-cells divided by mean somatic boutons per innervating Brainbow-axon (Mann Whitney test).
Attributions

- Regarding anti-XFP antibody production: Dawen Cai purified the XFPs (mTFP1.0 & mKate2, which has high homology with and labels TagBFP) and Luke J. Bogart prepared them for injection into animals, which was done by Covance Inc. Luke J. Bogart received the antibodies and tested all bleeds for specificity. Antibodies against GFP (labels EYFP) and mCherry were produced earlier in the laboratories of Drs. Jeff Lichtman and Josh Sanes and shared with us.

- The initial testing of the Brainbow-AAVs used in this work, including optimization of injection, histology, immunohistochemistry, and imaging protocols, was carried out by Luke J. Bogart (acknowledged in Cai et al., 2013), with advice from Dawen Cai.

- Ethan Glasserman, a Harvard college summer intern, performed the PV-axon convergence analysis in L2/3/4 (discussed below, Figure 5.4.a), using images acquired by Luke J. Bogart and under his supervision.
DISCUSSION

In Chapter 4, dark-rearing was shown to cause an experience-dependent decrease in PV-input to PV-cells. However, the circuit-level basis of this phenomenon could not be discerned from the techniques used. In this Chapter, we used Brainbow to distinguish whether reduced PV-PV connectivity arises from a drop in the number of boutons per innervating axon, or from a decrease in overall axon number. To study convergence in the PV-PV circuit, we performed semi-dense reconstruction of Brainbow-labeled axons targeting PV-cell bodies, quantifying the number of boutons each uniquely-colored axon contributed to the perisomatic basket. We found that bouton number per innervating axon was not sensitive to loss of visual experience, suggesting that dark-reared PV-cells receive fewer inputs because they are have fewer presynaptic partners. When we estimated how many PV-cells innervate a given PV-cell body, we found that this number was nearly 50% lower in dark-reared mice, indicating that the redundancy of recurrent inhibition is powerfully regulated by visual experience.

Experimental considerations

In order to study PV-PV convergence using Brainbow, we had to make a number of considerations when designing our experiment. First, the most reproducible pattern of labeling we achieved was one in which XFP-expressing cell bodies were mainly confined to the infragranular layers (Figure 5.2.a), with the great majority of PV-cells in these layers being labeled (data not shown). This material proved ideal because V1 regions of interest were far enough away from the injection site to have colors that were not “whited-out” due to too-high expression of XFPs (Figure 5.1.b), but yet were not too far away such that primary colors resulting from single AAV infections predominated. While basket cells exhibiting translaminar
axonal projections have been observed in sensory cortex of both cats (e.g., Somogyi et al., 1983) and rodents (e.g., Wang et al., 2002), most reports are of cells whose arbor is confined to the layer of origin (Dumitriu et al., 2007; Tremblay & Rudy, 2012), suggesting that we labeled a substantial fraction of the PV-inputs to L5 cells. However, as collaterals of both L2/3 (Helmstaedter et al., 2008) and L4 (Sun et al., 2006) basket cells can reach L5, we knew that we were not visualizing all PV-inputs to L5 PV-cells, as the density of our Brainbow-labeling tapered off above L5 (Figure 5.2.a+b). For this reason, we took the previously-determined number of synaptotagmin-2-positive inputs, which reflects the total number of PV-inputs received by the cell body, as the numerator in our estimate of PV-PV convergence (Figure 5.3.e).

The ability of this Syt2-based approach to estimate convergence accurately relies on the assumption that PV-cells across layers (i.e., those whose contacts onto L5 cells we are not visualizing) form similar numbers of synapses per target soma as the cells studied here. The available data, both from previous studies of ours using Brainbow in L2/3 & L4 as well as from the literature, strongly validate this assumption. In terms of their convergence onto cell bodies in L2/3 & L4, PV-Brainbow axons were found to form an average of 2.2 (LR) & 2.0 (DR) boutons/soma (Figure 5.4.a; P=0.27, Mann Whitney test; E. Glasserman, L.J. Bogart, & T.K. Hensch, unpublished observations), close to the values of 1.94 (LR) & 2.0 (DR) boutons/soma we observed in L5 (Figure 5.3.d). Similarly, considering divergence, or how single axons behave as they innervate multiple cells along their path, L2/3 & L4 axons contacted somata with an average of 1.98 (LR) & 1.99 (DR) boutons/soma (Figure 5.4.b; P=0.75, Mann Whitney test; L.J. Bogart & T.K. Hensch, unpublished observations), with additional contacts onto weakly-labeled, first-order dendrites often visible for pyramidal cells. Overall, this suggests that the population of PV-cell axons as a whole forms connections using a conserved number of somatic boutons.
across layers. Further support comes from the literature, where basket cells have been reported to allocate boutons as follows: 2.4 boutons/soma (cat, V1, L4, clutch cell, Kisvárday et al., 1985), ~2 boutons per PV+ soma (cat, V1, L3 & L5, large basket cells, Kisvárday et al., 1993), and 2.2 boutons per PV+ soma (rat, S1, L4, fast-spiking cells, Staiger et al., 2009). Thus, overall there is strong evidence indicating that more superficial basket cells, whose descending collaterals targeting L5 PV-cells would have been largely unlabeled in our Brainbow preparations, form connections using a similar number of boutons as the L5/6 axons predominantly assayed herein, and do so in both light-reared & dark-reared tissue.
Figure 5.4 = (a) Mean number of contacts made by PV-cell axons converging onto L2/3 & L4 neuronal somata. (b) A population of axons was reconstructed (LR, n = 25; DR, n = 15), and the number of boutons that each axon contributed to each neuronal somata it innervated along its length (i.e., divergence) was averaged. (c) The same type of analysis performed in (b) is shown, but was performed using a different analysis pipeline on a different dataset (also in L2/3 & L4) and at a later date. Note the consistency of the values obtained across studies (stats for (c), LR vs. DR: P=0.76, Mann Whitney test).
Technical considerations

A second consideration relates to our ability to identify axons as originating from distinct basket cells. Since we used sectioned tissue, nearly all of the axons in our images were severed from their parent somata. As a result, it was impossible to distinguish individual cells’ contributions to perisomatic baskets by structural information alone. To circumvent this, we relied on each axon’s Brainbow color to signal its uniqueness among a PV-cell’s complement of inputs. The validity of this approach therefore assumes the following: (1) there is sufficient color diversity, such that innervation of somata by non-physically contiguous axon segments of similar color is limited; and, (2) segments that are similar in color, but which lack physical contiguity, can be reconciled as being either uniquely-colored or belonging to a previously-identified input. In general, the first condition relies on aspects of sample preparation that were optimized earlier (i.e., virus titer, location of imaging relative to injection site), and largely holds true due to the high level of color diversity exhibited by the axonal population (Figure 5.2.c+d).

In contrast, judging the similarity of axonal colors can be less straightforward. Specifically, while the mixture of fluorescent proteins in a given cell is expressed uniformly throughout its processes (Livet et al., 2007), the color that this mixture gives rise to optically can vary. For example, XFPs were expressed cytosolically in Brainbow1/2, meaning that in narrower processes where less protein is present, signal intensity can dim and cause alterations of color. While in the Brainbow3 system that we used the XFPs are membrane-targeted and promote improved color constancy along axons of various caliber (Cai et al., 2013), additional challenges arise from the subtleties of how XFPs are distributed in the plasma membrane, as well as from the discretization & noisiness inherent to digital imaging. Specifically, while the color of many axons appears well-blended from a distance, on the nanoscopic, pixel-level these axons can
exhibit “islands” of color non-uniformity due to aggregation of XFPs (data not shown). These aggregates can hinder determination of an axon’s true color, as they generate variability in the RGBG values within the set of pixels representing an axon (red, green, blue, & gray, the ‘4th’ brainbow channel, usually left un-visualized on RGB displays; data not shown).

Overall, however, the variability contributed by these aggregates is minimal compared to that which is generated by the laser-scanning confocal microscope. In digital microscopy, images are made up of pixels representing the signal intensity measured at specific locations within the sample. Importantly, however, there is not a one-to-one relationship between the dye concentration at a given location and the intensity value of the corresponding pixel, as a Poisson distribution underlies photon absorption by the dye (Pawley, 2006, chapter 4). This means that on repeated acquisitions of the same image, the values of individual pixels will vary around a mean in a manner independent from one another. Critically, this variation in intensity is evident not only in time but also in space. Specifically, optimally-sized pixels are smaller than the resolution limit of light, meaning that adjacent pixels correspond to spatial locations so close to one another that their measured dye concentrations should be virtually identical. Because of Poisson noise, however, these pixel values can, and often do, differ substantially. While high levels of spatial-averaging (i.e., the repeated scanning of the same row of pixels 16 times before moving on to the next row) greatly reduces this pixel-to-pixel disparity in intensity values, the presence of 3-4 Brainbow channels, each of which has a signal level and resulting intensity values that are determined independently, work to compound inter-pixel variability even within single axons. The use of low laser-powers and short pixel dwell-times (0.62µs), essential for acquiring large, axially-oversampled stacks in a reasonable amount of time and without
significant photobleaching (Conchello & Lichtman, 2005), also increases the appearance of Poisson noise due to the limited number of photons emitted from the sample.

Due to these challenges, it is difficult to implement a pixel-based computational approach to discriminate similarly-colored axons from one another. For this reason, we decided to make color discriminations by eye using the strategies described in the Methods, which include tracking axonal segments back to branch points to check for physical contiguity, and toggling individual Brainbow channels on & off to examine the intensities of component colors. Since many axons contain 3-4 colors at varying levels, this latter strategy was particularly effective at identifying axons arising from different PV-cells. Specifically, the presence of dim colors – which can contribute imperceptibly little to an axon’s overall color profile – could often be used to distinguish two otherwise identically-colored axons from one another. Overall, only a very small minority of axons could not be definitively classified as being either uniquely-colored or part of a previously-identified input, and in these cases a judgment call was made.

Comparison to previous estimates of PV-cell convergence

As introduced above, convergence of PV-cells onto their target cells has previously been examined in both hippocampus and neocortex. In the study most similar to ours, Buhl et al. (1994) estimated that CA1 pyramidal cells receive somatic innervation from approximately 25 presynaptic basket cells. Using glutamate-uncaging to map connections between presynaptic PV-cells and patch-clamped postsynaptic pyramidal cells (PYR-cells), Packer & Yuste (2011) were able to estimate PV-cell convergence by modeling connectivity as a function of inter-somatic distance between cells. As they found that connections between PV-cells and PYR-cells were fairly dense for inter-somatic distances <200µm, they estimated convergence in both
somatosensory cortex (S1) and frontal cortex (FC) as follows: L2/3 of S1, 46 PV-cells per PYR-cell; L5 of S1, 184 PV-cells per PYR-cell; and, L2/3 of FC, 121 PV-cells per PYR-cell. As their physiology-based approach would have detected PV-cell connections formed onto PYR-cell dendrites (likely within ~30um of the soma; Kubota, 2014) that we did not address here, that their convergence estimate would be higher than ours is expected.

It is important to note that Packer & Yuste (2011) studied PV-PYR connections specifically and did not address connections between PV-cells. It is possible, if not likely, that PV-cell convergence may differ in a cell type-specific manner. As described above, Sik et al. (1995) found that CA1 PV-basket cells contact their PV+ targets with only one or a few boutons per connection, reserving more boutons for their innervations of pyramidal cells. With their dataset, Sik et al. (1995) estimated convergence in another way, which is by analyzing the divergence and target selection of PV-cell axons on an arbor-wide basis. In other words, by fully reconstructing the axonal arbor of a PV-cell and counting how many other PV+ neurons are contacted by that arbor, it can be reasoned that each PV+ neuron that is contacted itself contacts a similar number of PV+ neurons, allowing convergence to be estimated from measurements of divergence. Using this approach, Sik et al. (1995) estimated that individual CA1 PV-cells receive input from roughly 60 presynaptic basket cells. A similar study in cat V1 (Kisvárday et al., 1993) found that two fully-reconstructed large PV+ basket cells contacted the somata of 33 and 58 other PV+ cells, close to the numbers we have found in mouse V1 (Figure 5.3.e). In addition, a study specifically of nest basket cells in L2/3 of rat S1 found that each cell contacts roughly 25-35 other interneurons, divergence similar to that observed for large basket cells in the same region (Wang et al., 2002). Thus, overall, our estimate of PV-PV convergence is in good agreement with previous reports.
Interestingly, plasticity of PV-cells’ efferent connectivity on an arbor-wide basis has previously been reported (Bahé & Di Cristo, 2012). In organotypic slices of V1 in which isolated PV-basket cells were labeled by biolistic transfection, critical period-aged basket cells were found to innervate the somata of ~70% of the neuronal somata located within their arbor. Following cell-autonomous reduction of basket cell excitability, the percentage of cells innervated drops to ~50%, suggesting that activity-dependent mechanisms may regulate the density of innervation within arbors. As the identity of the postsynaptic targets that were lost was not investigated, it is unclear how exactly PV-cells reorganize their connectivity in the face of reduced activity. For example, PV-cells might preferentially reduce their connectivity to other PV-cells or may not do so at all, or may themselves lose inhibitory input from other PV-cells due to their own reduced excitability (Xue et al., 2014). As we have not yet analyzed PV-convergence onto non-PV+ cells, it will be interesting to see in future work whether the loss of sensory-driven activity on a network-wide level following dark-rearing may lead to circuit-level changes in PV-PYR connections.

**Relationship to previous reports of GABA-cell structural rewiring and potential impacts**

Changes in the inhibitory innervation of various cell-types have been reported across diverse regions of the nervous system. For example, Keck et al. (2011) report that following retinal lesions, the loss of sensory drive to adult V1 results in a 16% reduction in bouton density along the axons of neuropeptide-Y-expressing interneurons (NPY-cells), plasticity that is evident within 24hrs and which does not occur along excitatory axons. Interestingly, while the overall architecture of axons is unchanged (ibid), the loss of boutons may result in a given NPY-cell disconnecting from some of its postsynaptic partners (Flores & Méndez, 2014), reducing
convergence onto those cells. While it was not addressed, it is possible that this reduction in NPY-cell output could occur in a circuit-specific manner, paralleling what we have observed to take place at PV-PV connections following dark-rearing. Interestingly, PV-cells in other brain areas have been found to undergo target cell type-specific remodeling. For instance, striatal PV-cells normally exhibit two-fold higher connectivity rates to direct-pathway vs. indirect-pathway medium spiny neurons (Gittis et al., 2010), the principal cells of the striatum. Strikingly, these rates are plastic upon injury and are differentially-regulated, with PV-cell connectivity to direct-pathway medium spiny neurons remaining static but doubling to those belonging to the indirect-pathway (Gittis et al., 2011). That PV-cells across brain regions show the capability for precise remodeling of efferent connectivity suggests that this may be a conserved property within the cell-type as a whole.

Following sensory deprivation in adult mice induced by plucking of the facial whiskers, the cortical representation of the un-deprived whiskers expands into the territory previously driven by the plucked whiskers (Marik et al., 2010). Interestingly, excitatory and inhibitory axons involved in this process differ markedly in terms of the structural plasticity that they exhibit. Excitatory axons projecting from non-deprived to deprived areas display intense sprouting of new branches, leading to a strong net increase in deprived-barrel axonal density. In contrast, axons of inhibitory cells located within deprived columns undergo retraction near their somata but show a 250% increase in total length, leading to a more than doubling of the distance that they extend after just 2 days of plucking. Bouton turnover along pieces of stable axon shows a further divergence, with excitatory axons mainly adding boutons while inhibitory cells were biased toward bouton loss. That brief dark-rearing of adult mice re-opens plasticity (He et al., 2006) suggests that similar mechanisms could operate in sensory-deprived V1 (albeit in a
slightly different fashion due to selective whisker deprivation setting up competitive interactions between cortical inputs that would not be present following dark-rearing). Specifically, adult dark-rearing leads to a decrease in the ratio of GABA<sub>A</sub>R to AMPA-receptor protein (ibid), suggesting that inhibitory receptors are lost from synapses and that some synapses could be eliminated altogether. As PV-cells are especially responsive to changes in experience (Yazaki-Sugiyama et al., 2009), it is possible that their synapses, and potentially their synapses onto other PV-cells, may be weakened or lost following the reduction in sensory drive (Keck et al., 2011), thereby facilitating adult plasticity.

Interestingly, inhibitory interneurons in adult rat cortex have been found to express the polysialylated form of neural cell adhesion molecule (PSA-NCAM; Gomez-Climent et al., 2011), a marker typically confined to immature neurons & cortices and considered to have anti-adhesive properties that aid in the structural plasticity of developing cells (Di Cristo et al., 2007; Rutishauser, 2008). Specifically, NCAM function is associated with contact formation and synaptogenesis, processes that detract from the overall outgrowth of axons. Overexpression of NCAM disrupts axonal arborization in PV-cells, as well as the maturation of perisomatic inhibition onto pyramidal cells (Brennaman et al., 2008). As PSA negatively regulates NCAM, its expression in adult interneurons suggests that these cells retain a more juvenile-like capability for structural plasticity (Gomez-Climent et al., 2011). Although PV-cells comprise a relatively small number of adult PSA-NCAM(+) cells, it is possible that relatively minor changes in inhibitory output (Kuhlman et al., 2010) from the PV-cell network as a result of altered synapse number may be sufficient to re-open plasticity. Alternatively, or in addition, greater expression of PSA-NCAM in adult somatostatin-positive cells (Gomez-Climent et al., 2011) – an inhibitory
cell-type that strongly inhibits PV-cells in V1 (Pfeffer et al., 2013) – may provide a disinhibitory mechanism (e.g., Letzkus et al., 2011) by which plasticity is regulated in V1.
CHAPTER 6

PV-PV connectivity regulates cortical plasticity
ABSTRACT

In Chapter 4, we found that dark-rearing has specific effects at PV-PV vs. PV-pyramidal connections. In this Chapter, we sought to further explore the extent to which dark-rearing affects PV-circuitry. To do this, we FAC-sorted PV-cells from light- & dark-reared V1 and performed qPCR, finding that expression of Gabra1, which encodes the alpha-1 subunit of the GABA$_A$ receptor that is enriched at synapses formed by PV-cells (Klausberger et al., 2002), is reduced by nearly 20% selectively in dark-reared PV-cells. This reduction in alpha-1 expression had a physiological correlate in that PV-cell-mediated synaptic events were broadened only in PV+ cells, consistent with their expressing less of the fast-decaying alpha-1 subunit.

Dark-rearing has been found to produce a range of effects on inhibitory circuits (e.g., Morales et al., 2002; Di Cristo et al., 2007) and gene expression (e.g., Tropea et al., 2006) in V1. To test the hypothesis that PV-PV connections specifically are involved in regulating critical period plasticity, we next sought to model the deprived state of this circuit as it exists in dark-reared V1. To do this, we used the Cre-lox system to remove Gabra1 specifically from PV-cells, creating PV:GABA$_A$R-$\alpha_1^{(-/-)}$ mice. PV-PV connections in these mice were preserved structurally, but recapitulated the broader currents observed between PV-cells in dark-reared V1. Strikingly, this targeted manipulation of PV-PV connections was sufficient to extend V1 plasticity into adulthood, suggesting that experience-dependent maturation of recurrent PV-circuitry is important for normal closure of the critical period.
INTRODUCTION

In addition to maturing properties of the V1 inhibitory system on the circuit, cellular, and synaptic levels, experience also impacts GABAergic function by regulating the molecular phenotype of postsynaptic neurotransmitter receptors (Luscher et al., 2011). As individual presynaptic quanta are believed to largely saturate the population of receptors located postsynaptically from a given GABAergic release site (Edwards et al., 1990; Nusser et al., 1997, 1998), plasticity of the number (Nusser et al., 1998) and subunit-composition (Lavoie et al., 1997; Okada et al., 2000; Picton & Fisher, 2007) of these receptors is a key mechanism by which synaptic strength and function are regulated. After providing an overview of the developmental and experience-dependent changes exhibited by GABAergic postsynapses, our results on the cell type-specific plasticity of GABA receptor subunit-composition in V1 will be described.

Type A GABA receptors (GABA_ARs) show incredible diversity due to the large number of genes coding for their subunits (Luscher et al., 2011). Despite this, most GABA_ARs are composed of a common pentameric motif consisting of two alpha subunits, two beta subunits, and one gamma subunit. In the brain, the gamma-2 subunit is considered obligatory for postsynaptic receptors, with many of these receptors also containing beta-2/3 subunits (Fritschy et al., 1992). This leaves the alpha subunits, the expression of which are more variable than other subunit subtypes on the cellular level. For example, while cells in the basal forebrain show prominent expression of alpha-1, alpha-3 expression is more common in cells of the inferior olivary nucleus (ibid). Interestingly, cerebellar Purkinje cells demonstrate subcellular differences in alpha-subunit expression, with alpha-1 subunits restricted to somata, and both dendrites & somata expressing alpha-3. Further, hippocampal pyramidal cells exhibit synapse-specific localization of alpha subunits (Klausberger et al., 2002). Somatic contacts formed by PV+ basket
cells contain many times higher density of alpha-1 subunit-containing GABA\(_\text{AR}\) than adjacent contacts formed by PV-negative basket cells (ibid), which are enriched in alpha-2 (Nyíri et al., 2001). This enrichment of alpha-1 subunits at PV-synapses holds true at PV-PV connections, which actually exhibit alpha-1 densities that are three-fold higher than those at PV-pyramidal synapses (Klausberger et al., 2002).

Early in life, GABA\(_\text{AR}\)s throughout the brain undergo a switch in subunit-composition (Fritschy et al., 1994) as GABAergic neurotransmission shifts from mediating excitation to mediating inhibition (Ben-Ari, 2002). As a result, expression of the immature alpha-2 (Fritschy et al., 1994) & alpha-3 (Poulter et al., 1999) subunits declines while expression of the alpha-1 subunit increases, becoming the predominant variety in the adult brain. This switch is functionally relevant, as alpha-1 subunit-containing GABA\(_\text{AR}\)s deactivate faster and flux less current (Lavoie et al., 1997; Okada et al., 2000), allowing more precisely-timed inhibition. At PV-PV synapses, higher numbers of alpha-1 subunit-containing GABA\(_\text{AR}\)s (Klausberger et al., 2002) may enhance the function of PV-cell-networks, facilitating generation of gamma oscillations (Bartos et al., 2007).

In primary sensory areas, the overall level of GABA binding, and the subunit-composition of GABA\(_\text{AR}\)s, exhibit both developmental and experience-dependent phases of maturation. In cat V1, the overall density of GABA\(_\text{AR}\)s as revealed by tritiated-muscimol binding increases over the first several months of postnatal life (Shaw et al., 1985), a rise that parallels developmental increases in the expression of GABA synthetic enzymes glutamic acid decarboxylase (GAD) GAD-67 & GAD-65 (Guo et al., 1997). In rats dark-reared from birth, however, GABA\(_\text{AR}\) levels fail to fully mature (Gordon et al., 1997), an effect not mirrored
presynaptically as expression of both GAD67 & GAD65 reach adult levels in dark-reared cats (Mower & Guo, 2001).

On the level of GABA$_A$R subunits, rat V1 exhibits the same developmental switch in alpha-subunit expression as does the brain at large, with alpha-3 expression giving way to alpha-1 expression in advance of eye-opening (by P11, Heinen et al., 2004; in cats, over the first several weeks of life, Chen et al., 2001). Following dark-rearing, alpha-subunit expression responds in two ways. First, dark-rearing does not prevent the alpha-3 to alpha-1 switch (Heinen et al., 2004), which proceeds normally. Second, dark-rearing leads to increased expression of both alpha-1 & alpha-3 (Chen et al., 2001; only alpha-3, Heinen et al., 2004; Tropea et al., 2006; only alpha-1, Dahlhaus et al., 2011), with the non-developmentally-regulated alpha-2 gene (Heinen et al., 2004) also increasing (ibid; Tropea et al., 2006). While the increases in alpha-2/3 expression observed by Heinen et al. (2004) with DR until P21 did not significantly affect the decay kinetics of spontaneous IPSCs, it is possible that the greater levels of upregulation observed with DR until the peak of the critical period (P27; Tropea et al., 2006) may be sufficient to alter synaptic function. Alternatively, synaptic function may be altered in a cell type- or circuit-specific manner (Klausberger et al., 2002), with the L2/3 neurons randomly selected by Heinen et al. (2004) being too heterogeneous to show a strong effect.

While these published observations of GABA$_A$R alpha subunit plasticity suggest that V1 circuits may be exhibiting a homeostatic response to dark-rearing (Desai et al., 2002; Tropea et al., 2006), what is missing from this picture is a cell type-specific understanding of these effects. Pyramidal cells and interneurons differ widely in their afferent connectivity (Markram et al., 2004), and target cell type-specific synaptic specializations have been reported for FS-cells (e.g., Cruikshank et al., 2007; Bartos et al., 2007). Having obtained both anatomical and physiological
data indicating a selective weakening of PV-PV vs. PV-PYR connections following dark-rearing, we decided to investigate whether the specificity of this plasticity extends to the level of GABA<sub>A</sub>Rs.
RESULTS

Fluorescence-activated cell (FAC) sorting and Quantitative PCR (qPCR) of V1 from PV-EYFP mice

To compare the level of various GABA_A subunits expressed in V1 between light-reared (LR) and DR mice, we measured their gene expression by using qPCR to quantify mRNA levels. However, rather than extracting RNA from V1 homogenates (Heinen et al., 2004; Tropea et al., 2006; Dahlhaus et al., 2011), we first performed FAC sorting (Saxena et al., 2012) on V1 samples isolated from PV-EFYP-ChR2 mice. We used PV-EFYP-ChR2 mice rather than PV-EGFP mice (Meyer et al., 2002) because fluorescent protein expression in the latter is driven from the parvalbumin promoter, the activity of which is reduced in the dark (Tropea et al., 2006). In contrast, Cre-mediated expression of EYFP remains high even in dark-reared PV-EFYP-ChR2 mice, as the level of Cre is not a rate-limiting step (Morozov, 2008). As a result, cell counts were not significantly different between rearing-conditions for either the YFP(+) (PV-cell enriched; P = 0.29, Mann Whitney test) or YFP(-) (non-PV-cell enriched; P = 0.19, Mann Whitney test) fractions. RNA extraction and cDNA preparation were then carried out on each fraction individually, so that cell type-specific differences in the expression of the GABA_A subunit genes probed during qPCR could be determined. The qPCR experiment was designed such that differences in the expression of individual genes could be compared between rearing conditions for a given fraction, but that absolute levels of expression across different genes could not be compared.
Figure 6.1 = (a) GABA$_A$R $\alpha$-subunit mRNA levels from V1 of FAC-sorted PV-YFP mice. Both PV-cell enriched (‘+’) and non-PV-cells (‘-’) normalized to light-reared levels per gene. In 2way ANOVA of $\alpha l$ levels across cell-types (P<0.05), significant effect of rearing on YFP(+) PV-cells (asterisk, Bonferroni posttest). (b) GABA$_A$R $\gamma$2-subunit mRNA levels. (c) GABA$_A$R $\alpha$2-subunit mRNA levels. In 2way ANOVA of $\alpha 2$ levels across cell-types (P<0.001), significant effect of rearing on YFP(-) cells (asterisks, Bonferroni posttest).
We probed for four GABA\(\text{A}\)R subunit genes: alpha-1, alpha-2, alpha-3, & gamma-2, which is the obligatory postsynaptic subunit (Fritschy et al., 1992). We were most interested in the expression pattern of alpha-1, as this subunit is expressed with high-specificity at synapses formed by PV+ boutons onto both pyramidal cells and other PV-cells (Klausberger et al., 2002). We reasoned that a loss of PV+ inputs onto PV-cells in the dark (Chapter 4) may be accompanied by a reduction in alpha-1 expression in these cells. Indeed, when we compared the relative expression level of alpha-1 in the YFP(+) fraction between LR and DR V1, we found a clean, ~20% reduction in expression (Figure 6.1.a). When we assessed alpha-1 expression in the YFP(-) fraction as a control, we found that its expression in non-PV cells was unaffected by DR, indicating that regulation of alpha-1 by experience is selective for PV-cells (2-way ANOVA, P<0.05 effect of cell-type). Since alpha-1 subunits confer GABA\(\text{A}\)Rs with fast deactivation of channel opening (Lavoie et al., 1997; Okada et al., 2000), we compared expression of this subunit to one whose properties lie at the opposite extreme, the slow deactivation-mediating alpha-3 subunit (Picton & Fisher, 2007). In both YFP(+) and YFP(-) fractions, expression of alpha-3 did not change as a function of experience (Figure 6.1.a), indicating both cell type- & subunit-specific regulation of alpha-1 expression by PV-cells.

We then considered whether loss of visual experience impacts the expression of the other two GABA\(\text{A}\)R subunits that we probed for, gamma-2 and alpha-2. As one gamma-2 subunit is typically found in all postsynaptic GABA\(\text{A}\)Rs (Fritschy et al., 1992), its expression level may indicate whether there are changes in the overall number of GABA\(\text{A}\)Rs found in LR and DR cells. In the YFP(+) fraction, gamma-2 expression was not significantly affected by dark-rearing (Figure 6.1.b; P=0.39), though it trended lower. This trend was due to one LR sample having >2x higher levels of gamma-2 than the other samples, and excluding it as a potential outlier results in
LR & DR gamma-2 levels that are very similar (1.0 vs. 0.965). Gamma-2 levels did not differ significantly in the YFP(-) fraction either (P=0.39). As described above, synapses opposite PV-positive contacts onto hippocampal pyramidal cell somata rarely include alpha-2 subunit-containing GABA\textsubscript{A}Rs, which instead are found at synapses formed by cholecystokinin-positive basket cells (Nyíri et al., 2001). Alpha-2 subunits are also highly-enriched along the axon-initial segment of pyramidal cells (Fritschy et al., 1998), suggesting that these contacts are mediated by chandelier cells, the only cells that innervate this domain (Markram et al., 2004). While dark-rearing did not significantly alter alpha-2 levels in the YFP(+) fraction (P=0.09, unpaired t-test), it caused a sharp reduction of alpha-2 gene expression in YFP(-) cells (P=0.0007, unpaired t-test), specifically (Figure 6.1.c).

**Kinetics of sIPSCs in LR & DR V1**

Having found that PV-cells express less GABA\textsubscript{A}R alpha-1 subunit after dark-rearing, we next sought to understand the functional consequences of this change in gene expression. As alpha-1, -2, & -3 subunits confer GABA\textsubscript{A}Rs with fast, medium, & slow deactivation rates, respectively (Picton & Fisher, 2007), we reexamined our *in vitro* physiology data with a focus on the decay phase of the IPSC. Rather than quantify the length of the decay itself, which was often not very accurately detected by our analysis software, we measured the half-width (full-width at half-maximal amplitude) of each event. This metric is less vulnerable to inaccuracies that arise from trying to detect the exact point at which an IPSC decays, and yielded tighter data.

We first examined the half-width of spontaneous IPSCs (sIPSCs) recorded in PV-cells. Compared to sIPSC half-widths from LR PV-cells, those from DR PV-cells were significantly slower (Figure 6.2.a), both on a cell-averaged basis (inset, black bars) and when events sampled
from the entire population were compared using KS tests (black lines). This effect was specific to PV-cells, as pyramidal cell sIPSC half-widths failed to show a change with dark-rearing (Figure 6.2.b) using either statistical test.

Having obtained synaptic physiology data consistent with a reduction in PV-cell alpha-1 content following dark-rearing, we next wanted to know whether this shift to slower sIPSC half-widths was due to PV-cell-mediated events. To do this we analyzed PV-IPSCs, quantal events recorded after channelrhodopsin-activation of the PV-cell-network, which were desynchronized by the presence of extracellular strontium (Chapter 4). Similar to sIPSCs recorded in PV-cells, PV-IPSCs recorded in these cells were significantly slower in the dark (Figure 6.2.a, green bars & lines), suggesting that the sIPSC effect is mediated largely by events originating from PV-cells. This effect was again specific to PV-cells, as pyramidal cell PV-IPSC half-widths were unchanged in the dark (Figure 6.2.b, green bars & lines). Average waveforms of PV-IPSCs recorded in PV-cells (Figure 6.2.c) and pyramidal cells (Figure 6.2.d) highlight the selective effect of visual deprivation on PV-cell kinetics.
Figure 6.2 = (a) Cumulative distribution of half-widths of sIPSCs (black lines) & PV-IPSCs (green lines) recorded in light-reared (dashed lines) & dark-reared (solid lines) PV+ cells. Asterisk, results from KS tests comparing events sampled from their broader populations (LR PV-IPSC vs. DR PV-IPSC). (Inset) Cell-averaged representation of the same data shown in the cumulative distributions. Red symbols, Mann Whitney tests. (b) Half-widths for PYR cells. (Inset) Cell-averaged data for PYR cells. (c) Average PV-IPSC waveforms for an LR & DR PV+ cell, highlighting the faster half-widths seen in LR cells. Scale bars, 5pA, 5ms. (d) Average PV-IPSC waveforms for an LR & DR PYR cell.
PV:GABA<sub>A</sub>R-α1<sup>(−/−)</sup> mice as a model of PV-cell connectivity following dark-rearing

Using both qPCR and synaptic physiology, we found that PV-cells exhibit a deficit in fast GABAergic transmission following dark-rearing. This effect is specific to PV-cells, as non-PV cells show neither a decrease in GABA<sub>A</sub>R alpha-1 subunit level, nor a lengthening of sIPSC or PV-IPSC half-width. Importantly, this plasticity at PV-PV connections is only one of many changes taking place in V1 in response to dark-rearing (Tropea et al., 2006; Dahlhaus et al., 2011). To test the hypothesis that a deficit specifically at PV-PV connections underlies the adult plasticity observed in dark-reared mice, we aimed to model this deficit – and only this deficit – in otherwise-normal, light-reared mice. We reasoned that we could do so genetically by removing the alpha-1 subunit-encoding gene (Gabral) specifically from PV-cells using the Cre-lox system (Morozov, 2008). Critically, as both PV-PV and PV-pyramidal connections are enriched in alpha-1 subunit-containing GABA<sub>A</sub>Rs (Klausberger et al., 2002), deleting Gabral only from PV-cells leaves the PV-pyramidal connection intact (Figure 6.3.a). Further, as expression of Cre under control of the parvalbumin promoter does not reach appreciable levels until the peak of the critical period (Morishita et al., 2015), we figured that PV:GABA<sub>A</sub>R-α1<sup>(−/−)</sup> mice (“KO”) would develop normally and undergo an endogenous critical period for ocular dominance plasticity before Gabral is deleted from PV-cells. Thus, we reasoned that KO mice would be useful to investigate the role of PV-PV connections in adult plasticity.
Figure 6.3 = (a) Postsynaptic receptors opposite PV+ boutons are enriched in α1-containing GABAₐRs at both PV-PYR and PV-PV synapses. We modeled the loss of α1 in PV-cells of dark-reared mice by deleting the α1-encoding gene selectively from PV-cells (PV:GABAₐR-α₁⁻/⁻, KO), leaving PV-PYR connections intact. (b) (Left) PV+ cells from KO mice & wild-type littermate controls (WT), from which mean somatic α1 immunofluorescence signal was measured. Scale bar, 5µm. (Right) Relative α1 intensity of both PV(+) and PV(-) cells in KO mice and controls, normalized to PV(-) cell intensity per genotype (Kruskal-Wallis test P<0.0001; KO PV(+) cells P<0.001 vs. all other conditions, Dunn’s multiple comparison tests). (c) GABAₐR subunit mRNA levels from anterior forebrain of FAC-sorted PV:GABAₐR-α₁⁻/⁻-tdTomato mice and controls. Shown are results from the tdTomato(+) cell fraction, which are normalized to WT levels per gene. Asterisks, unpaired t test. (d) The density of synaptotagmin-2+ (Syt2+) boutons per PV-cell body in KO mice is unchanged from that of WT (Mann Whitney test, P=0.7958), and is not different vs. light-reared mice (Kruskal-Wallis test & Dunn’s multiple comparison tests).
We first characterized KO mice in several ways. First, we performed immunostaining for PV and alpha-1 in order to assay whether alpha-1 protein disappears from PV-cells and not from pyramidal cells (Figure 6.3.b). While PV-cells in wildtype mice (“WT”, PV:GABA_A-α1(+/+)

littermates) show a ring of alpha-1 signal around the inner edge of the somatic membrane (Figure 6.3.b, lower-left image), in KO mice this staining is abolished, with only background signal remaining (Figure 6.3.b, lower-right image). Quantification of mean somatic fluorescence intensity across genotypes revealed that KO mice exhibit a loss of alpha-1 signal selectively from PV-cells, with pyramidal cell-levels unchanged (Figure 6.3.b, right). As the alpha-1 antibody used in these experiments is capable of detecting the truncated form of the alpha-1 protein that results following Cre-mediated recombination (data not shown), these immunostaining results validated our targeted gene manipulation strategy.

In order to confirm this immunostaining result as well as assay for compensatory changes in the expression of other GABA_A subunits following alpha-1 deletion (Kralic et al., 2002a) from PV-cells, we next performed FAC sorting and qPCR analysis on anterior forebrain samples of PV-Cre x GABA_A-α1(+/+) x tdTomato(+/+) mice (n: WT, 6; KO, 5). In the PV-cell-enriched tdTomato(+) fraction, alpha-1 expression in KO samples was only ~14% of that measured in WT samples (Figure 6.3.c), confirming a dramatic reduction in alpha-1 expression. This effect was specific for PV-cells, as alpha-1 expression in the tdTomato(-) fraction was unchanged in KO mice (data not shown; WT, 1.0; KO, 1.048; P=0.35, unpaired t test). As for compensatory changes in other subunits found in PV-cells (i.e., in the tdTomato(+) fraction; Figure 6.3.c), no significant change in expression was observed for alpha-3 (P=0.27, unpaired t tests) or gamma-2 (P=0.18). Although alpha-2 expression was ~50% lower in KO mice, this reduction was not significant (P=0.25). This lack of significance was not due to one WT sample showing >4x more
alpha-2 expression than the mean of the remaining samples, as exclusion of this sample produces mean relative expression levels of 1.0 for WT and 0.77 for KO, which yields a P-value of 0.36 (data not shown). Taken together, these data suggest that following deletion of Gabra1 selectively from PV-cells, no major compensatory changes in the expression level of other GABA_A R subunits by PV-cells results. Although both alpha-3 and gamma-2 show expression that is increased by ~13% in KO mice, these changes are mild compared to the significant increases in alpha-2 & -3 expression reported in alpha-1 total knockout animals (~30-40%, Kralic et al., 2002b).

Given that alpha-1 is the major alpha-subunit found at PV-PV connections (Klausberger et al., 2002), we were unsure what effect its deletion from PV-cells in the developed brain would have on existing synapses. Further, it was possible that pre- & post-synaptic structures could be differentially affected. For example, deletion of the obligatory postsynaptic subunit gamma-2 from adult hippocampal neurons results in degradation of GABAergic postsynapses, with scaffolding molecule gephyrin also lost in addition to gamma-2 itself (Schweizer et al., 2003). Interestingly, however, presynaptic terminals remain in place despite postsynaptic disruption. Following deletion of the alpha-1 subunit itself, cerebellar circuits respond in a host of unexpected ways (Fritschy et al., 2006). Normally, Purkinje cells receive dendritic contacts from stellate cells and somatic contacts from basket cells, both of which use the alpha-1 subunit postsynaptically. Following germ-line deletion of alpha-1, Purkinje cells no longer receive functional inhibition. This is mediated by a 75% reduction in symmetric synapses formed onto Purkinje dendrites by stellate cells, which instead form asymmetric synapses onto Purkinje dendritic spines. In contrast, somatic synapses formed by basket cells are unchanged on an ultrastructural level, despite being non-functional. Given the range of outcomes following
deletion of various GABA\textsubscript{A}R subunits, we decided to characterize the connectivity of PV:GABA\textsubscript{A}R-\textalpha{1}{(-/-)} mice on the synaptic level.

We first examined PV-PV connectivity in KO mice using the synaptotagmin-2 assay introduced in Chapter 3. Compared to the density of Syt2\textsuperscript{+} inputs received by PV-cell somata in WT (n=2 mice), cells in KO (n=3 mice) received an identical level of innervation (Figure 6.3.d; P=0.8, Mann Whitney test). In a 1-way ANOVA including PV-cells from all other conditions (Chapter 4, Figure 4.1.b), WT and KO did not differ significantly from one another, nor did they differ from light-reared PV-cells (Kruskal-Wallis test, P<0.0001; Dunn’s multiple comparison tests). Overall, these results suggest that deletion of alpha-1 from PV-cells in the juvenile brain has no effect on PV-PV connectivity in V1, as assayed anatomically using a presynaptic marker.

In terms of postsynaptic changes, A.E. Takesian & T.K. Hensch (unpublished observations) found that sIPSCs onto PV-cells in frontal cortex were unchanged in frequency, but did exhibit both decreased amplitude and longer half-widths, consistent with that observed for sIPSCs recorded in DR PV-cells in V1. This effect is specific for PV-cells, as events onto pyramidal cells have amplitudes and kinetics that are unaltered (ibid). Thus, as KO mice displayed a synaptic phenotype consistent with what might be expected following loss of alpha-1 subunits, we were satisfied that our targeted gene-deletion strategy was executed faithfully. Further, as KO mice reproduced the postsynaptic deficits of dark-reared mice that we observed specifically in PV-cells (Figure 6.2), we concluded that we had modeled this aspect of the DR PV-cell-network in our creation of KO mice.
Figure 6.4 = (a) Visual evoked potentials (VEPs, inset traces in black & red; scale bars, 50µV, 100ms) recorded from L4 of V1 in vivo decrease in amplitude as spatial frequency of the visual stimulus increases (inset black & white gratings), indicating visual acuity of the mouse at X-intercept. (b) In contrast to wild-type mice (WT, left), PV:GABA$_A$R-α1$^{/-}$ mice (KO, right) display acuity loss at adult ages in response to brief monocular deprivation (MD, 4 day). Asterisks, unpaired t tests.
Adult plasticity following PV-PV disruption in PV:GABA_A R-α1(-/-) mice

In dark-reared animals, the critical period for ocular dominance plasticity is delayed until adulthood and occurs only upon exposure to light (Mower et al., 1983; Fagiolini et al., 1994). In Chapter 4, we examined V1 of dark-reared mice and found that interconnections between PV-cells fail to mature in the absence of visual experience. As adult light-reared mice – which lack ocular dominance plasticity – show robust PV-PV connectivity, we hypothesized that the PV-PV connection may be involved in regulating the state of the critical period. To test this hypothesis we examined PV:GABA_A R-α1(-/-) mice, reasoning that disruption of PV-PV connectivity may result in adult plasticity.

To assay KO mice for adult plasticity, we recorded visual evoked potentials (VEPs) in vivo. Following anesthetization, a craniotomy was performed and an electrode was lowered to cortical L4, from which field recordings were obtained. Presentation of sine wave gratings generated VEPs, the amplitude of which decreased as spatial frequency of the stimulus increased (Figure 6.4a). The spatial frequency at which VEP amplitude reached zero identified the visual acuity of the mouse. In adult WT mice, a brief period of monocular deprivation (MD, 4 days) does not lead to loss of acuity in the deprived eye (Figure 6.4.b, left), consistent with closure of the critical period (Kang et al., 2013). However, MD in KO mice yields a striking reduction in deprived-eye acuity (Figure 6.4.b, right), an effect seen not only at young adult ages (P60), but also well after closure of the endogenous critical period (P150). This finding suggests that critical period regulation is disrupted in PV:GABA_A R-α1(-/-) mice, and that plasticity may even be open-ended.
Attributions

Luke J. Bogart and Henry Lee (H.H.C. Lee) performed FACS & qPCR on light-reared & dark-reared mice together. Luke J. Bogart performed *in vitro* physiology on LR & DR mice and synaptotagmin-2 immunohistochemistry. Henry Lee performed all *in vivo* physiology, FACS/qPCR for PV:α1-KO mice, and PV/alpha-1 immunostaining. Anne E. Takesian performed the *in vitro* physiology experiments in PV:α1-KO mice that are discussed.
DISCUSSION

As described in Chapter 4, we found that dark-rearing produces an array of both cellular and synaptic effects on the V1 inhibitory system, most notable of which is a selective weakening of PV-PV connections. In Chapter 5, we found that dark-rearing leads to circuit-level changes in PV-cells’ connections with one another, manifest as a dramatic decrease in convergence within the network. However, missing from this picture was more detailed knowledge of DR’s effects on synaptic function, and an understanding of whether any experience-dependent changes that occur are likely to regulate critical period plasticity. To this end, we identified a postsynaptic deficit present selectively in PV-cells: following dark-rearing, expression of the GABA\(_A\)R alpha subunit most highly-expressed at PV-PV synapses, alpha-1 (Klausberger et al., 2002), is significantly reduced (Figure 6.1.a). This loss of alpha-1 had a functional impact on PV-cells, resulting in broader half-widths of both the sIPSCs and PV-IPSCs that PV-cells receive (Figure 6.2.b). When we modeled these effects by genetically removing alpha-1 subunits from mature PV-cells, we found that this recapitulated the kinetic effects of dark-rearing on PV-PV connections postsynaptically, and that this was sufficient to extend plasticity into adulthood in these mice. Taken together, these results indicate that PV-PV connections regulate V1 plasticity.

Technical and experimental considerations

Our results extend previous efforts to measure the expression and plasticity of GABA\(_A\)R subunit mRNAs in visual cortex from the regional level to the cell type-specific level. Among these earlier reports, there is broad agreement that expression of alpha-1 and/or alpha-3 subunit mRNA is upregulated in the absence of visual experience (Chen et al., 2001; only alpha-3, Heinen et al., 2004; Tropea et al., 2006; only alpha-1, Dahlhaus et al., 2011), an effect also seen
on the protein level (Chen et al., 2001). In our study, however, we did not observe increased expression of any subunit following dark-rearing (Figure 6.1). There are numerous experimental differences between our study and past studies which may explain this discrepancy.

First and foremost, whereas previous studies used homogenates of light- & dark-reared V1, the samples used in our study were first FAC sorted into YFP(+) (PV-cell-enriched) and YFP(-) (non-PV cell containing) fractions before mRNA levels were analyzed using qPCR. Through this approach, we were able to detect a change in alpha-1 expression that occurred specifically in YFP(+) PV-cells, a cell type-specific effect that would have been missed using V1 homogenates. Nevertheless, on the surface it is somewhat surprising that our YFP(-) fraction, which putatively includes all of the cells and cell-types (except for PV-cells) that would be found in V1 homogenates, did not exhibit increased alpha-1/3 expression as per previous reports. However, that our samples were first FAC sorted before being subjected to qPCR constitutes a key methodological difference.

In preparing samples for FACS (Saxena et al., 2012), dissociated cells are passed through a strainer containing finely-sized pores, which may shear dendrites known to store mRNA for local translation (Bramham & Wells, 2007). Because of this, most of the mRNA remaining in our samples may have originated from proximally-biased locations with respect to the soma, a region containing only a small minority of a cell’s cytoplasm and synaptic real estate (Gulyás et al., 1999). Further, as the FACS step itself systematically rejects many of the items interrogated on the basis of size and other criteria (e.g., survivability), it is possible that our YFP(-) fractions may have been enriched with a non-random population of cells, with various glial cell-types known to express GABA_{A}Rs (Vélez-Fort et al., 2012) potentially excluded. Additionally, whereas past work has mainly used younger animals (cats, DR to 5 & 20 weeks, Chen et al.,
2001; rats, DR to P21, Heinen et al., 2004; mice, DR to P27, Tropea et al., 2006; mice, DR to P46, Dahlhaus et al., 2011), our mice were dark-reared until ~3 months of age. That our YFP(-) fractions from adult DR mice did not exhibit increased expression of GABA$_A$R alpha subunit mRNAs suggests that, if expression were higher near the peak of the critical period (e.g., Tropea et al., 2006), it may have moderated with continued development of the visual system in the dark (Kang et al., 2013). Overall, due to these differences in the source material from which mRNA was extracted, it is difficult to directly compare our results to those of previous studies.

While PV-cells exhibited a clean, significant reduction in GABA$_A$R alpha-1 subunit expression following dark-rearing, the magnitude of this effect was fairly modest (~20%, Figure 6.1.a). Despite our cell type-specific approach, there are numerous factors that may have contributed to a dilution of this effect (or, to be fair, could have generated a false-positive, but this is unlikely for reasons discussed below). First, while our acute dissections aimed to collect tissue solely from V1, it is likely that some tissue from bordering, non-primary visual areas such as V2 and retrosplenial cortex was also harvested, as dissections were performed by eye. As these areas would be expected to be less responsive or unresponsive to the effects of DR than V1, their contaminating presence may have lessened the magnitude of alpha-1’s reduction. In the future, more precise targeting of V1 by first cutting vibratome sections and/or using laser micro-dissection methods would be helpful refinements, with the latter potentially useful to examine inter-laminar differences.

Second, while basket cells comprise a large majority of the PV+ cell population in V1 (Rudy et al., 2011), chandelier cells and multipolar bursting cells also express PV, and therefore were likely included in our YFP(+) fraction. While the afferent connectivity of these cell-types and their response to visual deprivation are not well characterized, their low abundance make
them unlikely to have contributed substantially to these qPCR results. If in the future markers identifying PV-basket cells, or sub-types of PV-basket cells (Rossier et al., 2015; Donato et al., 2015), with high-specificity are obtained, it will be interesting to reassess these cells’ plasticity of alpha-1 expression and other metrics on a finer level.

Finally, regarding the qPCR analysis of GABA$_A$R subunits in PV:GABA$_A$R-$\alpha$1$^{(-/-)}$ mice (Figure 6.3.c), there are number of reasons why results indicating an “incomplete knockout” of alpha-1 may have been obtained. Specifically, we found that compared to the expression level of alpha-1 in tdTomato(+) PV-cells of WT mice, the tdTomato(+) fraction in KO mice showed alpha-1 expression at ~14% of the WT level. First, assuming that this result is accurate, it is possible that some alpha-1 mRNA does remain in ~P60 KO mice. If Cre does not reach appreciable levels in V1 until the peak of the critical period (Morishita et al., 2015), onset of Cre expression from the activity-dependent PV locus would likely occur even later in frontal cortex (Hensch, 2005) from which samples were obtained, and may not be the same in all cells. If some recombination events may have only occurred in the days leading up to tissue collection, then it is possible that mRNA for highly-expressed functional proteins – which can have half-lives ranging several days (Yu & Russell, 2001) – like alpha-1 may still be present in KO samples. Future qPCR experiments examining cells from older mice would reveal how much alpha-1 expression persists after longer post-recombination delays. Second, it is possible that the tdTomato(+) fractions may have been contaminated with non-PV+ cells. While some level of contamination is unavoidable, the selection criteria for fluorescent cells may not have been sufficiently stringent. Probing for a gene known not to be expressed in PV-cells, such Ca$^{2+}$/calmodulin-dependent protein kinase II alpha which is present only in excitatory cells (Liu & Murray, 2012), would help calibrate the level of contamination.
Functional impact of dark-rearing and GABA\textsubscript{A}R alpha-1 subunit deletion on PV-cells

In our FACS/qPCR experiment investigating cell type-specific changes in the expression of GABA\textsubscript{A}R subunits after dark-rearing, the only significant change we observed in YFP(+) PV-cells was a ~20% reduction in alpha-1 expression (Figure 6.1.a). While alpha-1 subunit-containing GABA\textsubscript{A}Rs ("alpha-1 receptors") are the predominant "mature" form in the adult brain (Fritschy et al., 1994) and are found at sites throughout the dendritic tree (Hensch, 2005), PV-PV connections contain very high levels of alpha-1 receptors (Klausberger et al., 2002), and we have focused our interpretation of this result with respect to this synapse. As adjacent, PV-negative terminals onto hippocampal pyramidal cell somata are often also immuno-negative for alpha-1 (ibid), reduction of alpha-1 expression in DR PV-cells may have a fairly specific effect on PV-PV connections.

Since changes in the expression of subunit mRNAs are often paralleled by changes in the surface content and function of GABA\textsubscript{A}Rs (Luscher et al., 2011), it is likely that reduction of PV-cells’ principal alpha-subunit mRNA by dark-rearing indicates a functional impact on PV-PV synapses. Specifically, unless alpha-1 mRNA expression is reduced simply due to PV-cells receiving fewer PV+ inputs in the dark (Figure 4.1.b), there may be physiological deficits at remaining PV-PV synapses. For example, alpha-1 receptor-containing synapses could exhibit a reduction in amplitude and/or a lengthening of decay kinetics, depending on whether or not there is compensation in receptor number at individual synapses. In Chapter 4, we found that PV-IPSCs onto dark-reared PV-cells are unchanged in amplitude vs. those onto LR cells (Figure 4.4.c), suggesting that total receptor number at these synapses was largely unchanged by DR. However, as we observed in Figure 6.2.a that the half-width of PV-IPSCs recorded in DR PV-cells is longer than in LR cells, this suggests that a switch to slower-deactivating alpha-subunits
(Picton & Fisher, 2007) occurred at these synapses. Indeed, despite the aforementioned enrichment of alpha-1 receptors at PV-synapses (Klausberger et al., 2002), PV-cell synapses onto hippocampal pyramidal cells have recently been shown to contain alpha-2/3 receptors (albeit less often; Strüber et al., 2015), suggesting that the same may occur at PV-PV connections.

Despite the potential of some residual alpha-1 expression in KO PV-cells, the exact state and subunit-composition of GABA$_A$Rs at PV-PV synapses remains unknown. Nevertheless, as A.E. Takesian & T.K. Hensch (unpublished) found that KO mice retain functional PV-PV connectivity and that sIPSCs received by PV-cells are broadened, it is likely that non-alpha-1-containing GABA$_A$Rs are located at these synapses. Lacking strong compensatory increases in the expression of other alpha-subunit mRNAs (Figure 6.3.c), it is likely that post-transcriptional mechanisms may be operating in KO mice in order to drive insertion of enough functional receptor at PV-PV synapses (Luscher et al., 2011). Interestingly, only a small minority of total GABA$_A$R protein is present on the cell-surface at any given time, and these synaptic GABA$_A$Rs turn-over at a very high rate. It is possible that the pool of alpha-2/3-containing GABA$_A$Rs, which have slower kinetics (Picton & Fisher, 2007), may have sufficient capacity to accommodate some receptors being targeted to PV-PV synapses that would normally express alpha-1. However, as changes in subunit mRNA expression are often paralleled by changes in the surface content and function of GABA$_A$Rs (Luscher et al., 2011), it is likely that IPSC amplitude at synapses normally enriched with alpha-2/3-containing GABA$_A$Rs would decline without additional receptors being translated and assembled. As Takesian & Hensch (unpublished) have already found that PV-PV connections are reduced in amplitude in KO mice
(in addition to a reduction in amplitude in the broader sIPSC population), it would be informative to investigate whether other distinct sources of inhibitory input to PV-cells are weaker as well.
CHAPTER 7

Discussion
Summary of findings

In this work, we have studied the mechanisms of ocular dominance (OD) plasticity in primary visual cortex (V1), the premier model of experience-dependent plasticity. A growing body of work from our lab and others has identified the maturation of GABAergic inhibition as central to this process (Hensch, 2004, 2005; Maffei & Turrigiano, 2008; Takesian & Hensch, 2013), but how this regulates plasticity remains unknown. Previously, GABA_A receptors (GABA_ARs) containing the alpha-1 subunit have been implicated in gating V1 plasticity (Fagiolini et al., 2004), but the precise location of the operant synapses within the cortical microcircuit has not been identified. Alpha-1 subunit-containing GABA_ARs are present at synapses formed by presynaptic boutons expressing PV (Klausberger et al., 2002), a marker of a molecularly-distinct class of “basket” interneuron (Rudy et al., 2011) that innervates the perisomatic domain of both pyramidal cells and other PV-expressing cells.

Here, we began by investigating both of these PV-cell-mediated connections. Using an antibody for a presynaptic marker recently characterized as a specific label of PV+ boutons, synaptotagmin-2 (‘Syt2’; Sommeijer & Levelt, 2012), we found that PV-cells provide strong innervation to the somata of both pyramidal cells and other PV-cells (Chapter 3). We next assayed these connections for their response to dark-rearing, a classical method to deprive animals of sensory experience and maintain V1 plasticity into adulthood (Fagiolini et al., 1994). Strikingly, we found that dark-rearing delays the maturation of PV-PV connections specifically, while allowing PV-pyramidal innervation to develop largely as normal (Chapter 4). We next addressed the circuit-level basis of this plasticity by labeling PV-cells with Brainbow, a neuroanatomical tool allowing axons of individual cells to be distinguished via their genetically-encoded color (Livet et al., 2007; Cai et al., 2013). Surprisingly, we found that the loss of PV-
inputs to dark-reared PV-cells was the result of dramatically reduced convergence within the network, rather than due to individual presynaptic axons each contributing fewer boutons (Chapter 5).

PV-PV connections also exhibited sensitivity to dark-rearing on the molecular and synaptic levels. PV-cells isolated from dark-reared V1 expressed less of the fast inhibition-mediating alpha-1 subunit (Lavoie et al., 1997) of the GABA$_A$R, an effect not seen in non-PV cells (Chapter 6). The loss of alpha-1 was manifest as a broadening of spontaneous inhibitory postsynaptic currents (sIPSCs) selectively in PV-cells, an effect determined optogenetically to be mediated by presynaptic PV-cells. Modeling of this synaptic deficit via genetic deletion of the alpha-1 subunit specifically from PV-cells (PV:GABA$_A$R-α1$^{(-/-)}$ mice, “PV:α1-KO”) recapitulated this broadening of IPSCs, while leaving PV-pyramidal connections intact. Remarkably, this targeted, postsynaptic manipulation of PV-PV connectivity was sufficient to extend V1 plasticity into adulthood, with brief monocular deprivation at both P60 & P150 able to induce amblyopia in PV:α1-KO mice. Overall, these results argue for a role of recurrent PV-cell connections in critical period regulation, and suggest that experience-dependent maturation of PV-PV synapses may mark the end of plasticity.

**Parameters of recurrent PV-connections and potential role for experience in their development**

As summarized above, each Chapter of this thesis has uncovered another dimension along which reciprocal connections between PV-cells are regulated by visual experience. In response to experience, innervation of PV-somata by other PV-cells rises (Chapter 4), increasing the level of convergence within the network (Chapter 5). With this comes increased expression
of the GABAAR alpha-1 subunit, narrowing the half-width of inhibitory events at recurrent PV-synapses (Chapter 6). Each of these specializations enhances the function of PV-PV connections, which have been extensively studied outside of the context of experience-dependent plasticity (Bartos et al., 2007).

Paired recordings of GABAergic synapses between FS-basket cells reveal that these connections are characterized by low latencies, high amplitudes, and fast rise-times & decay taus (Bartos et al., 2007). This latter property, decay tau, is twice as fast at FS-FS connections than at FS-granule cell connections, and is a synaptic property especially important for promoting synchronous firing among FS-cells. PV-cells are extensively connected in sensory cortex (mouse S1; Galaretta & Hestrin, 2002), with 35% of pairs demonstrating a unidirectional GABAergic connection and an additional 43% of pairs showing reciprocal connectivity. These chemical synapses often co-exist with electrical synapses mediated by gap junctions (over 60% of pairs; Galaretta & Hestrin, 1999; Gibson et al., 1999), the coupling coefficient of which can be strong enough to synchronize spiking between cells. The fact that connections are sheared during the slicing process suggests that both of these values are likely even higher in vivo, and our Brainbow results would suggest that PV-PV connection probability is regulated in an experience-dependent manner. The joint presence of chemical and electrical synapses in FS-cell networks promotes firing at gamma-frequency (30-70 Hz; Tamás et al., 2000), with the physical proximity of GABAergic synapses to gap junctions found to curtail junctional potential and facilitate entrainment. That increased perisomatic innervation of PV-cells with experience may function specifically to modulate the decay of coupling potentials and promote synchronous firing of PV-cell networks is intriguing.
In addition, autapses are commonly found on GABAergic cells and appear to be the rule for basket cells (Tamás et al., 1997a), which form an average of 12 self-innervations each in V1 of adult cats. These contacts are biased close to the soma (~12um away on average; ibid), where they regulate the temporal precision of spike output (Bacci & Huguenard, 2006). As IPSCs at PV-PV connections are broadened in dark-reared mice, it is likely that the rate and precision of spiking in these cells may be affected by the same change in kinetics at PV-autapses. While the time-course of autaptic currents, as measured by the asynchronous release of transmitter following spike trains, narrows with age in rat frontal cortex (Jiang et al., 2015), a role for experience specifically in this maturation has not been determined. As a concomitant decrease in amplitude suggests that autapse number may decline with age and/or experience, this question may be readily addressed using Brainbow.

However, as this decrease in autaptic amplitude may rely on a developmental shortening of the decay of presynaptic calcium transients (ibid), experience-dependent increases in the expression of PV (Sugiyama et al., 2008), a calcium-binding protein, may also be involved. Interestingly, that PV expression level has been found to negatively correlate with the level of transmitter release (Eggermann & Jonas, 2012) suggests that emerging subpopulations of basket cell (Donato et al., 2013, 2015), which express different amounts of PV, may exhibit differences in autapse function. Future examination of various subpopulations of PV-cell may elucidate their differential involvement in the experience-dependent maturational paradigm described herein. As we have not yet addressed which presynaptic PV-partners are “missing” from the complement of inputs to DR PV-cells (Chapter 5), it is possible that changes to the wiring diagram may involve a reweighting of the level of interconnectedness between different PV-subnetworks.
Emergent properties of recurrent PV-connections and their role in OD plasticity

As mentioned earlier, the combination of electrical and chemical connections between PV-cells endows these circuits with specialized properties, such as the ability to detect the synchrony of excitatory inputs (Galaretta & Hestrin, 2001). Specifically, electrical-coupling permits the depolarization caused by nearly coincident inputs to distinct PV-cells to bring both cells closer to threshold, promoting synchronous firing. The coincidence of inputs is key, as premature spiking of one cell can generate an inhibitory response in the other cell, due to both the GABAergic connection and infiltration of the afterhyperpolarization.

As shown by Bruno & Sakmann (2006), individual thalamocortical inputs are ineffective at driving spiking in principal cells in vivo. This outcome is likely due to a failure of these inputs to overcome the feedforward inhibition mediated by thalamorecipient PV-cells (Cruikshank et al., 2007), which gate the flow of information into cortex by employing the synchrony detection mechanism described above. Weak inputs, which activate only a small, local pool of PV-cells (which are more excitable than principal cells), trigger feedforward inhibition that suppresses excitatory cell responses. However, due to high convergence within the pathway, synchronous activation of thalamocortical inputs can drive responses in excitatory cells (Bruno & Sakmann, 2006). This suggests that broad activation of the PV-cell-network by coincident inputs “opens the gate,” by creating a temporal window between PV-cell spikes through which more slowly mediated excitation of principal cells can occur (Cruikshank et al., 2007).

Synchrony detection by PV-cell networks has been hypothesized to play a role in experience-dependent plasticity (Hensch, 2005). Following monocular deprivation (MD), thalamocortical input along the deprived-eye pathway no longer transmits activity driven by the viewing of real-world scenes, which is characterized by phasic, temporally-precise firing.
Instead, deprived-eye signals reflect variations in the level of light and other more slowly-changing stimulus parameters, producing cortical input patterns characterized by jittery, asynchronous firing. This disparity in input patterns from the two eyes leads to differential activation of the synchrony detection mechanism in the PV-cell-network, which may be involved in mediating ocular dominance plasticity through subsequent spike-timing-dependent plasticity (STDP) mechanisms. STDP is a refinement of Hebbian theory, and posits that inputs active a short time before postsynaptic firing become strengthened at the expense of those inputs active shortly after firing, which become weakened (Zhang et al., 1998).

In a modeling study, Kuhlman et al. (2010) argue that beyond a role for inhibition in shaping the integration window for spike generation (Pouille & Scanziani, 2001), the level of PV-cell-mediated inhibition determines whether the synaptic drive or temporal coherence of competing inputs wins out to control postsynaptic spike times (ibid). Specifically, when inhibition is weak, the pronounced contralateral bias of thalamocortical projections into binocular V1 enables those inputs – even if controlled by the deprived eye – to outcompete via STDP the smaller population of highly-coherent open-eye inputs. Consistent with this, MD performed before critical period onset, when inhibition is weak, does not produce an OD shift (Fagiolini & Hensch, 2000). However, upon its maturation to critical period levels, inhibition reduces the efficacy of less-coherent inputs driven by the deprived eye, guiding STDP toward favoring the more-coherent inputs from the open eye (Kuhlman et al., 2010). As such, MD during the critical period, when inhibition is more developed, can induce an OD shift. Finally, further strengthening of inhibition in the model prevents plasticity. Although unspecified, this may be because the integration window that inhibition shapes (Pouille & Scanziani, 2001) may become too narrow for even the temporally-coherent inputs arriving from the open eye to effect
plasticity in the circuit. This suggests that despite maturation of PV-cell intrinsic physiology being largely complete by the peak of the critical period (Kuhlman et al., 2010; Quast & Hensch, 2012), the possibility that late-developing elements of the circuitry, which may include PV-PV connections, might ultimately signal the end of the critical period remains unknown.

**Disruption of PV-PV connections in DR & PV:α1-KO mice and its impact on synchrony detection**

Despite this framework (Hensch, 2005; Kuhlman et al., 2010), how exactly the parameters of inhibition, and PV-PV inhibition in particular, influence synchrony detection and overall PV-cell-network function remain incompletely understood. However, that both DR & PV:α1-KO mice undergo an OD shift in response to MD in adulthood – when mature inhibition normally prevents plasticity – suggests that a disruption in synchrony detection may prevent critical period closure. In other words, inhibition in each of these models functions at the maturity level seen during the critical period (Kuhlman et al., 2010), in that it permits STDP mechanisms to select for coherent inputs. As such, consideration of the synaptic deficits in each model may be useful for understanding how this phenotype comes about.

The nature of PV-PV disruption is slightly different in each case. In DR mice, there is decreased physical connectivity between PV-cells, along with reductions in the frequency and amplitude of the sIPSCs that PV-cells receive (Chapter 4). These sIPSCs are also broader, an effect found to be PV-cell-mediated (Figure 6.2.a). Further, this physical disconnection results in dramatically lower PV-PV convergence in DR V1 (Chapter 5), decreasing the recurrence of inhibition within the network. In contrast, PV-cells in PV:α1-KO mice receive a normal number of afferent inputs from other PV-cells (Figure 6.3.d), leaving PV-PV convergence also
presumably unchanged. However, PV:α1-KO mice do show deficits in inhibitory synaptic transmission, as PV-cells receive sIPSCs that are both decreased in amplitude and that have slower kinetics, effects found via optogenetics experiments to also affect PV-cell-mediated maximal IPSCs (Takesian & Hensch, unpublished). Taken together, these two models of adult plasticity show a common synaptic phenotype, which can be characterized as a change in the timecourse of PV-mediated inhibition onto PV-cells. This is also accompanied by a decrease in the overall strength of PV-PV connections, a change which arises in slightly different ways in each model\(^1\).

These changes in PV-PV connections are likely to affect network function in key ways, and may result in changes to the thalamocortical (TC) gate that permit ectopic plasticity (Kuhlman et al., 2010). Perhaps most plausibly, broadening of the half-width of PV-cell-mediated IPSCs (“PV-IPSCs”) in both models would lengthen the window of time during which PV-cells are suppressed following TC activation. Recall from earlier that in response to TC input, PV-cells generate an action potential before principal cells do, which results in both principal cells and other PV-cells receiving strong feedforward inhibition (Cruikshank et al., 2007). This inhibitory conductance overlaps with the excitation that principal cells receive, effectively suppressing their firing. However, in response to strong TC activation, principal cells can fire during the time in which PV-cells are suppressed by recurrently-delivered IPSCs. If these IPSCs are broader, PV-cells are suppressed for longer, and principal cells have a greater chance of firing during this lengthened window. In Kuhlman et al. (2010)’s model, this may

\(^1\) In PV:GABA\(_\text{A}\)R-α1\(^{-/-}\) mice virally-expressing channelrhodopsin in PV-cells, the amplitude of maximal, light-evoked inhibitory current onto patched PV-cells is lower than in α1\(^{+/+}\) control littermates (A.E. Takesian & T.K. Hensch, unpublished). sIPSCs onto PV-cells are also reduced in amplitude in PV:α1-KO mice, an effect which may be mediated by events originating from PV-cells. In dark-reared PV-cells, strontium-desynchronized PV-IPSCs are static in amplitude vs. light-reared controls, but sIPSCs are decreased in amplitude in dark-reared mice. That the frequency of sIPSCs, along with the density of synaptotagmin-2+ boutons, is decreased in dark-reared mice offers indirect evidence that the decrease in sIPSC amplitude observed in DR PV-cells is mediated by the presence of fewer large, PV-cell-mediated events.
grant highly-coherent open-eye inputs the ability to drive principal cell spiking more effectively than less-coherent inputs belonging to the deprived eye, causing an ocular dominance shift. In PV:α1-KO mice, the constitutive lengthening of PV-IPSCs at PV-PV connections may permit open-ended plasticity. In dark-reared mice, subsequent experience-dependent maturation of PV-PV-connections following exposure to light may eventually lead this “last mile” of development (i.e., the speeding of PV-IPSCs via a switch to alpha-1 GABA_ARs) to occur, closing plasticity.

**Further impact of disrupted PV-PV connectivity on DR & PV:α1-KO networks**

Other changes at PV-PV connections observed in DR & PV:α1-KO mice may also be involved. Considering the reduced interconnectivity of PV-cells in DR mice, one can imagine an extreme scenario in which lateral inhibitory connections between PV-cells (Cruikshank et al., 2007) are completely absent. In this case, activation of (basally more excitable) PV-cells by thalamic inputs may result in principal cell activation never occurring, due to their unchecked suppression by PV-cells and without a consistently-timed window during which they can spike. With an intermediate level of PV-PV connectivity like that seen in DR mice, individual PV-cells may receive an inhibitory conductance that is either too small or rises too slowly. This may result in a failure to constrain the timing of subsequent action potentials, which is regulated by the decay of the IPSC (Bartos et al., 2007), and lead to a disruption of network synchrony due to each PV-cell responding and signaling more independently.

As lateral inhibition within the network would also be sparser due to DR PV-cells having fewer synaptic partners, this may result in a breakdown of the specificity of cortical activation (i.e., at a certain retinotopic location). Indeed, maturation of perisomatic inhibition in the hippocampus has been found to lead to sparser activation of cells in the dentate gyrus, creating
more parcellated processing streams (Yu et al., 2013). In sensory cortex, this may result in the response to a given TC input spreading further laterally than it normally would, without strong, lateral inhibition to contain the spread of excitation (Miller et al., 1989). As we have not investigated the architecture of DR PV-cell arbors and the distribution of cells of various types within them (Kisvárday et al., 1993; Budd & Kisvárday, 2001), it is unknown whether PV-cell targets may be lost at non-random locations within the arbor. Preferential loss at either central or peripheral arbor locations would alter the topography of lateral inhibition in distinct ways (Hensch & Stryker, 2004).

Additionally, as we have not examined the topography of gap junctions in the DR PV-cell network, it is unclear whether alterations in PV-PV convergence may have created a mismatch in the size and/or architecture of the electrical and chemical networks. In layer 2/3 of cat V1, immunostaining indicates that PV-cells form an average of 60 gap junctions with other nearby PV-cells, creating networks of connections that can be laterally traced in a “boundless manner” (Fukuda et al., 2006; also, Amitai et al., 2002). As there are no previous reports of gap junction development in interneuron networks occurring in an experience-dependent manner, it is likely that the electrical network maintains its size while the chemical network becomes sparser as we have shown (however, activity-dependent regulation of gap junctions has been reported; Haas & Landisman, 2012). Due to there being less PV-cell-mediated inhibition within the network, it is possible that static electrical connectivity may bias the PV-network further toward excitability (Amitai et al., 2002).
Impact of altered PV-PV connectivity on cortical excitability

PV-cells in both DR & PV:α1-KO mice may be thought of as receiving less inhibition overall, and less inhibition from other PV-cells specifically. What effect does this have on the excitability of these cells, and on the excitability of cortex overall? Normally, inhibition of PV-cells by other PV-cells is thought to cause disinhibition (Kisvárday et al., 1993), the relief from inhibition that would otherwise be received by the inhibited PV-cell’s synaptic targets. As a result, disinhibition has a facilitatory effect, enabling the greater excitability of these downstream cells. If the PV-cells in these models receive fewer and/or weaker inhibitory inputs from other PV-cells, we might expect that this relief from inhibition onto PV-cells results in the PV-cells themselves becoming more excitable. Greater excitability of PV-cells, in turn, would lead to greater inhibition of the downstream targets of these PV-cells, which would presumably result in a dampening of cortical activity due to the highly-divergent nature of PV-cell axons (Markram et al., 2004).

To test this hypothesis, we employed voltage-sensitive dye imaging (VSDI) of acute V1 slices (E.E. Diel & T.K. Hensch, unpublished). By incubating slices in a dye (Di-4-ANEPPS) that reads out subthreshold changes in membrane voltage, we reasoned that we would be able to observe disinhibition of the PV-cell-network as a change in the spatial spread of activity within cortex (Tanifuji et al., 1994). Following stimulation of the white matter below V1 (Durand et al., 2012), high-resolution video captured the propagation of activity with millisecond-scale resolution. Inspection of videos from light-reared (LR) slices revealed that activity traveled ‘on-beam’ towards the pial surface in the milliseconds after the pulse, as shown via the time-series in Figure 7.1.a (LR, top row). Further, activity in LR slices rapidly left infragranular layers, only spreading laterally once it had passed through L4.
We next examined V1 slices from DR mice, expecting to find that cortex was less excitable. Surprisingly, activity in DR slices spread further and persisted longer in infragranular layers, before moving superficially as a broad front (Figure 7.1.a, DR). A similar pattern was observed in PV:α1-KO mice, in which activity spread broadly from the stimulation site rather than being funneled narrowly through L4 (Figure 7.1.a, KO). We measured what appeared to be a differential lateral spread of activity between conditions by placing a series of horizontally-arrayed regions of interest within both L2/3 & L5 (Figure 7.1.b, left). Quantifying the change in fluorescence over the first 100ms following stimulation yielded an activation vs. position curve (Figure 7.1.b, right), which highlighted the greater activation of DR tissue at lateral positions. When we compared the level of activation between all conditions at the most lateral position (~1100µm from stimulation; Figure 7.1.c), we found that activity was indeed higher in both DR & PV:α1-KO slices.
Figure 7.1 = (a) Time series of voltage-sensitive dye imaging of coronal V1 slices. Within milliseconds of white matter stimulation (marked by asterisks), activity in light-reared slices rapidly leaves lower layers and proceeds superficially in a spatially-restricted manner (LR, top). In contrast, enhanced lateral spread and perseveration of activity is observed in lower layers, which then propagates upward as a broad front in dark-reared (DR, middle) and PV:α1-KO mice (KO, bottom). Scale bar, 400µm. WM, white matter. (b) (Left) Most lateral of a series of horizontally-arrayed regions of interest (box) spanning L5 of V1, used for quantification of integrated fluorescence change (first 100ms post-stimulation). (Middle) Change in fluorescence vs. time curves obtained from L5 regions of interest. (Right) Summary curves showing marked elevation of activity in DR slices over light-reared across loci lateral to stimulus site. (c) Integrated L5 fluorescence change at most lateral region of interest (unpaired t tests). (d) Model of “local” PV-basket synapses enriched for GABA$_A$R α1-subunits, while synapses on more distal targets utilize slower α3-subunits (“long-range”). A reduction of local PV-PV inhibition in DR & PV:α1-KO mice may strengthen longer-range PV-PV transmission, enabling further lateral spread of PYR cell activation.
Mechanisms of enhanced cortical excitability following alteration of PV-PV connectivity

Overall, the results of our VSDI experiment suggest that, contrary to our expectations, alteration of PV-PV connectivity in DR & PV:α1-KO mice results in a paradoxical increase in V1 excitability. This increase in excitability suggests that rather than being less inhibited in these models, PV-cells may actually be more inhibited. This would result in less inhibitory drive onto principal cells and other synaptic targets of PV-cells, rendering them more excitable. This effectively increased inhibition of PV-cells may result from some combination of the synaptic disruptions detailed earlier. For instance, in models of interneuron networks that contain FS-cells whose connections with one another are slow and weak, heterogeneity in the strength and timing of excitatory inputs to the network reveals a lack of robustness (Bartos et al., 2007). This may be the case in dark-reared and PV:α1-KO mice, with deprived connectivity and signaling in PV-cell networks resulting in neurons that fail to fire synchronously, and that instead inhibit each other at off times. This would in turn lead to less output from the PV-cell-network, facilitating the increased excitability of principal cells.

Recent results from hippocampus suggest that distance-dependent properties of PV-cell connections to their target cells may be involved. Specifically, sequential paired-recordings between presynaptic PV-cells and postsynaptic dentate gyrus granule cells revealed that inhibition was greater in pairs with smaller inter-somatic distances (Strüber et al., 2015). This was related to a distance-dependent fall-off in density of the presynaptic PV-cell’s axon, which resulted in cells further away receiving fewer inputs. Additionally, GABA_A R puncta contacted by the PV-cell axon exhibited a distance-dependent distribution of their alpha-subunit content, with proximal contacts containing alpha-1 subunits more frequently than distal contacts (Figure 7.1.d). Further, the levels of both alpha-2 & alpha-3 subunits did not change with distance. These
results suggest that lateral inhibition within individual PV-cell arbors may show differences in kinetics at different positions within the arbor, with signaling onto cells located more proximal to the soma being faster than onto those located more distal.

This paradigm has implications for our understanding of how inhibition may be altered in DR & PV:α1-KO mice. While PV-PV contacts are enriched with alpha-1 subunit-containing GABA\_Rs (Klausberger et al., 2002), it is possible that PV-cell contacts formed onto PV-cells may exhibit this axonal distance-dependent recruitment (Strüber et al., 2015) of non-alpha-1 subunits distally to some degree as well. If this is the case, down-regulation of alpha-1 expression in DR PV-cells, or its deletion in PV:α1-KO mice, may have a greater effect on PV-PV connections proximal to the soma, as these connections are more likely to be mediated by alpha-1 receptors. This may result in a re-balancing of inhibitory output from the PV-cell arbor, with an initial bias toward faster ‘local’ inhibition being mitigated and replaced with a more uniform output profile. In effect, such re-weighting may lead to a relative strengthening of ‘long-range’ inhibition onto more distal PV-cell targets, as these connections would more likely be mediated by non-alpha-1 subunits whose expression is unchanged (Figure 7.1.d, right). Due to this distal PV-PV inhibition, principal cells at lateral locations may be disinhibited, resulting in the greater excitability that we observed.

Overall, the importance of lateral inhibition in a species that lacks a clear columnar structure in its cortex remains unknown. In species such as cats that do have ocular dominance columns, basket cell arbors have been observed to span opposing regions of these columns (Buzas et al., 2001), suggesting that the lateral inhibition they provide may be involved in mediating competition in some way. Indeed, modulating the strength of intracortical inhibition pharmacologically can produce bidirectional changes in column width (Hensch & Stryker,
2004), indicating that activity in local cortical circuits can shape domains of competition. The lateral disinhibition that we have observed here – which would exist on the level of individual cells in vivo – may provide a spatial mechanism by which competition can play out in an acolumnar space. Interestingly, the arrangement of Ctip2+ pyramidal cells in layer 5 of mouse visual cortex does show a column-like periodicity (Maruoka et al., 2011), raising the possibility that PV-cell arbors may wire-up to these cells in yet-undiscovered ways to mediate competition on a finer scale.

**Attributions**
Erin E. Diel performed the VSDI experiments discussed in this chapter.
References


