

Characterization of VIP+ interneurons in the mouse whisker barrel cortex during development



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Abstract

GABAergic interneurons (INs) are thought to be important players in normal cortical circuit development, with a body of data highlighting the role of two major IN classes – parvalbumin and somatostatin-positive (PV+ and SST+) subtypes – in neonatal mouse sensory cortices (Marques-Smith et al., 2016; Takesian and Hensch, 2013; Tuncdemir et al., 2016). More recent evidence supports a developmental role for a third class of IN, defined by the expression of vasoactive intestinal peptide (VIP+ INs) (Batista-Brito et al., 2017; Goff and Goldberg, 2019; Mossner et al., 2017). This IN subtype has been shown to play a role in sensorimotor integration via preferential targeting of SST+ cells and consequent disinhibition of pyramidal cells in the mature cortex (e.g., Lee et al., 2013). However, it is unknown how and when VIP+ INs integrate into the local and long-range circuitry during early development.

To address this, I first investigated the integration of VIP+ cells into the local circuit in postnatal whisker barrel cortex (S1BF). Data show that, while VIP+ INs acquire mature electrophysiological properties and integrate in the local glutamatergic network over the first two postnatal weeks, they engage with other INs and pyramidal cells already in the first postnatal week.

Second, I employed a viral optogenetic strategy to test the emergence of long-range inputs from anterior-motor areas onto S1BF VIP+ cells. Data show that VIP+ INs start to be recruited already in the first postnatal week, but they are fully integrated in the long-range circuitry only at the end of the second postnatal week.

Finally, I have used the *VIP-Cre;Prox1^{c/c}* conditional knockout model (Miyoshi et al., 2015) to investigate whether genetic perturbation of VIP+ INs has an impact on synaptic integration and *in vivo* activity. Data suggest that conditional deletion of *Prox1* leads to reorganisation of the local but not long-range glutamatergic input, and increased activity upon whisker stimulation.

These findings define the emergence of the VIP+ circuitry and show their early influence in circuit maturation, further supporting the importance of IN signalling in cortical development.

Statement of Authorship

The text and figures presented in this thesis represent my own work. I collected the vast majority of the experimental data reported here, but received help for specific aspects. In particular, Sara Ratti – an MSc student in Neuroscience from the University of Trieste, supervised by me between February 2018 and June 2019 – helped with the collection of the *VIP-Cre;Ai32* dataset, performed immunofluorescence in P1-P3 *VIP-Cre;Ai9* animals; and optimized and performed the immunofluorescence protocol for PROX1 staining. Filippo Ghezzi developed the first draft of the analysis code for LSPS experiments, which I modified for glutamatergic mapping. He also introduced me to linear mixed effects analysis to compare the models described in Chapter 3. The *in vivo* experiments were performed in collaboration with Dr Liad J. Baruchin: he trained me to perform the experiments, developed the code used for their analysis and provided the visual representation of the methodology employed for the analysis of baseline activity. I independently performed surgery, electrode placement and recordings in 6 data points of the *in vivo* dataset here presented. For the entire *in vivo* dataset, I confirmed electrode placement through immunohistochemistry. I also performed spike sorting analysis, ROUT outliers analysis, data visualization and statistical analysis. Other data collection and analysis not specified here represent entirely my own work.

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Chapter 3 – Integration of VIP+ INs in the local circuitry

Table 3.1 Intrinsic properties of VIP+ INs across development. 81

Abbreviations

4-Aminopyridine	4-AP
Serotonin 3a Receptor	5HT3aR
Anterior Cingulate Cortex	ACC
Artificial cerebrospinal fluid	ACSF
Afterhyperpolarization	AHP
α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid	AMPA
Bacterial artificial chromosome	BAC
Basal ganglia	BG
Base pair	bp
Cajal-Retzius	C-R
Cholecystokinin	CCK
Caudal Ganglionic Eminence	CGE
Choline acetyltransferase	ChAT
Channelrhodopsin-2	ChR-2
Calretinin	CR
Current-source density	CSD
Differential interference contrast	DIC
Deoxyribonucleic acid	DNA
Days post injection	dpi
Embryonic day 12.5, Embryonic day 15.5..	E12.5, E15.5..
Enhanced Green Fluorescent Protein	EGFP
Excitatory post-synaptic current	EPSC
Excitatory post-synaptic potential	EPSP
Gamma-aminobutyric acid	GABA
Giant Depolarizing Potentials	GDPs
High divalent cation	HDC
Heterozygous	Het
Internal Capsule	IC
Insulin Growth Factor-1	IGF-1
Interneuron	IN
Inhibitory post-synaptic current	IPSC
Inhibitory post-synaptic potential	IPSP
Inter-stimulus interval	ISI

<i>In utero</i> electroporation	IUE
Knockout	KO
Layer 1, Layer 2/3...	L1, L2/3...
Local field potential	LFP
Lateral Ganglionic Eminence	LGE
Laser Scanning Photostimulation	LSPS
Long-term potentiation	LTP
Primary Motor Cortex	M1
Medial Ganglionic Eminence	MGE
Multi-unit activity	MUA
N-methyl-D-aspartate receptor	NMDAR
Neuropeptide Y	NPY
Optimal cutting temperature	OCT
Postnatal day 1, Postnatal day 2...	P1, P2...
Phosphate-buffered solution	PBS
Paraformaldehyde	PFA
Posterior medial nucleus/ posteromedial complex	POm
Parvalbumin	PV
Pyramidal cell	Pyr
Reelin	Re
Resting Membrane potential	RMP
Root mean square	rms
Ribonucleic acid sequencing	RNA-seq
Region of interest	ROI
Room temperature	RT
Somatosensory Barrel Field	S1BF
Sensory-Motor cortex related Thalamus	SMTh
Subplate	SP
Somatostatin	SST
Single unit	SU
Subventricular Zone	SVZ
Slow Wave Activity	SWA
Tetrodotoxin	TTX
Primary Visual Cortex	V1
Vasointestinal peptide	VIP
Vasointestinal peptide receptor 2	VIPR2
Ventral posteromedial nucleus	VPM
Ventricular Zone	VZ
Wild-type	WT
Yellow Fluorescent Protein	YFP

Chapter 1 – Introduction

The brain performs a variety of computations that allow animals to perceive the world around them and interact with it through appropriate behaviours. Systems neuroscience can be defined as understanding the cellular and circuit basics of these behaviours. The adult brain is hugely complex: in humans, it contains 86.1 billion neurons (Azevedo et al., 2009; Herculano-Houzel, 2012), which are estimated to form between several hundred trillion to over a quadrillion synaptic connections (Silbereis et al., 2016). In the mouse brain, neurons number approximately 70 million (Herculano-Houzel et al., 2006) and form $\sim 10^{11}$ synapses (Zhu et al., 2018). To untangle the complexity of neural networks underlying behaviour, a developmental approach to system neuroscience is needed. Understanding how neuronal diversity appears and how these cells start to integrate in the network, sculpting emerging perception and behaviours, is key not only to provide insights into neurological or psychiatric disorders (Connor, 2018; Ilieva et al., 2018; Silbereis et al., 2016; Vadodaria et al., 2018), but also into adult brain function.

For decades, sensorimotor systems have been studied to map input-output relationships from perception to behaviour (see for a recent review

Petersen, 2019). We have made progress mapping the local and long-range circuits linking perception and motor output, but are still far from grasping its entire complexity. In recent years, there has been a growing interest in the field in cortical vasoactive intestinal peptide positive (VIP+) interneurons (INs), a subpopulation of INs that occupy a very peculiar node in the cortical sensorimotor circuit. They are placed at the interface between local and long-range modulation and integrate cortical and subcortical inputs (Audette et al., 2018; Fu et al., 2014; Hajós et al., 1997; Ji et al., 2016; Lee et al., 2013; Staiger et al., 1996; Wall et al., 2016; Williams and Holtmaat, 2019). Their function appears to be conserved across brain areas and has been linked to top-down and bottom-up modulation, as well as behavioural state transitions (Audette et al., 2018; Batista-Brito et al., 2017; Fu et al., 2014; Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013; Turi et al., 2019; Williams and Holtmaat, 2019; Zhang et al., 2014). However, the details of the circuitry in which VIP+ INs are involved are still not fully understood. In particular, it is unknown how and when VIP+ INs integrate in the cortical network to become a crucial node in the sensory-motor circuitry. This thesis aims to uncover VIP+ INs development and circuit integration in the local and long-range sensory-motor network, with the final goal to unravel their function in emergent perception and sensory processing.

In the following section, after a brief description of adult cortical circuits, the present understanding of the biological mechanisms involved in cortical development and circuit formation will be reviewed, with a focus on the role of INs in this process. Subsequently, our current knowledge regarding VIP+ INs connectivity and function will be summarised. Finally, the three main aims of this thesis will be highlighted: (1) to unravel how VIP+ cells integrate into the local

circuit; (2) to investigate how VIP+ cells are engaged by long-range connections; (3) to test the consequences of genetic impairment of VIP+ INs at circuit and network levels. As my entire work is based on the use of mice as model organisms, all the literature discussed refers to rodent studies unless otherwise stated.

1.1 – Cortical circuits in the adult brain

From an anatomical perspective, the brain is composed of cerebrum, cerebellum and brainstem. The surface of the largest part of the brain, the cerebrum, is called ‘cerebral cortex’ and is involved in the most complex brain functions, such as attention, memory and language (Kandel et al., 2013).

Although the cortex is highly specialised, anatomical and connectivity studies allowed to identify the basic architecture common to all primary sensory cortices, the “canonical cortical microcircuit”, which is based on the laminar organisation of this structure (Armstrong-James et al., 1992; Douglas and Martin, 2007; Lefort et al., 2009; Sheperd and Grillner; 2018). According to this model, thalamic input drives activity to layer (L)4 cells, that sequentially activate supragranular layers, which in turn activate infragranular ones. Supragranular layers project to other areas of the cortex, while infragranular ones project to the thalamus, the midbrain and the spinal cord. This layer-specific connectivity allows information to be sent to other cortical and subcortical structures, respectively.

More recent analysis of cortical microcircuits has revealed a more intricate network of connections beyond the canonical ones, redefining to certain extent the function of cortical layers, thus their differential contribution to cortical computation and perception (Adesnik and Naka, 2018; Constantinople and Bruno, 2013; Pluta et al., 2015; Quiquempoix et al., 2018).

From a cellular perspective, the cortex is characterised by two broad neuronal classes: excitatory pyramidal cells and gamma-aminobutyric acid (GABA) expressing inhibitory INs, representing respectively 70-80% and 20-30% of the entire neuronal population (Markram et al., 2004). GABAergic INs can be further divided into three main populations, based on the selective expression of molecular markers: the calcium-binding protein parvalbumin (PV+), the neuropeptide somatostatin (SST+) and the serotonin ionotropic receptor 5HT3a (5HT3aR+) (Rudy et al., 2011) (Figure 1.1). PV+ INs represent 40% of the total population, SST+ cells 30% and the 5HT3aR+ subtype the remaining 30% (Rudy et al., 2011). 5HT3aR+ INs can be further divided into two groups, the vasointestinal peptide positive (VIP+) and the reelin positive (Re+) subpopulations (Rudy et al., 2011) (Figure 1.1).

1.2 – The development of cortical circuits

Cortical development starts embryonically and continues postnatally. Neurogenesis, neuronal migration and synaptogenesis are the three main events that overlap during the generation of the cerebral cortex (see for review: Cadwell et al., 2019; Jiang and Nardelli, 2016). One of the most fascinating

aspects of these processes is the incredible diversity generated throughout – diversity in cell types, connectivity patterns, functions, and anatomical structures.

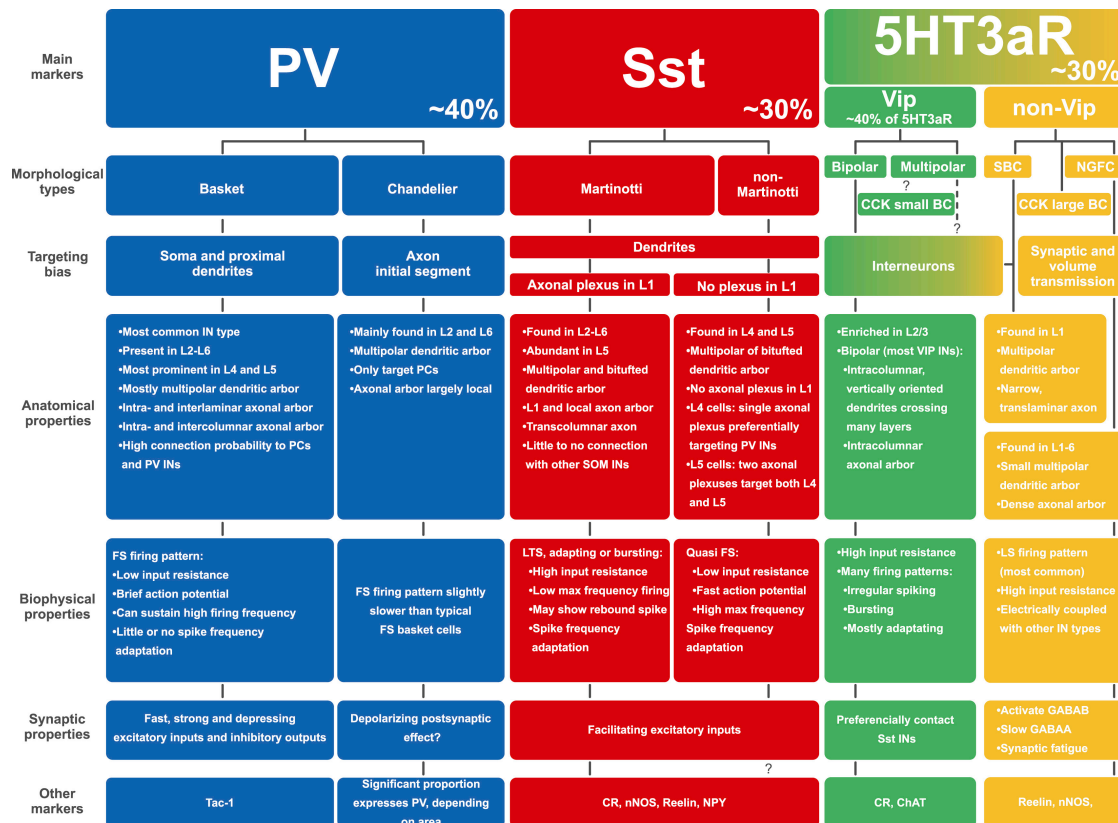


Figure 1.1 Diversity of cortical GABAergic INs. Schematics of the anatomical, electrophysiological and molecular features of parvalbumin (PV, blue), somatostatin (Sst, red), and the ionotropic serotonin receptor 5HT3aR (5HT3aR, green-yellow) positive INs. From Tremblay et al (2016), with permission (see Appendix 4).

Diversity between excitatory and inhibitory neurons lies not only in their function, but also in their origin: cortical pyramidal cells originate locally from progenitor cells in germinative zones of the dorsal telencephalon (pallium) called the ventricular and subventricular zones (VZ and SVZ respectively) (Hevner, 2006; Rakic and Sidman, 1968; Reillo et al., 2011; Shitamukai et al., 2011; Tan et al., 1998). INs in contrast originate from subpallial structures called

the ganglionic eminences, and need to migrate longer tangentially to reach the cortex (Faux et al., 2012; Harwell et al., 2015; Lim et al., 2018a; Mayer et al., 2015; Tan et al., 1998). The majority of PV+ and SST+ cells originate from the medial ganglionic eminence (MGE), while 5HT3aR+ INs derive preferentially from the caudal ganglionic eminence (CGE) (Butt et al., 2005; Miyoshi et al., 2010).

1.3 – The role of INs in wiring the brain

INs are key players in brain development. GABA – which is thought to be depolarising in the immature brain (Ben-Ari, 2002; Ben-Ari et al., 2007, 2012) – has been shown to regulate proliferation of neuronal progenitors, neuronal migration and maturation, and *de novo* synaptogenesis, exerting its action already during the embryonic stages (Behar et al., 2000; Bolteus and Bordey, 2004; Cellot and Cherubini, 2013; LoTurco et al., 1995; Manent et al., 2005; Oh et al., 2016; Owens and Kriegstein, 2002; Wang and Kriegstein, 2009). Transient circuits between excitatory and GABAergic cells represent the earliest connections established in the brain and, at least in part, the substrate for synchronized network activity (Cosgrove and Maccaferri, 2012; Garaschuk et al., 2000; Hanganu et al., 2009; Kilb and Luhmann, 2001; Luhmann et al., 2014; Myakhar et al., 2011; Radnikow et al., 2002; Soda et al., 2003).

1.3.1 – The earliest cortical circuits

The first-born neuronal populations are Cajal-Retzius (C-R) and subplate (SP) cells, respectively located in the outermost and the innermost lamina of the developing cortex (namely, the marginal zone and the subplate, respectively). C-R cells are glutamatergic cells secreting extracellular signals, including reelin, to promote cortical lamination (Frotscher, 1998). GABAergic cells innervate C-R cells, providing excitatory inputs as well as apoptotic signalling to trigger their disappearance around the second postnatal week (Blanquie et al., 2017a; Kilb and Luhmann, 2001; Mienville, 1998; Radnikow et al., 2002; Soda et al., 2003).

The subplate was originally defined as a synaptic zone containing various cell types, including GABAergic and glutamatergic neurons (Kostovic and Rakic, 1980). GABAergic cells, located either in the subplate or in the cortical plate, innervate SP cells and provide them with depolarizing signals (Hanganu et al., 2002, 2009). In contrast to the C-R population, not all the SP cells die during development, but a remnant of this structure, L6b, forms later in life (Hoerder-Suabedissen et al., 2018; Marx et al., 2017; Price et al., 1997). SP cells are located in a strategic node of the developing circuitry: they receive thalamic inputs and project to the developing L4 (Allendoerfer and Shatz, 1994; Kanold and Luhmann, 2010; Piñon et al., 2009; Zhao et al., 2009). It has been shown that SP cells are responsible for relaying early oscillatory activity to the developing cortex, enabling thalamocortical and GABAergic IN maturation (Kanold, 2009; Kanold and Luhmann, 2010; Kanold et al., 2019; Tolner et al., 2012).

1.3.2 – INs and early oscillatory activity

Early oscillatory activity is key for the maturation of cortical circuits as it influences synaptogenesis and synaptic plasticity (Khazipov and Luhmann, 2006). IN contribution in early oscillations has been demonstrated both *in vivo* and *in vitro*.

GABA-driven giant depolarizing potentials (GDP) synchronising local neuronal assemblies have been observed in the somatosensory cortex already at the end of the first postnatal week (Allène et al., 2008). Wang et al. (2019) recently demonstrated that early-generated L5 INs (putative SST+) are able to drive GDPs in the postnatal somatosensory cortex. Minlebaev et al. (2011) showed that oscillatory activity synchronising the developing thalamocortical network progressively required INs for its generation. Similarly, Bitzenhofer et al. (2015) showed an interplay between pyramidal and GABAergic network to promote oscillatory activity contributing to the maturation of prefrontal-hippocampal communication.

In immature hippocampal slices it has been shown that widely connected GABAergic cells are able to shape network synchrony during development by generating GDPs. For this reason, they are defined as ‘operational hub cells’ (Ben-Ari et al., 2007; Bonifazi et al., 2009; Cossart, 2014; Picardo et al., 2011). Operational hub cells represent a diverse class of GABAergic neurons that can be divided into two categories, based on their anatomical connectivity (Cossart, 2014). Basket-like, perisomatic-targeting cells are defined as ‘provincial hubs’, as they show a dense local arborisation pattern. Although their origin and adult fate is still poorly understood (Bonifazi et al., 2009; Cossart, 2014), recent evidence

suggests that these cells are MGE-derived (Wester and McBain, 2016). Contrarily, 'connector hubs' link the hippocampus to distant structures via long-range projecting axons (Bonifazi et al., 2009; Picardo et al., 2011). These are early born GABAergic cells, which in significant proportion express SST and continue into adulthood as long-range projecting GABAergic neurons (Picardo et al., 2011; Villette et al., 2016). They are also able to mediate fast oscillatory activity by synchronising other interneurons (Quilichini et al., 2012).

1.3.3 – INs and critical period plasticity

Further evidence obtained from other anatomical structures supports the role of INs in cortical circuits development. Many studies focused on critical periods in sensory systems, temporal windows in early postnatal life characterised by reshaping of neuronal circuits under the control of sensory-driven, activity-dependent mechanisms (Hensch, 2005).

The role of MGE-derived populations has been investigated across sensory modalities: in the developing somatosensory and auditory cortices, it has been shown that MGE-derived INs integrate in the local glutamatergic network as early as the first postnatal week, forming both translaminar and local circuits which evolve over time in a subtype- and layer-specific fashion (Anastasiades et al., 2016; Deng et al., 2017).

The contribution of PV+ cells to mediate critical period plasticity has been extensively studied in visual cortex (Hensch, 2005; Takesian and Hensch, 2013), and further studies in auditory and barrel cortex have confirmed their role in

mediating plasticity across sensory modalities (Chittajallu and Isaac, 2010; Daw et al., 2007; Gainey et al., 2018; Vickers et al., 2018).

Two recent pieces of work demonstrated the role of SST+ INs in the developing somatosensory barrel cortex (S1BF): it has been shown that infragranular SST+ Martinotti cells receive thalamic innervation in the first postnatal week and mediate the establishment of thalamic connections to PV+ and L4 spiny stellate cells (Marques-Smith et al., 2016; Tuncdemir et al., 2016) (Figure 1.2).

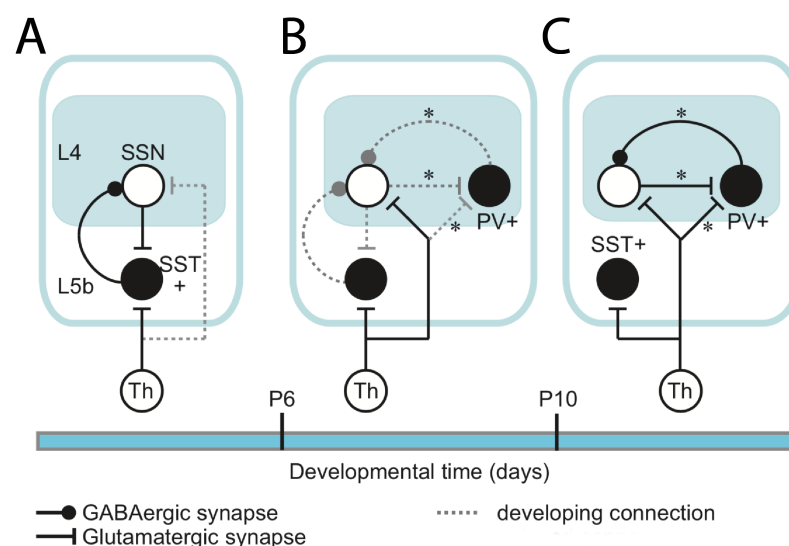


Figure 1.2 Transient circuits involving infragranular SST+ INs in the early postnatal S1BF. Diagram of the circuit identified by Marques-Smith et al. (2016). Black circle = GABAergic IN; filled circle ending = GABAergic synapse; white circle = glutamatergic neuron; flat line ending = glutamatergic synapse; grey dotted line connector = connection undergoing remodelling. L=layer; SSN, spiny stellate neuron; Th= VPM nucleus. * = connections previously reported in the literature. At the beginning of the first postnatal week, thalamo-recipient L5b SST+ INs and L4 SSNs form reciprocal connections (A), which act as a scaffold for the developing thalamic input onto L4 cells. L5b-L4 connections gradually disappear during the second postnatal week (B) until thalamic inputs onto L4 SSNs and PV+ INs are established, and PV+ INs-mediated local inhibition onto SSNs emerges (C). Adapted from Marques-Smith et al. (2016), Creative Commons Attribution License (CC BY).

In the last years, interest has grown towards understanding the function of CGE-derived 5HT3aR+ INs in circuit formation. In the somatosensory cortex, rabies virus-based monosynaptic retrograde tracing experiments and optogenetic studies showed that L1 Re+ INs receive cortical and thalamic inputs during the first postnatal week (De Marco García et al., 2015). Further tracing experiments showed that these connections are dynamically remodelled during the second postnatal week: cortical inputs to L1 Re+ INs increase, while thalamic ones decrease (Che et al., 2018). However, the functionality of these connections during the second postnatal week has not been confirmed by optogenetic studies.

Sensory inputs were shown to have a role in Re+ INs development and function: sensory deprivation by chronic whisker plucking leads to a reduced synchronization of spontaneous L1 Re+ INs activity and affects their morphological development (Che et al., 2018; De Marco García et al., 2015). In parallel, whisker stimulation elicits L1 Re+ INs activation, already in the first postnatal week (Che et al., 2018). These findings were also confirmed in the auditory cortex: L1 5HT3aR+ INs receive direct connections from the thalamus and chemogenetic silencing of 5HT3aR+ cells prevents tonotopic plasticity (Takesian et al., 2018). Interestingly, this is specific to the Re+ population, as chemogenetic silencing of VIP+ INs does not lead to changes in tonotopic plasticity (Takesian et al., 2018). Before discussing the emerging role of VIP+ INs in developing circuits (Batista-Brito et al., 2017; Goff and Goldberg, 2019; Mossner et al., 2017), the next section will focus on their development and maturation.

1.4 – VIP+ INs: from the embryo to adulthood

Starting at embryonic day (E) 12.5 (Miyoshi et al., 2010), the CGE gives rise to a heterogeneous population of INs sharing one common feature, the expression of the 5HT3a receptor (Lee et al., 2010). 5HT3aR+ INs can be further delineated into specific subtypes by the expression two main markers: VIP and Re, which represent 30-40% and 25-50% of this population respectively (Lee et al., 2010; Rudy et al., 2011).

The combinatorial expression of VIP and Re with the peptide cholecystokinin (CCK), the calcium binding protein calretinin (CR) and the neuropeptide Y (NPY) increases 5HT3aR+ IN heterogeneity, which is reflected (at least partially) at a morphological and electrophysiological level (Lee et al., 2010).

Re+ cells are mostly late-spiking cells and a considerable proportion of them have small soma, short ramifying dendrites and dense local axonal plexus. In contrast, most irregular spiking cells have bipolar/tripolar morphology, axons projecting to deep layers and are positive for VIP and CR (Butt et al., 2005; Lee et al., 2010; Miyoshi et al., 2010; Vucurovic et al., 2010).

Temporal origin of CGE-INs predicts their physiological features: at E13.5, the CGE produces mainly dense plexus (putative Re+) and bitufted no-rebound spike regular spiking cells; while at E15.5 it generates double bouquet/bitufted rapidly adapting regular-spiking INs. Bipolar, VIP+ regular-spiking cells are instead produced throughout (Butt et al., 2005; Miyoshi et al., 2010), with a peak at E14.5 (Miyoshi et al., 2010).

After birth, CGE-derived INs tangentially migrate to the cortex, above and below the cortical plate (the marginal zone and the ventricular zone, respectively), to then radially sort themselves within the different cortical layers by postnatal day (P)7 (Miyoshi and Fishell, 2011). Birth date does not control the final location of CGE-INs, unlike the MGE-counterpart (Miyoshi and Fishell, 2011; Miyoshi et al., 2010). All CGE-derived cells preferentially occupy supragranular layers. However, recent *in utero* electroporation (IUE) experiments suggested that laminar fate is instead dependent on progenitor domains. Torigoe et al. (2016) hypothesised that Re⁺ INs (which are mainly located in L1) derive from the most caudal part of the CGE, while VIP⁺ INs derive from the most rostral compartment, adjacent to the lateral ganglionic eminence (LGE). While this hypothesis is compelling, it has not been directly tested, as the authors inferred the target site of IUE retrospectively, by observing the INs labelled in cortical and subcortical structures.

Fate-mapping experiments of CGE-INs shed some light on VIP⁺ INs; however, the creation of a Cre-driver line for genetic targeting of VIP⁺ INs allowed a targeted and more meticulous characterisation of their morphological and electrophysiological features (Goff and Goldberg, 2019; Prönneke et al., 2015, 2018; Taniguchi et al., 2011). The *VIP-Cre* line revealed that at P0 VIP⁺ INs are migrating in the white matter, while at P2 they are radially migrating towards the pia surface (Taniguchi et al., 2011). VIP⁺ INs are ~13% of the GABAergic cells in the adult barrel cortex: 60% of this population is located in L2/3, while the other 40% is scattered across the other layers (Prönneke et al., 2015).

Several morphological and anatomical properties distinguish L2/3 from L4-6 VIP+ INs. The dendrites of L2/3 VIP+ cells are restricted to upper layers, while their axons reach all layers of the cortex; on the contrary, L4-6 VIP+ INs spread their dendrites throughout the cortex, while their axons are mainly targeting infragranular layers (Prönneke et al., 2015). The axonal terminal boutons of L2/3 VIP+ INs target mostly L2/3 and L5a; while the boutons of deep-layer VIP+ cells are localised mainly in L5b and 6, suggesting different input-output domains for these two subpopulations (Prönneke et al., 2015). Some differences have been also observed at the soma level: while VIP+ INs could be multipolar, bipolar or modified bipolar across the depth of the cortex, Prönneke et al. (2015) found bitufted cells only in L4-6.

The differences between these populations are also reflected in their electrophysiology: L2/3 cells are characterized by more depolarized resting membrane potential than L4-6; have smaller inward rectification indexes (i.e. a measure of their ability to stabilize resting membrane potentials in more hyperpolarized states) and a smaller after-hyperpolarization (Prönneke et al., 2015). Moreover, continuous adapting, irregular spiking and high threshold bursting non-adapting cells are found in all cortical layers, while bursting cells are only present in supragranular layers of the adult cortex (Prönneke et al., 2015).

In terms of molecular markers, it has been reported that VIP+ INs can express calretinin (CR), choline acetyltransferase (ChAT) (Engelhardt et al., 2007; Porter et al., 1998), neuropeptide Y (NPY) (Xu et al., 2010) and cholecystokinin (CCK) (Kubota and Kawaguchi, 1997).

Efforts to tackle the variety of firing patterns, morphologies and molecular markers showed that there is no clear segregation of these properties into different cell types (Tremblay et al., 2016). For example, bipolar VIP+ cells are typically associated with an irregular spiking profile, and can be classified in two main subtypes based on duration of initial burst and CR or ChAT expression (Cauli et al., 2014; Porter et al., 1998). In contrast, small VIP+ multipolar basket cells often express CCK, although VIP+ CCK+ bipolar cells can also be found (Kubota and Kawaguchi, 1997).

Finally, several factors increase the complexity of VIP+ INs classification: the recent discovery that serotonergic and cholinergic neuromodulation leads to a burst-tonic switch in bursting VIP+ INs (Prönnke et al., 2018); the lack of a standardized nomenclature to describe IN intrinsic electrophysiological properties (see however PING et al., 2008); and the growing characterization of new molecular markers using single-cell sequencing (Tasic et al., 2016, 2018).

1.5 – Interneuron development: tracking the emergence of diversity

Despite recent progress in combinatorial genetics and single-cell sequencing, addressing how IN diversity is established during development is still a challenge (Lim et al., 2018; Mayer et al., 2018; Mi et al., 2018; Miyoshi, 2019; Wamsley and Fishell, 2017). While it is recognised that both intrinsic and environmental mechanisms have a role in this process, their relative influence is still unclear. There are two main hypotheses: (1) the ‘progenitor specification model’, which posits that IN identity is already established at the progenitor

stage, and (2) the ‘progressive specification model’, which suggests that, while INs are restricted into a general class at birth, they acquire a definitive identity later in development through interaction with the environment (Wamsley and Fishell, 2017). MGE-derived INs have been extensively studied in this regard (see Lim et al., 2018 for a recent review). In the next section, our current understanding of the origin and specification of CGE- derived INs will be reviewed.

1.5.1 – Molecular Determinants of CGE-INs diversity

Fate mapping, *in situ* hybridisation and genetic ablation experiments identified several transcription factors involved in the CGE genetic programme, such as *Dlx1/2*, *Mash-1*, *COUP-TFI* and *COUP-TFII* (See Table 1.1- Genetic Factors).

Notably, while the majority of the studies summarised in Table 1.1 involves cell autonomous mechanisms, it has been recently demonstrated that specification of CGE-derived subtypes is also dependent on non-cell autonomous mechanisms. For example, modifying pyramidal neuron identity by genetically ablating the transcription factor *Satb2*, thereby converting intratelencephalic pyramidal neurons to pyramidal tract ones, affects CGE-INs lamination, density and connectivity (Wester et al., 2019). This is in line with what previously reported for MGE INs following alteration of pyramidal neuron identity by deletion of the transcription factor *Fezf2* (Lodato et al., 2011b).

Table 1.1 Regulation of CGE interneuron development (with a specific focus on VIP+ INs) – Genetic Factors¹

Genetic Factors			
Manipulation	Outcome	Genes	References
<i>Dlx1</i> knockout	Preferential reduction of CR+ bipolar cells and SST+ cells. PV+ INs are unaffected	<i>Dlx1</i>	(Cobos et al., 2005)
siRNA-mediated <i>COUP-TFII</i> knockdown in transplanted cell	Inhibition of caudal migration of CGE-derived cells	<i>COUP-TFII</i>	(Kanatani et al., 2008)
<i>COUP-TFI</i> knockout in subventricular precursors and postmitotic cells (using <i>Dlx5-6 Cre</i> line)	Decrease of VIP+ and CR+ INs, compensated by increased PV+ INs; higher resistance to pharmacologically induced seizure	<i>COUP-TFI</i>	(Lodato et al., 2011a)
<i>Mash1BAC-CreER</i> driver to selectively fate map CGE-derived cortical INs	- CGE INs: ~30% of all cortical interneurons, at least 9 subtypes. Mostly Re+ or VIP+ - Initiation (E12.5) and peak production (E16.5) of CGE INs occurs 3 days later than MGE INs - CGE INs do not integrate into the cortex in an inside-out manner - CGE INs preferentially (75%) occupy superficial cortical layers independently of birthdate	<i>Mash1</i>	(Miyoshi et al., 2010)
In situ hybridization to identify combinatorial transcription codes and define a continuum of subpallial progenitor domains	- CGE identified as 'caudal intermediate subpallium' and 'caudal subpallium' - CGE expression of <i>Dlx2</i>, <i>Pax6</i>, <i>Gsh2</i>, <i>Er81</i>, <i>COUP-TFI</i> and <i>Lhx2</i>	<i>Dlx2</i> , <i>Pax6</i> , <i>Gsh2</i> , <i>Er81</i> , <i>COUP-TFI</i> and <i>Lhx2</i>	(Flames et al., 2007)
Genetic combinatorial strategies to identify <i>Prox1</i> + precursors and specifically delete this gene in them	<i>Prox1</i> promotes migration, differentiation, circuit integration, and maintenance of CGE INs	<i>Prox1</i>	(Miyoshi et al., 2015; Rubin and Kessaris, 2013)
In situ hybridization in <i>Dlx1/2</i> or <i>Mash1</i> knock out animals to reveal genetic programmes underlying CGE development	<i>Dlx1/2</i> controls the expression of: ↑ <i>Arx</i>, <i>Brn4</i>, <i>Dlx1,2,5,6</i>, <i>ESRG</i>, <i>FoxP1</i>, <i>FoxP4</i>, <i>Meis1</i>, <i>Meis2</i>, <i>Oct6 (POU3F1)</i>, <i>Pbx1</i>, <i>Pbx3</i>, <i>Prox1</i>, <i>Six3</i>, <i>Sox4</i>, <i>Sox11</i>, <i>Sp8</i>, <i>Tle4</i>, <i>Tshz1</i>, and <i>Vax1</i>; <i>CXCR4</i>, <i>CXCR7 (RDC1)</i>, <i>ErbB4</i>, <i>Gad67 (Gad1)</i>, <i>Gucy1a3</i>, <i>Robo2</i>, <i>Shb</i>, <i>Tiam2</i>, and <i>Thbs</i>. ↓ antisense-<i>Dlx6</i>, <i>COUP-TFI (NR2F1)</i>, <i>Ctip2 (Bcl11b)</i>, <i>Gbx1</i>, <i>Gsh1</i>, <i>Gsh2</i>, <i>Id2</i>, <i>Ikaros</i>, <i>Islet1</i>, <i>Lmo1</i>, <i>Mash1</i>, <i>Olig2</i>, <i>Otp</i>, and <i>Sall3</i>; <i>Dact1</i> and <i>PKR1</i>	<i>Dlx1&2</i> , <i>Mash1</i>	(Long et al., 2009)
Loss of <i>Gsx1</i> and <i>Gsx2</i> function in <i>Dlx1/2</i> mutants	Rescue of the <i>Dlx1/2</i> mutants phenotype by increased expression of <i>Ascl1</i> and <i>Hes5</i> (Notch signalling mediators), and <i>Olig2</i> (oligodendrogenesis mediator)	<i>Gsx1</i> and <i>Gsx2</i>	(Wang et al., 2013)
<i>NPAS1</i> , <i>NPAS3</i> knockout and <i>NPAS1/3</i> double knockout	<i>NPAS1</i> ^{-/-} mutants have increased proliferation, ERK signaling, and expression of <i>Arx</i> . VIP+ INs increase in <i>NPAS1</i> ^{-/-} mice but decrease in <i>NPAS3</i> ^{-/-} and <i>NPAS1/3</i> ^{-/-}	<i>NPAS1</i> , <i>NPAS3</i> , <i>Arx</i>	(Stanco et al., 2014)
<i>Arx</i> conditional knockout in <i>Dlx5/6Cre</i> animals	Changes in number and distribution of CR+ cells	<i>Arx</i>	(Marsh et al., 2009)
Immunohistochemistry in <i>Dlx5/6</i> GFP animals	<i>Sp8</i> is expressed by a subpopulation of dCGE INs	<i>Sp8</i>	(Ma et al., 2012)
<i>Satb2</i> cKO mouse in pyramidal neurons	Affected lamination of VIP+ INs and of Re+ cell density, disrupted synaptic connectivity between pyramidal cells and CGE-INs in superficial cortex	Non cell autonomous mechanism	(Wester et al., 2019)

¹ Note: CR+ bipolar cells are considered putative VIP+ INs

For simplicity, all the new studies on single cell RNA-seq were omitted (i.e. Frazer et al., 2017, Paul et al., 2017, Mayer et al., 2018, Mi et al., 2018)

↑: high expression levels; ↓: low expression levels.

Recent single cell RNA-sequencing (RNA-seq) experiments allowed further investigation of transcriptional profiles in manually dissected CGE and in genetically defined neuronal populations. Mi et al. (2018) sequenced manually dissected CGE-derived cells and found that the CGE is able to generate neurons with a molecular profile typical of SST+ cells, corroborating the hypothesis that the CGE contains distinct progenitor domains (Flames et al., 2007). Similarly, Mayer et al. (2018) reported heterogeneity in manually dissected early CGE progenitors, that have similar transcriptional patterns to those located in other ganglionic eminences, suggesting a common set of progenitor states.

RNA-seq of genetically defined neuronal populations represents a complementary approach to studying IN transcriptomics. Using a combinatorial genetic approach based on Cre and Flp recombinase driver lines to identify IN subpopulations, Paul et al. (2017) confirmed that INs retain a 'transcription resume' of their developmental history, contributing to the maintenance of their phenotypes. By analysing the transcriptome of early postnatal 5HT3aR+ INs, Frazer et al. (2017) identified different IN subtypes according to their expression profiles. These include a Re+/COUP-TFII+ group, preferentially distributed in L1, and a Re-negative/COUP-TFII-negative group, whose transcriptome resembles that of VIP+ INs identified by previous work (Tasic et al., 2016; Zeisel et al., 2015). A much more detailed understanding of the transcriptional factors that are involved in VIP+ INs specification and identity maintenance emerged from these studies.

1.5.2 – *Prox1* (*Prospero Homeobox 1*)

Among all the transcription factors, PROX1 is of special interest for VIP+ INs as it has been demonstrated to affect their maturation and synaptic development (Miyoshi et al., 2015). *Prox1* is a homeobox gene homolog to the *Drosophila melanogaster* gene *prospero*. In the mouse, it is expressed in the developing central nervous system (CNS), eye lens, liver, pancreas, cochlea, and in the endothelial cells that will generate the lymphatic vasculature (Bermingham-McDonogh et al., 2006; Burke and Oliver, 2002; Oliver et al., 1993; Wang et al., 2005; Wigle and Oliver, 1999; Wigle et al., 1999). During adulthood, *Prox1* is expressed by the dentate gyrus of the hippocampus and the cerebellum (Lavado and Oliver, 2007; Pleasure et al., 2000).

Early *in situ* hybridization and immunohistochemistry analyses showed *Prox1* expression in the ganglionic eminences already at E12.5, and its co-expression with *Mash-1*, a basic helix-loop-helix transcription factor expressed by the CGE (Lavado and Oliver, 2007; Oliver et al., 1993; Torii et al., 1999).

While Lavado and Oliver (2007) reported the presence of PROX1 in postnatal neurons in the outer layer of the cortex, only later PROX1 was identified as a marker for CGE interneurons (Rubin and Kessaris, 2013). Rubin and Kessaris (2013) also confirmed PROX1 postnatal expression by a variety of IN subtypes, including calretinin (CR)-, neuropeptide Y (NPY)- and Re-positive cells, as well as 100% of the VIP-expressing population.

This figure has been reproduced by Miyoshi et al. (2015), who selectively depleted *Prox1* in VIP+ INs and showed a comparable density and layer distribution of VIP+ INs at P7, but a layer-specific decrease of L2/3 VIP+ cells at

P21. Moreover, they reported a 30% decrease of VIP/CR co-expression at P7, that further decreased at P21. In addition, some morphological defects on the bipolar VIP+ cells were reported in the *Prox1* conditional knockout (cKO) model, suggesting that altering *Prox1* expression could be a reliable model to affect VIP+ INs development. In Chapter 5, I employ this model to further study the effect of VIP+ INs impairment on development in S1BF.

1.5.3 – The role of activity in CGE-INs specification

Manipulation of activity in CGE-derived cells has been investigated in regards to migration, maturation, cell death and synaptic connectivity (Azim et al., 2009; Batista-Brito et al., 2009; De Marco García et al., 2011, 2015; Frazer et al., 2015; Karayannis et al., 2012; Lodato et al., 2011a; Mardinly et al., 2016; Murthy et al., 2014; Priya et al., 2018). Current evidence suggests that altering neuronal activity has subtype-specific effects in CGE-INs, with Re+ cells appearing to be generally more sensitive to activity perturbations, such as chronic whisker plucking or hyperpolarisation (See Table 1.2 – Activity).

There is contrasting evidence about the role of activity on CGE-IN migration. De Marco García et al. (2015) showed that suppression of neuronal excitability through overexpression of the inward rectifying potassium channel Kir2.1 (promoting neuronal hyperpolarisation (Doupnik et al., 1995)) leads to altered migration of CR+ and Re+ cells. However, another set of activity manipulations targeting the blockade of thalamic inputs either via chronic whisker plucking or abolition of activity from N-methyl-D-aspartate receptors (NMDARs, specifically employed by thalamic synapses) in *Dlx6*-positive INs did not affect migration in CGE-derived cells (De Marco García et al., 2011, 2015).

Table 1.2 Regulation of CGE interneuron development (with a specific focus on VIP+ INs) – Activity

Activity and migration			
Manipulation	Outcome	Genes	References
Overexpression of <i>Kir2.1</i> under the control of the <i>Dlx5/6</i> enhancer element from P0 (<i>electroporation at E15.5</i>)	ectopic settling of CR+ and Re+ cells within the cortical lamina. Rescued by <i>Elmo1</i> overexpression	<i>Elmo1</i>	(De Marco García et al., 2011)
Whisker plucking/NMDAR conditional knock out in <i>Dlx5/6 Cre</i> animals	No migration defects	<i>Grin1</i>	(De Marco García et al., 2015)
Genetic loss-of-function approaches and in vivo grafts	5HT3aR cell autonomously required for migration and proper positioning of Re+ INs	<i>Htr3a</i>	(Murthy et al., 2014)
<i>SERT</i> knock out mice or exposure to SERT inhibitor fluoxetine	Altered cortical laminar distribution of CGE-derived INs, especially VIP+ INs		(Frazer et al., 2015)
Activity and maturation			
Manipulation	Outcome	Genes	References
Overexpression of <i>Kir2.1</i> under the control of the <i>Dlx5/6</i> enhancer element from P3	Deficit in morphogenesis of Re+ and CR+ cells (including putative VIP+ CR+ bipolar cells), but not of VIP+ multipolar cells * No change in amplitude or frequency, but slower kinetics of sEPSCs in Re+ cells (not in CR+ or VIP+ cells).		(De Marco García et al., 2011; Karayannis et al., 2012)
Kynurenic acid at P3 (NMDA and AMPA/kainate receptor blocker)	Deficit in morphogenesis of Re+ and CR+ cells (including putative VIP+ CR+ bipolar cells), but not of VIP+ multipolar cells *		(De Marco García et al., 2011)
Whisker plucking	Deficit in morphogenesis of Re+ INs, but not VIP+ multipolar cells (no data on CR+ cells)*		(De Marco García et al., 2015)
Activity and cell death			
Manipulation	Outcome	Genes	References
Genetic ablation of transcription factors important either for MGE or CGE development	Reduced number of MGE INs leads to increased number of CGE INs and vice versa	<i>Sox6, Nkx2, COUP-TFI</i>	(Azim et al., 2009; Batista-Brito et al., 2009; Butt et al., 2008; Lodato et al., 2011)
Conditional <i>Lhx6</i> knock out in <i>Nkx2.1-Cre</i> animals to target MGE INs	Death of MGE INs leads to lower death levels of CGE INs (but not VIP+ cells)	<i>Lhx6</i>	(Denaxa et al., 2018)
Overexpression of <i>Kir2.1</i> from P5	Regulation of IN cell death through a calcineurin-dependent mechanism, but not for VIP+ INs		(Priya et al., 2018)
Activity and synaptic plasticity			
Manipulation	Outcome	Genes	References
Exposure of dark-reared animals to light	IGF-1 functions cell-autonomously in VIP+ neurons to increase inhibitory synaptic input onto these neurons and regulate visual acuity	<i>Igf1</i>	(Mardinly et al., 2016)

* VIP+ INs are not only multipolar cells

These discrepancies can be related to the different overall effect of the manipulations, as Kir2.1 overexpression leads to hyperpolarisation of CGE-INS, while blockade of thalamic input does not affect CGE electrophysiological properties (De Marco García et al., 2015). The fact that forced hyperpolarization reduces IN motility (Bortone and Polleux, 2009) could underlie the differences between those manipulations.

Alterations in serotonergic signalling have opposite effects on the migration of Re+ and VIP+ CGE-INS (Frazer et al., 2015; Murthy et al., 2014). In particular, knocking out the *5Htr3a* gene, thereby blocking 5HT3aR-mediated signalling, impairs laminar positioning of Re+ cells, but not VIP+ (Murthy et al., 2014). Conversely, increase in extracellular levels of serotonin through pharmacological blockade or genetic deletion of SERT, the serotonin transporter, affects VIP+ (but not Re+) INs migration, likely through an excessive activation of 5HT3aR (Frazer et al., 2015).

Perturbation of neuronal activity, whether through overexpression of Kir2.1, pharmacological blockade of glutamate-mediated activity or sensory deprivation, has distinct effects on Re+ and VIP+ INs (De Marco García et al., 2011, 2015; Karayannis et al., 2012). In particular, it seems that Re+ cells are more prone to maturation defects than VIP+ INs. However, there is an important caveat in these studies: they showed that VIP+ multipolar cells were not affected by activity, but CR+ cells were. As mentioned above, VIP+ bipolar INs also express CR (see 1.4) Therefore the conclusion that activity does not affect VIP+ INs should be reconsidered, in light of the morphological and molecular diversity of this population.

Programmed cell death is a key process in circuit maturation. It controls appropriate neuronal targeting and circuit refinement, affecting both pyramidal cells and INs (Kuan et al., 2000; Wong et al., 2018; Yamaguchi and Miura, 2015). Activity has an important role in controlling programmed cell death in cortical networks, and this is thought to be mediated by the balance between excitation and inhibition (Blanquie et al., 2017; Wong et al., 2018). This is not only mediated by the ratio between pyramidal cells and INs, but also via the balance between different IN subtypes.

Several genetic ablation experiments interfering with the molecular machinery controlling MGE-INs specification report an increase of CGE-derived populations following MGE-INs death, and *vice versa* (Azim et al., 2009; Batista-Brito et al., 2009; Butt et al., 2008; Lodato et al., 2011a). More recently, it has been shown that depletion of MGE-derived INs reduces cell death in CGE-INs in an activity-dependent manner, without perturbing VIP+ cells (Denaxa et al., 2018). The fact that VIP+ INs survival does not seem to be dependent on an activity-related mechanism has been recently confirmed by Priya et al. (2018). However, activity does have an effect on VIP+ INs: dark rearing experiments have shown that VIP+ INs express insulin-like growth factor (IGF-1) in an experience-dependent manner, and that this molecular programme promotes inhibitory synaptic input onto these cells and visual acuity (Mardinly et al., 2016).

Genetic- and activity- dependent mechanisms control CGE-INs development to different extents. There is increasing evidence that maturation or survival of VIP+ INs (or at least of the VIP+/CR- multipolar population) are hardwired in the circuit and are not affected by activity perturbations (De Marco García et al., 2015). This suggests a more profound contribution of cell-

autonomous mechanisms in influencing VIP+ INs integration in cortical circuit development (Miyoshi et al., 2015).

1.6 – VIP+ INs local and long-range connectivity

In recent years, there has been an increased interest in studying VIP+ INs, as several papers demonstrated that they occupy a key node in the cortical network, mediating disinhibition of pyramidal cells via preferential inhibition of SST+ INs (Fu et al., 2014; Karnani et al., 2016; Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013). Interestingly, this disinhibitory motif has been found in several cortical and subcortical areas, suggesting its role across modalities and functions (Krabbe et al., 2018; Turi et al., 2019). Moreover, VIP+ INs are recruited not only by local pyramidal cells from across the depth of the cortex (Staiger et al., 2002; Xu and Callaway, 2009), but also by subcortical and long-range cortical inputs, suggesting a complex role for this cell population, spanning bottom-up and top-down regulation, cross-modal communication and behavioural state transitions (Audette et al., 2018; Batista-Brito et al., 2017; Fu et al., 2014; Hajós et al., 1997; Ji et al., 2016; Lee et al., 2013; Staiger et al., 1996; Wall et al., 2016; Williams and Holtmaat, 2019; Zhang et al., 2014).

1.6.1 – VIP+ INs in sensory-motor integration

VIP+ INs are emerging as key players in the sensory-motor circuitry. Electrophysiology experiments have demonstrated that S1BF VIP+ INs receive

input from the thalamus (Audette et al., 2018; Ji et al., 2016; Staiger et al., 1996; Williams and Holtmaat, 2019). Moreover, optogenetic and viral tracing technologies have shown that S1BF VIP+ INs are recruited from the primary motor cortex (M1) and through this connection modulate S1BF activity (Lee et al., 2013; Wall et al., 2016). Finally, complementary *in vivo* experiments have shown that VIP+ INs are responsive during active whisking (Barson et al., 2018; Sachidhanandam et al., 2016; Yu et al., 2019). All these pieces of evidence corroborate the hypothesis that VIP+ INs mediate motor integration in the somatosensory cortex via local disinhibition (Lee et al., 2013).

In parallel, it has been hypothesized that VIP+ INs mediate locomotion-related modulation of cortical responses via cholinergic inputs from the basal forebrain (Dipoppa et al., 2018; Fu et al., 2014; Pakan et al., 2016). Intriguingly, rabies virus experiments suggested that the basal forebrain input is present as early as the first postnatal week (De Marco García et al., 2015), although no study has confirmed the functionality of this connection. RNA-seq data showed that 5HT3aR+ cells express the alpha 1 subunit of the cholinergic nicotinic receptor already at E18 and maintain its expression at P2 and P5, suggesting that VIP+ INs may be able to respond to acetylcholine already at late embryonic stages (Frazer et al., 2017).

The possibility of a sensory-motor integrative circuit, in which VIP+ INs combine long-range cortical and subcortical inputs from motor cortex, thalamus and basal forebrain to trigger local disinhibition, is definitely intriguing. However, extensive evidence suggests a higher degree of complexity in this model.

1.6.2 – Further complexity in the model

While the specificity of the VIP+ to SST+ connection is well known (Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013), VIP+ INs also target other INs, such as PV+ cells and other VIP+ INs, even though to a smaller degree (Dávid et al., 2007; Hioki et al., 2013; Lee et al., 2013; Pfeffer et al., 2013; Staiger et al., 1997, 2002). In turn, VIP+ INs are targeted by SST+ INs and to a lesser extent by PV+ cells (Pfeffer et al., 2013; Sohn et al., 2016). Moreover, PV+ and VIP+ INs exert differential control over SST+ cells (Walker et al., 2016). Finally, VIP+ INs directly target pyramidal cells, combining direct VIP-to-pyramidal inhibition and indirect VIP-to-SST mediated disinhibition, thereby establishing an inhibitory pull-push circuit on this cell population (Chiu et al., 2018; Garcia-Junco-Clemente et al., 2017; Jiang et al., 2013, 2015; Lee et al., 2013; Pfeffer et al., 2013; Staiger et al., 2002).

Layer differences add another level of complexity to VIP+ INs connectivity: while the majority of the above studies focused on L2/3, recent evidence suggest that VIP-to-SST and VIP-to-pyramidal connections are layer specific (Muñoz et al., 2017; Zhou et al., 2017). Moreover, VIP+ INs in specific layers are engaged by different inputs: L4 VIP+ INs are targeted by the ventral posteromedial nucleus (VPM) of the thalamus, while L2/3 VIP+ INs are targeted by the posteromedial complex (POm) (Audette et al., 2018; Ji et al., 2016; Staiger et al., 1996; Williams and Holtmaat, 2019). These two thalamic nuclei respectively belong to the lemniscal and paralemniscal pathways, two functionally segregated circuits in the somatosensory pathway, which provide distinct types of sensory information to the cortex (touch and whisking

information, respectively) through anatomically interdigitated connections in S1BF (Bureau et al., 2006). This differential connectivity may reflect different functionality between L4 and L2/3 VIP+ cells.

Finally, the role of VIP+ INs is even more intricate when considering state- and context- dependent differences in cortical circuit engagement. It is well known that cortical function varies with behavioural states such as arousal, locomotion and quiescence, and that this modulation is mediated via local excitatory and inhibitory circuits (Batista-Brito et al., 2018; Busse et al., 2017; Poulet and Crochet, 2019; Vinck et al., 2015).

Previous work has demonstrated a role for VIP+ INs in state-dependent modulation, showing that primary visual cortex (V1) VIP+ INs are activated by locomotion and in turn trigger pyramidal cells disinhibition by inhibiting SST+ INs (Batista-Brito et al., 2017; Fu et al., 2014). However, a recent paper by Bigelow et al. (2019) showed different effects in auditory cortex: while activation of VIP+ INs increase overall cortical firing, locomotion decrease it. The authors proposed that these contrasting results are due to a shift in resource allocation during movement in favour of spatial perception rather than hearing (Bigelow et al., 2019). This suggests modality-specific effects of locomotion and VIP+ INs activation on cortical networks.

Additional work in V1 further challenged the simple disinhibitory motif, based on the activation of VIP+ INs triggering inhibition of SST+ cells and thus release of inhibition to pyramidal cells. For instance, Jackson et al. (2016) showed that V1 VIP+ INs are active across multiple brain states, such as locomotion, non-locomotion, visual stimulation, and under isoflurane anaesthesia.

Moreover, recent experiments highlighted the context-dependency of the networks revolving around VIP+ INs. In visual cortex, Pakan et al. (2016) and Dipoppa et al. (2018) showed that the effect of locomotion combined with sensory stimulation on VIP+ and other INs does not fit with the simple disinhibition model, highlighting the flexibility of this circuit. They demonstrated that VIP+ INs are context-independent, remaining responsive to locomotion regardless of the presence of sensory stimuli, while SST+INs are not: their activity increase with locomotion during visual stimulation, but decrease with locomotion in absence of sensory stimulation (Pakan et al. 2016). Furthermore, the effect of locomotion onto VIP+ and SST+ INs activity varies with sensory stimulation of different intensity (Dipoppa et al. 2018). This supports the hypothesis of a more complex, context-dependent circuit, that flexibly adapts to a variety of sensory stimuli.

Computational work showed that the VIP-mediated disinibitory circuit can operate in different regimes (Garcia del Molino et al., 2017) and it has been proposed as a mechanism to promote sensory gating (Khan and Hofer, 2018; Wang and Yang, 2018; Yang et al., 2016a).

Together, these recent works challenge the notion of a simple disinhibitory motif. However, a caveat to consider is that most experiments (Bigelow et al., 2019; Dipoppa et al., 2018; Pakan et al., 2016) have not been performed in S1BF.

Cytoarchitectonic differences between auditory, visual and somatosensory cortices have been found in terms of IN populations. These areas show differences in IN morphology, densities and subtypes proportions (Keller et al., 2018; Kim et al., 2017; Scala et al., 2019). In particular, S1BF has less VIP+

INs and a higher PV/SST INs ratio in comparison to the primary auditory and visual cortices (Kim et al., 2017). Furthermore, auditory and visual cortices seem to have a higher proportion of VIP+/CR+ INs, those which are thought to provide inhibition to SST+ INs (Cauli et al., 2014). These cytoarchitectural differences may have an impact on net circuit responses and therefore explain why a purely disinhibitory model does not work for auditory and visual cortices but may still be valid for S1BF.

Recent experiments confirmed that L2/3 S1BF VIP+ INs are modulated by running, whisking or both (Barson et al., 2018; Sachidhanandam et al., 2016). In addition, Yu et al. (2019) showed that passive touch does not drive VIP+ INs, but activates PV+ and SST+ INs, at shorter and longer latencies, respectively. Intriguingly, some VIP+ INs show a suppression following passive touch, at a latency that suggests direct inhibition from SST+ INs. Active whisking instead activates VIP+ INs, while SST+ INs are modulated in a layer-specific manner. In L2/3 for instance, the majority of SST+ cells are inhibited, confirming the purely disinhibitory motif (Yu et al., 2019).

These findings support the idea that activation of S1BF VIP+ INs during whisking is driven not by the VPM (i.e. the 'sensory component'), but by long-range excitation from the motor cortex and/or the POM (Audette et al., 2018; Lee et al., 2013; Williams and Holtmaat, 2019) or by cholinergic inputs (Fu et al., 2014), which not only increase during running, but also during active whisking (Eggermann et al., 2014). Altogether, these data support the simple disinhibitory motif following state-dependent modulation of VIP+ INs in S1BF, at least in L2/3 (Barson et al., 2018; Yu et al., 2019).

1.7 – VIP+ INs in development and plasticity: exploring a role in emergent perception

Seminal papers characterised VIP+ cells in embryonic and postnatal brain development, focusing on expression changes of the vasoactive intestinal peptide, distribution in the cortex and morphological analysis (Emson et al., 1979; Götz and Bolz, 1989; McDonald et al., 1982). More recently, emerging evidence has proven a functional role for VIP+ INs in development and plasticity (Batista-Brito et al., 2017; Fu et al., 2015; Goff and Goldberg, 2019; Mossner et al., 2017; Williams and Holtmaat, 2019).

Several papers investigated the effects of perturbation of developmental disorder-associated genes in VIP+ INs (Batista-Brito et al., 2017; Goff and Goldberg, 2019; Mossner et al., 2017). Mossner et al. (2017) deleted the Rett Syndrome gene *Mecp2* (methyl-CpG binding protein 2) in VIP+ INs. Rett Syndrome is a neurodevelopmental disorder characterised by loss of motor skills, social withdrawal, loss of language and other autistic features (Chahrour and Zoghbi, 2007). *Mecp2* deficient VIP+ INs lead to impairments in locomotor activity in the open field test and in marble burying, a task to measure anxiety and exploratory behaviour (Mossner et al., 2017). Moreover, mice lacking *Mecp2* in VIP+ INs show sociability deficits in the three-chamber sociability task (Mossner et al., 2017). These findings suggest that VIP+ INs may contribute to motor and sociability deficits caused by *MECP2* loss in Rett Syndrome.

Batista-Brito et al. (2017) exploited a model where VIP+ INs lacked the receptor tyrosine kinase ERBB4, which is thought to regulate GABAergic circuits

development (Fazzari et al., 2010). By performing *in vivo* recordings in awake animals, they showed that *ErbB4* deletion in VIP+ INs affects cortical network dynamics. In particular, it leads to increased spontaneous activity in regular spiking neurons, abolishment of cortical state transitions between quiescent and active periods, sensory processing alterations and compromised learning (Batista-Brito et al., 2017). Moreover, they showed that impairments in sensory processing emerge around P25-28, period that can be considered ‘adolescence’ for mice and matches with the onset of schizophrenia in humans (Silbereis et al., 2016). Their findings suggest that VIP+ INs contribute to generate the behavioural and sensory processing impairments observed in murine genetic models of human developmental disorders (Batista-Brito et al., 2017; Leung and Jia, 2016).

Finally, Goff and Goldberg (2019) specifically recorded VIP+ INs in a mouse model of Dravet Syndrome, a neurodevelopmental disorder characterised by severe epilepsy and intellectual disability. It is caused by loss of function mutations in *SCN1A*, the gene encoding the voltage gated sodium (Na⁺) channel subunit Nav1.1. The authors found that VIP+ INs express Nav1.1, and that irregular spiking (but not continuous adapting) VIP+ cells are hypoexcitable in *Scn1a*^{+/-} mice (Goff and Goldberg, 2019). They suggested that irregular spiking VIP+ INs might be relevant to contribute to epilepsy and cognitive impairments typical of Dravet Syndrome (Goff and Goldberg, 2019).

All together, these last findings (Batista-Brito et al., 2017; Goff and Goldberg, 2019; Mossner et al., 2017) suggest that VIP+ INs might have a critical role in neurodevelopmental disorders. Intriguingly, the receptor 2 for VIP (VIPR2, also known as VPAC2) has been identified as a schizophrenia risk gene

and recently suggested as a potential target for this pathology (Piggins, 2011; Sakamoto et al., 2018; Vacic et al., 2011). However, the impairments in the VIP-circuitry leading to these disorders, as well as the contribution of peptidergic transmission, are still unknown.

There are a few potential mechanisms that may underpin the role of VIP+ INs in developmental disorders. Firstly, VIP+ INs have been linked to plasticity mechanisms (Audette et al., 2018; Fu et al., 2015; Mardinly et al., 2016; Williams and Holtmaat, 2019). In visual cortex, it has been shown that VIP+ INs promote adult plasticity following monocular deprivation, potentiating the visual response of the open-eye (Fu et al., 2015). Moreover, exposure of dark-housed mice to light promotes IGF-1 expression in VIP+ INs, which leads to a cell-autonomous increase in inhibitory synaptic input, thereby regulating visual acuity (Mardinly et al., 2016). In the somatosensory cortex, VIP+ INs are thought to act as a plasticity gate, integrating POM derived input to induce synaptic long-term potentiation (LTP) in pyramidal cells via inhibition of SST+ INs (Audette et al., 2018; Williams and Holtmaat, 2019). Intriguingly, the role of SST+ INs in development of the S1BF columnar circuit starts at the beginning of the first postnatal week (P4-P6) (Marques-Smith et al., 2016; Tuncdemir et al., 2016) (Figure 1.2).

The specificity of the VIP-to-SST connection suggests an early role for VIP+ INs in regulating cortical column development. As aforementioned, they are located in a key node of the S1BF cortical column, since they mediate motor integration via local disinhibition (Lee et al., 2013; Figure 1.3 B).

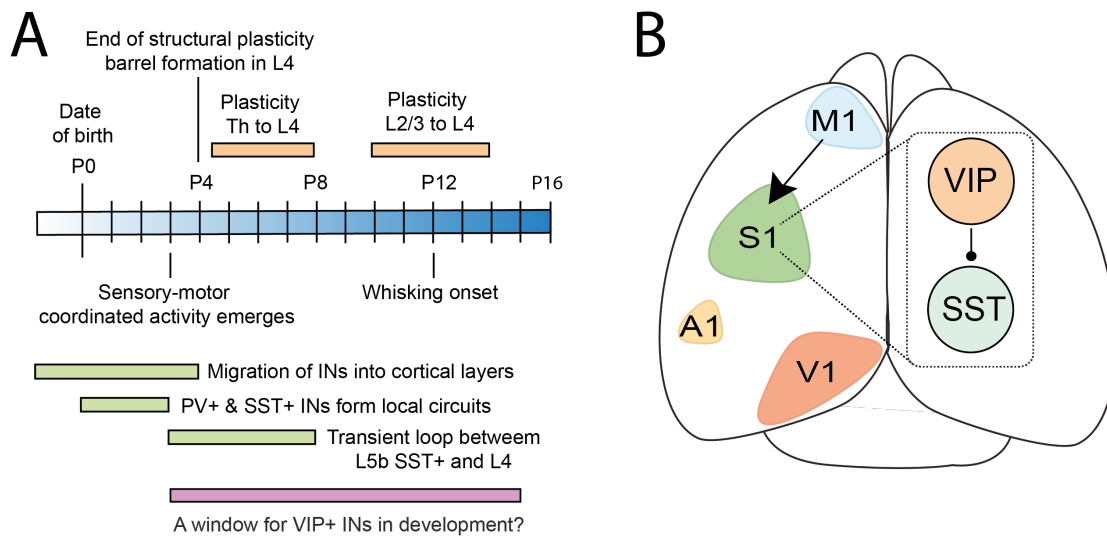


Figure 1.3 Do VIP+ INs have a role in developing circuits? (A) Timeline of S1BF development, with emphasis on IN-mediated circuits (green bars). Pink bar = window for a potential developmental role for VIP+ INs. Adapted from Butt et al., (2017). (B) Basic schematics of the circuits supporting the role of VIP+ INs in development. VIP+ INs mediate sensorimotor integration by receiving M1 input (arrowhead) and inhibit specifically SST+ INs (inset), which are known to have a role in establishing thalamocortical connections in S1BF (see A). M1= primary motor cortex, S1= primary somatosensory cortex, A1= primary auditory cortex, V1= primary visual cortex.

Reciprocal interactions between sensory and motor systems establish during the first postnatal week . Spontaneous activity synchronises sensory and motor areas as early as P3 (An et al, 2014; McVea et al., 2012, 2017). An et al. (2014) showed that the primary motor cortex is able to elicit ~30% of spontaneous forepaw movements observed at P3-5, subsequently eliciting activity in the somatosensory cortex. McVea et al. (2017) demonstrated that spontaneous (but not whisker-evoked) activity in S1BF correlates with activity in motor cortex already at P5, time at which no functional or anatomical S1BF-to-M1 connections are found (McVea et al., 2017). By P8, sensory-evoked activity starts to synchronise S1BF and M1 (McVea et al., 2017). By P12, when active whisking behavior emerges, activity correlation between these areas becomes

more pronounced (Arakawa and Erzurumlu, 2015; van der Bourg et al., 2016; McVea et al., 2017) (Figure 1.3 A). These findings support the contribution of early activity patterns in development of functional sensory-motor connectivity prior to active whisking.

Considering that adult VIP+ INs are engaged during active whisking (Barson et al., 2018; Yu et al., 2019), this raises the question of whether VIP+ INs could coordinate the development of sensory-motor integration, and thus emergent perception, through the establishment of local disinhibition and long-range M1 to S1BF connectivity within the first two postnatal weeks (Figure 1.3). Chapters 3 and 4 investigate the local GABAergic output from VIP+ INs and the establishment of long-range connectivity to S1BF VIP+ INs, respectively.

1.8 – Assessment of in vivo activity

To address the role of VIP+ INs at the network level, *in vivo* electrophysiological recordings combined with whisker stimulation in urethane-anaesthetised animals will be discussed in Chapter 5. During urethane-induced anaesthesia, rhythmic patterns of activity can be observed, which mimic sleep-like activity (Clement et al., 2008). The following section will cover sleep oscillations observed during the non rapid-eye-movement (NREM) stage of sleep, focusing on the underlying mechanisms and the current understanding of INs function in these processes. Next, the different stages of the development of whisking behaviour will be reviewed, as well as our knowledge of INs role in touch processing. All of this will set the ground for the interpretation of the results

presented at the end of this thesis: the analysis of baseline oscillations and whisker-triggered activity in the *Prox1* cKO model.

1.8.1 – Non rapid-eye-movement sleep oscillations

In the brain, coordinated electrical activity in cortical networks results in neuronal oscillations whose frequency ranges approximately from 0.05 Hz to 500Hz. These oscillations emerge from a combination of properties at the cellular and circuit level and are often associated with different brain states (Buzsaki, 2004). The non rapid-eye-movement (NREM) stage of sleep is characterised by three rhythms: (1) sleep spindles in the range of ~10–15 Hz, (2) delta waves between 1.5-4 Hz, and (3) slow oscillations at <1.5 Hz, which alternate between active and silent periods of neuronal activity, also known as up and down state respectively (Vantomme et al., 2019).

Slow and delta oscillations can be described jointly as slow-wave activity (SWA), ranging from 0.5 to 4 Hz. Most of slow waves and spindles are local, but it has been shown that slow waves can propagate along the fronto-caudal axis (Nir et al., 2011). While SWA is initiated by cortical neurons, and propagated by cortico-cortical and cortico-thalamo-cortical connections (Steriade, 2006), spindles are generated by the reticular nucleus of the thalamus (TRN) and arise from thalamo-cortical loops (Huguenard and McCormick, 2007).

SWA is a reliable marker of sleep need: SWA power increases after wakefulness periods and decreases after periods of sleep (Tobler and Borbély, 1986; Werth et al., 1996). SWA is modulated by cholinergic, glutamatergic,

monoaminergic, purinergic, and GABAergic transmission, as well as developmental processes (Greene and Frank, 2010). For instance, SWA amplitude is low at younger ages, increases before adolescence and declines afterwards, reaching its minimum during senescence (Greene and Frank, 2010). From a functional perspective, the synaptic homeostasis hypothesis – which proposes that sleep is ‘the price we pay for plasticity’ (Tononi and Cirelli, 2003, 2006, 2014) – suggests that SWA is not only an index of synaptic homeostasis, but also actively contributes to synaptic renormalisation, defined as the process to decrease the total synaptic strength through synaptic depression in order to restore neuronal selectivity (Tononi and Cirelli, 2014).

Conversely, spindles have been related to potentiation of synapses, learning, cognitive function and performance (Clawson et al., 2016). Sleep spindles are not present in the developing brain, which is instead characterised by spindle-like oscillations called ‘delta brushes’ in humans and ‘spindle bursts’ in rodents (Whitehead et al., 2017; Yang et al., 2016b).

In humans, delta brushes are oscillations in the range of 8-20 Hz and represent the predominant activity *in utero* and in premature babies. The triggering mechanisms are unclear; however, they can be initiated from sensory-evoked activity via the same thalamo-cortical circuits underlying adult spindles (Milh et al., 2007).

‘Spindle bursts’ (5–25 Hz) are thought to be the rodent equivalent of delta brushes: they are elicited by early motor activity in somatosensory cortex (Khazipov et al., 2004) and by retinal waves in the visual cortex (Colonnese and Khazipov, 2010; Hanganu et al., 2006). It has been proposed that they trigger the activation of silent thalamocortical synapses, therefore leading to somatotopic

mapping in somatosensory cortex (Isaac et al., 1997) and to the formation of visual pathways to the visual cortex (Colonnese and Khazipov, 2010; Colonnese et al., 2010; Hanganu et al., 2006).

In humans, mature sleep spindles emerge between 6 weeks and 3 months after birth (Louis et al., 1992): they start appearing around primary motor and primary somatosensory cortices, then spread to frontotemporal areas, acquiring higher synchrony over time (Hagne, 1972). After peaking during adolescence, spindle density decreases across lifetime. Duration and amplitude also show a decrease correlated with aging (Clawson et al., 2016). These changes have been associated with cognitive decline and are more prominent in pathologies such as Alzheimer's and Parkinson's diseases (Latreille et al., 2015; Rauchs et al., 2008).

Data from animal models show instead that spindle bursts are present during the first postnatal week in the rat whisker barrel cortex (Minlebaev et al., 2007, 2009), but then quickly disappear between P8 and P9, just few days before active whisking (Colonnese et al., 2010). Similarly, in rat visual cortex bursting activity disappears by P12, just before eye opening (Colonnese et al., 2010). These abrupt switches to more mature responses correspond to the emergence of continuous cortical activity and modulation of cortical activity by vigilance state (Colonnese et al., 2010).

Surprisingly, it has been shown that changes in spindle activity correlated with age are dependent on the rodent genetic background, suggesting the importance of genetic factors in these processes (Hasan et al., 2012).

1.8.1.2 – Role of interneurons in SWA and spindles

INs are involved in generation and maintenance of slow wave and spindle activity, even though there is contrasting evidence regarding their specific role. Considering SWA, *in vitro* work previously demonstrated that PV+, SST+ and VIP+ INs increase spiking activity during up states (Neske et al., 2015), and that silencing SST+ INs increases pyramidal cell excitability during up states (Neske and Connors, 2016). However, *in vivo* evidence added complexity to this model: for example, activation of PV+ INs decreases SWA and cortical firing (Funk et al., 2017), promoting cortical suppression during down states upon thalamic recruitment (Zucca et al., 2019). Recent *in vivo* work showed that SST+ INs activity increases before down states but decreases during up states (Niethard et al. 2018), while other work showed that *in vivo* activation of SST+ INs triggers SWA and increases NREM sleep duration (Funk et al., 2017).

There is evidence supporting a role for VIP+ INs in SWA. While a recent paper claimed that VIP+ INs modulate pyramidal cells activity regardless of the brain state (Jackson et al., 2016), it must be noted that the authors recorded VIP+ INs activity only during locomotion, non locomotion, visual stimuli and light isoflurane-induced anaesthesia, without testing specifically for up and down states.

It was demonstrated that vasoactive intestinal peptide deficient animals show smaller NREM rhythms amplitude, but no alteration of the total amount of daily NREM sleep (Hu et al., 2011). However, it cannot be concluded that this phenotype is caused by cortical VIP+ INs since vasoactive intestinal peptide-mediated signalling and VIP+ cells in the suprachiasmatic nucleus of the

hypothalamus have a role in sleep homeostasis and circadian rhythms (Herzog et al., 2017; Liu et al., 2018; Ono et al., 2016).

In support of the role of VIP+ INs in slow wave activity, it was shown that enhanced inhibition mediated by superficial VIP+ INs in the cortex is required for generating cortical SWA (Hall et al., 2015). In contrast, *in vitro* work showed that VIP+ INs do not contribute to pyramidal cell excitability during up states either when excited or inhibited (Neske and Connors, 2016). The precise role of INs in SWA is still under investigation and the contrasting evidence reported here may be simply explained by intrinsic differences between *in vitro* and *in vivo* works.

The literature linking INs and spindles mainly focuses on the role of subcortical INs: the thalamic reticular nucleus (TRN), the pacemaker of sleep spindles, is a GABAergic structure (Pinault, 2004) and activation of INs in the TRN has been shown to lead to cortical spindles (Halassa et al., 2011). Moreover, it has been demonstrated that the peptide SST inhibits spindle-like oscillation in the TRN (Sun et al., 2002), and activation of PV+ INs in the TRN elicits sleep spindles in the cortex, while optogenetic stimulation of TRN-targeting PV+ INs in the basal forebrain reduces cortical spindle density (Thankachan et al., 2019).

In the cortex, it has been demonstrated that PV+ INs activity increases during spindles, while SST+ INs activity does not change (Niethard et al. 2018).

So far, there is no evidence linking VIP+ INs to sleep spindles, neither in the cortex nor in the TRN, even though VIP mRNAs have been previously found in this structure (Burgunder et al., 1999). However, given that VIP+ INs are located in a key node in the cortical circuit – mediating both direct inhibition and disinhibition of pyramidal cells– and that their role in SWA has been already

suggested, it is conceivable they are players in spindle generation or maintenance.

1.8.2 – Whisking behaviour development and the role of INs

The anatomical changes underlying barrel formation have been extensively studied, prenatally and postnatally (for a review, see Erzurumlu and Gaspar, 2012). Before birth, thalamocortical axons reach the cortex (Senft and Woolsey, 1991), and at P4 the critical period for structural plasticity terminates (Durham and Woolsey, 1984). At P5-7 barrels become visible cellular aggregates (Rice et al., 1985), between P10 and P14 L4 to L2/3 synapses emerge (Lendvai et al., 2000; Maravall et al., 2004a, 2004b; Shoykhet et al., 2005; Stern et al., 2001), and between P13-P16 horizontal connections between layer 2/3 neurons are formed (Wen and Barth, 2011). The development of whisking behaviour spans the first three postnatal weeks, with the onset of active whisking emerging at end of the second postnatal week (Arakawa and Erzurumlu, 2015).

Recent work shed light on the maturation of whisker-evoked sensory processing *in vivo* before, during and after active whisking onset (van der Bourg et al., 2017, 2019). Van der Bourg et al. (2017) showed that over development there is a layer-specific refinement of whisker-evoked responses, with a decrease of whisker-evoked multi-unit activity in L2/3 and L4, and an increase in L5 and 6. Moreover, around active whisking onset, response selectivity, sparsification and decorrelation of evoked responses emerge in L2/3 neurons (van der Bourg et al., 2017). Finally, van der Bourg et al. (2019) demonstrated that whisking evoked multi-unit activity responses sharpen over development,

with more temporally- and spatially- locked responses upon single whisker stimulation.

While the *in vivo* role of different INs classes in the development of whisker-evoked sensory processing has yet to be fully understood (Che et al., 2018; Baruchin et al., *in preparation*), it is clear that in the mature circuit INs play an important role in this process. For instance, it has been demonstrated that L4 PV+ INs directly integrate thalamic inputs (Gutnisky et al., 2017; Yu et al., 2016), or that SST+ INs show layer-specific activity modulation upon whisker stimulation according to the relative weight of VIP+ INs mediated inhibition (Muñoz et al., 2017). Lee et al. (2013) reported that firing rate increases in VIP+ INs after whisking stimulation and, recently, Yu et al. (2019) specified that VIP+ INs do not exhibit touch-evoked activity, but respond to whisker movements per se, suggesting a role for this cells in integrating either cholinergic inputs (Fu et al., 2014) or long-range excitatory inputs from the motor cortex (Lee et al., 2013).

Given our current understanding of the role of VIP+ INs in the developing sensorimotor systems, it is possible that they play a role in early sensory processing and whisking onset.

1.9 – Thesis objectives

Despite the recent efforts to understand the functional role of VIP+ INs in development (Batista-Brito et al., 2018; Goff and Goldberg, 2019; Mossner et al., 2017), it is still unknown how and when VIP+ INs integrate into the local and

long-range circuits, as well as their contribution to early sensorimotor integration. This thesis seeks to probe the integration of VIP + INs in the postnatal mouse whisker barrel cortex (S1BF), employing optical and electrophysiological methods in combination with developmental genetic and viral transduction approaches.

The three main aims of this thesis, corresponding to the three results chapters, are:

- Aim 1: To investigate the integration of VIP+ cells into the local S1BF circuit, focusing on (1) the distribution of VIP+ cells across the first three postnatal weeks, (2) the maturation of their intrinsic properties, (3) the emergence of glutamatergic inputs onto VIP+ cells, and (4) the development of GABAergic outputs from VIP+ INs to other cells.
- Aim 2: To probe how VIP+ cells integrate into the long-range M1 to S1BF circuitry at early time points, establishing a viral optogenetic strategy allowing the recruitment of long-range afferents in less than a week from neonatal channelrhodopsin injection. This allowed me to test the emergence of long-range cortico-cortical inputs from the anterior-motor areas onto S1BF VIP+ cells.
- Aim 3: To test how perturbation of VIP+ INs affects their integration in the local and long-range circuitry, as well as early sensory processing. This has been achieved by employing the *VIP-Cre;Prox1^{c/c}* model (Miyoshi

et al., 2015), in which the postnatal maturation of VIP+ INs is genetically perturbed.

1.9.1 – Integration of VIP+ INs in the local S1BF circuitry: Chapter 3

Considering the early role of SST+ INs in S1BF development (Marques-Smith et al., 2016; Tuncdemir et al., 2016) and the specificity of the VIP-to-SST INs input (Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013), it was hypothesised that VIP+ INs might start to integrate in the cortical network already in the first postnatal week, despite their late birthdate (Butt et al., 2005; Miyoshi and Fishell, 2011; Miyoshi et al., 2010). To test this, distribution of VIP+ cells across development was assessed by immunofluorescence experiments, while electrophysiological approaches were employed to probe the maturation of these cells and their connectivity profiles.

In particular, patch-clamp recordings combined with optical techniques (namely laser scanning photostimulation combined with glutamate uncaging and optogenetics) were used to map local glutamatergic inputs onto VIP+ INs and their output to other cells in S1BF during the first two postnatal weeks.

Reporter lines such as the previously characterised *VIP-Cre;Ai9* (tdTomato) line (Proenneke et al., 2018; Pröenneke et al., 2015; Taniguchi et al., 2011) and the *VIP-Cre;Ai32;Lhx6-EGFP* (where all VIP+ cells express channelrhodopsin while PV+ and SST+ cells are labelled by the green fluorescent

protein) were used to map excitatory input onto VIP+ INs and their output respectively.

1.9.2 – Integration of VIP+ cells into the long-range sensorimotor circuitry: Chapter 4

A key function of VIP+ INs in S1BF is to mediate sensorimotor integration via local disinhibition upon recruitment from M1 long-range afferents (Lee et al., 2013). Considering that active whisking emerges only towards the end of the second postnatal week (Arakawa and Erzurumlu, 2015; van der Bourg et al., 2016), but that synchronised activity between sensory and motor areas can be observed earlier (An et al., 2014; Khazipov and Milh, 2018; Luhmann et al., 2016; McVea et al., 2012, 2017), it was hypothesised that the M1 to VIP+ INs connection might be established already during the first postnatal week, to be able to mediate sensorimotor integration by the time active whisking emerges.

To test this, I chose a viral optogenetic approach: different injection protocols were first tested to establish a reliable method to recruit specifically the long-range afferents from anterior-motor areas, and elicit monosynaptic excitatory post-synaptic currents in S1BF cells already at the end of the first postnatal week. Then, I tested the development of long-range input on L2/3 VIP+ INs and pyramidal cells across the first two postnatal weeks in the *VIP-Cre;Ai9* model.

1.9.3 – Effect of genetic perturbation of VIP+ cells in their integration in the sensorimotor circuitry: Chapter 5

Evidence from previous literature suggests that VIP+ INs are, to certain extent, hardwired in the cortical circuitry and less susceptible to activity perturbations (De Marco García et al., 2011, 2015; Karayannis et al., 2012). I employed a genetic approach to specifically perturb this population: the previously characterised *VIP-Cre;Prox1^{c/c}* model (Miyoshi et al., 2015). In this model, cell density, marker co-expression and morphology of VIP+ INs are impaired (Miyoshi et al., 2015).

It was hypothesised that perturbing VIP+ INs could affect their integration in the local or long-range circuitry, and therefore impair early sensory processing. To test this, experimental paradigms established in the previous chapters (namely laser scanning photostimulation combined with glutamate uncaging and long-range optogenetics) were employed, together with *in vivo* extracellular recording to assess network functionality following VIP+ INs impairment.

Chapter 2 – Materials and Methods

2.1 – Subjects

The following mouse lines were maintained on a mixed background, comprising C57BL/6J and CD1: *VIP-ires-Cre* (*Vip*^{tm1(cre)Zjh}/J) (Taniguchi et al., 2011); *Lhx6-EGFP* (Tg(Lhx6-EGFP)BP221Gsat) (Gong et al., 2003); *Ai9* (B6.Cg-*Gt(ROSA)26Sor*^{tm9(CAG-tdTomato)Hze}/J) (The Jackson Laboratory); *Ai32* (B6;129S-*Gt(ROSA)26Sor*^{tm32(CAG-COP4*H134R/EYFP)Hze}/J) (The Jackson Laboratory); *Prox1* (*Prox1*^{tm1.1Fuma}) (Iwano et al., 2012). *Prox1* mice were kindly provided by Professor Paul Riley (Department of Physiology, Anatomy and Genetics, Oxford, UK), but they originally derived from the colony of Dr Fumio Matsuzaki (RIKEN Centre for Developmental Biology, Minatojima Minamimachi Chuo-ku Kobe Japan). Animals were kept with the dam until weaning, housed in groups of 3–5 following weaning on a 12 h light/dark cycle and given food and water *ad libitum*.

Experimental animals were heterozygous for *VIP-Cre* and the reporter (either *Ai32* or *Ai9*), and, when applicable, hemizygous for *Lhx6-EGFP*. *Prox1* animals were either homozygous or heterozygous for the *Prox1* allele. *VIP-*

Cre;Ai9, *VIP-Cre;Ai32;Lhx6-EGFP*, *VIP-Cre;Ai9;Prox1^{c/c}* and *VIP-Cre;Ai9;Prox1^{c/+}* neonates were obtained from appropriate breeding pairs and used for experiments between postnatal day 0 and 22 (P0 and P22). Animal use was approved by the local ethical review board at Oxford University and conducted according to the Home Office personal and project (I9DE382C2; 30/3052) licenses under the UK Animals (Scientific Procedure) 1986 Act.

2.2 – Genotyping

All experiments involving the *Prox1* line (*Prox1^{tm1.1Fuma}*) (Iwano et al., 2012) were performed blind to the genotype, which was identified by polymerase chain reaction (PCR) after data analysis completion. To amplify the DNA of interest, the following primers were used:

- Forward: CAG CCC TTT TGT TCT GTT GGC CAG
- Reverse: GCA GAT GCT GTC CCT ACC GTC C

PCR was performed as follows: after 2 min of denaturation at 94 °C, 35 amplification cycles were performed (94 °C for 30 s, 60 °C for 30 s, 72 °C for 45 s), followed by a final extension stage at 72 °C for 5min. PCR products were kept at 4°C and then run on a 1.5% agarose gel in TAE (Tris-acetate-EDTA) buffer (Sigma). Two bands were obtained: a larger transgenic band (220bp) and lower wild-type (WT) band (184bp), as previously described (Iwano et al., 2012) (see Figure 5.1C).

2.3 – Immunohistochemistry

Tissue preparation and staining for cell counting

Following induction with 4% isofluorane in 100% O₂, pups of both sexes were sacrificed with an overdose (>200mg/kg) of 20% pentobarbital sodium solution (J.M. Loveridge Ltd) and transcardially perfused with 4% paraformaldehyde (PFA, Alfa Aesar) in phosphate-buffered solution (PBS, Sigma). Brains were dissected out, post-fixed in 4% PFA in PBS for 1 hour at 4°C, washed in PBS and stored in PBS-Azide (0.02-0.05%) at 4°C for few days.

Depending on the staining, brains were either prepared for vibratome or cryostat cutting. In the first case (i.e. tdTomato, GFP, calretinin and PROX1 staining), brains were embedded in a 5% low-gelling agarose gel (in PBS) and 50 µm coronal slices were cut through a vibratome (Leica VT1000S). Slices were then stored in PBS-Azide (0.02-0.05%) at 4°C.

Alternatively (i.e. VIP staining), brains were cryoprotected using increasing concentrations of sucrose (10% and then 30%) in PBS at 4°C overnight. Brains were then embedded in O.C.T. compound (VWR) on dry ice and kept at -80°C until they were sectioned on a cryostat (Leica Biosystems) into 20-25 µm serial coronal sections and mounted on SuperFrost® Plus slides (VWR).

Before immunohistochemistry, slices were washed in PBS at room temperature (RT). After being permeabilised with PBS-T (0.1M PBS + 0.2% Triton-X100, Sigma) for 30 minutes and blocked with 5% normal goat serum (NGS, Invitrogen) in PBS-T for 1-2 hours at RT, tissue was then incubated with the primary antibody (polyclonal rabbit anti-dsRed antibody, 632496, Clontech, dilution 1:500; monoclonal mouse anti-dsRed antibody, 3994-100, BioVision,

dilution 1:500; polyclonal chicken anti-GFP, ab13970, Abcam, dilution 1:2000; mouse monoclonal anti-Calretinin clone 6B8.2, MAB1568, Sigma-Aldrich, dilution 1:100; polyclonal rabbit anti-VIP, ab8556, Abcam, dilution 1:500; polyclonal rabbit anti-PROX1, AB5475, Sigma-Aldrich, dilution 1:1000) in the blocking solution overnight at 4°C.

After being washed in PBS and left additional 30 minutes in the blocking solution at RT, slices were incubated with a 1:500-1000 dilution of secondary antibody (goat anti-rabbit IgG (H+L) Alexa Fluor® 546 conjugate, A-11035, Molecular Probes; goat anti-chicken Alexa Fluor® 488 conjugate, ab150169, Abcam; goat anti-mouse IgG (H+L) Alexa Fluor® 568 conjugate, ab175473, Abcam; goat anti-rabbit Alexa Fluor® 633, A-21070, Molecular Probes) for 2 hours at RT.

After 3x5 min washes in PBS, sections were counterstained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI, D3571, Molecular Probes, dilution 1:1000), re-washed, mounted when appropriate and cover-slipped with Fluoromount™ aqueous mounting medium (Sigma). Finally, they were permanently sealed using nail polish.

Image acquisition and analysis

Microscopy imaging for cell counting was performed with a Zeiss laser scanning confocal microscope (LSM710), using a UPLS APO (air) 10x/0.40 objective at a pixel resolution of 1024 x 1024. Anatomically matched sections of the somatosensory barrel cortex were selected for cell counting, using the Atlas of the Developing Mouse Brain (Paxinos et al., 2007). For each image, cells were

counted across all layers in regions of interest (ROI)s of fixed width (either 500 or 1000 μm).

For quantification of tdTomato+ cells, a customised macro developed in Fiji (Schindelin et al., 2012) was used. Briefly, the boundaries of the different cortical layers were manually determined by DAPI counterstaining (Figure 2.1A). TdTomato signal was then used to automatically build a binary mask identifying only somata (Figure 2.1B). To normalize the distribution of positive cells, each selected column was divided into 10 equal bins (Figure 2.1B, yellow boundaries).

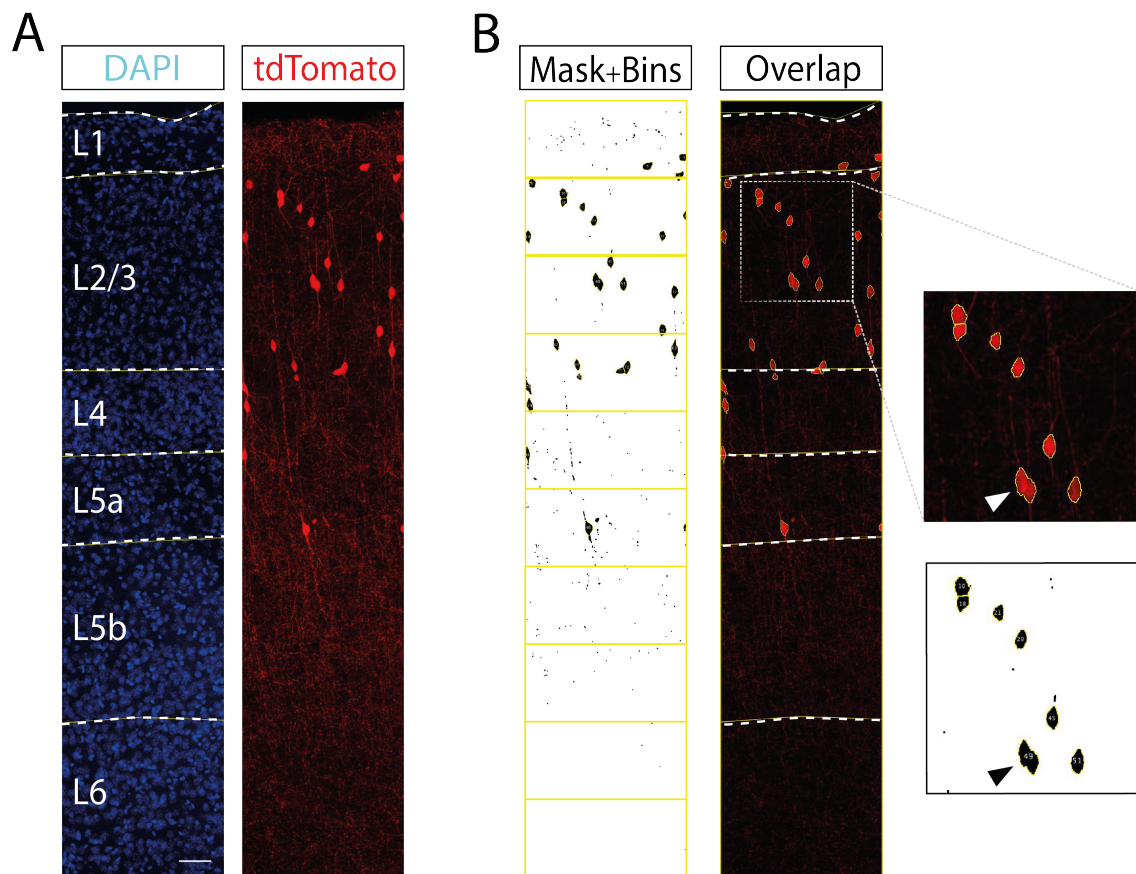


Figure 2.1 Key steps in quantification of tdTomato+ cells. (A) Exemplary immunofluorescence picture of *VIP-Cre;Ai9* somatosensory cortex. Layer boundaries were identified by the user through DAPI counterstaining (dotted lines) (B) The customised macro identified tdTomato signal by building a binary mask (left panel) and selected only somata (right panel, yellow outline). Somata were then assigned to layers or bins (left panel, Yellow boxes) according to their location. Occasionally, two somata were identified as a single one (arrowhead magnification). Scale bar= 50 μm ; L= layer.

Based on their centroid location, somata were then assigned to layers and bins. In less than 1-2 cells per area of interest this strategy was not able to distinguish partially overlapping cells (Figure 2.1B, magnification).

To quantify cell density, a similar strategy to the one reported by Prönneke et al. (2015) was followed. The area of the cortical depth in which cells were counted was measured (μm^2) and multiplied by the thickness of the section, to obtain a volume value in μm^3 . To allow direct comparison with previous literature (Prönneke et al., 2015), results were converted in mm^3 values. Cell density was then measured as ratio of the number of cell over the volume in mm^3 .

To quantify EGFP+/PROX1+ cells, the Trainable Weka Segmentation plugin was employed (Arganda-Carreras et al., 2017). This Fiji plugin allowed the automatic segmentation of both PROX1+ and EGFP+ cells using a machine learning approach. EGFP+/PROX1+ cells were determined from the overlapping of Prox1+ and EGFP+ regions of interest (ROIs).

Microsoft Excel (2010, Microsoft, UK) and Prism 8 (Graphpad) were used for data organisation and data analysis, respectively. Given that multiple counts were obtained within the same animal, mixed effect analysis was performed on the data obtained, using 'age' as fixed effect and 'subject' as mixed effect. Statistical analysis was not performed on the EGFP+/Prox1+ dataset as only few animals were analysed. Data are presented as overall mean $\pm$ standard deviation.

2.4 – Viral injections

P0-P1 pups were separated from the dam, anesthetized through hypothermia for 5-8 min, and moved to a customised stereotaxic set-up. To ensure early channelrhodopsin-2 expression, the following viruses were used: AAV1.CaMKIIa.hChr2(H134R)-eYFP.WPRE.hGH virus (Penn Vector Core, Addgene 269696P), Lot CS1025-5CS, titre 1.99×10^{13} genome copies (GC)/ml (first trial injections) and Lot CS1322, titre 3.388×10^{13} GC/ml (experimental injections), both suspended in PBS with 5% glycerol (Arruda-Carvalho et al., 2017).

A small volume of virus (49.9 nL x 3 cycles; rate: 0.23 nL/s; delay: 3 s) was injected with the Nanoject III (Programmable Nanoliter Injector, Drummond) using a pulled glass pipette (~ 20 µm inner tip diameter, bevelled at a 45° angle). To ensure targeting of the developing anterior-motor areas, the following coordinates were used (calculated from vascular lambda and expressed as mm): AP -3.12; ML 1.1; DV 0.8 mm (Figure 2.2A). No incisions were made, but once reached the right AP and ML positions, the pipette tip was used to pierce through the skull and then positioned in the correct DV coordinates to perform the injection. The pipette was withdrawn ~30 seconds after completion of injection.

Subjects were closely monitored following surgery and left in a mini-Thermacage to recover for 10-20 minutes. To prevent rejection by the dam, pups were rubbed with dirty bedding before reintroducing the dam in their home cage, and left there for a minimum of 6 days until further manipulation (recording or immunohistochemistry). After 6-7 days post-injection (dpi) it was possible to observe good viral spread in anterior-motor areas (Figure 2.2B), as

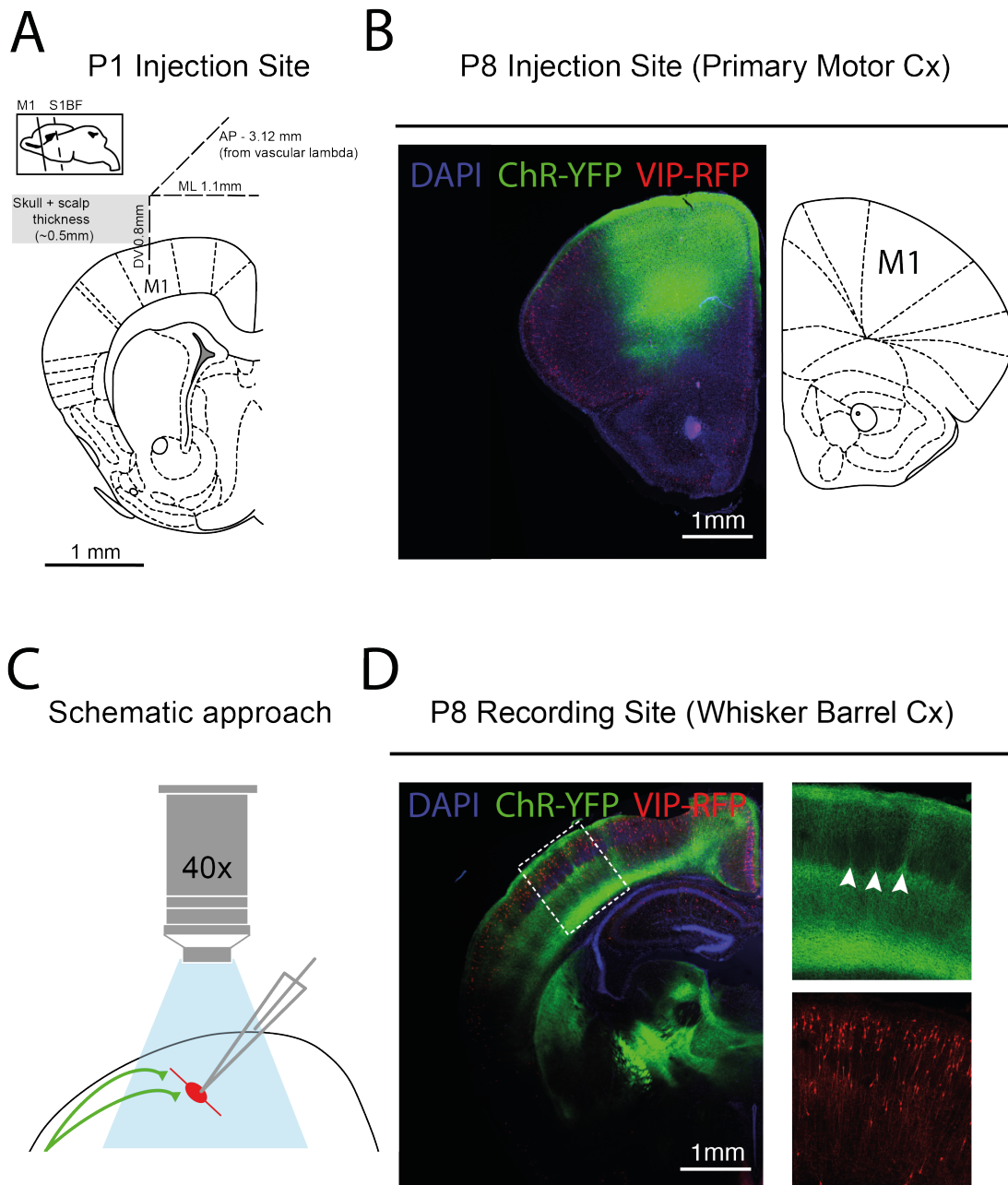


Figure 2.2 Strategy to target long-range afferents from anterior-motor areas. (A) Injection coordinates in P1 brain. Adapted from Paxinos et al. (2007). (B) Injection area at P8, 7 days post injection. The right panel is adapted from Paxinos et al. (2007). (C) Schematic approach of *in vitro* experiments. A blue light pulse (470 nm) activates the ChR+ long-range projecting fibres (in green) while a VIP+ INs (in red) is recorded with a glass electrode. (D) Recording site at P8, 7 days post injection. Arrowheads indicate the fibres passing between barrels. Blue= DAPI; Green= ChR2+ cells and fibers; Red= VIP+ cells in the *VIP-Cre;Ai9* line.

well as S1BF targeting fibres (Figure 2.2D), allowing stimulation of the terminals while recording of post-synaptic cells (Figure 2.2C).

Confirmation of target of viral injection

To refine the injection strategy and confirm the targeting of anterior-motor areas, mice were sacrificed as previously described (see Materials and Methods 2.3). Brains were post-fixed in 4% PFA in PBS for a maximum of 24 hour at 4°C, washed in PBS and embedded in 5% low gelling agarose (Sigma). Brains were sectioned coronally at 50µm on a vibrating microtome (Leica, VT1000S), and kept in PBS-Azide (0.02-0.05%) for longer storage. After 1 hour in blocking solution (5% NGS in 0.25% Triton in PBS), floating slices were incubated with polyclonal chicken anti-GFP antibody (ab13970, Abcam, dilution 1:2000) and polyclonal rabbit anti-dsRed (632496, Clontech, dilution 1:500) in blocking solution overnight at 4°C. Following 3 washes in PBS for 10min, slices were incubated with goat anti-chicken Alexa Fluor® 488 conjugate (ab150169, Abcam, dilution 1:1000) and goat anti-rabbit Alexa Fluor®546 conjugate (A11035, Life Technologies, dilution 1:1000) for 2 h in blocking solution at RT.

Next, they were washed 3x10 min in PBS and counterstained with DAPI (D3571, Molecular Probes, dilution 1:1000) for 3-5 min. Slices were mounted on slides (VWR) after a final 5 min wash in PBS. They were let dry for ~2 h, cover-slipped with Fluoromount™ aqueous mounting medium (Sigma) and permanently sealed using nail polish. Fluorescence images were acquired with a Zeiss 800 Airyscan using a 10x 0.45NA Plan-APOCHROMAT (Zeiss) objective.

2.5 – In vitro Electrophysiology

Slice preparation

Acute brain slices were prepared as previously described (Anastasiades et al., 2016a; Marques-Smith et al., 2016). Mice of both sexes (P5-P16) were anaesthetised using 4% isoflurane in 100% O₂ and decapitated. The brain was rapidly dissected in ice-cold, artificial cerebral spinal fluid (ACSF) of the following composition (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1 MgCl₂, 2 CaCl₂, 20 glucose (300-310 mOsm); pH equilibrated with 95%O₂/5% CO₂ (i.e. 'carbogenated') to pH 7.2-7.4. All reagents were provided by Sigma unless otherwise stated. Coronal cortical slices (400µm) were cut in ice-cold carbogenated ACSF using a vibratome (Vibratome 3000 Plus; The Vibratome Company) and allowed to recover at room temperature (RT) in carbogenated ACSF for at least one hour before recordings.

Whole-cell patch-clamp electrophysiology

Slices containing the somatosensory whisker barrel cortex (S1BF) were selected for electrophysiology experiments after having assessed a good preservation of the columnar structure under a low magnification objective (Olympus 10X UPLFLN objective).

Neurons were selected ~50µm below the slice surface and targeted for whole-cell patch-clamp recordings guided through infrared-differential interference contrast (DIC) microscopy using a 40x water-immersion objective (Olympus LUMPLFLN 40XW objective). Recordings were performed at RT using a MultiClamp 700B amplifier (Molecular Devices) and a Digidata 1440A digitiser.

Borosilicate glass microelectrodes (6 – 9M Ω resistance, Harvard Apparatus) were obtained using a Narishige PC- 10 micro-electrode puller.

To obtain intrinsic electrophysiological properties, as well as mapping of local and long-range glutamatergic inputs, patch electrodes were filled with a standard potassium-based solution of the following composition (in mM): 128 K-gluconate, 4 NaCl, 0.3 Li-GTP, 5 Mg-ATP, 0.1 CaCl₂, 10 HEPES and 1 glucose (pH 7.2 with KOH; 270-280 mOsm). To record GABAergic inputs, electrodes were filled with a Cesium-based intracellular solution, containing (in mM): 100 gluconic acid, 0.2 EGTA, 5 MgCl₂, 40 HEPES, 2 Mg-ATP, 0.3 Li-GTP (pH 7.2 using CsOH; 270-280 mOsm). Intracellular solutions contained Neurobiotin Tracer (SP-1120, Vector Laboratories, final concentration: 8 mM) to enable recovery of the morphology of recorded cells.

In order to record glutamatergic excitatory post-synaptic currents (EPSCs), neurons were voltage clamped at -60mV (Anastasiades and Butt, 2012). To record GABAergic inhibitory post-synaptic currents (IPSCs), neurons were kept at the reversal potential for glutamate (E_{glut}), set to 0 mV (corrected for calculated liquid junction potential of ≈ 13 mV) (Anastasiades and Butt, 2012; Anastasiades et al., 2016). Cortical layers were distinguished in the DIC image based on changes in cell size and density.

Recordings were sampled at 20 kHz and low-pass filtered online at 0.5 kHz. Cell input and series resistance were monitored during the recordings without applying compensation; recordings were discarded when series resistance exceeded 20% of its initial value.

Analysis of Intrinsic Properties

Recordings of intrinsic properties were performed in current clamp, using the potassium-based internal solution. Depending on the parameter of interest, depolarising or hyperpolarising current pulses of 500 ms were delivered at 0.5Hz via Clampex (v.10.1, Molecular Devices).

Electrophysiological data were analysed using a Matlab custom-written script. Firstly, the resting membrane potential was evaluated. The input resistance was evaluated according to the Ohm's law using the following formula: $R=V/I$, where R = input resistance; V = the maximum amplitude of the membrane potential response (always $<-10\text{mV}$); I = amplitude of the injected negative current. The membrane time constant (τ) was determined by fitting an exponential to the membrane potential response to a current injection leading to a hyperpolarisation of $<-10\text{ mV}$ ($f(x) = Ae^{-x/\tau} + B$; A = maximum amplitude, B = membrane potential at the maximum amplitude of response).

Traces with a single action potential evoked at rheobased were used to analyse firing threshold (evaluated as the membrane potential where the time derivative exceeded 5 mV/ms (Platkiewicz and Brette, 2011; Vera et al., 2017)), spike delay at rheobase, spike height, spike half width, 10-90% rising time and amplitude (measured from firing threshold to peak) of action potentials.

After hyperpolarization (AHP) amplitude was determined by measuring the difference in voltage from firing threshold to the maximum deflection of the repolarization. The delay of AHP was measured from the time point when the repolarization of the action potential crossed the firing threshold to the maximum amplitude of the AHP (see Prönneke et al, 2015). Maximum firing frequency was calculating by applying increasing depolarising current until

spike failures could be observed. The adaptation in firing frequency was measured at the maximum firing rate as the relative difference between the number of spikes in the first 100 ms and those in the last 100 ms of the spike train.

Cells that did not meet the quality criteria of good access and stable recordings (see above) across the different protocols to evaluate all the intrinsic properties were excluded from further analysis.

Data was tested for normality with the D'Agostino-Pearson normality test and for equal variance. If they both passed, data was tested with a one-way analysis of variance (ANOVA), if not with a Kruskal–Wallis one-way ANOVA.

Laser scanning photostimulation (LSPS) and mapping of columnar excitation

LSPS allows to map local input sources onto the neuron of interest (Dantzker and Callaway, 2000; Yoshimura and Callaway, 2005; Yoshimura et al., 2005). LSPS protocols were performed as previously described (Anastasiades and Butt, 2012; Anastasiades et al., 2016; Marques-Smith et al., 2016). Glutamatergic post-synaptic currents in the targeted VIP+ INs were recorded while simultaneously stimulating excitatory neurons in a small portion of the cortical column. Stimulation was performed using a low intensity (max. 2mW), long duration (100ms) laser pulse generated by an ultraviolet (UV) laser (DPSL-355/30) together with a UGA-42 targeting module (Rapp OptoElectronic GmbH) and focused through Zeiss Axioskop FS2-plus microscope equipped with a 10X UPLFLN objective (Olympus).

The laser intensity was adjusted to allow to evoke three action potentials when the laser was directed at the soma of the presynaptic cell, while minimizing suprathreshold responses at distal sites (Anastasiades and Butt, 2012; Anastasiades et al., 2018). This allowed to obtain maps with a final resolution of $\sim 50\mu\text{m}$.

Prior to LSPS, slices were incubated for at least 6 min with high divalent cation (HDC) ACSF (ACSF with 4mM MgCl_2 and 4mM CaCl_2 , to reduce all polysynaptic and spontaneous activity), containing 100 μM of 4-Methoxy-7-nitroindolyl-caged-L-glutamate (MNI glutamate, Tocris Bioscience).

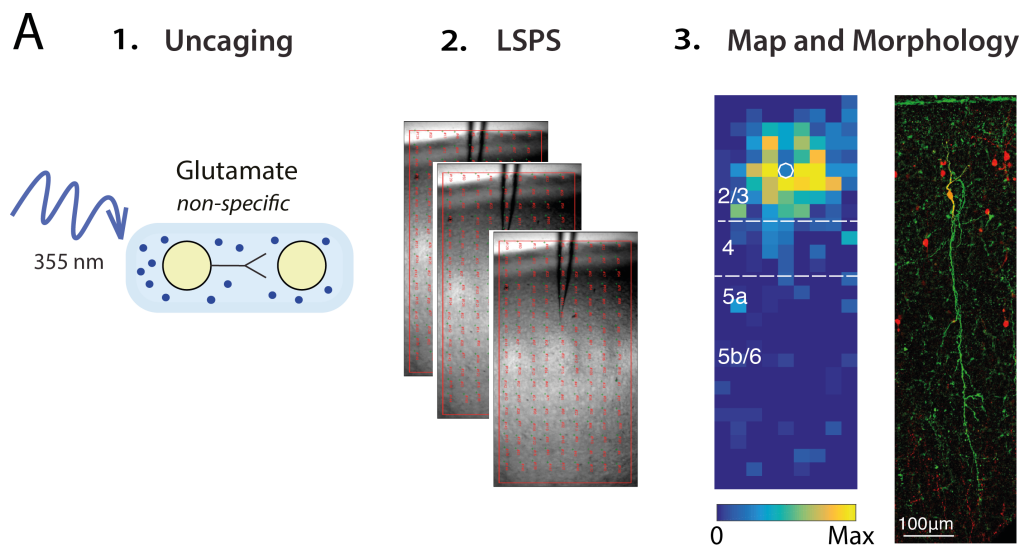


Figure 2.3 Key steps in laser scanning stimulation (LSPS) experiments. (A) 1 - Caged glutamate is activated by the UV laser and not-specifically triggers action potentials in the surrounding cells. 2 - The laser is fired at different positions (see LSPS grid in red) to uncage glutamate and activate different cells across the depth of the cortex, while signals are recorded through a patch pipette. 3- A connectivity map is obtained, and the morphology of the recorded cell is recovered (Red= tdTomato; green= Biocytin filling)

The LSPS stimulation grid consisted of 17x9 laser target points (850 x 450 μm), triggered in a pseudorandom pattern to prevent sequential stimulation of adjacent sites at 1Hz (Anastasiades and Butt, 2012; Shepherd et al., 2003). To

cover the entire depth of the cortex, 2-3 LSPS grid were run and properly aligned offline. LSPS grids at each locations were run multiple times, aiming to obtain a minimum of two-three repeat runs per cell without noticeable deterioration in the health of the cell. An IR-DIC photomicrograph of the LSPS grid relative to the patched cell was taken and used as a reference to reconstruct the pixel positions relative to the layer boundaries (Figure 2.3)

Analysis of LSPS data

Offline analysis of the current traces was performed adapting a customised Matlab script developed in the lab (Matlab2018a). Using a long duration, low intensity laser pulse to uncage glutamate leads to two kind of responses at -60 mV: (1) a short-latency, consistent, slow and large inward current, whose rise time is locked to the laser duration (i.e. 'direct response'), originating from the glutamate-mediated depolarisation of the recorded neuron (Figure 2.4B, traces in the first row) and (2) smaller excitatory post-synaptic currents (EPSCs) originating from connected surrounding pyramidal cells (Figure 2.4B, traces in the second and third row). In some cases, EPSCs ride on top of the direct response (Figure 2.4B, arrowheads on first row traces), therefore posing the question of how to automatically analyse these traces. A possibility is to exclude them completely, given the challenge of separating the two components (see for example Deng et al., 2017; Sheikh et al., 2019; Sturm et al., 2014).

Here, events were defined as deflections of the current traces exceeding for at least 3 standard deviations the baseline mean (i.e. a 100ms window before laser onset). Previously published criteria (Anastasiades and Butt, 2012) were used to determine the putative monosynaptic event detection window, defined

as a 200 ms long window starting 40 ms after the laser onset (Figure 2.4 B, grey shadow).

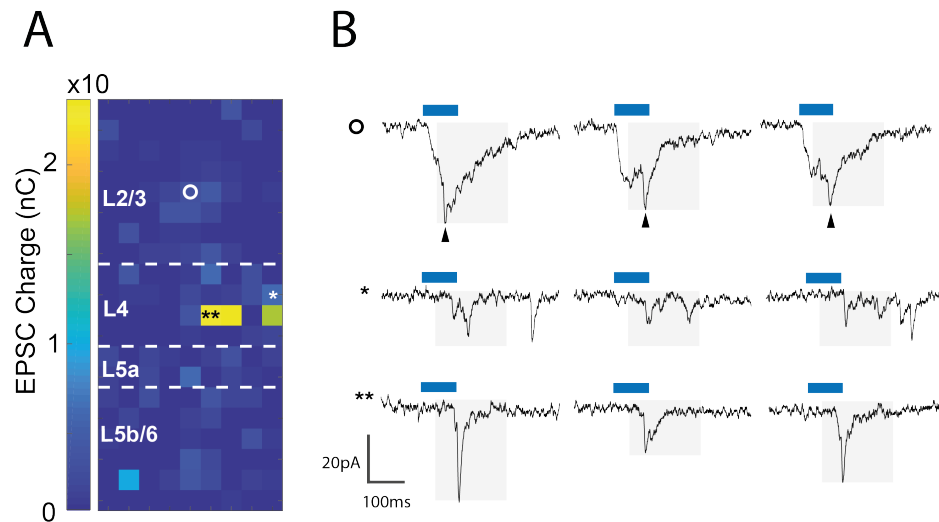


Figure 2.4 EPSCs originating from laser scanning stimulation (LSPS) experiments at -60mV. (A) Exemplary glutamatergic connectivity map obtained from a single cell as indicated in Figure 2.3. The circle represents the cell body position, the dotted lines the layer boundaries. * and ** are two distinct L4 pixels in the map. (B) Current traces from different positions in the map in A (3 repetitions per position). Circle: Glutamate uncaging at the cell body leads always to short-latency direct responses, often combined with EPSCs (arrowheads). * and **: direct responses cannot be observed when stimulating spots in L4. Also note the variability of elicited EPSCs in different trials. Grey = monosynaptic window; Blue= Laser pulse.

For events with onset within the monosynaptic window, the total charge in this window was extracted. Events with onsets before the monosynaptic window were considered to contain a direct-response component. In this case, the charge value was interpolated from all the other surrounding points using the Matlab function 'griddata' with linear interpolation (Weiler et al., 2018). This allowed to reduce overestimation of local connectivity caused by partially including the direct response. Figure 2.5 shows a comparison between maps built by manually identifying responses with the software Minianalysis 6.0 (Synaptosoft Inc.) and those built with automatic detection with interpolation (Figure 2.5A vs B).

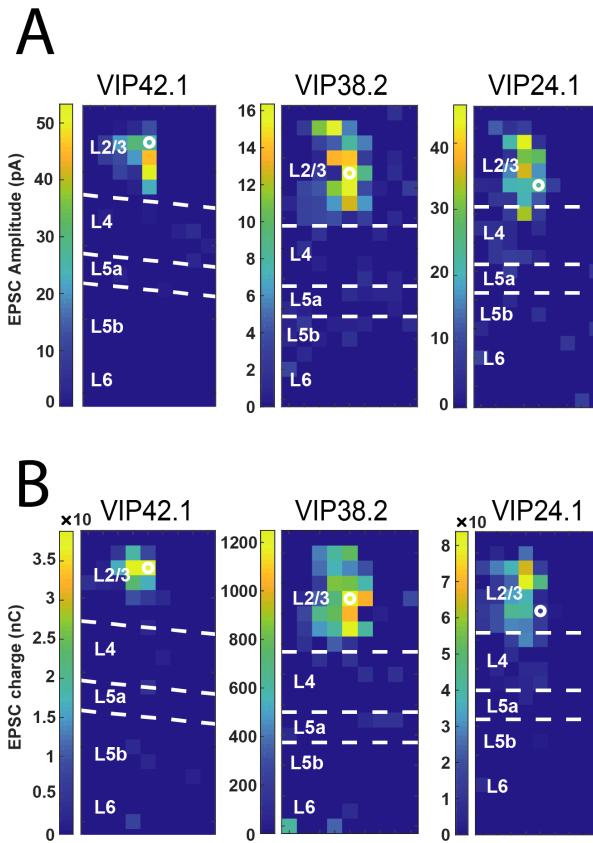


Figure 2.5 Comparison of maps obtained from manual and automatic detection of EPSCs. (A) Exemplary glutamatergic maps of VIP+ INs obtained following manual detection of EPSCs using the software Minianalysis (Synaptosoft). Raw values are presented in the maps and the input is expressed in pA. Each cell is identified by an ID. VIP42.1 = P7, VIP38.2 and VIP24.1=P9. (B) Same maps as in (A), but obtained with the custom-written automatic detection algorithm. Raw values are presented in the maps and the input is expressed in nC. Connectivity patterns do not change according to the method used (compare A vs B), an indication that the custom-written algorithm can be used reliably for EPSCs detection.

Charge values from different runs of the same map were then averaged. Map obtained across the depth of the cortical column were then combined to obtain a final map. For each cell, the absolute (pC/pixel) and normalised (%pC/pixel) afferent input was measured. Excitatory maps were presented as normalised maps: each pixel indicates the amplitude of the average evoked EPSCs expressed as a percentage of the overall input evoked across the extent of the LSPS grid. Laminar input profiles were evaluated by summing each

normalized input evoked from each horizontal row. Average maps were plotted aligning individual maps to the L4-L5a border.

A 2way-ANOVA was used to compare the raw input per layer across time points and across different layers, while single way ANOVAs were used to compare normalised inputs across development. Data was tested for normality with the D'Agostino-Pearson normality test and for equal variance. If they both passed, data was tested with a one-way analysis of variance (ANOVA), if not with a Kruskal–Wallis one-way ANOVA.

Cluster analysis and modelling of LSPS data

Principal component analysis (PCA) was performed on the normalised afferent input obtained for each cell. Following identification of the first three principal components, cluster analysis was performed employing k-means clustering, using squared Euclidean distance as the metric and a maximum number of 100 iterations. To determine the optimal number of clusters, silhouette analysis was performed.

R (R Core Team, 2019) and lmer (Bates et al., 2015) was used to perform a linear mixed effects model analysis to explain the variability in VIP+ INs LSPS data. Visual inspection of residual plots did not reveal any obvious deviation from homoscedasticity or normality. A likelihood ratio test was then used to verify the role of clusters in explaining VIP+ INs variability.

Optogenetic stimulation of VIP+ INs output

Activation of VIP+ INs somata and their terminals in the *VIP-Cre;Ai32* line was achieved by wide-field blue light stimulation (Cool-LED pE-100, 470 nm)

through a 40x objective (Olympus LUMPLFLN 40XW Objective). 10 ms square light pulses were delivered at multiple LED power intensity (0.082, 0.38, 0.7, 1.33, 2.83 and 5.52 mW/cm²). In each recording, stimulations were repeated five times every 20 seconds. This protocol allowed to test channelrhodopsin-2 (ChR-2) functionality and its working range during extracellular recordings of VIP+ INs somata. Moreover, in synaptic terminals stimulation, it ensured to elicit postsynaptic currents across multiple cell types and layers, given that VIP+ boutons density onto postsynaptic cells is both layer- and cell type- specific (Zhou et al., 2017). Post-synaptic cells were considered responsive only if light-evoked IPSCs were present in at least 2 out of 5 trials. The minimal stimulation, defined as the minimal LED power able to elicit at least 2 IPSCs in 5 trials, was calculated for each cell.

A customised Matlab script (Matlab2018a) was used to extract multiple features of the response, such as percentage of failure, average response transferred charge, average latency and latency jitter. In particular, latency was calculated as the time of the onset of the response starting to from the LED onset; while latency jitter was defined as the standard deviation of the latency across trials.

Optogenetic stimulation of anterior-motor afferents in S1BF

Activation of virally expressed channelrhodopsin either in the soma of anterior-motor pyramidal cells or in their terminals in S1BF was achieved as discussed above. In this case, 1ms and 10 ms blue light pulses were delivered at different LED intensities (0.082, 0.38, 0.7, 1.33, 2.83 and 5.52 mW/cm²), to ensure to find the minimal stimulation required to engage postsynaptic cells.

To confirm the monosynaptic nature of the responses and calculate the monosynaptic window, tetrodotoxin (TTX, 1 μ M) and 4-aminopyridine (4-AP, 100 μ M) were added to the bath solution (Petreanu et al., 2009; Suter and Shepherd, 2015; Yamawaki and Shepherd, 2015). TTX is a voltage-gated sodium channels blocker that prevents propagation of the action potential therefore blocking polysynaptic transmission. 4-AP blocks instead Kv1 voltage-activated potassium channels, which are critical for axon repolarization (Petreanu et al., 2009). Together, they ensure that polysynaptic transmission is abolished, while light-induced vesicles release from ChR-2+ terminals is facilitated by an increased depolarization at the terminal level.

Whenever EPSCs were not detected in L2/3 neurons, the presence of functional ChR-2+ terminals was confirmed by recording a L6 pyramidal cell, an internal positive control as demonstrated by the abundant presence of ChR-2+ fibres in L6 (See Chapter 4.1). Data analysis was performed as described above.

Neuronal Morphology: Staining and Image Acquisition

After the recordings, slices with neurobiotin-filled neurons were fixed overnight at 4°C with 4% PFA in PBS. In the majority of cases, after being washed in PBS, they were incubated with PBS-T (0.1M PBS + 0.2% Triton-X100) for 30 minutes at RT and then left overnight at 4°C in a solution of PBS-T and streptavidin Alexa Fluor® 488 or 568 conjugate (S11223 and S-11226 respectively, Molecular Probes, dilution 1:500).

To allow recovery of the morphology in ChR-2 injected *Prox1* animals, DAB (3,3'-Diaminobenzidine) staining was performed on these slices. After they were incubated with streptavidin conjugate as described above, slices were

washed in PBS and incubated with PBS-Triton (0.5%) for 20 min at RT. Following 3x5 minutes washings in PBS, slices were then incubated in 1% hydrogen peroxide (H₂O₂) in 75% methanol for 20min at RT, to quench the endogenous peroxidase. Slices were washed 5 minutes in PBS for three times and then incubated for 30min at RT in blocking solution, consisting of 5% Normal Goat Serum (NGS) in PBS. Next, they were incubated overnight at 4°C with byotinated goat anti-streptavidin antibody (Vectastain Ba-0500, ZD0113, 1:200) in blocking solution. After being washed in PBS for 3x10 minutes, slices were incubated with ABC (avidin/biotinylated enzyme complex) solution for 2 hours (Vectastain ABC Kit Elite PK6100 Standard). After being washed at least 3x10 minutes in PBS, slices were incubated with the enzyme substrate, the DAB solution with Nickel. The reaction was closely monitored and once developed as expected, slices were put in PBS or water to stop the reaction.

After completion of the either staining protocol, slices were mounted with Fluoromount™ aqueous mounting medium (Sigma) and sealed using nail polish. Images of recorded neurons were acquired with a Zeiss laser scanning confocal microscope (LSM710), using a UPLS APO 20X/0.75 objective at a pixel resolution of 1024 x 1024.

2.6 – In vivo electrophysiology

Electrode implantation

Mice of both sexes aged between P14 and P22 were anaesthetised with 10% urethane in PBS (Sigma), at different doses according to their age (1-1.5 g/kg for P≤15 and 2g/kg for P19-22). Subcutaneous administration of atropine

(600UI/kg, Atrocare 600 μ g/ml solution for injection, Atropine Sulphate) prevented breathing failure during the recordings.

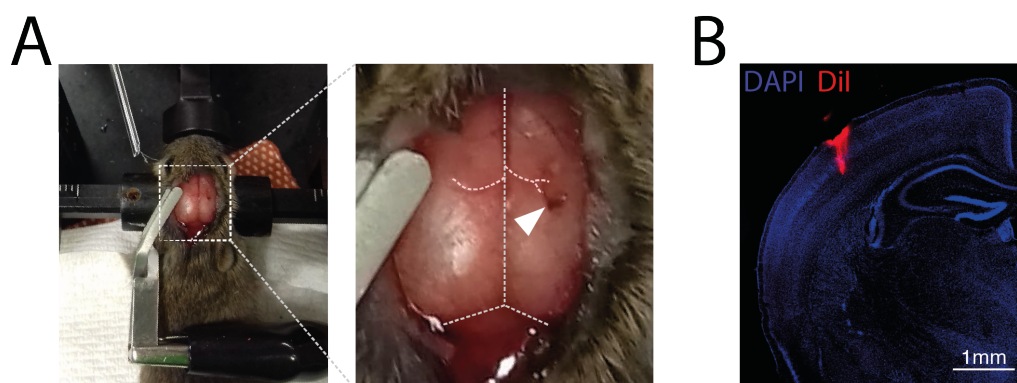


Figure 2.6 Key steps in electrode implantation. (A) After exposing the skull, a craniotomy of ~2mm was performed in order to insert the electrode in S1BF (see arrowhead in magnification) White dashed lines= sutures; pink dashed lines= vasculature (B) The position of the electrode was checked through *post-hoc* histological analysis. Blue = DAPI, Red= Dil. Scale bar=1mm.

Mice were then moved to the stereotaxic frame (Kopf, with Stoelting pup adaptor), their head was secured with ear cuffs, and the scalp was removed to expose the skull. To ensure that recording sites included L2/3 neurons, the following coordinates were used for S1BF at different time points, with lambda used as reference: P13-P14: AP 3.46 mm, ML 2.7 mm, DV -750 μ m; P20-P21: AP 3.30 mm, ML 3.125 mm, DV -750 μ m. Universal super-glue (Loctite) was used to harden the skull at the younger ages ($P \leq 15$), then a craniotomy of ~2mm in diameter was obtained with a drill (Volvere i7, NSK Gx35EM-B OBJ30013 and NSK VR-RB OBJ10007) equipped with a 0.5 mm tip. A 32-channel single shank electrode (A1x32-Poly2-5mm-50s-177, Neuronexus) was inserted in the brain.

To ensure *post-hoc* confirmation of the electrode location (see Figure 2.6), it was submerged in Dil (DiI_{C18}(3), 2.5 mg/ml, D282 Lot 1990320 in 70%

ethanol, Sigma) for 10-20 minutes before insertion. During the entire duration of the experiments, depth of anaesthesia and breathing were constantly monitored. The animal temperature was maintained with a heating pad (Watlow 025037500, 120 Volts, 46 Watts, 1JR 1529C-14) set at 37 °C.

29 mice were recorded in total, but 14 were excluded from the analysis for the following reasons: electrode misplacement (n=7), incomplete protocols (n=2), failure in assigning layers (n=1) or excessive noise level in the recordings due to a sub-optimal *in vivo* set up (i.e. lack of an air table and a Faraday cage) (n=4).

Experimental paradigm

A 20 min baseline was recorded few minutes after electrode insertion (Open Ephys acquisition board). A piezo stimulator (PB4NB2W Piezoelectric Bimorph Bending Actuator with Wires, Thorlabs) was used to perform the whisker pad stimulation protocol (1 s stimulation at 10 Hz with 20 s inter stimulus interval, for a total of 40-50 trials). To allow simultaneous stimulation of all the contralateral whiskers, they were glued together using a small amount of super-glue (Loctite), before placing the animal in the stereotaxic frame (Kopf). This stimulation protocol was chosen to mimic natural, rhythmic patterns of whisker activity (Cao et al., 2012; Mégevand et al., 2009). In case of excessive grooming of the animal and subsequent lack of whiskers, the stimulation was performed directly at the level of the whisker pad, with no apparent difference in response comparing to the rest of the animals.

Analysis of baseline activity

To analyse baseline activity, oscillations were detected as increase in amplitude of the root mean square (rms) of the band-pass filtered voltage signal (1.5-4 Hz for delta waves and 7-15 Hz for spindles) that exceeded a recording-specific threshold (Cichon et al., 2014). This threshold was calculated from a histogram of voltage filtered traces (divided in 0.1 μ V bins) built from the entire baseline recording (20min), starting from its onset. A double exponential ($y=ae^{bx}+ce^{dx}$) was then fitted to the voltage histogram and time points containing voltage values higher than the mean + 2 standard deviations (SD) of the fitted curve were considered oscillatory events (see Figure 2.7).

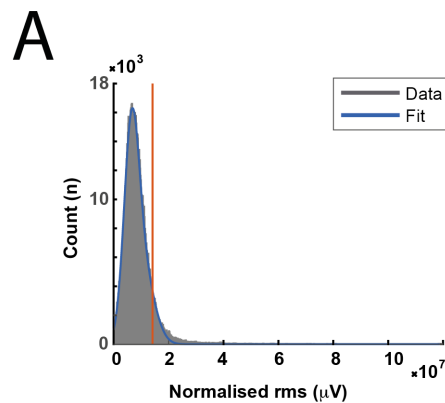


Figure 2.7 Strategy to identify oscillatory activity. (A) Representative histogram of root-mean-squared (rms) amplitude (grey) of recorded LFP during baseline. Blue curve= double exponential function fitted to the LFP values; red line= threshold for oscillatory events. All the voltage values higher than the threshold were considered oscillatory events.

To avoid fragmented detection of events, only events that contained at least 3 cycles were kept for further analysis. Manual definition of baseline levels confirmed the reliability of this method, with a low numbers of false positive

(Baruchin et al, *in preparation*). Recordings with more than 50% of the power in the noise frequency ($\sim 50\text{Hz}$) were excluded from further analysis.

Analysis of Local Field Potential

Local field potential (LFP) traces were first filtered with a 500 Hz low pass filter. To analyse baseline activity, LFP power spectra were computed using a fast Fourier transform focusing on frequencies up to 20Hz. To analyse whisker-evoked responses, the LFP response after the first whisker deflection was extracted, averaged across channels within the same layers (defined from the current-source density maps, see below) and analysed (See Figure 2.8). Amplitude was defined as the absolute difference in μV between the baseline and the peak of the LFP; latency as the time to peak from the whisker deflection, and half width was calculated as the width of the LFP response at half-maximal amplitude. Before any further analysis, ROUT outlier test ($Q=1\%$) was performed within each age, layer and genotype group (only if $n \geq 3$ per data points).

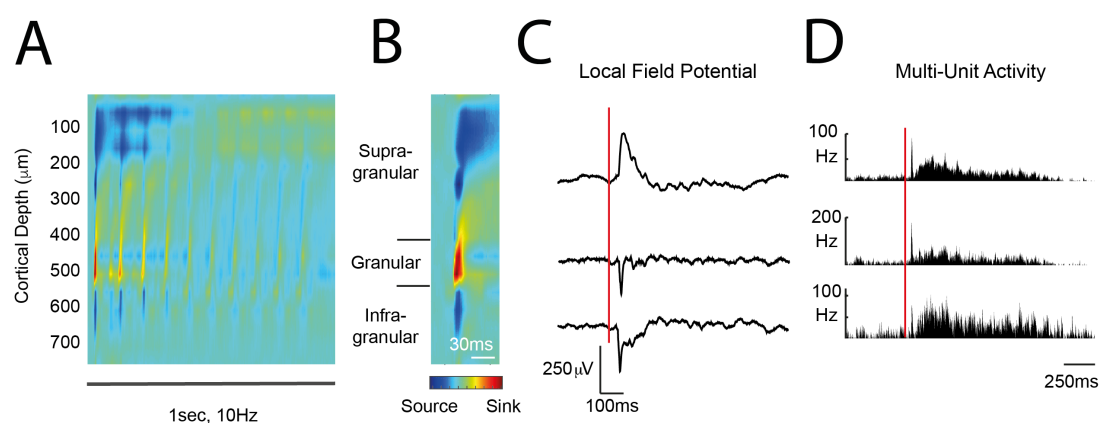


Figure 2.8 Representative recordings of neuronal activity following multi-whisker stimulation. (A) Representative current-source density (CDS) map across layers during the 10 Hz, 1 second stimulation (B) Magnification of (A) at the beginning of the stimulation. The granular layer is identified as a sink following whisker stimulation. Signal above and below is classified as deriving from supragranular and infragranular respectively.

Continues from previous page Representative Local Field Potential traces (C) and multi unit activity traces (D) following multi-whisker stimulation (indicated as red bar). CSD maps are visualised as pseudocolor images, with red representing sinks and blue indicating sources.

Current source density maps and layer assignment to recording sites

The cortical depth of each recording was evaluated using current-source density (CSD) maps, calculated as the second spatial derivative of the LFP averaged across all the trials. The first CSD sink evoked upon sensory stimulation was used to define the granular layer. Channels above and below that were assigned to supragranular and infragranular layers respectively (See Figure 2.8).

One recording was excluded from analysis because the CSD map did not show a clear sensory stimulation evoked sink, therefore it did not allow reliable layer assignment. No normalisation for differences in cortical thickness across development was applied neither the number of recording sites per cortical layer was kept constant for all age groups.

Spike detection and sorting

Prior to analysis, recordings were spike sorted with Kilosort (Pachitariu et al., 2016) and inspected in Phy (Rossant et al., 2016) to differentiate between multi-unit activity (MUA), single unit (SU) or noise.

Spike waveforms and correlograms were evaluated to ensure a proper classification, considering in particular: (1) shape of the waveform, its amplitude, jitter and reproducibility, (2) a refractory period in the autocorrelograms, present only in case of single units. On occasion, clusters were merged according to the following the criteria: (1) waveform similarity, (2) physical distance, (3) similarity coefficient, (4) autocorrelograms and crosscorrelograms similarity, (5)

overlap between the principal component analysis of the clusters, (6) change of waveform amplitude over time. Considering the low number of SU detected overall, analysis focused only on MUA.

Analysis of multiunit activity: detection of sensory evoked responses

To analyse whisker-evoked MUA, spike rate was averaged across trials for each MUA. Averaged MUAs identified within the same layer were then averaged together (See figure 2.8). The peak response was identified within a 50 ms window after the first whisker deflection as a response 3 times bigger than the standard deviation of the baseline activity (evaluated as the mean activity 50 ms before stimulation).

The following parameters were extracted for each layer: (1) Whisker-evoked firing rate, evaluated as the difference between firing rate at baseline and maximal firing rate after stimulation of all the layer-averaged MUAs, (2) latency to reach the maximum firing rate from the onset of the first whisker deflection and (3) half-width, i.e. the width of the response at half-maximal amplitude, that was used as a measure of the duration of the stimulus-triggered response.

Before any further analysis, ROUT outlier test (Q=1%) was performed within each age, layer and genotype group (only if $n \geq 3$ per data points).

Post hoc confirmation of electrode placement following in vivo recordings

After the recordings, animals were sacrificed and perfused as previously described (see Materials and Methods 2.3). The brain was extracted and kept for a maximum of 24h in 4% PFA in PBS for post-fixation. After embedding in 5% low gelling agarose (Sigma) in PBS, the brain was sliced at 80 μm and all the

slices were kept. To ensure a more correct localization of the probe, the slices were counterstained with DAPI (D3571, Molecular Probes, dilution 1:500-1:1000 in PBS) for 5-10 minutes. After 3 washes in PBS, slices were mounted on a slide, let dry for ~2 h, coverslipped using Fluoromount as mounting media and sealed with nail-varnish. Slices were routinely analysed at the wide-field fluorescent microscope (Leica DMR Trinocular Industrial Research Microscope) equipped with a Leica DFC500 3.2 digital camera (Leica Microsystems). Representative pictures were taken at the confocal microscope (Zeiss 800 Airyscan, equipped with a 10x 0.45NA Plan-APOCHROMAT (Zeiss) objective) (Figure 2.6)

2.7 – Statistical Analysis

All results are expressed as mean $\pm$ standard deviation of the mean; n indicates the number of cells recorded in each independent experiment, unless otherwise stated. Statistical analysis was performed mainly with GraphPad Prism version 8.0 (GraphPad).

To compare proportions of responsive cells between two datasets, the Fisher's exact test was employed. Chi-square analysis was used to analyse cumulative distributions of responsive cells at different LED powers, while log-linear analysis (a version of chi-square analysis) was applied to compare proportions of responsive cells between multiple datasets. These analyses were performed using online programs (VassarStat, US).

Continuous data were assessed for normality with the D'Agostino-Pearson normality test and for equal variance, in order to apply the appropriate

parametric and not-parametric tests. Statistical significance was evaluated at $p \leq 0.05$. Precise p values are reported in the text. Details of all the tests performed are available in Appendix 1-3 (relative to Chapters 3-5).

Chapter 3 – Integration of VIP+ INs in the local circuitry

Previous work has determined the distribution of VIP+ INs in the juvenile and adult cortex, together with their morphological, electrophysiological and molecular properties (Butt et al., 2005; Emson et al., 1979; Frazer et al., 2017; Goff and Goldberg, 2019; Götz and Bolz, 1989; Lee et al., 2010; McDonald et al., 1982; Miyoshi et al., 2010; Paul et al., 2017; Prönneke et al., 2015, 2018; Taniguchi et al., 2011; Tasic et al., 2016, 2018; Vucurovic et al., 2010; Zeisel et al., 2015); however, the emergence of VIP+ INs circuitry and their role in neonatal cortex is still unknown. To gain a better understanding of the role of VIP+ cells in neonates, the recently developed *VIP-Cre* reporter line was employed (Taniguchi et al., 2011). I characterised the distribution of VIP+ INs in primary somatosensory whisker barrel cortex (S1BF), their electrophysiological properties, their recruitment from the local glutamatergic network, and their output to local INs and pyramidal cells during the first two postnatal weeks.

3.1 – Distribution of VIP+ cells in early cortex and their maturation

3.1.1 – Confirmation of basic VIP+ INs molecular markers in the Vip-Cre;Ai9 line

In order to effectively target VIP+ INs in the neonatal cerebral cortex, the previously described *Vip-Cre;Ai9* (tdTomato) mouse line was employed (Goff and Goldberg, 2019; Proenneke et al., 2018; Pröenneke et al., 2015; Taniguchi et al., 2011). *Vip* mRNAs can be detected in developing INs as early as E18.5 (Frazer et al., 2017; Mayer et al., 2018), an age when it is possible to identify very few tdTomato+ cells in *Vip-Cre;Ai9* animals (Miyoshi et al., 2015). Previous literature suggests that *Vip-Cre* mediated tdTomato expression can be reliably observed between P0 and P7 in developing INs (Miyoshi et al., 2015; Taniguchi et al., 2011). Here, I used immunohistochemistry to confirm the co-expression of tdTomato and VIP in P21 *Vip-Cre;Ai9* animals (Figure 3.1A and 3.1B, upper panel). Although the sample size precluded statistical analysis, I could observe that sporadically (i.e. less than 1 cell per field of view) tdTomato did not co-localise with VIP (Figure 3.1B, arrowhead in upper panel). Yet, all the VIP+ cells detected in this test experiment also expressed tdTomato.

I also confirmed the presence of the endogenous calcium-binding protein calretinin (CR), another marker co-expressed in a portion VIP+ INs (Butt et al., 2005; Engelhardt et al., 2007; Miyoshi et al., 2010; Porter et al., 1998; Vucurovic et al., 2010). Even though statistical analysis could not be performed due to small sample size, both calretinin (CR)+ and CR-negative/tdTomato+ cells were

observed in this test experiment, indicating that the diversity of VIP+ INs could be captured by the *VIP-Cre;Ai9* line (Figure 3.1B, lower panel).

Thus, I confirmed that this genetic line labels VIP+ INs and captures their diversity, as previously published (Prönneke et al., 2015). Therefore, tdTomato+ cells could be considered VIP+ INs.

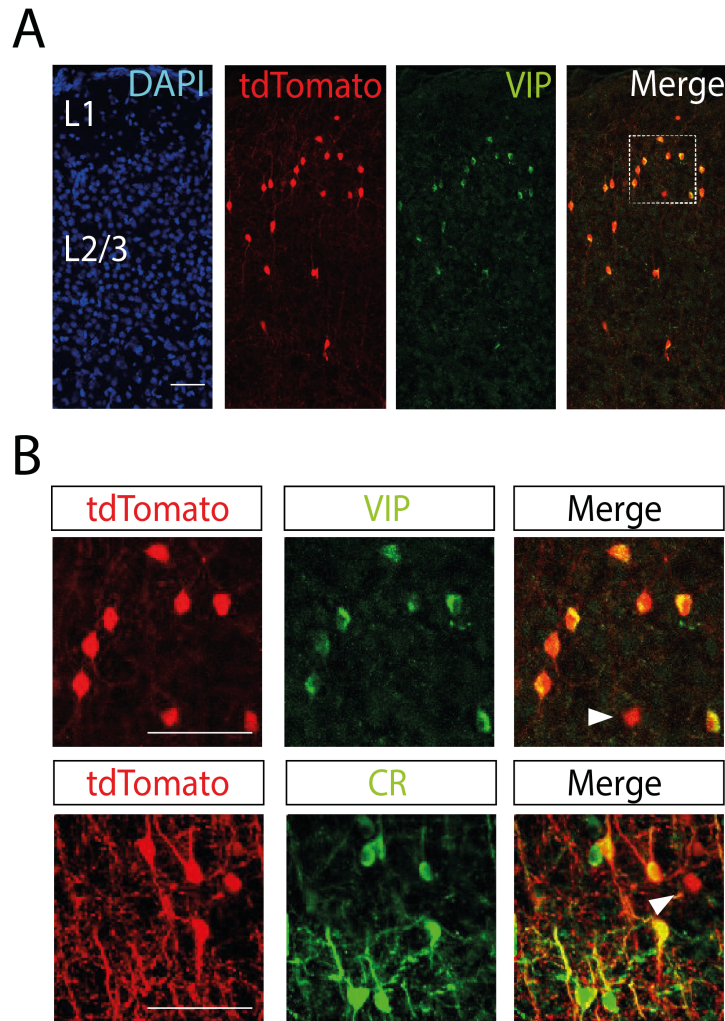


Figure 3.1 The *VIP-Cre;Ai9* line expresses vasoactive intestinal peptide and calretinin. (A) Representative images of supragranular layers in P21 *VIP-Cre;Ai9* animals obtained from 20-25 μ m cryosections. Blue: DAPI, red: tdTomato, green: VIP. Scale bar = 50 μ m. The dotted box represent the area magnified in (B), top panel. (B) Representative magnified images of supragranular layers in *VIP-Cre;Ai9* animals. The top panel is a magnified image from the dashed box in (A). In this test experiment, all VIP+ cells express tdTomato, but not *vice-versa*. In few cases, I found tdTomato+ cells that were not VIP+ (white arrow head in the merge panel). Red: tdTomato, green: VIP. Scale bar = 50 μ m. In the bottom panel, representative magnified image showing tdTomato and calretinin (CR) staining in P21 *VIP-Cre;Ai9* animals obtained from 50 μ m floating section. Note that not all the VIP+ cells were also CR+ (white arrow head in the merge panel). Red: tdTomato, green: CR. Scale bar = 50 μ m. n=1 animal per combination of stainings.

3.1.2 – Quantification of VIP+ INs distribution across development

I next quantified the distribution of VIP+ cells across the depth of the cortex through postnatal development (P1, P3, P7-8, P14-15 and P21) in S1BF (Figure 3.2A). Cortical expansion and layer maturation characterise the first postnatal week in S1BF (Rice and Loos, 1977): in particular, L5 and L6 are already present between P1 and P3, but L2/3 and 4 cannot be distinguished yet and thus are still referred to as ‘cortical plate’ (CP) (Figure 3.2A, P1-P3). Barrels become visible between P5 and P7 (Rice et al., 1985) and therefore L2/3 and 4 can be reliably distinguished in DAPI counterstaining profiles.

To compare across all the data points, I analysed the normalised distribution of cells in a layer independent manner (Figure 3.2B) and confirmed that the majority of VIP+ INs localised superficially already at P1. No significant differences were found in normalised cell distribution at any developmental point (Mixed effect linear model analysis, see Appendix 1 with details).

I then quantified the overall number of cells in a predefined region of interest (ROI) in S1BF (see Chapter 2 – Materials and Methods) and normalised it as cells/mm³ in order to compare across time points. A significant difference in the number of VIP+ INs over time was observed (Mixed liner effect analysis, P1: 1703±535; P3: 2824±1495; P7-8: 7062±672; P14-15: 7247±959; P21: 7457±577 cells/mm³, $p<0.0001$), in particular between P1-3 and P7-21 (Figure 3.2C, see Appendix 1 with details of the Tukey’s multiple comparisons test).

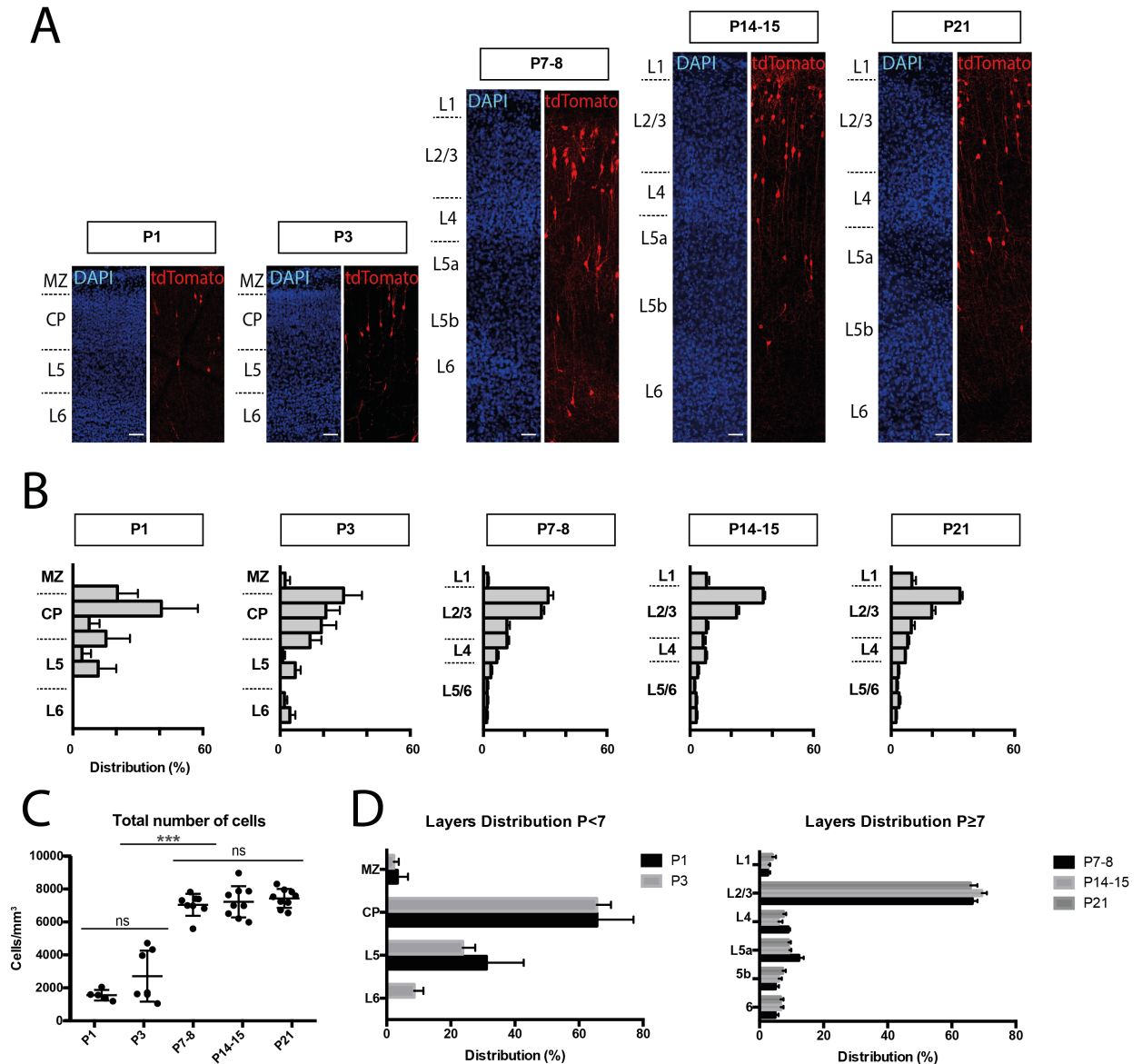


Figure 3.2 Quantification of tdTomato positive cells in the *VIP-Cre;Ai9* line. (A) Representative images of cortical columns from the *VIP-Cre;Ai9* line at different postnatal days (P), namely P1, P3, P7-8, P14-15 and P21. Blue: DAPI, red: tdTomato. Scale bar = 50 μ m. MZ: marginal zone, CP: cortical plate. L: layer. (B) Normalised distribution of tdTomato+ cells across the cortical depth from the pial surface to the white matter tract at each time point. Bars represent the mean, error bars the standard deviation. The majority of tdTomato+ cells is localised superficially already at P1 without changes across development. (C) Overall number of tdTomato+ cells across time points indicated in cells/mm³. Lines represent the mean, error bars the standard deviation. Density of tdTomato+ cells increases during development. (D) Normalised distribution of tdTomato+ cells across layers at P<7 and P≥7. Bars represent the mean, error bars the standard deviation. Layer distribution of tdTomato+ cells does not change in either developmental group.

P1: 6 counts from 2 animals, P3: 8 counts from 3 animals, P7-8: 8 counts from 3 animals, P14-15: 9 counts from 3 animals, P21: 9 counts from 3 animals. ***= $p \leq 0.001$

Finally, to compare the distribution of VIP+ INs in a layer specific manner, I divided the time windows analysed in two subset: $P < 7$ (i.e. P1 and P3) and $P \geq 7$ (P7-P21). No differences in layer distribution were observed in either of the subset identified (Figure 3.2D left and right panels, respectively. Details in Appendix 1).

This data could suggest either that VIP+ INs are still migrating to the cortex up to the end of the first postnatal week (Miyoshi and Fishell, 2011; Miyoshi et al., 2010; Taniguchi et al., 2011) or that the tdTomato reporter under the control of the *Vip-Cre* promoter is fully expressed only by the end of the first postnatal week (Miyoshi et al., 2015). The overall laminar distribution of VIP+ INs does not change throughout the first three postnatal weeks.

3.1.3 – Characterisation of intrinsic electrophysiological properties in developing VIP+ INs

In parallel with the histological analysis, I investigated intrinsic electrophysiological profiles of VIP+ INs through postnatal development using *in vitro* whole-cell patch-clamp ($n = 52$ cells, from P5 to P15). To be able to capture the majority of VIP+ INs and avoid sampling bias potentially due to low tdTomato expression, I focused my analysis on L2/3 VIP+ INs from P5 onwards.

I binned intrinsic properties according to three developmental age groups: P5-P8, P9-11 and P12-P15 (See Table 3.1, Figure 3.3 and Figure 3.4). These windows cover key steps in S1BF development: the critical period plasticity for L4 (Fox, 1992), the maturation of the L4 to L2/3 connections (Bureau et al., 2004; Lendvai et al., 2000; Maravall et al., 2004a, 2004b; Shoykhet

et al., 2005; Stern et al., 2001) and the critical period for horizontal connections between L2/3 neurons (Wen and Barth, 2011; Anastasiades et al., 2016; Erzurumlu and Gaspar, 2012).

Electrophysiological properties are the result of combination of ion channels on the cell membrane, cell size, morphology and synaptic integration (Kandel et al., 2013; Llinas, 1988; Mainen and Sejnowski, 1996). I measured both passive and active properties of VIP+ INs, to describe how these cells behave upon negative and positive charge injection, respectively (See Table 3.1, Figure 3.3 and 3.4).

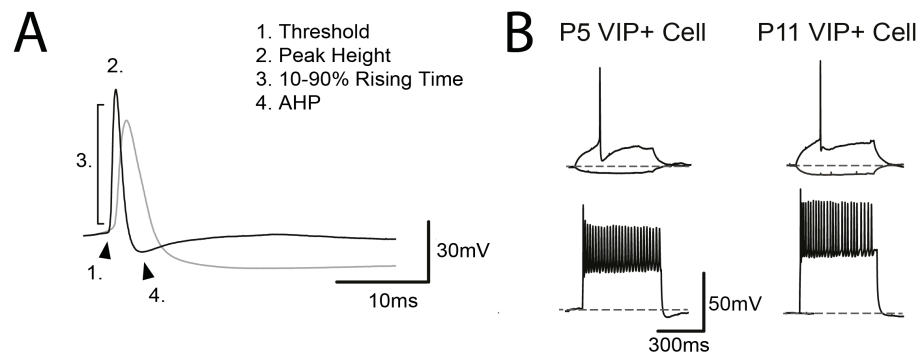


Figure 3.3 Representative electrophysiological traces recorded in developing VIP+ INs (A) Action potential in young (P5, grey) and old (P11, black) VIP+ INs. Basic parameters (1-4) are indicated on the older trace as reference (B) Spiking patterns of representative young (P5) and old (P11) VIP+ INs.

Age	RMP (mV)	Input Resistance (MΩ)	Membrane constant (tau) (ms)	Rheobase (pA)	Spike							Max. firing frequency (Hz)	Adaptation in firing frequency (%)
					Threshold (mV)	Spike delay at Rheobase (ms)	Height (mV)	Half width (ms)	10-90% time (ms)	AHP Amplitude (mV)	AHP delay (ms)		
P5-P8 (n=26)	-57±7	1598±759	74±17	13±8	-32±7	173.8±88.8	45±8	2.8±0.9	1.1±0.3	18±5	23.0±10.1	38±15	27±25
P9- P11 (n=12)	-58±7	949±480	52±26	22±10	-34±4	198.1±130.8	57±14	2.1±0.7	0.8±0.3	17±4	19.3±9.9	41±15	27±26
P12+ (n=14)	-61±5	667±227	41±18	34±21	-35±3	238.6±151.1	54±13	1.7±0.5	0.6±0.2	12±5	12.9±11.9	50±18	51±17

* vs P5-P8
 ** vs P5-P8
 *** vs P5-P8
 **** vs P5-P8

Table 3.1 Intrinsic properties of VIP+ INs across development. All data is reported as mean ± standard deviation. RMP: Resting membrane potential. 10-90% time: time from the 10% to the 90% of the action potential height. AHP: after hyperpolarization. Multiple comparisons corrected results following one-way ANOVA or Kruskal Wallis one-way ANOVA are color-coded (see legend). See Figure 3.4 for visual representation of parameters.

Passive properties such as input resistance and membrane time constant significantly decreased over time; active properties such as rheobase, spike height and percentage of adaptation significantly increased over time, while spike half width, 10-90% rising time, and after hyperpolarization amplitude (AHP) decreased (see colour coding in the Table 3.1 for p values and detailed statistics in Appendix 1). Overall, the observed changes in active and passive properties agreed with an acquisition of mature electrophysiological properties by VIP+ INs by the end of the second postnatal week. Intriguingly, high variability could be observed at P12-P15 in active properties such as rheobase, AHP amplitude and AHP delay. This raises the question whether the recently identified electrophysiological subtypes of L2/3 VIP+ INs (Prönneke et al., 2018) could be already distinguished at this time point (Figure 3.4). However, further analysis is needed to confirm this hypothesis. Overall, these data show the maturation of VIP+ INs across the first two postnatal weeks.

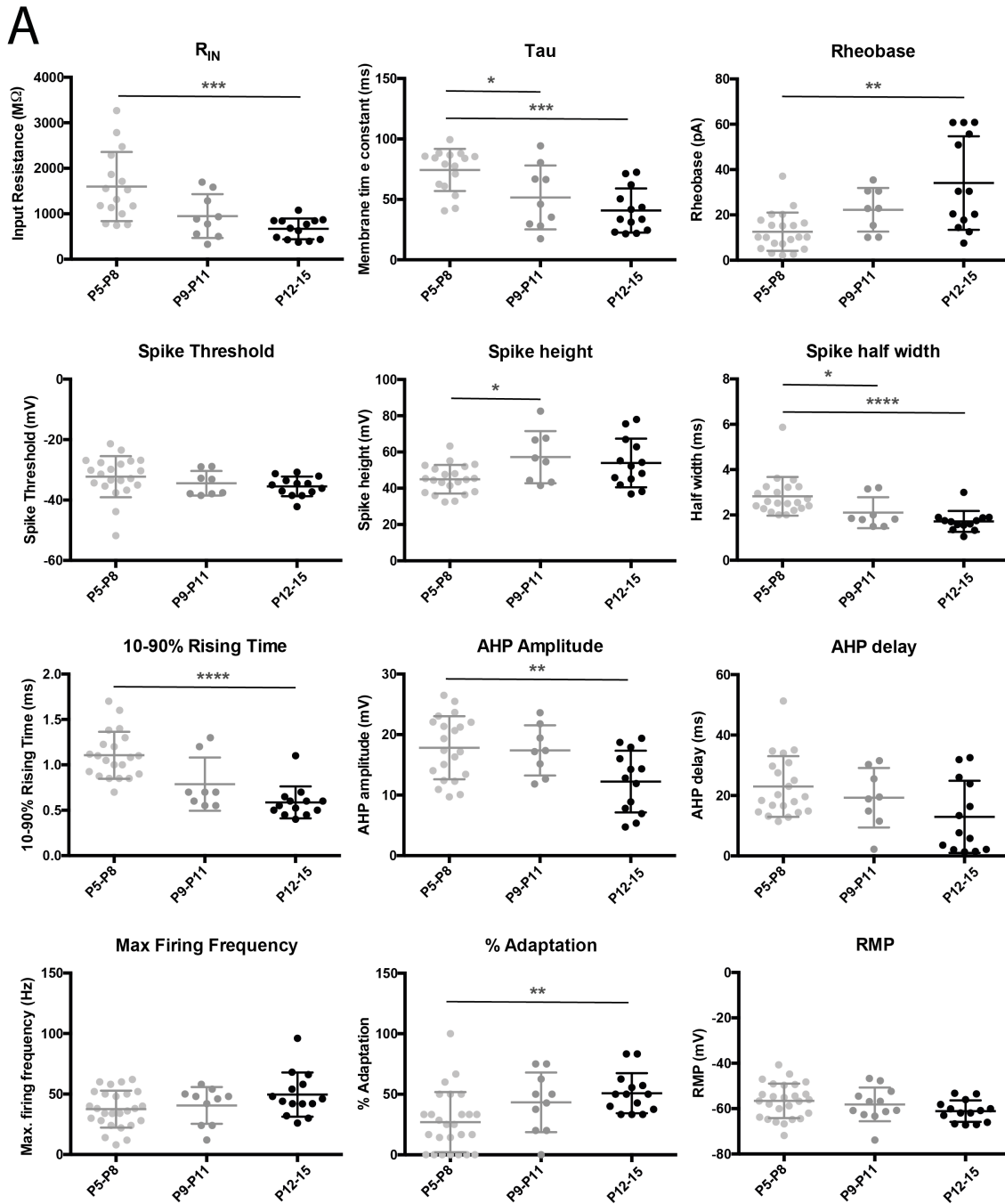


Figure 3.4 Intrinsic properties of VIP+ INs across development. This figure represents some of the data reported in Table 3.1. Single dots represent single data points, horizontal lines the mean and error bars the standard deviation. Multiple comparisons corrected results following one-way ANOVA or Kruskal Wallis one-way ANOVA are reported as follows *: $p \leq 0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$; ****: $p \leq 0.0001$. Full details of statistical analysis in Appendix 1. Changes in passive and active properties demonstrate the progressive development of VIP+ INs during the first two postnatal weeks.

3.1.4 – Discussion and Limitations

In this first section, I confirmed that the *Vip-Cre;Ai9* mouse is a valid mouse line to investigate VIP+ INs in neonatal mice, as previously published for older animals (Goff and Goldberg, 2019; Proenneke et al., 2018; Prönneke et al., 2015; Taniguchi et al., 2011). In this model, it was possible to observe both CR+ and CR-negative VIP+ cells, identifying two previously characterised subtypes of VIP+ INs (Butt et al., 2005; Engelhardt et al., 2007; Miyoshi et al., 2010; Porter et al., 1998; Vucurovic et al., 2010).

The observation of odd VIP-negative/tdTomato+ cells could be explained by the different cellular localization of VIP and tdTomato: the former localises in the Golgi apparatus (Järvi et al., 1989), the latter in the cytosol. Therefore VIP-negative/tdTomato+ cells could simply be VIP+/tdTomato+ cells lacking the Golgi apparatus due to the slicing process, rather than the result of recombination of the tdTomato reporter not mediated by the Cre recombinase.

Nevertheless, my data appears to agree with what has been reported in the literature. In the previous characterization of the *VIP-Cre;Ai9* line, Prönneke et al. (2015) found that 88.9% of tdTomato+ cells co-localised with *in situ* hybridisation probes targeting *Vip* RNA; while 99.1% of these cells co-localised with VIP antibody staining, demonstrating that this line is a reliable model to track VIP+ INs in the adult brain.

The results presented here confirmed that VIP+ INs are preferentially located in superficial layers already at early developmental time points, as previously described (Taniguchi et al., 2011). A developmental increase in the total number of VIP+ INs in S1BF was observed, suggesting either a late

migration of these cells to the cortex or a delayed expression of tdTomato in some VIP+ IN subtypes. Although these results did not allow to confirm either hypothesis, previous work has demonstrated that VIP+ INs migrate to the cortex postnatally and find their final location from P7-8 onwards (Miyoshi and Fishell, 2011; Taniguchi et al., 2011). While VIP+ INs were identified here as early as P1, seminal work using immunohistochemistry in the developing rat visual cortex reported the presence of cortical VIP+ cells only from the end of the first postnatal week (McDonald et al., 1982; McGregor et al., 1982). This could be due to methodological differences or differential brain maturation timelines in the two models (Clancy et al., 2001; Pressler and Auvin, 2013).

The total amount of VIP+ INs across the depth of the cortex identified here is not in agreement with what showed by Prönneke et al. (2015). The authors reported ~ 2500 tdTomato+ cells/mm³, almost a third of what quantified at P ≥ 7 . There are a number of plausible reasons for this apparent discrepancy, such as differences in methodologies: while I used an antibody to enhance the tdTomato fluorescence, Prönneke et al. (2015) evaluated the native fluorescence of tdTomato cells, therefore tissue quenching cannot be totally excluded in their preparation. Moreover, the authors analysed brains of 20–30 weeks old animals, while I focused on the first three postnatal weeks. Mice are considered ‘mature adults’ between 3 and 6 months (i.e. 12 to 24 weeks) (Flurkey et al., 2007). After that, their maturation progresses really fast: at 10–14 months they are considered ‘middle aged’, between 18–24 months they become ‘old’ (Flurkey et al., 2007). Considering the fast maturation occurring after 24 weeks, cell death and other age dependent changes in the brain cannot be excluded (Flurkey et al., 2007). An age-related decrease of VIP+ INs has been previously identified,

although only in the rat suprachiasmatic nucleus of 32-33 months old animals (Chee et al., 1988). Radioimmunoassays performed in the rat brain from P21 to adulthood ($\sim P>56$) identified a peak in VIP content between P28 and P42, followed by a decline from P42 to adulthood (Emson et al., 1979), suggesting that age-related changes may happen already in mature adults.

Finally, I showed the gradual maturation of VIP+ INs across the first two postnatal weeks by analysing their electrophysiological properties. A decrease of both input resistance and membrane time constant was observed over time, together with an increase in rheobase, spike height and percentage of adaptation. This was mirrored by a decrease of spike half width, 10-90% rising time, and after hyperpolarization amplitude (AHP). These changes are in line with previous work characterising the maturation of developing neurons (Anastasiades et al., 2016; Ehrlich et al., 2012; Kroon et al., 2019; McCormick and Prince, 1987; Nomura et al., 2017; Zhang, 2004; Zhou and Hablitz, 1996).

A developmental decrease in input resistance may be associated with an increase in soma size and a more complex dendritic arbour – morphological changes that have been previously observed in developing VIP+ cells (Götz and Bolz, 1989; McDonald et al., 1982; McGregor et al., 1982). Moreover, it could be a mechanism to reduce the responsiveness to synaptic input and therefore homeostatically compensate for increasing synaptic strength (Ehrlich et al., 2012; Zhang, 2004). The parallel decrease in membrane time constant may be due to insertion in the membrane of ion channels open at the cell resting membrane potential, such as ‘leak’ channels (McCormick and Prince, 1987). The fact that younger VIP+ INs have larger membrane time constants implies slower voltage responses following synaptic input, and therefore promotes temporal

summation of inputs (Ehrlich et al., 2012). This would allow their temporal integration in an excitatory network dominated by slower synaptic inputs, such as the developing cortex in the first postnatal week (Kim et al., 1995; Zhang, 2004).

The changes in active properties reported are associated with spiking maturation and suggest changes in ion channel expression. In particular, the age-effect on spike height, spike half-width and 10-90% rising time suggests a developmental up-regulation of voltage-dependent sodium and potassium conductances, as previously reported for excitatory cells (Huguenard et al., 1988; Picken Bahrey and Moody, 2003; Zhang, 2004). Many channels could underlie the AHP amplitude changes here reported (see for review Robinson and Siegelbaum, 2003), but the specific conductances present in VIP+ INs have not been described yet.

Recent papers characterised electrophysiological properties of adult VIP+ INs by employing the *VIP-Cre;Ai9* line (Goff and Goldberg, 2019; Prönneke et al., 2015, 2018). The values identified by the authors suggest that VIP+ INs complete their maturation in the third postnatal week, without further changes (Goff and Goldberg, 2019). The results reported here characterise the onset of VIP+ INs maturation in the *VIP-Cre;Ai9* line during the first two postnatal weeks, extending the current understanding about the timeline of the development of their electrophysiological properties (Goff and Goldberg, 2019; Prönneke et al., 2015, 2018).

3.2 - Development of glutamatergic afferent input onto L2/3

VIP+ interneurons

Intrinsic properties are a tool to help elucidate neuronal function, as they provide information about how each neuron transforms its input into its output (Connors and Gutnick, 1990). However, to have a more complete understanding of a neuron's role in the network, it is necessary to capture the overall information flow, from input to output.

Optical stimulation techniques allow investigating local and long-range input-output relationships (Anastasiades et al., 2016, 2018; Ashby and Isaac, 2011; Dalva and Katz, 1994; Dantzker and Callaway, 2000; Petreanu et al., 2007, 2009; Shepherd et al., 2005). Laser scanning photostimulation (LSPS) combined with photolysis of caged compounds is an established method to investigate local IN connectivity, revealing connections that are preserved in a reduced *in vitro* preparation (Anastasiades et al., 2016, 2018; Apicella et al., 2012; Fino and Yuste, 2011; Marques-Smith et al., 2016; Packer and Yuste, 2011; Xu and Callaway, 2009). Moreover, LSPS has helped unravel the dynamics of developing networks (Anastasiades and Butt, 2012; Anastasiades et al., 2016; Ashby and Isaac, 2011; Deng et al., 2017; Marques-Smith et al., 2016; Sheikh et al., 2019; Sturm et al., 2014). To complete the functional characterisation of VIP+ INs during development, I set out to study their local glutamatergic input performing LSPS during the two first postnatal weeks.

3.2.1 – Analysis of local glutamatergic connectivity with LSPS

To map local glutamatergic inputs to VIP+ INs in S1BF, I performed whole-cell patch-clamp combined with LSPS of caged glutamate, focusing on the previously described developmental windows: P5-P8, P9-P11 and P12-P15 (Figure 3.5A).

For each cell, I measured the absolute (pC/pixel) and normalised (%pC/pixel) afferent input. I then identified its vertical input profile, overall input, and absolute and normalised input components from each layer (Figure 3.5B-D). Average normalized heatmaps obtained from LSPS experiments at the three developmental windows are shown in Figure 3.5A.

The total amount of glutamatergic input onto VIP+ INs did not change across development (Figure 3.5C, left panel, Kruskal–Wallis one way ANOVA, P5-8: 255 ± 411 nC, P9-11: 376 ± 280 nC, P12-16: 313 ± 217 nC; $p=0.24$). Local L2/3 input represented the principal source of excitation to VIP+ INs at any time point (Figure 3.5B and C, right panel; two-way ANOVA, layer effect: $F(3,84) = 14.17$, $p \leq 0.0001$; *post-hoc* multiple comparisons with Tukey's correction: L2/3 vs L4 $p \leq 0.01$; L2/3 vs L5a $p \leq 0.0001$; L2/3 vs L5b/6 $p \leq 0.0001$, see Appendix 1 for details).

However, while at P5-8 L2/3 VIP+ INs received mainly local glutamatergic input, from P9 onwards inputs from other layers started to appear (Figure 3.5A-B). In particular, a significant decrease of local input could be observed between P5-8 and P12-16 (Figure 3.5D; one way-ANOVA, $F(2, 29) = 3.359$, $p=0.0487$; Tukey's multiple comparisons test, $p \leq 0.01$). This reduction in the normalised input component from L2/3 suggests a developmental increase of glutamatergic input from granular and infragranular layers, mirroring the

gradual network integration of VIP+ INs suggested by the maturation of their intrinsic properties.

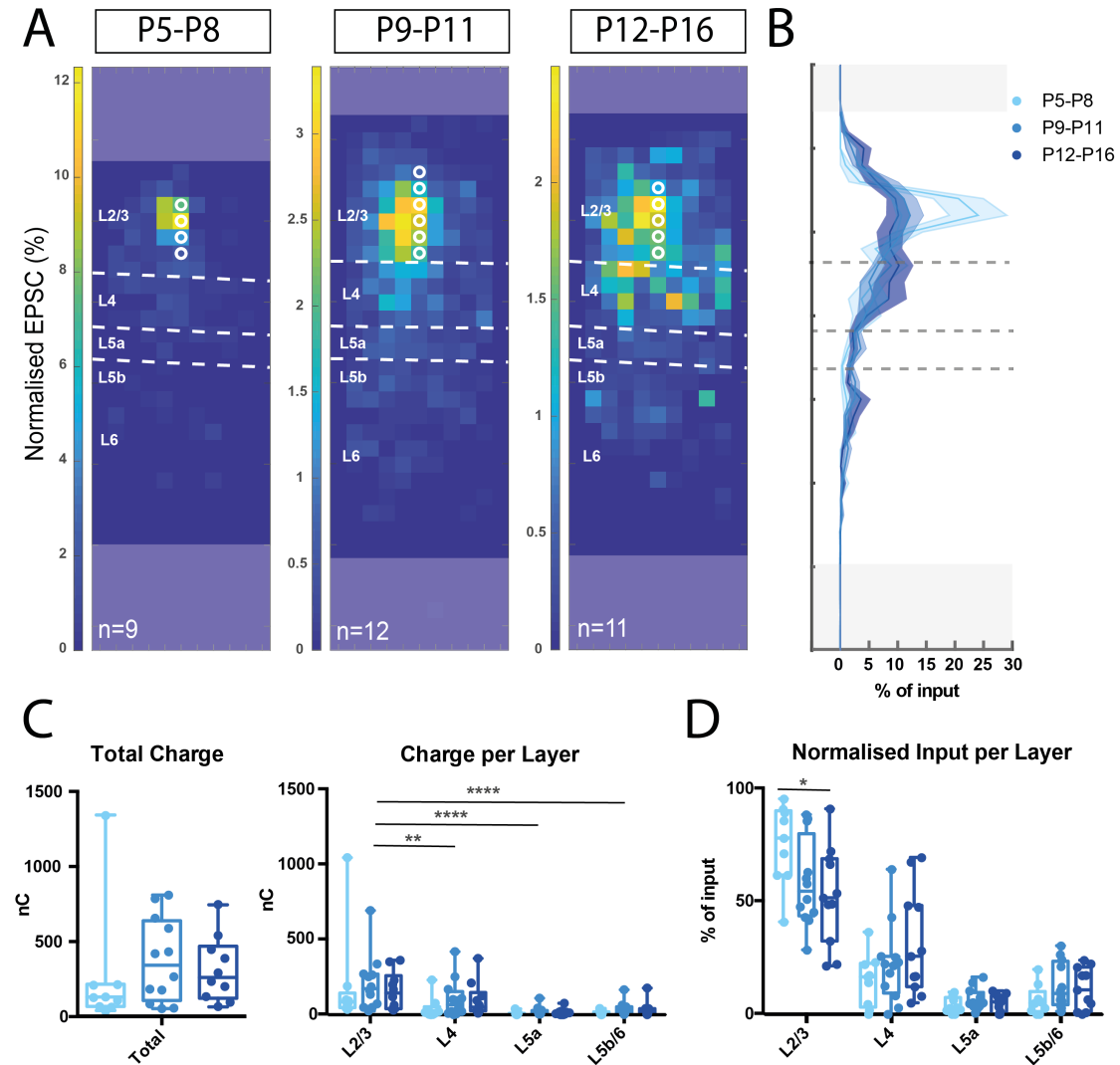


Figure 3.5 LSPS of caged glutamate reveals the laminar organisation of glutamatergic synaptic inputs onto L2/3 VIP+ INs during development. (A) Average normalised glutamatergic inputs onto L2/3 VIP+ INs across the developmental time points analysed. Within each age group, maps of individual cells were aligned by the L4/5a border. White circle: cell position. When two or more cells occupy the same position, only one circle is shown for simplicity. Dashed white lines: average layer boundaries. Transparency: areas outside the cortical depth. (B) Average normalised laminar profiles of L2/3 VIP+ INs across development. (C) Left panel: Total LSPS-evoked glutamatergic synaptic input onto L2/3 VIP+ INs across development. Boxplots: circles=single data points; horizontal line=mean of the data, box=standard deviation; error bars = spread of the data. Right panel: Laminar distribution of glutamatergic synaptic input onto L2/3 VIP+ INs across development. (D) Normalised laminar distribution of glutamatergic inputs onto L2/3 VIP+ INs. L2/3 pyramidal cells provide the majority of glutamatergic input onto L2/3 VIP+ INs (A,B,C), but their contribution to the overall normalised input decreases by P12-16 (D)

*, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$

Although analysis of average maps allows to understand trends occurring at a population level, it can also hide individual differences, especially when using the *VIP-Cre* line, which captures a heterogeneous population of cells (see Figure 3.1). Previous efforts to classify VIP+ INs employed a combination of electrophysiological properties, anatomical features and expression of molecular markers (Cauli et al., 2014; He et al., 2016; Kubota and Kawaguchi, 1997; Porter et al., 1998; Prönneke et al., 2015, 2018). However, identifying specific subtypes of VIP+ INs has been challenging, considering their high heterogeneity (Rhomberg et al., 2018; Tasic et al., 2016, 2018; Tremblay et al., 2016). Diversity of afferent input reflects INs subtype diversity (Anastasiades et al., 2016; Naka et al., 2019). Therefore, local glutamatergic profiles could potentially be used to identify VIP+ INs subtypes across development. I performed cluster analysis to test this hypothesis, aiming to describe subpopulations of VIP+ INs characterised by different local connectivity patterns.

To reduce dimensions and prevent correlations between the different measured values, I first performed principal component analysis (PCA) on the normalised layer component values of all recorded cells (n=32), regardless of the developmental time point. The first 3 principal components obtained from the PCA were able to describe 100% of the variance across the data (Figure 3.6A). The PCA scores in those components were used to find clusters. Figure 3.6B shows the first two PCA components, able to explain 98.8% of the variance across the data.

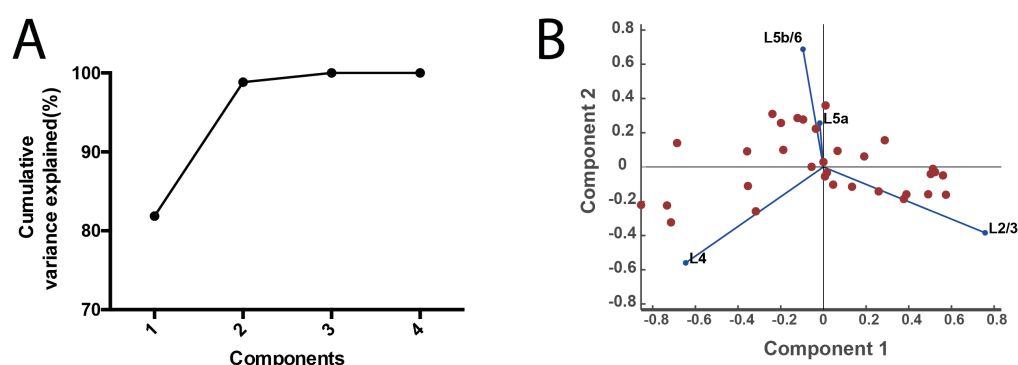


Figure 3.6 Principal component analysis (PCA) of normalised glutamatergic input profiles of VIP+ INs (A) Quantification of cumulative explained variance in relationship with the number of components identified by PCA (B) Scatter plot of the first two principal components scores of each data point. The lines indicate different variables inserted in the analysis (i.e. the relative layer component). This analysis allows to dissect clusters of cells characterised by different input profiles.

Then, I run k-means clustering and evaluated silhouette values obtained from different numbers of clusters (up to 6, Figure 3.7A-F): according to this analysis, VIP+ INs seemed to cluster optimally in 3 different groups (compare the silhouette analysis with the dendrogram in Figure 3.8A and 3.8B).

To confirm that describing VIP+ INs variability through clusters was informative, I performed a linear mixed effects analysis focusing on the relationship between normalised input per layer, developmental time and clusters. I ran two models, one taking into account clusters and developmental time points to explain variance in the data and one considering only development. As fixed effects, I entered the normalised layer component, developmental age groups and cluster identity into the model. As a random effect, I had intercepts for each recorded cell ('subject'). I found that the model

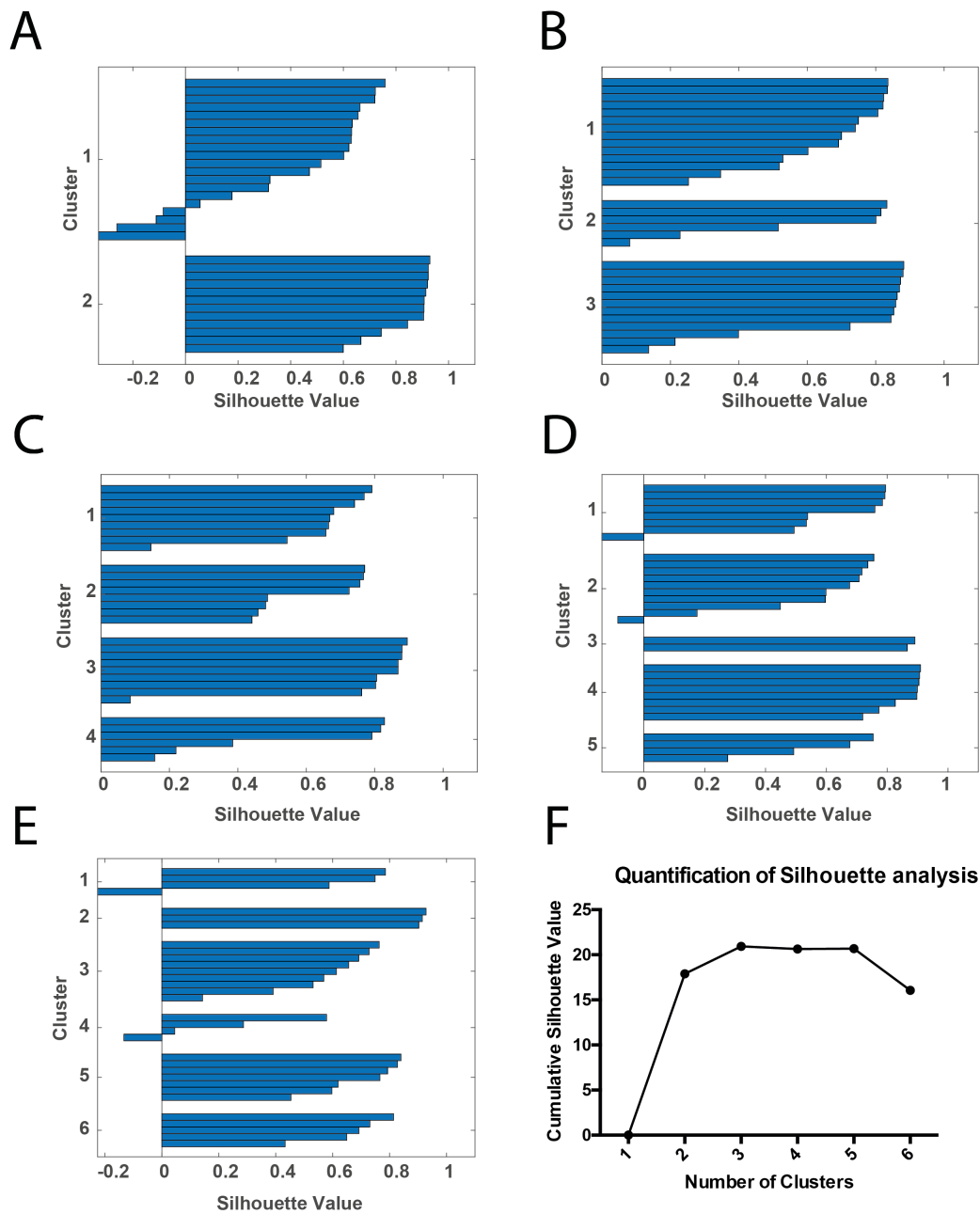


Figure 3.7 Silhouette analysis on k-means clustering following PCA on normalised glutamatergic input profiles of VIP+ INs. (A-E) Silhouette analysis on k-means clustering from k=2 to k=6. (F) Quantification of cumulative silhouette values relative to the different number of clusters identified. k=3 (B, and observe the relative cumulative silhouette value in F) seems to be the optimal number of clusters to classify VIP+ INs input profiles.

considering clusters was better than the one with only development (likelihood ratio test, $\chi^2(8) = 180.29$, $p = 2.2 \times 10^{-16}$), suggesting that cluster information captured variance in the data. This suggests that a classification into clusters could be a powerful tool to describe VIP+ INs diversity.

To characterise these clusters, several features were analysed: normalised input across layers, age, distance from the pial surface, and soma location of recorded cells in the cortical column (Figure 3.8C-F). In particular, if the VIP+ cell patched was localised above a barrel it was classified as 'barrel cell', while if it was above the septum was classified as 'septal cell' (Figure 3.8F).

The normalised local input, the variable used to perform the PCA, was significantly different across clusters, confirming the success of the clustering strategy (Figure 3.8C). Cluster 1 was characterised by a significantly more prominent L5b/6 component, cluster 2 had higher L4 input and cluster 3 presented a larger L2/3 input (Figure 3.8C and Appendix 1 for details).

Further cluster-specific differences were identified: age in cluster 2 was significantly higher than in cluster 3 (Figure 3.8D, 12.8 ± 2.3 and 9.2 ± 2.4 postnatal days respectively; One way ANOVA, $F(2, 29) = 3.650$, $p = 0.0386$; $p \leq 0.05$ following *post-hoc* multiple comparisons). Similarly, distance from the pial surface was significantly greater in cluster 2 than cluster 3 cells (Figure 3.8E, $284 \pm 23 \mu\text{m}$ and $205 \pm 49 \mu\text{m}$ respectively; Kruskal-Wallis one way ANOVA, $p = 0.0144$; $p \leq 0.05$ following *post-hoc* multiple comparisons). No significant differences were found across clusters in soma location (Figure 3.8F, see Appendix 1 for results of the Fisher's exact test).

Representative cells in each cluster are shown in Figure 3.9, highlighting clear differences between their glutamatergic network connectivity profiles.

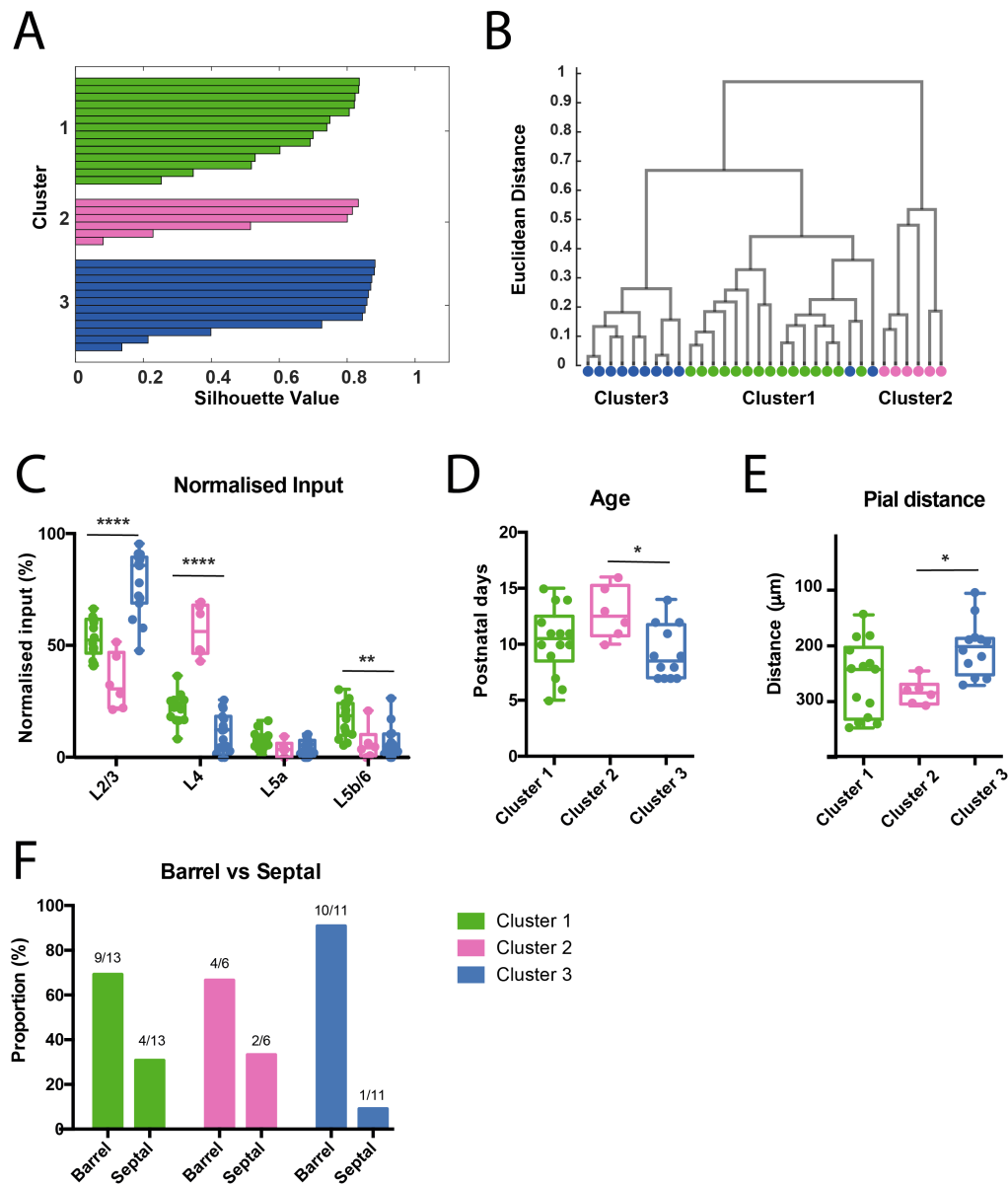


Figure 3.8 Characterisation of clusters of VIP+ INs. (A) Silhouette analysis on k-means clustering with $k=3$. (B) Dendrogram based on the Euclidean distance among the data points. Each cell is represented by a circle. Each cluster is identified by a different colour. (C) Normalised laminar distribution of glutamatergic inputs onto L2/3 VIP+ INs divided in clusters. Data is shown as described in Figure 3.5 (D) Age analysis of VIP+ INs according to the three clusters identified. (E) Distance from pial surface of VIP+ INs according to the three clusters identified. (F) Proportion of location of the cell body of VIP+ INs in the three clusters identified. Barrel= if the cell body is above the barrel; Septal: if the cell body is above the septum. The three clusters can be distinguished based on their normalised input (C). While Cluster 1 does not differ from the others for age or pial distance, Cluster 2 and 3 can be distinguished based on age and distance from the pia (D,E). No significant differences can be observed in cell body location in different clusters (F).

While morphology differences across clusters could be observed, low successful rate in the recovery of the morphology did not make this further analysis possible. A bigger dataset would be needed to address any cluster-specific morphological differences in VIP+ INs.

3.2.2 – Discussion and Limitations

In this section, I characterised the local glutamatergic connectivity of L2/3 VIP+ INs across the first two postnatal weeks. The initial analysis based on developmental time points identifies L2/3 as the major input to VIP+ INs. A gradual integration in the glutamatergic granular and infragranular network is observed from P9 onwards and mirrors the gradual changes in electrophysiological properties previously reported (Chapter 3.1.3).

Local glutamatergic connectivity onto adult VIP+ INs has been previously demonstrated (Porter et al., 1998; Rozov et al., 2001; Staiger et al., 2002). Through immunohistochemistry, Sohn et al. (2016) showed that glutamatergic terminals on L2/3 VIP+ INs are mainly localised in their distal-dendritic compartment and therefore thought to be deriving from supragranular neurons. Moreover, experiments investigating the synaptic connectivity of L2/3 INs identified local pyramidal inputs to INs that for morphological features (i.e. bitufted or bipolar morphology) or molecular markers expression (i.e. CR) resembled VIP+ cells (Avermann et al., 2012; Caputi et al., 2009; Jiang et al., 2015).

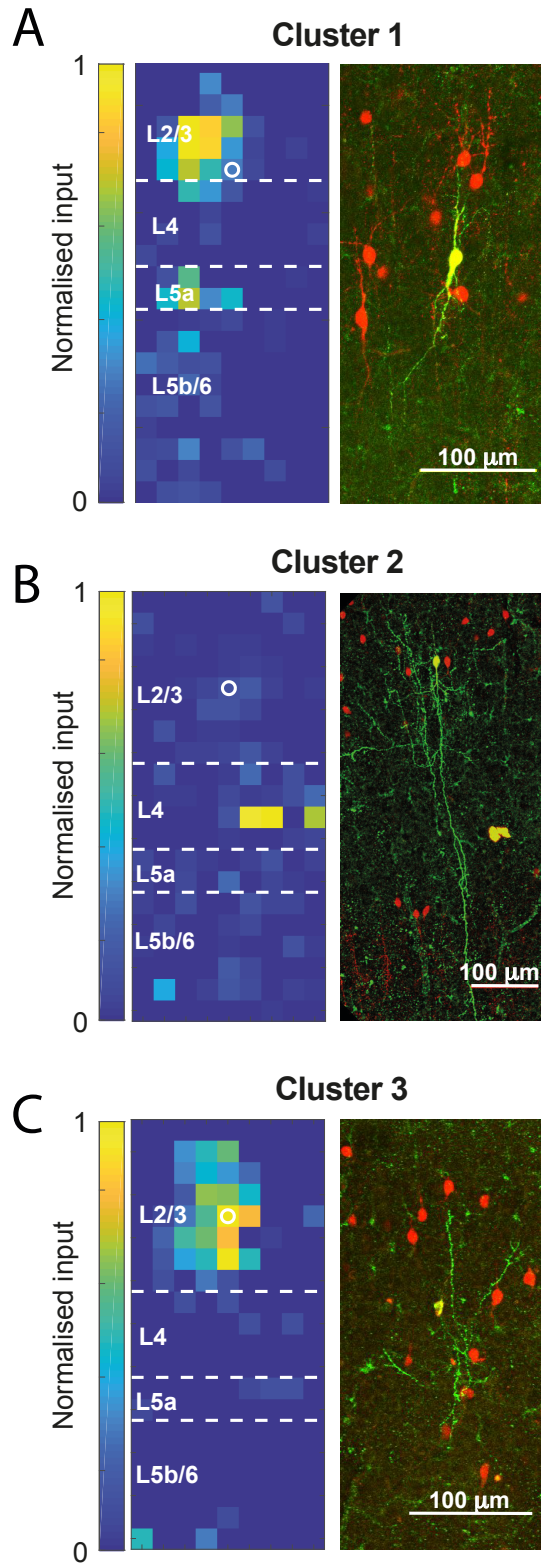


Figure 3.9 Examples of VIP+ INs belonging to each cluster. (A) Glutamatergic connectivity map and morphology of a VIP+ IN in Cluster 1, with inputs from infragranular layers. (B) Glutamatergic connectivity map and morphology of a VIP+ IN in Cluster 2, with prominent L4 input. (C) Glutamatergic connectivity map and morphology of a VIP+ IN in Cluster 3, with mainly local input.

The presence of granular and infragranular glutamatergic inputs to L2/3 VIP+ INs has not been previously demonstrated; however, glutamatergic connectivity of L2/3 INs whose morphology resembled VIP+ cells has been previously characterised (Dantzker and Callaway, 2000; Helmstaedter et al., 2008; House et al., 2011; Jiang et al., 2015; Xu and Callaway, 2009). Jiang et al. (2015) did not find this input while performing multiple paired recordings in adult V1, but similar experiments performed in S1BF reported the presence of glutamatergic connections between L4 glutamatergic neurons and L2/3 bipolar INs (Helmstaedter et al., 2008; House et al., 2011).

LSPS experiments performed in L2/3 INs further support the presence of granular and infragranular input to INs that can potentially be defined as VIP+ cells. Dantzker and Callaway (2000) identified a subtype of adapting, non-fast spiking INs which received L4 and L5 glutamatergic input. Similarly, Xu and Callaway (2009) found L2/3 irregular spiking INs with a prominent infragranular component and L2/3 bipolar cells with a strong granular and infragranular component. Although the authors did not classify these cells specifically as VIP+ INs, the spiking profiles and morphologies resemble what is known about VIP+ cells (Prönneke et al., 2018; Prönneke et al., 2015).

An important factor to take into account when comparing these studies with the results here presented is that spiking patterns such as adapting or irregular spiking can be found in other CGE-derived INs subtypes (Butt et al., 2005; Lee et al., 2010; Miyoshi et al., 2010). Therefore, further experiments combining LSPS, intrinsic properties characterisation and morphological reconstruction are needed to support comparisons with the previous literature.

VIP+ INs are characterised by a high degree of heterogeneity (Cauli et al., 2014; He et al., 2016; Kubota and Kawaguchi, 1997; Porter et al., 1998; Prönneke et al., 2015, 2018; Rhomberg et al., 2018; Tasic et al., 2016, 2018; Tremblay et al., 2016). To have some insights onto whether specific connectivity profiles could underlie subtypes differences, I performed k-means clustering and identified 3 main populations of VIP+ INs that differ for their input profiles.

The first cluster presents a higher proportion of glutamatergic input coming from infragranular layers, and contains cells across all developmental points (ranging from P7 to P14) without any preferential location in L2/3. Intriguingly, it was possible to observe in this population occasional cells (i.e. 1-2) with a L5a component (Figure 3.9A), suggesting a potential engagement from the paralemniscal pathway. Cells from the second cluster have a larger L4 component (i.e. lemniscal), a higher average age ($P12.8 \pm 2.3$) and are located further from the pial surface (i.e. lower L2/3, putative L3). Finally, the third cluster is characterized by a stronger local input, its cells are generally younger ($P9.2 \pm 2.4$) and closer to the pial surface (i.e. upper L2/3, putative L2 cells) when compared with cells belonging to cluster 2.

These results suggest both developmental and layer-specific effects on local connectivity of VIP+ INs. Infragranular and local input are the first emerging during development, while L4 connectivity establishes later. This is in line with the dynamics of cortical development. The cerebral cortex develops in an inside-out fashion: infragranular pyramidal neurons generation and maturation precede supragranular ones (Greig et al., 2013; Marin-Padilla, 1978;

Raedler and Raedler, 1978). This could justify why infragranular input onto VIP+ INs could be observed early in the first postnatal week.

Although only occasionally cells with a L5a component could be identified in this cluster, it would be interesting to further explore this population and the role of VIP+ INs in the paralemniscal pathway, especially at early time points. It has been reported that the posteromedial complex (POm) engages with L2/3 VIP+ INs in the adult brain (Audette et al., 2018; Williams and Holtmaat, 2019). While the majority of studies characterising the establishment of thalamocortical connectivity did not focus on distinction between ventral posteromedial nucleus (VPM) and POm, a recent paper suggested that the paralemniscal pathway could develop as early as P3 (Liu et al., 2014). Moreover, it has been previously reported that the thalamus engages with infragranular layers at early time points (Marques-Smith et al., 2016; Tuncdemir et al., 2016). Therefore the possibility of an early role for VIP+ INs in the paralemniscal pathway cannot be excluded, even though more experiments are needed to confirm this hypothesis.

L4-to-L2/3 connections establish later in development, following the maturation of the lemniscal pathway (Bureau et al., 2004; Lendvai et al., 2000; Maravall et al., 2004a, 2004b; Shoykhet et al., 2005; Stern et al., 2001). Thus, it is not surprising that cluster 2 cells, characterised by a prominent L4 component, start appearing from P10 onwards. On the contrary, local connections are present already at P5, in line with an initial local integration in the network (Anastasiades and Butt, 2012).

Another interesting feature of cluster 2 and 3 is their preferential location in lower L2/3 (putative L3) and upper L2/3 (putative L2), respectively. Connectivity differences between L2 and L3 neurons have been previously

characterised *in vitro* and *in vivo*, supporting a depth-dependency of L4 input, at least for pyramidal cells (Bureau et al., 2004, 2006; Gur and Snodderly, 2008; Meng et al., 2017; Shepherd and Svoboda, 2005; Staiger et al., 2015).

Layer-specific connectivity differences have also been found between L2/3 barrel or septal pyramidal cells (Bureau et al., 2006; Shepherd and Svoboda, 2005); however, differences in soma location did not emerge from the cluster analysis performed here. A possibility to further investigate the barrel vs septal glutamatergic component onto VIP +INs is to perform LSPS with a two-photon stimulation (rather than a single photon, as employed here) and increase the spatial resolution, allowing the differentiation of septal vs. barrel inputs (e.g. Ashby and Isaac, 2011; Izquierdo-Serra et al., 2018).

A final consideration about this cluster analysis is concerning the number of chosen clusters. I defined the ideal number of clusters based on silhouette analysis of k-means clustering, a technique based on the random generation of initial values that are representing the 'means' of the data (i.e. the centroid of the cluster). Considering this random element and the fact that, according to the silhouette profile analysis, the values of $k=3$ are not extremely different from $k=5$ (see Figure 3.7F), it cannot be excluded that 5 clusters are as good as 3 in identifying diversity in VIP+ INs. Perhaps with 5 clusters it would be possible to differentiate cells not only by their connectivity profile, but also by their age, splitting for example the cluster 1 in multiple clusters. More data points and further analysis are needed to support this conclusion.

3.3 – Development of GABAergic output from VIP+ cells

3.3.1 – Investigating the development of VIP+ cells output using the VIP-Cre;Ai32 mouse line

Previous studies have documented VIP+ INs output onto pyramidal cells and other INs (Dávid et al., 2007; Garcia-Junco-Clemente et al., 2017; Hioki et al., 2013; Jiang et al., 2013, 2015; Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013; Staiger et al., 1997, 2002, 2004). However, it is currently unknown when VIP+ INs first engage with the surrounding network. To investigate this, the *VIP-Cre* line was crossed with the *Ai32* reporter (Madisen et al., 2012). This allowed the expression of channelrhodopsin-2(ChR-2)/EYFP fusion protein following exposure to Cre recombinase in all VIP+ INs.

I first performed extracellular recordings from VIP+;EYFP+ INs and confirmed the ability to evoke action potentials in these cells upon blue light illumination (470nm) (Figure 3.10). Higher LED powers were able to elicit action potentials at shorter latencies. Overall, spike latencies ranged between ~10 and ~17ms from laser onset at the maximal stimulation tested (Figure 3.10B).

Next, to be able to target specifically INs or pyramidal cells, the *VIP-Cre;Ai32* line was bred on a background hemizygous for *Lhx6-EGFP* (Gong et al., 2003). This transgene contains the enhanced green fluorescent protein (EGFP) inserted into a bacterial artificial chromosome (BAC) at the transcription initiation codon of the *Lhx6* gene, a transcription factor important for specification, survival and laminar position of PV+ and SST+ cells (Liodis et al., 2007; Vogt et al., 2014; Yuan et al., 2018; Zhao et al., 2008). Thus, in the *Lhx6-*

EGFP line, PV+ and SST+ cells express EGFP and can be targeted during whole cell patch clamp experiments (Abraham et al., 2017; Gittis et al., 2010; Maroof et al., 2010).

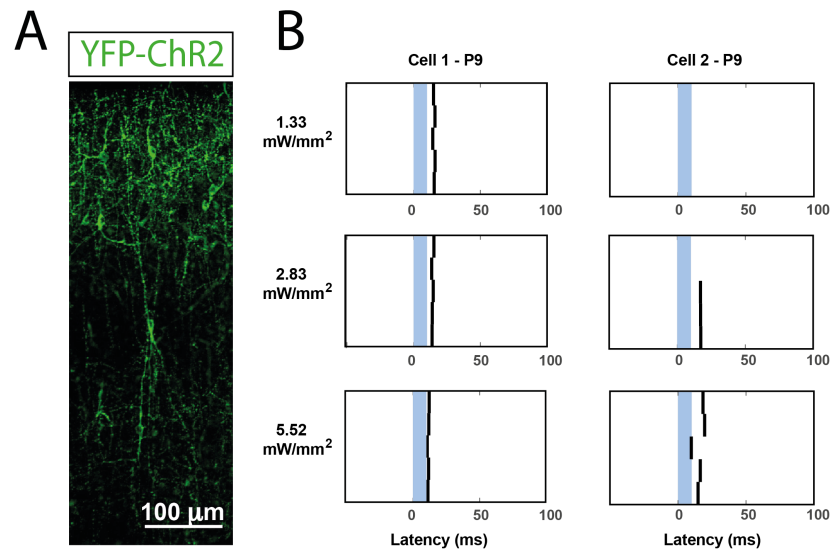


Figure 3.10 The *VIP-Cre;Ai32* line expresses functional channelrhodopsin. (A) Immunohistochemistry from a floating section showing YFP+ cells in the *VIP-Cre;Ai32* line. Green=YFP; scale bar= 100μm (B) Raster plots obtained from loose cell attached recordings in two P9 VIP+ INs showing time-locked responses to a 10 ms stimulation on different LED powers. Each line in the raster plot represents a single sweep. Compare left and right panels and note the variability of VIP+ INs responses.

To study the development of VIP+ INs output, light-evoked inhibitory post synaptic currents (IPSCs) were recorded in either pyramidal or Lhx6-EGFP+ cells across the depth of the cortex during the first two postnatal weeks. To have a more precise picture of the development of VIP+ INs output, the following time points were used: P5-6 and P7-8 (respectively an early and late window for the critical period plasticity for L4 (Fox, 1992)), and P12-P15, the age when VIP+ INs are fully integrated in the glutamatergic network (see Chapter 3.2).

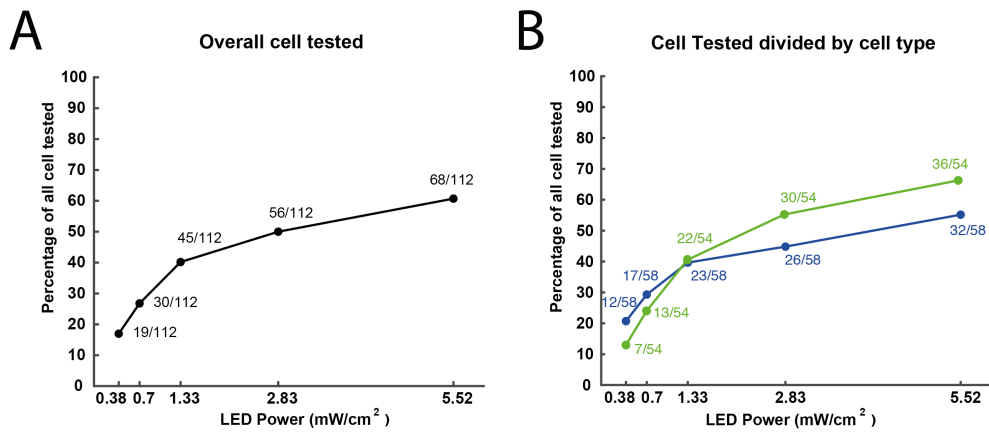


Figure 3.11 Cumulative distribution of responsive cells while increasing LED power. (A) Increasing LED power leads to a higher proportion of responsive cells (B) Same cells as in (A), but divided according to their identity. Lhx6-GFP+ cells are in green, pyramidal cells in blue.

The overall number of responsive cells increased by increasing LED power (Figure 3.11), without significant differences between Lhx6-EGFP+ and pyramidal cells (Figure 3.11, B, $\chi^2(4) = 4.1387$, $p = 0.38756$).

Responses elicited at the minimal stimulation presented more scattered average latency and latency jitter values than those elicited at the maximum stimulation (i.e. 5.52 mW/cm²) (compare Figure 3.12A and B). In both cases it was possible to observe short-latency/low-jitter and long-latency/high-jitter events. This suggested two alternative scenarios:

(1) the recruitment of both VIP+ terminals and cell bodies upon LED stimulation, with terminals stimulation eliciting short-latency responses, and soma stimulation leading to long-latency events. This hypothesis is in part supported by the fact that evoked spikes could have latencies up to 17 ms (see Figure 3.10). However, previous work based on paired recordings suggests 1- 2

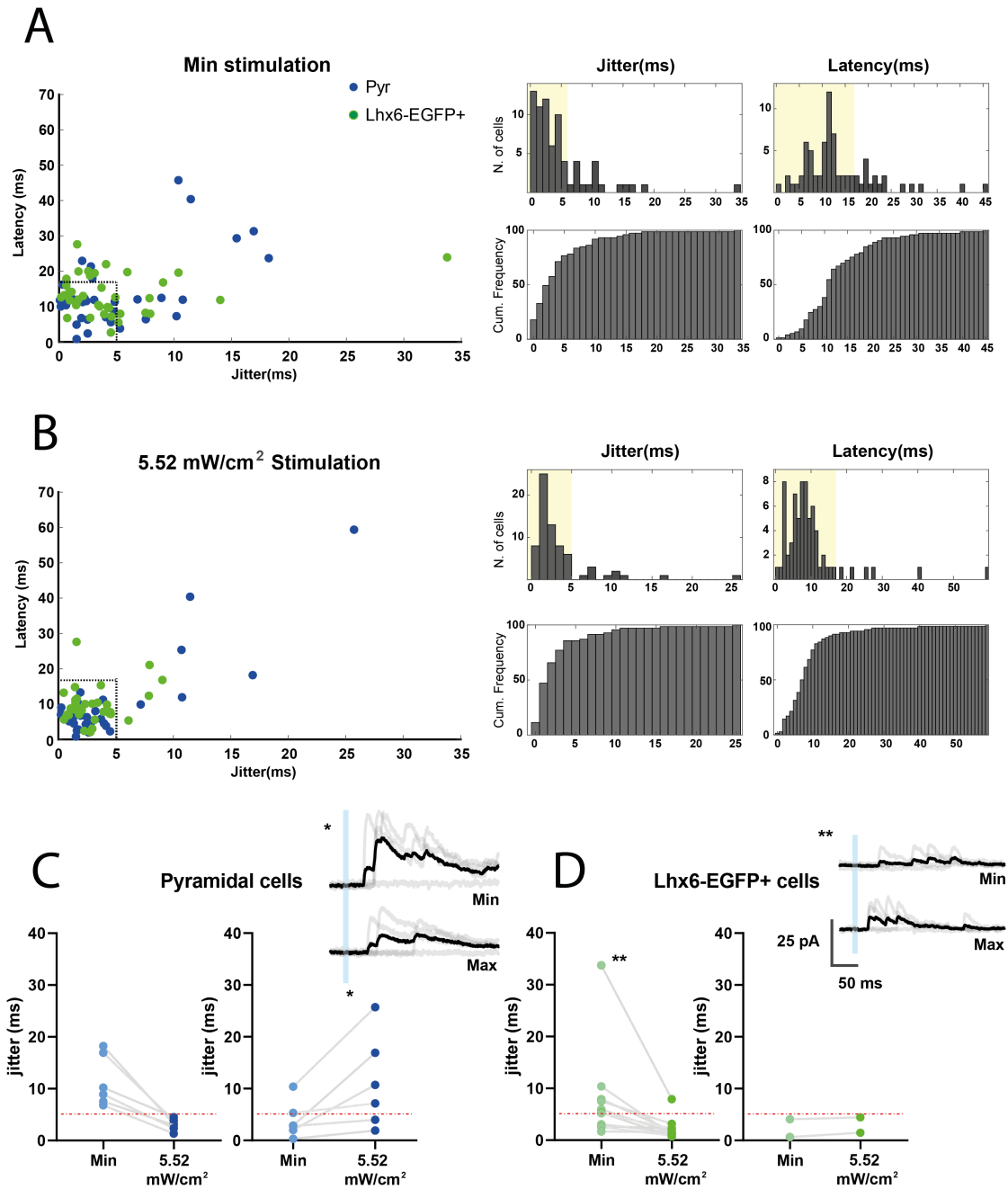


Figure 3.12 Investigating the variation of jitter and latency at minimum and maximal stimulation. (A) Left panel: Scatter plot of jitter and latency of Lhx6-EGFP+ cells (green) and pyramidal cells (blue) at minimum stimulation. Right Panel: histograms (top) and cumulative distributions (bottom) of latency and jitter shown in the left panel. Dotted lines in scatter plot and yellow shading in histograms: values used to evaluate the putative monosynaptic window. (B) As (A) at the maximum stimulation. (C) Left panel: pyramidal cells with long-latency/high-jitter at the minimum stimulation that showed a decrease in jitter at maximum stimulation ($n=6/12$). Right panel: pyramidal cells with long-latency/high-jitter at the minimum stimulation that showed either an increase in jitter or no changes at maximum stimulation ($n=6/12$). (D) Left panel: Lhx6-EGFP+ cells with long-latency/high-jitter at the minimum stimulation that showed a decrease in jitter at maximum stimulation. Only 9.09% (1/11) of these cells decreased its jitter at higher LED power, without reaching the threshold of 5ms (see **).

Continues from previous page. Right panel: Lhx6-EGFP+ cells with long-latency/high-jitter at the minimum stimulation that showed no changes in jitter at maximum stimulation. Exemplary traces from single cells in (C) and (D) are reported in the corners of the panels. Blue line: 10ms LED stimulation, grey traces: individual sweeps, black trace: average trace. Top traces: minimal stimulations. Bottom traces: maximal stimulation.

ms delay for VIP-to-IN and VIP-to-Pyr connections (Walker et al., 2016; Pfeffer et al., 2013), which would not explain events with latencies >20ms.

(2) the presence of both monosynaptic and polysynaptic events (short-latency/low-jitter and long-latency/high-jitter, respectively). This is conceivable given the known depolarizing nature of GABA during early postnatal development (Ben-Ari et al., 2007); hence, upon blue light stimulation, VIP+ INs could excite an IN, in turn monosynaptically connected to the recorded cell.

The common approach to overcome this issue is to perform such experiments in the presence of TTX and 4-AP to isolate monosynaptic events. This approach was attempted in a small subset of recorded cells ($n = 4$; data not shown) but no light-evoked IPSC could be recorded, in line with previous observations in our lab (Marques-Smith, 2014; Stacey, 2018). As an alternative, I employed empirical values obtained in the present dataset, namely the latency and jitter values that defined the cluster of short-latency/low-jitter events occurring at the maximum stimulation (Figure 3.12B, dotted lines and yellow shading). Therefore, responsive cells were considered monosynaptically connected to VIP+ INs if characterized by average latency <17 ms and latency jitter <5 ms at the maximal stimulation tested.

Increasing the LED power increased the overall number of cells considered responsive and monosynaptically connected (compare Figure 3.13 and 3.11), with significant differences between Lhx6-EGFP+ INs and pyramidal

cells (Figure 3.13B, $\chi^2(4) = 15.2216$, $p = 0.004263$). In particular, VIP+ INs preferentially recruited Lhx6-EGFP+ cells over pyramidal cells (Lhx6-EGFP+: 59%, $n=54$ vs pyramidal: 46% $n=58$ at 5.52 mW/cm^2). Both PV+ and SST+ subtypes were identified by *post-hoc* morphological reconstruction of the recorded cells.

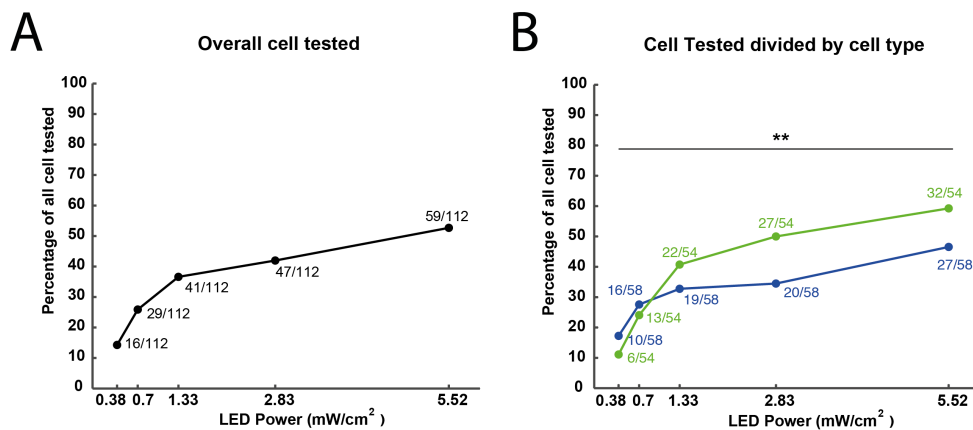


Figure 3.13. Cumulative distribution of responsive cells with average latency <17ms and jitter<5ms while increasing LED power. (A) Increasing LED power leads to a higher proportion of responsive cells (B) Same cells as in (A), but divided according to their identity. Lhx6-EGFP+ cells are in green, pyramidal cells in blue.

To avoid underestimation of the number of cells recruited by VIP+ INs, data obtained with a 5.52 mW/cm^2 LED power were used for further analysis. A developmental increase could be observed in the overall proportion of recruited cells (Log-linear analysis, interaction between responsiveness and age $p=0.0125$ Figure 3.14A). At P7-8, Lhx6-EGFP+ cells were preferentially recruited by VIP+ INs as compared to pyramidal cells, an effect nearly reaching statistical significance (Fisher's exact test, $p = 0.0531$).

To further characterise the reliability of IPSCs in Lhx6-EGFP+ and pyramidal cells, I analysed jitter and latency at minimal and maximal stimulation across development (Figure 3.15). Jitter at minimal or maximal stimulation did not change at any time point neither in Lhx6-EGFP+ INs (Figure 3.15A) nor in pyramidal cells (Figure 3.15B). In Lhx6-EGFP+ cells, stimulation intensity significantly reduced the average latency at all ages tested (Figure 3.15C). A similar effect was observed on pyramidal cells, but not at all ages (Figure 3.15D, see Appendix 1 for details).

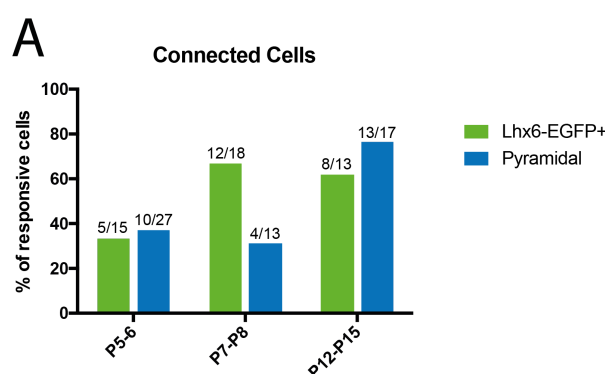


Figure 3.14 Developmental increase in the proportion of Lhx6-EGFP+ and Pyramidal cells recruited across the first two postnatal weeks. (A) Proportion of Lhx6-EGFP+ and pyramidal cells recruited at the time points identified. Note the peak in responsive Lhx6-EGFP+ cells at P7-8.

Finally, I directly compared responses in Lhx6-EGFP+ and pyramidal cells and I did not find any cell-specific difference in either jitter or latency at minimal or maximal stimulation (see three-way ANOVA results in the Appendix 1). Overall, this data suggest that VIP+ INs preferentially target Lhx6-EGFP+ cells mainly between P7 and P8; however, no differences in average latency and in

latency jitter were found between Lhx6-EGFP+ and pyramidal cells across development.

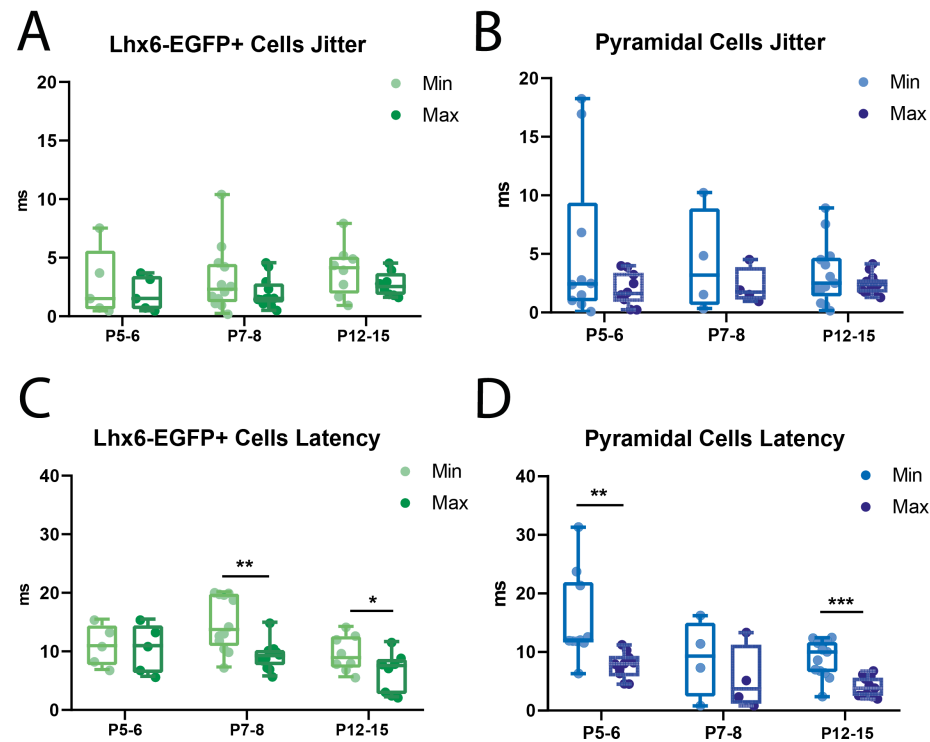


Figure 3.15 Changes in jitter and latency at minimal and maximal stimulation during development. (A) Jitter in Lhx6-EGFP+ cells at the minimal and maximal LED stimulation at the different ages tested. (B) Jitter in pyramidal cells at the minimal and maximal LED stimulation at the different ages tested (C) Latency in Lhx6-EGFP+ cells at the different ages tested. (D) Latency in pyramidal cells at the different ages tested. Boxplots as previously described.

While increasing LED stimulation does not change the jitter of evoked responses in either Lhx6-EGFP+ or pyramidal cells at any time point (A, B), latency is affected in an age and cell-type dependent fashion (C, D). However, no significant differences in latency and jitter are found across cell types at any stimulation.

Lhx6-EGFP+cells: P7-8: n=9 cells; P12-15 n=7 cells. Note: I couldn't perform statistical tests at P5-6 as only 1/5 cells was responsive at LED power < 5.52 mW/cm²

Pyramidal cells: P5-6: n= 9 cells; P7-8: n=3 cells; P12-15 n=11 cells

*: p≤0.05; ** p≤0.01; *** p≤0.001

3.3.2 – Layer-specific differences in the output from VIP+ cells

Layer-specific differences in VIP+ INs connectivity have been previously reported (Muñoz et al., 2017; Zhou et al., 2017). To examine how VIP+ cells engage with the columnar circuit over time, recorded cells were analysed also according to the position of their soma (i.e. within supragranular vs. infragranular layers) (Figure 3.16).

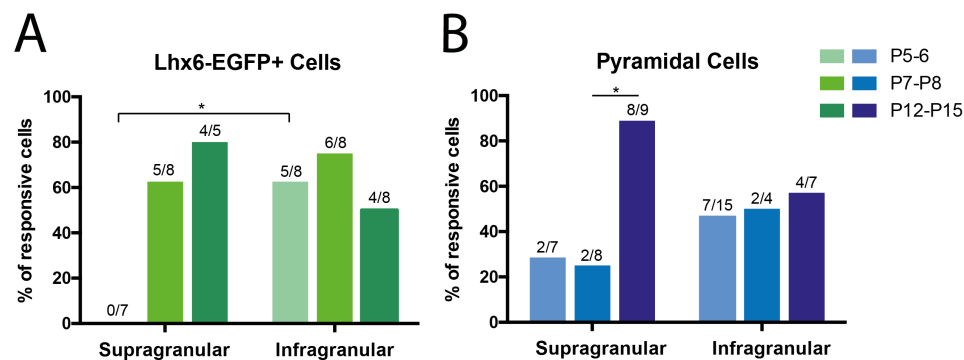


Figure 3.16 VIP+ INs engage differently the Lhx6-EGFP+ and pyramidal networks in infragranular and supragranular layers. (A) Proportion of Lhx6-EGFP+ INs recruited by VIP+ cells over the developmental time points analysed according to the location of the cell body. (B) Proportion of pyramidal cells recruited by VIP+ cells over the developmental time points analysed divided by location of the cell body. Infragranular Lhx6-EGFP+ INs are recruited by VIP+ cells since the earliest time points analysed, while supragranular INs are only recruited from P7 onwards (A). Infragranular pyramidal cells are recruited constantly throughout development, while supragranular ones are recruited more prominently from P12-15 (B). Only results from Fisher's Exact test are reported. *: $p \leq 0.05$.

Overall, supragranular layers were incrementally engaged over time (Log-linear analysis, $G^2=16.02$; $p=0.0003$), while VIP+ connections to infragranular layers remained stable over development, regardless of cell type (Log-linear analysis, $G^2=0.74$; $p=0.6907$. See Appendix 1 for details).

Lhx6-EGFP+ INs were differently recruited in supragranular and infragranular layers (Log-linear analysis, $G^2=10.04$; $p=0.0182$). In particular, more Lhx6-EGFP+ INs were recruited in infragranular layers at P5-6 (Figure

3.16A Fisher's exact test, $p=0.0256$). Pyramidal cells were not preferentially recruited in either layer (Figure 16B, see Appendix 1 for detailed statistics); however, the proportion of supragranular cells targeted at P12-15 significantly increased from P7-8 (25% vs 88%, Fisher's exact test, $p=0.0152$)

On occasion, infragranular Lhx6-EGFP+ INs were recorded at earlier time points (P3-P4) (Figure 3.17). Strikingly, 75% of the recorded cells (3/4) received putative monosynaptic input, suggesting that VIP+ INs engage with other interneurons as early as 3 days after birth, a time point in which they migrate to the cortex (Miyoshi and Fishell, 2011; Miyoshi et al., 2010; Taniguchi et al., 2011).

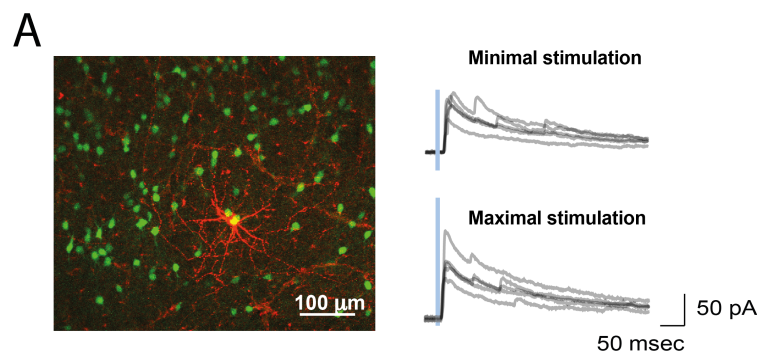


Figure 3.17 Example of Lhx6-GFP+ IN recruited at P3 by VIP+ INs. (A) Recovered morphology and (B) IPSCs at minimal and maximal stimulation from a P3 Lhx6-GFP+ cell recruited by VIP+ INs. Scale bar=100 μm.

To better understand the different level of engagement of VIP+ INs with the infragranular and the supragranular networks, the VIP+ input received by Lhx6-EGFP+ and pyramidal cells at the maximal stimulation was quantified during development, according to their location (see Figure 3.18).

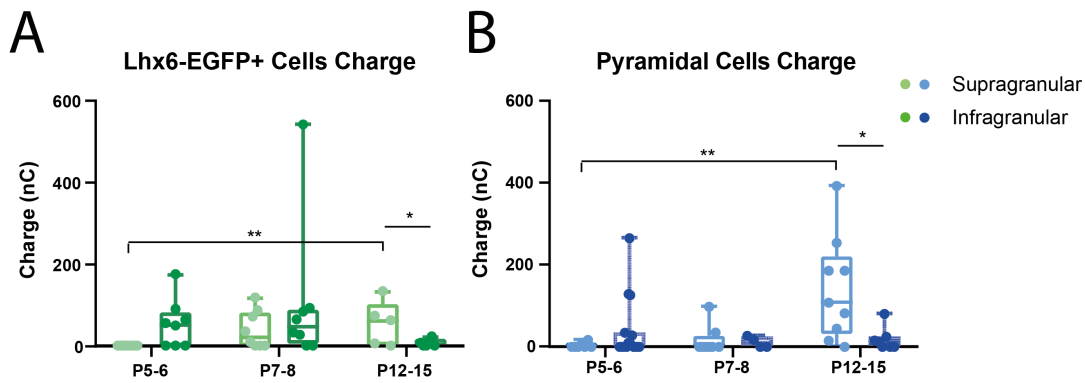


Figure 3.18 Quantification of transferred charge suggests the differential engagement of VIP+ INs with the supragranular and infragranular networks. (A) Quantification of transferred charge from VIP+ INs to Lhx6-GFP+ cells in different layers and at different time points (B) Quantification of transferred charge from VIP+ INs to pyramidal cells in different layers and at different time points. Boxplots as previously described. Each data point indicates the average input of each cell recorded, calculated as the mean IPSC charge from 5 stimulations. Not responsive cells were included in this analysis and indicated as charge = 0pC.

VIP-input onto supragranular cells significantly increases over time regardless of cell type, with P12-15 cells receiving more input than P5-6 cells. Moreover, VIP-input onto supragranular P12-15 cells is significantly higher than the infragranular counterpart, regardless of cell type.

*: $p \leq 0.05$; **: $p \leq 0.01$

Three-way ANOVA identified an interaction between age and location of the cell ($F(2, 82) = 5.881$, $p = 0.0041$) and an interaction between age and cell type ($F(2, 82) = 3.128$, $p = 0.0491$). Supragranular layers at P12-P15 received a significantly higher input than supragranular layers at P5-6, regardless of cell type (45.04 ± 68.19 nC vs. 1.73 ± 4.67 nC $n = 23$ and $n = 14$ respectively, $p = 0.0065$, Tukey correction). Moreover, all supragranular cells at P12-P15 received a larger VIP-mediated input than infragranular cells at the same developmental point (45.04 ± 68.19 nC vs. 12.98 ± 20.28 nC $n = 23$ and $n = 15$ respectively, $p = 0.0172$, Tukey correction). The interaction between age and cell type was not robust enough to survive multiple comparisons corrections (Sidak's multiple comparison test, $p > 0.9999$, $p = 0.1997$, and $p = 0.1374$ for comparison between Lhx6-EGFP+ and pyramidal cells at each age group).

The data shown so far suggest that VIP+ INs engage differently with infragranular and supragranular cells. In particular, they do not contact supragranular Lhx6-EGFP+ cells until P7-8, but already engage with infragranular ones at P3. Over time, VIP+ INs engage more and more with the supragranular network, contacting first a higher proportion of Lhx6-EGFP+ (P7-P8) and then pyramidal cells (P12-P15). Cell-specific differences in VIP+ input size between INs and pyramidal cells were not found.

3.3.3 – Discussion and Limitations

In this final section, the *VIP-Cre;Ai32* line was used to assess how VIP+ INs engage with local Lhx6-EGFP+ and pyramidal cells. Previous developmental studies successfully employed the *Ai32* line (De Marco García et al., 2015; Stacey, 2018) and the *VIP-Cre;Ai32* line has been already used to unravel VIP+ INs output in adult cortical and subcortical structures, both *in vivo* and *in vitro* (Bigelow et al., 2019; David and Topolnik, 2017; Fan et al., 2015; Francavilla et al., 2018; Garcia-Junco-Clemente et al., 2017; Pi et al., 2013; Rhomberg et al., 2018). The data presented here further demonstrate that this line is a reliable tool to investigate VIP+ INs output, already during the first two postnatal weeks.

Results show that VIP+ INs recruit a higher proportion of Lhx6-EGFP+ INs (i.e. both PV+ and SST+ cells) than pyramidal cells, in particular at P7-8. Moreover, responsive infragranular Lhx6-EGFP+ cells are observed as early as P3. Considering the role of infragranular INs (especially SST+ cells) in scaffolding the thalamocortical network (Marques-Smith et al., 2016; Tuncdemir et al., 2016), and the importance of IN-IN communication over development

(Anastasiades et al., 2016), this could suggest an early role for VIP in shaping emerging cortical circuits. Further experiments combined with morphological reconstruction at the earliest time points will help clarifying this hypothesis.

Data show that VIP+ INs engage differently with the infragranular and supragranular networks in an age-dependent fashion. In particular, VIP+ INs preferentially target infragranular neurons including Lhx6-EGFP INs at the earliest ages tested (P5-6). In the following days (P7-8), VIP+ cells start targeting supragranular Lhx6-EGFP+ cells, while infragranular INs and pyramidal cells are equally connected. Finally, following the complete integration of VIP+ INs in the local glutamatergic network (Chapter 3.2.1), VIP+ cells form synaptic connection onto supragranular pyramidal cells. The magnitude of input to the supragranular layers increase over development, regardless of cell type.

The fact that infragranular layers are recruited constantly from the earliest time points, while supragranular layers are recruited incrementally over time, is in line with their differential maturation (Greig et al., 2013; Marin-Padilla, 1978; Raedler and Raedler, 1978). Moreover, P12-15 is the developmental window for supragranular layers maturation following whisking onset (van der Bourg et al., 2017; Erzurumlu and Gaspar, 2012; Wen and Barth, 2011), suggesting that VIP+ INs may play a role in this process.

Layer specific differences in VIP+ INs connectivity have been already demonstrated (Muñoz et al., 2017; Zhou et al., 2017). Zhou et al. (2017) showed that VIP+ boutons preferentially target non-GABAergic dendrites across the depth of the cortex. Moreover, these structures are preferentially targeted in supragranular layers rather than infragranular ones. In contrast, infragranular GABAergic dendrites, known to belong to infragranular INs (Feldmeyer et al.,

2018), are preferentially targeted by VIP+ boutons. Muñoz et al. (2017) reported instead that VIP+ INs form stronger synaptic inputs onto supragranular SST+ INs than infragranular ones. Given the differences in techniques, area under study, and age of the animals, these works cannot be directly compared with the results here reported.

The majority of the literature on VIP+ INs connectivity to date has focused on L2/3 circuitry, emphasising that VIP+ INs preferentially innervate SST+INs (Lee et al., 2013; Pfeffer et al., 2013; Pi et al., 2013; Staiger et al., 2004; Zhang et al., 2014). It has been also shown that VIP+ INs (or cells that can be identified as such by morpho-physiological properties) innervate PV+ cells (Dávid et al., 2007; Hioki et al., 2013; Lee et al., 2013; Pfeffer et al., 2013; Staiger et al., 1997, 2002) and pyramidal cells in both supragranular and infragranular layers (Jiang et al., 2013, 2015; Lee et al., 2013; Pfeffer et al., 2013; Yetman et al., 2019). The results here presented are overall in line with these previous reports. The fact that no difference in VIP+ input magnitude was found between cell types is potentially in contrast with other papers in the adult brain (Lee et al., 2013; Pfeffer et al., 2013). Area and age-related differences could explain this discrepancy.

A limitation of the experiments discussed in this section is that the stimulation protocol employed did not allow to make any claim about the laminar distribution of presynaptic VIP+ INs. In Chapter 3.2.1 cluster analysis suggested layer-dependent heterogeneity between VIP+ cell global glutamatergic profiles (i.e. putative L2 vs. putative L3). Moreover, morphological analysis performed by Prönneke et al. (2015) indicates that supragranular and infragranular VIP+ INs are involved in different circuits. In particular, axons of L2/3 VIP+ INs extend to the entire cortical depth, with axonal boutons

preferentially in L2/3 and L5a; while axons of L4-6 VIP+ cells only target infragranular layers, with boutons preferentially in L5b and 6. This suggests that L2/3 VIP+ INs recruit supragranular cells (data recently confirmed by Yetman et al., 2019), but the origin of VIP+ input to infragranular cells cannot be defined. Considering that infragranular layers are engaged as early as P3, time point when VIP+ INs are still migrating to the cortex (Miyoshi and Fishell, 2011; Miyoshi et al., 2010; Taniguchi et al., 2011), it is possible that early VIP+ inputs originate from migrating VIP+ INs.

To assess the location of VIP+ inputs, several strategies could be employed. For instance, LSPS combined with blue light ramp stimulation could be performed in the *Vip-Cre;Ai32* mouse line. This technique would allow stimulating only cell bodies of VIP+ INs and therefore finding the input location (Adesnik and Scanziani, 2010; Naka et al., 2019). It has been previously employed in adults to unravel local VIP+ connectivity in the suprachiasmatic nucleus (Fan et al., 2015).

Alternatively, the *VIP-Cre* line could be crossed with the *rP2X2* line to map the location of VIP+ inputs (Marques-Smith et al., 2016; Anastasiades et al., 2016). In the *rP2X2* line the ATP receptor P2X2 is expressed under the control of the Cre recombinase. Focal uncaging of caged-ATP allows to trigger action potentials at the level of the soma, therefore enabling the localisation of the presynaptic cell (Zemelman et al., 2003). I previously tested the effect of the expression of the P2X2 receptor in VIP+ INs and observed that its activation led to sustained depolarisation of VIP+ cells and subsequent death, possibly for calcium mediated excitotoxicity. Therefore, this approach could not have been used with VIP+ INs. Alternatively, double-patching experiments or *in vitro* time-

lapse imaging could be performed. The latter strategy has been recently employed by Lim et al. (2018) to show that axonal targeting is coupled with migration in Martinotti SST+ cells at early postnatal days. A similar phenomenon cannot be excluded for other classes of INs such as VIP+ cells.

In conclusion, these results shed light on VIP+ INs connectivity over development and suggest that VIP+ INs are engaged early in the local columnar circuitry, developing synaptic connections with both INs and pyramidal cells.

Chapter 4 – Integration of VIP+ cells into the long-range cortical circuit

Previous research has shown that S1BF VIP+ INs in the adult brain receive long-range connections from both cortical and subcortical structures (Audette et al., 2018; Lee et al., 2013; Staiger et al., 1996; Wall et al., 2016; Williams and Holtmaat, 2019). In particular, Lee et al. (2013) demonstrated that long-range glutamatergic projections from the primary vibrissal motor cortex (vM1) preferentially recruit L2/3 S1BF VIP+ INs among other excitatory and inhibitory neuronal populations. Through this connection, VIP+ INs provide feedforward inhibition of SST+ INs, mediating motor integration in S1BF (Lee et al., 2013). Viral tracing studies confirmed the M1 to S1BF VIP+ INs connectivity, showing that, overall, individual VIP+ INs receive a higher number of inputs from primary motor cortex than any other INs classes (Wall et al., 2016). This connectivity pattern supports a major role for VIP+ INs in integrating sensory-motor information; however, the developmental timeline for the establishment of these long-range connections is currently unknown. To address this, I employed a viral

optogenetic approach to investigate when the connectivity between motor cortex and L2/3 VIP+ INs in S1BF is established, analysing monosynaptic inputs across development and comparing them with L2/3 pyramidal cells.

4.1 – Establishment of an early-expressing viral strategy to allow developmental studies

4.1.2 – Background

Long-range connectivity studies in the adult brain employ either anatomical or functional approaches (Helmstaedter, 2013; Lim et al., 2013; Nassi et al., 2015; Osten and Margrie, 2013; Scanziani and Häusser, 2009; Zeng, 2018). Anatomical approaches, either through molecular or viral tracers, efficiently highlight the presence of terminals across brain areas (Oh et al., 2014; Osten and Margrie, 2013; Zeng, 2018) and thanks to recent advances in microscopy and automatic annotation, many connectivity atlases are available today (e.g. Li et al., 2018; Oh et al., 2014; Pollak Dorocic et al., 2014). An important caveat of these studies is that they can lead to false positives. For instance, these techniques do not inform about functional synapses (nor synaptic strength) and, in cases of monosynaptic mapping through rabies virus, viral particles can also spread via non-synaptic mechanisms (Svoboda K., 2019).

Functional approaches allow instead to probe synaptic connectivity between areas, either through electrical stimulation or the use of opsins, techniques that can be considered complementary (Lim et al., 2013; Scanziani

and Häusser, 2009). Electrical stimulation offers excellent temporal resolution and reproducibility, but requires the integrity of the fibres connecting two areas (Agmon and Connors, 1991; Rocco-Donovan et al., 2011). Moreover, it is non-selective, as it activates cells and axons of passage without discrimination (Lim et al., 2013; Scanziani and Häusser, 2009).

In contrast, opsins can be selectively expressed only in the neuronal population of interest and allow to probe long-range connections without preserving the axons. Indeed, stimulating opsins expressed in synaptic terminals and recording post-synaptic currents is an established method to reveal functional long-range connectivity at the cellular level between areas of interest (e.g. Petreanu et al., 2007), and it has been extensively adopted to probe connectivity within the sensorimotor system (Hooks et al., 2015; Mao et al., 2011).

However, opsins do not offer the same temporal resolution as electrical stimulation due to variant-specific kinetics and desensitization (Lin, 2011). In addition, the majority of adeno-associated viral vectors encoding opsins require at least 2-3 weeks to obtain reliable expression of functional protein (Zhang et al., 2010). However, serotype-specific differences in expression patterns and strength have been previously reported (Taymans et al., 2007).

Given the reduced selectivity of electrical stimulation strategies, I sought to employ an opsin-based approach. There are currently no studies investigating *in vitro* functional long-range connectivity from M1 to S1BF during development through this technique, as the previous literature relied on *in vivo* approaches that preserved the connectivity between these areas (Dooley and Blumberg, 2018; Luhmann, 2017; Tiriach et al., 2014).

Two main challenges have to be faced when applying this technique to the developing brain: (1) spatial resolution, i.e. the expression of opsins only in the region of interest; and (2) timing, i.e. the temporal delay in the expression of functional opsins.

To probe the functional connectivity from M1 to S1BF, I needed to express channelrhodopsin-2 (ChR-2) in the motor cortex, with minimal contamination from surrounding areas. Unfortunately, to the best of our knowledge, there were no mouse lines expressing M1 specific markers and I could not employ conditional expression of the *Ai32* construct as described previously (see Chapter 3.3). Therefore, a viral approach was employed to target ChR-2 expression exclusively to the area of interest, i.e. M1. This presented an issue: how to get functional ChR-2 expression in early development, given that a three-week time window is too broad for investigating sensorimotor development. In fact, by the second postnatal week, S1BF is fully developed and mature sensory-motor behaviours appear (Arakawa and Erzurumlu, 2015; Erzurumlu and Gaspar, 2012).

In utero electroporation could be a solution for allowing enough time for the expression of functional ChR-2. However, it is challenging to target specific structures with this technique (dal Maschio et al., 2012), the targeted cells depend on the time of the procedure (Bitzenhofer et al., 2017) and developmental effects can still be observed (Marques-Smith, 2014).

The need for early developmental expression of ChR-2 has been addressed previously in Arruda-Carvalho et al. (2017), where the authors investigated the functional development of prefrontal cortex-amygdala connectivity using optogenetics. In this paper, they showed ChR-2+ terminals to

be able to elicit post-synaptic currents already after 6 days post injections, using the AAV1.CaMKIIa.hChR2(H134R)-eYFP.WPRE.hGH virus (Penn Vector Core). This is a serotype 1 adeno-associated virus (AAV1), encoding for the H134R ChR-2 variant fused with the yellow fluorescence protein (YFP) reporter under the control of the CamKIIa promoter, which is specific for excitatory cells (see Materials and Methods). Thus, this viral construct would allow me to perform injections around the time of birth into motor cortex and investigate the development of functional connectivity onto VIP+ INs in S1BF from the end of the first postnatal week in *VIP-Cre;Ai9* animals.

4.1.2 – Testing differential viral strategies

I first confirmed the optimal stereotaxic coordinates to perform injections in the motor cortex of P0-1 mice (see Materials and Methods). Several injection protocols were tested, to ensure observable viral expression at early time points not only in M1 neuron somata, but also in projecting fibres to S1BF.

Arruda-Carvalho et al. (2017) used 150 nL of virus in their protocols; however, I was concerned that this quantity was excessive considering the size of the region I aimed to target. I set out to try three injection strategies in independent litters: (1) a low dose of virus (75 nL); (2) a 1:2 dilution of virus (100 nL of virus diluted in PBS for a total volume of 200 nL) and (3) a high dose injection (150 nL).

Low dose injections (75 nL of virus, Figure 4.1) led to good YFP expression in the injected area 7 days post-injection (dpi, equivalent to P7-8 Figure 4.1A), as well as to few YFP+ fibres in the corpus callosum (Figure 4.1B).

Only high background noise was visible in S1BF, suggesting lack of YFP+ fibres (compare signal in magnification in Figure 4.1B and Figure 4.1C), and no signal was detectable in visual cortex (Figure 4.1D).

In a second attempt, 100 nL of virus diluted in 1:2 in PBS was used. This protocol allowed only very low YFP expression at the injection site at 7dpi (Figure 4.2A). At 14dpi (equivalent to P14-15) I could observe a brighter YFP signal in the injection site (Figure 4.3A), yet few YFP+ fibres in S1BF (Figure 4.3C) and none in visual cortex (Figure 4.3D).

Finally, a higher volume of virus without any dilution was tested (150nL); in these conditions, strong YFP expression at the injection site (i.e. M1) was observed (Figure 4.4A) as well as YFP+ fibres in S1BF, the internal capsule and the sensory-motor cortex related thalamus (Figure 4.4C) but not in other areas such as primary visual cortex (V1) (Figure 4.4D). The presence of these structures suggested an effective viral targeting of primary motor cortex efferent pathways, comparable with previous tracing in adults (Allen Brain Atlas; Kinnischtzke et al., 2014).

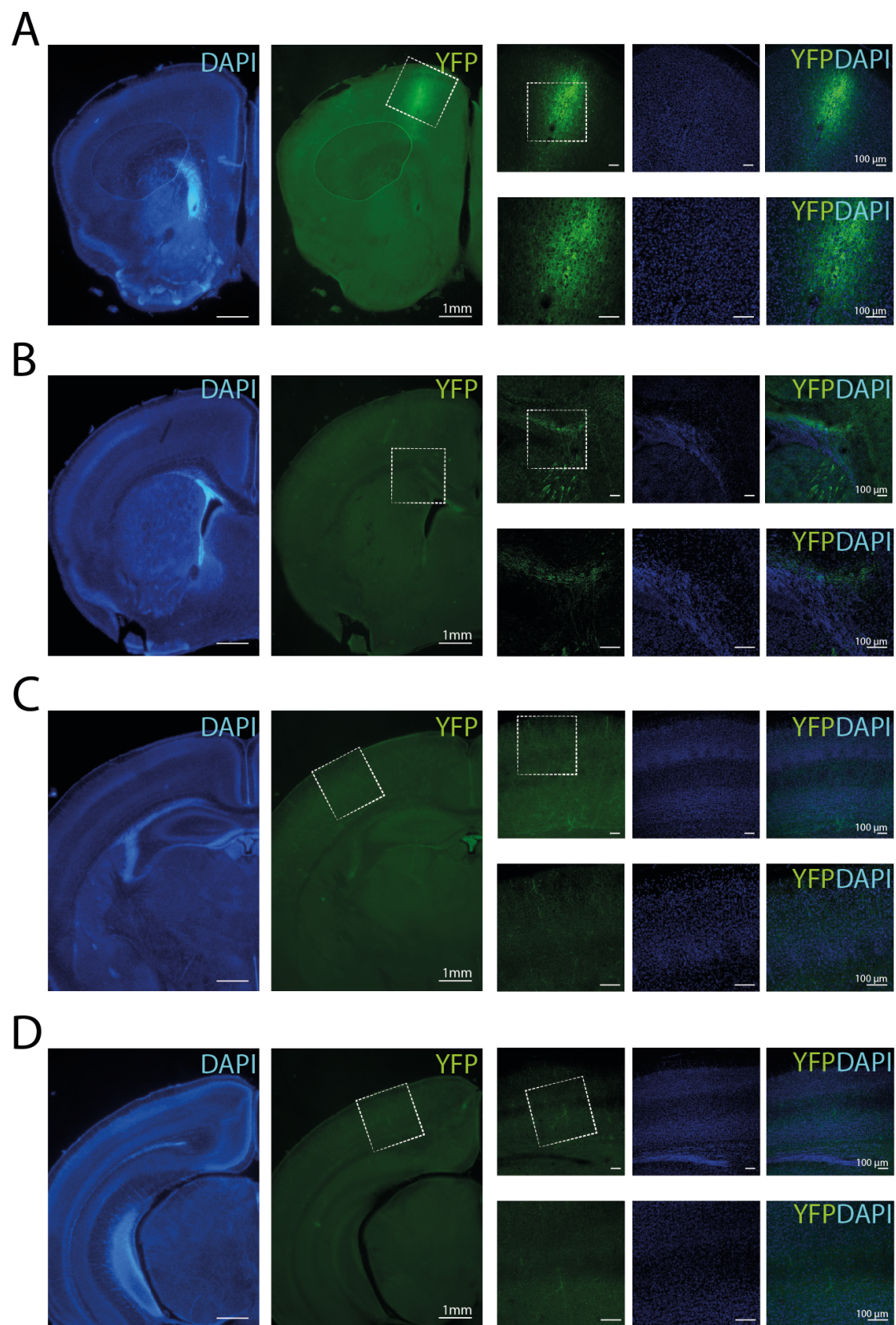


Figure 4.1 Expression level and viral spread at 7 days post injection (dpi, i.e. P7-8) with 75 nL of virus - See page 126 for description.

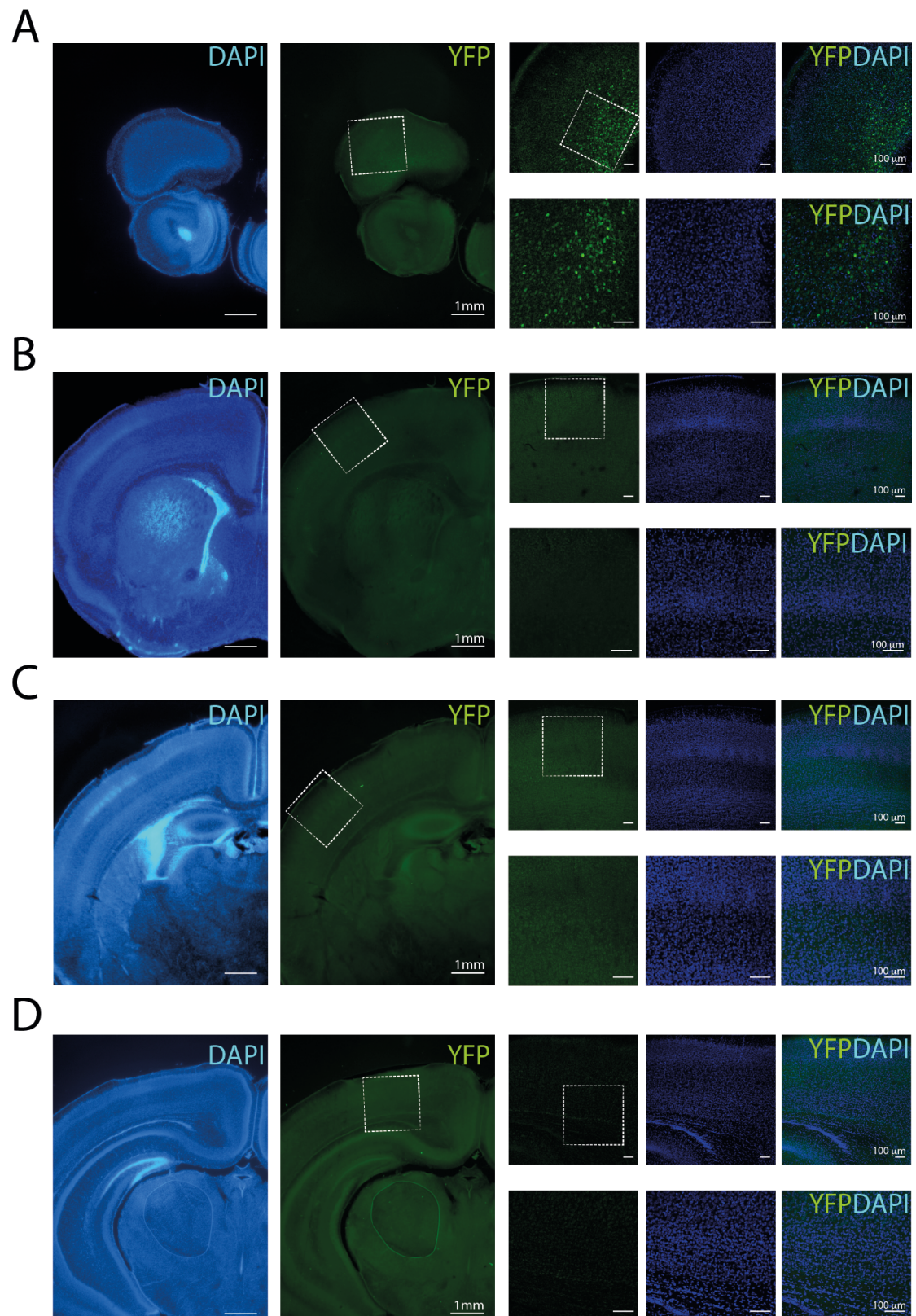


Figure 4. 2 Expression level and viral spread at 7 days post injection (dpi, i.e. P7-8) with 100 nL of virus diluted 1:2 in PBS - See page 126 for description.

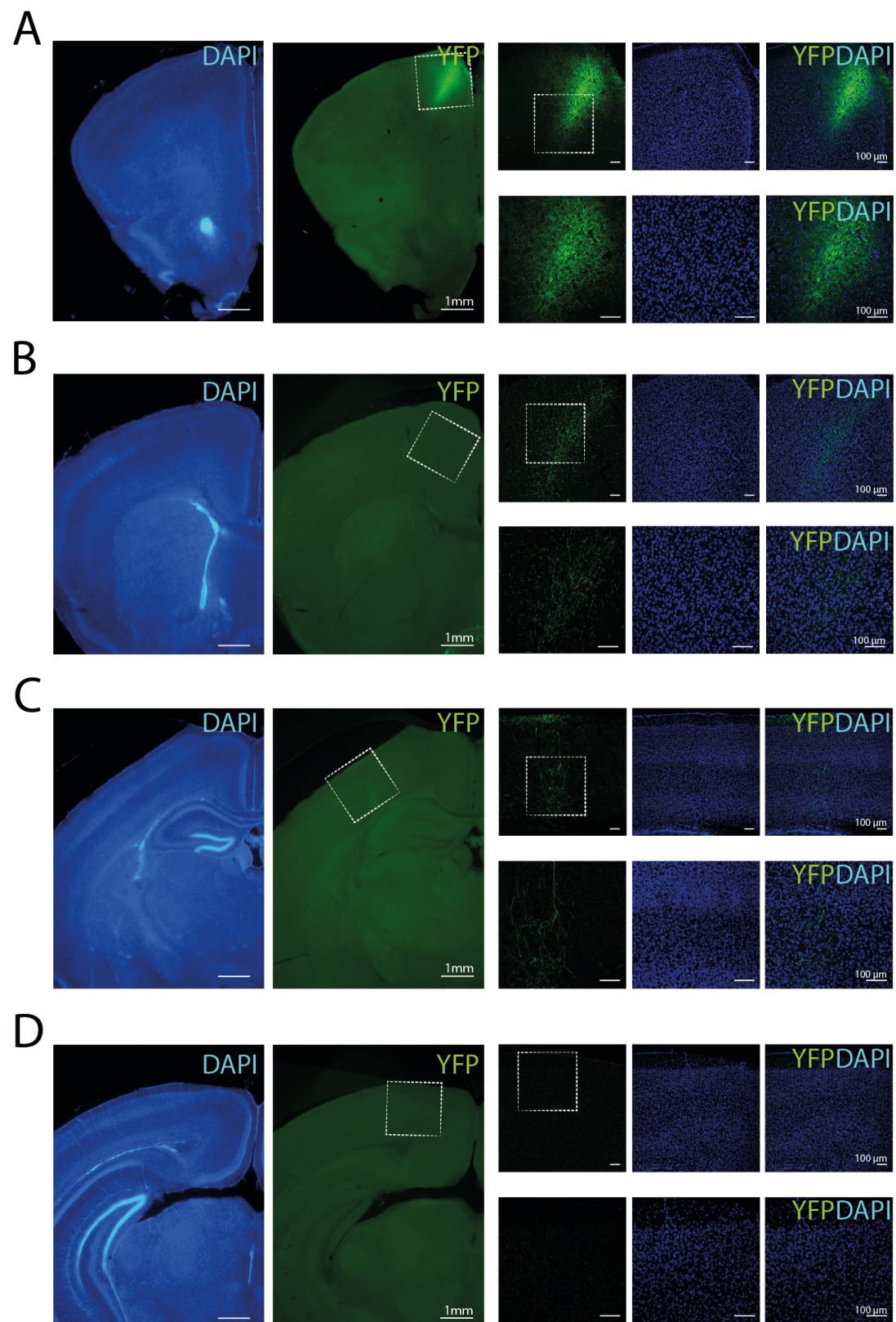


Figure 4. 3 Expression level and viral spread at 14 days post injection (dpi, i.e. P14-15) with 100 nL of virus diluted 1:2 in PBS - See page 126 for description.

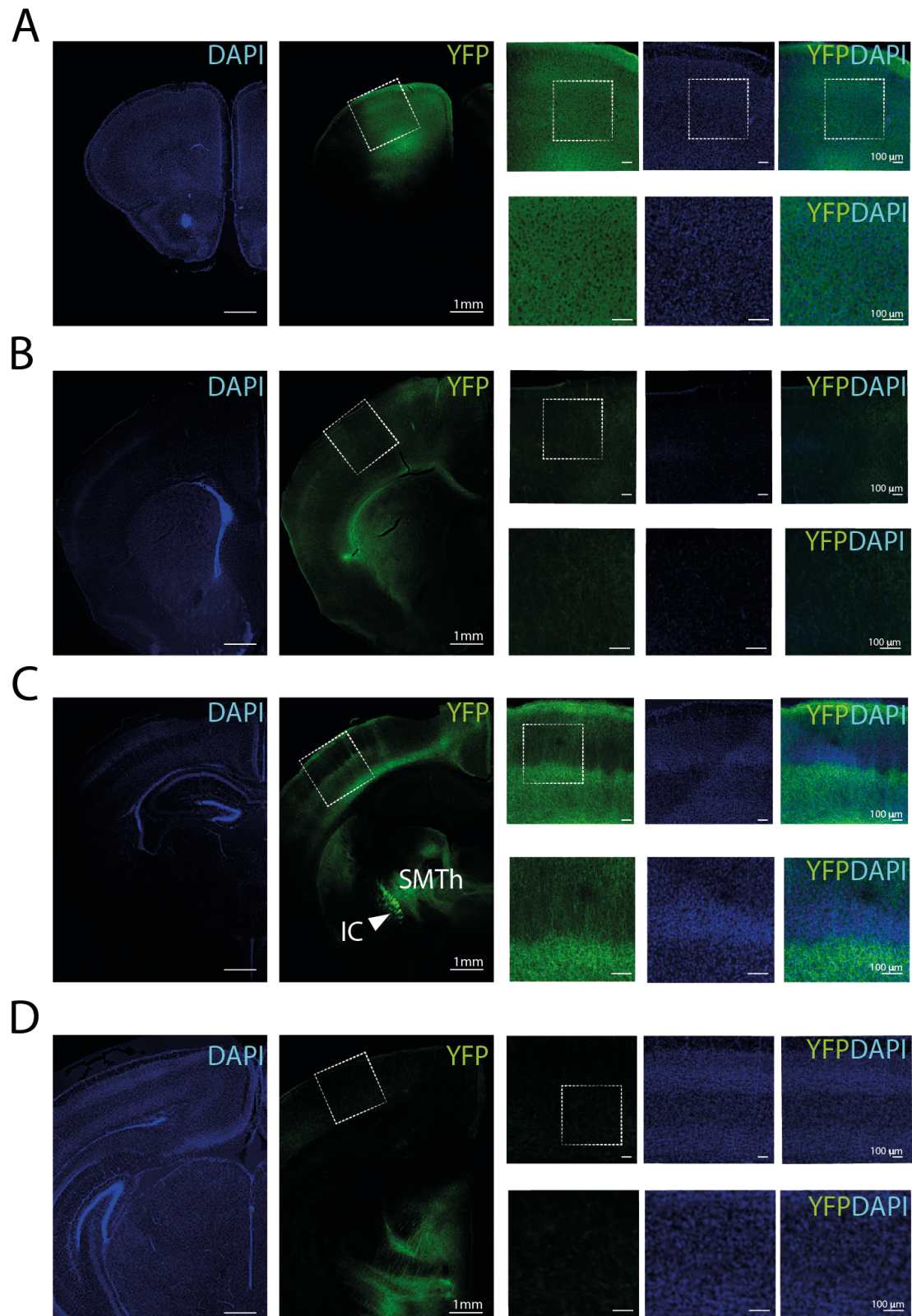


Figure 4.4 Expression level and viral spread at 7 days post injection (dpi, i.e. P7-8) with 150 nL of virus - See page 126 for description.

Figure 4.1 Expression level and viral spread at 7 days post injection (dpi, i.e. P7-8) with 75 nL of virus (A-D) Immunofluorescence pictures of neonatal brain at 7dpi (i.e. P7-8) are taken across the rostro-caudal axis with a wide-field fluorescent microscope, in order to visualise spread of YFP+ fibres from the injection site (A) to visual cortex (D). The right panels represent confocal magnifications of the area highlighted on the left panels. The images at the bottom on the right panels represent a magnification of the top panels. YFP+ signal can be observed mainly at the injection site (A) and at the level of the corpus callosum (B). Scale bar on the left: 1mm; Scale bar on the right: 100 μ m. Green:YFP; Blue:DAPI.

Figure 4.2 Expression level and viral spread at 7 days post injection (dpi, i.e. P7-8) with 100 nL of virus diluted 1:2 in PBS (A-D) Immunofluorescence pictures of neonatal brain at 7dpi (i.e. P7-8) are taken across the rostro-caudal axis as described in Figure 4.1. By 7dpi the presence of YFP+ signal is extremely low at the level of the injection site (A). Scale bar on the left: 1mm; Scale bar on the right: 100 μ m. Green:YFP; Blue:DAPI.

Figure 4.3 Expression level and viral spread at 14 days post injection (dpi, i.e. P14-15) with 100 nL of virus diluted 1:2 in PBS(A-D) Immunofluorescence pictures of neonatal brain at 14 dpi (i.e. P14-15) are taken across the rostro-caudal axis as described in Figure 4.1. By 14 dpi YFP+ signal can be detected the level of the injection site (A), but only few fibres are visible in S1FB (C). Scale bar on the left: 1mm; Scale bar on the right: 100 μ m. Green:YFP; Blue:DAPI.

Figure 4.4 Expression level and viral spread at 7 days post injection (dpi, i.e. P7-8) with 150 nL of virus(A-D) Immunofluorescence pictures of neonatal brain at 7dpi (i.e. P7-8) are taken across the rostro-caudal axis with a confocal microscope, in order to visualise spread of YFP+ fibres from the injection site (A) to visual cortex (D). The right panels represent magnifications of the area highlighted on the left panels. The images at the bottom on the right panels represent a magnification of the top panels. By 7 dpi YFP+ signal can be detected the level of the injection site (A), in S1BF and other subcortical structures, IC=internal capsule; SMTh=Sensory-Motor cortex related thalamus (C). Scale bar on the left: 1mm; Scale bar on the right: 100 μ m. Green:YFP; Blue:DAPI.

4.1.3 – Injection of 150nL of virus allows to perform early developmental studies

Following 150 nL injection of virus, I observed viral spread from the injection site to neighbouring areas such as secondary motor cortex (M2) or anterior cingulate cortex (see Figure 4.5A, first panel). YFP+ projections in S1FB and in subcortical structures such as the basal ganglia, the internal capsule and the sensory-motor cortex related thalamus confirmed the successful target of M1 (Emos and Agarwal, 2019; Jeong et al., 2016).

Variability between injections could be observed, either due to small differences in terms of injection site or viral spread (see Figure 4.5A, second and third panel). For example, following a too lateral injection I observed a reduction of fibres in the sensory-motor cortex related thalamus and S1BF, with a more prominent YFP+ signal from S1ULp (primary somatosensory cortex, upper lip region) and the secondary somatosensory cortex (S2) (see Figure 4.5A second panel).

After a more medial injection, probably targeting preferentially M2, I could not observe a prominent S1BF signal, although I still observed S2 labelling (see Figure 4.5A third panel). Animals that didn't show clear labelling of anterior-motor areas (i.e. M1 and surroundings), together with projections in S1BF, basal ganglia, sensory-motor thalamus and internal capsule were excluded from further analysis.

I confirmed that YFP+ fibres in S1BF surrounded VIP+ INs (Figure 4.5B) and that the conditions employed did not lead to retrograde labelling of S1BF neurons, as demonstrated by the lack of somatic staining in S1BF (Figure 4.5C).

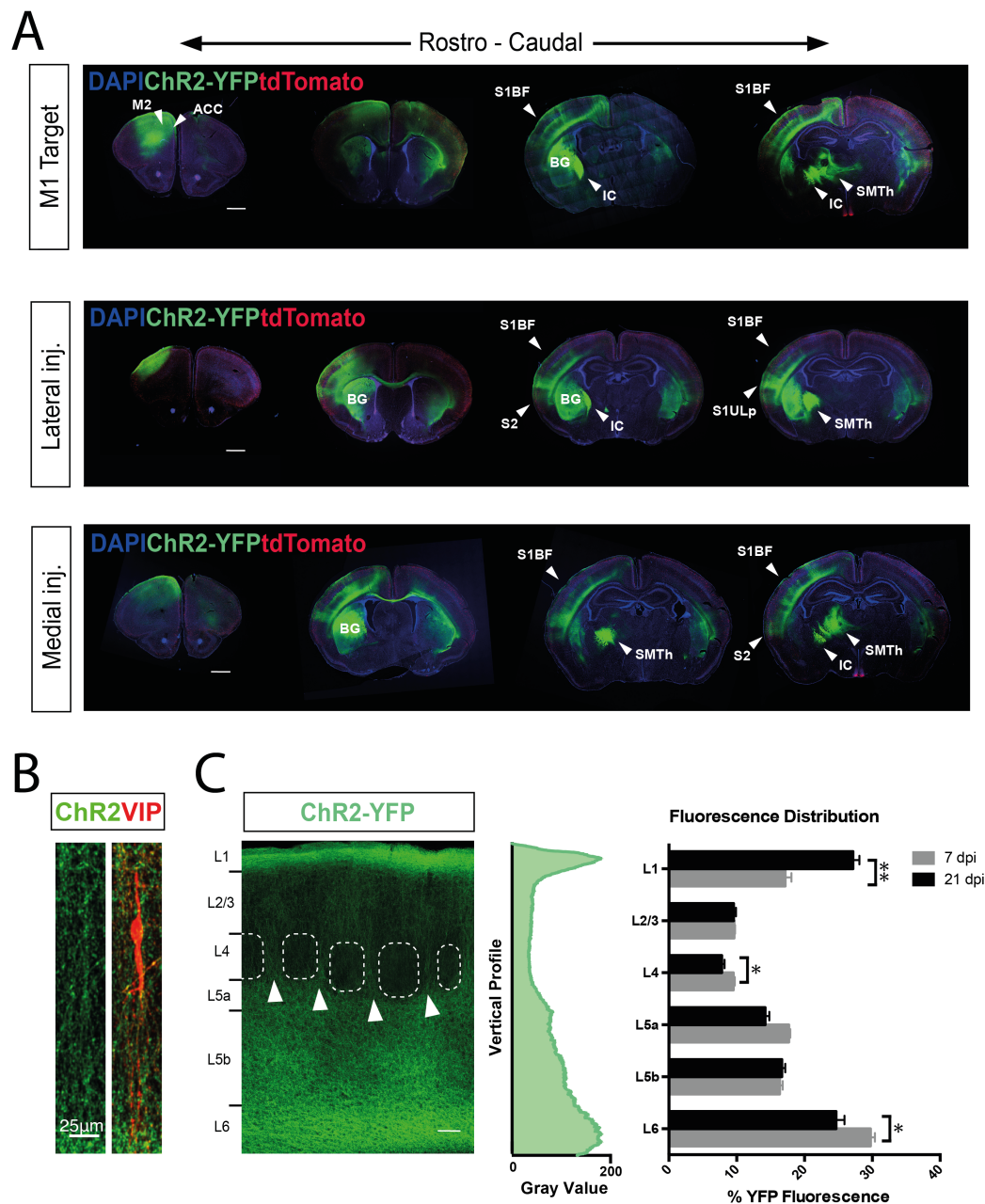


Figure 4.5 Analysis of viral spread: cross-animal variability and quantification of YFP fluorescence in S1BF (A) Examples of different structures targeted by subtle variations during injections. The first panel represents an ideal injection, with targeting of primary motor cortex (M1). The second panel shows a more lateral injection while the third a more medial one. Targeted areas are indicated with the following acronyms: M2=secondary motor cortex; ACC=anterior cingulate cortex; BG=basal ganglia; IC=internal capsule; SMTh=Sensory-Motor cortex related thalamus; S1ULp = primary somatosensory cortex, upper lip region; S2=secondary somatosensory cortex. Scale bar=1 mm. Green:YFP; Red:tdTomato; Blue:DAPI. (B) Magnification at the level of S1BF, showing a L2/3 VIP+ INs surrounded by YFP+fibers. Scale bar=25 μm(C) YFP+ signal in S1BF. Left: Immunofluorescence of YFP+ fibers in S1BF. Layers are indicated on the side, dotted lines represent the barrels and arrowheads the septa. Scale bar=100 μm. Central panel: Intensity profile of the picture on the left. Right panel: quantification relative fluorescence signals in different layers at 7 and 21 dpi (P7-8 and P21-22 respectively). YFP+ signal in L2/3 remains constant at the different time points analysed. 7dpi: n=3 animals, 8 counts. 21dpi: n=2 animals, 6 counts. *:p≤0.05; **:p≤0.001

In successful injections, I could observe fibres mainly in L1 and L6, as well as in L4 septa, as previously reported in the adult brain (Kinnischtzke et al., 2014) (Figure 4.5C). Following immunofluorescence, I quantified the YFP signal in S1BF and observed an overall similar distribution across time points. However, some age-related differences in relative fluorescence across layers were present. In particular, there was a significantly higher proportion of YFP+ signal in L1 at later time points (21dpi, equivalent to P21-22) when compared to 7dpi, together with smaller L4 and L6 component. (Mixed effect analysis: L1: 7dpi= 17.2±2.6% vs. 21dpi= 27.1±2.3%, $F(1, 3) = 95.90$, $p=0.0023$; L4: 7dpi=9.5±0.6%; 21dpi=7.8±1.0%, $F(1, 3) = 18.25$, $p=0.0235$; L6: 7dpi=29.7±1.9%; 21dpi=24.7±3.1%, $p=0.0452$, see Appendix 2 for detailed statistics). The presence of edge artefacts was excluded, given that the YFP+ signal was only present ipsilaterally to the injection site (Figure 4.5C).

This might suggest either a developmental or a viral expression effect on fluorescence distribution. However, no differences in YFP fluorescent intensity was observed in L2/3 at the two time point investigated, suggesting that viral expression in these layers may be already maximal after 7 dpi. This data show that the viral approach employed here successfully labels projecting fibers in S1BF already at the end of the first postnatal week. Once the functionality of ChR-2 is confirmed, it could be used to assess the development of long-range connections to S1BF.

4.1.4 – Discussion and limitations

I tested several injection strategies in P0-1 animals to see whether I was able to express YFP-fused ChR-2 in primary motor cortex to evoke suprathreshold optical response and therefore probe connections to S1BF during development.

I identified the necessary conditions to ensure effective YFP+ labelling of the injection target and presence of fibres in S1BF surrounding VIP+ INs, without retrograde labelling of S1BF soma. I was able to observe subcortical structures such as the basal ganglia, the internal capsule and the sensory-motor cortex related thalamus, in line with what previously published in the adult brain (Alloway et al., 2009; Jeong et al., 2016). All these structures are involved in motor cortex function and output: the basal ganglia is responsible for motor control (Lanciego et al., 2012); the internal capsule is a white matter structure that contains the corticospinal tract originating from the motor and premotor areas (Emos and Agarwal, 2019); the sensory-motor cortex related thalamus includes structures such as the POm and it has been shown as a key element in the modulation of somatosensory processing by the motor cortex (Lohse et al., 2018). Thus, I confirmed the successful staining of fibres originating from M1 as well as many of its target areas in the mouse brain.

Neonatal stereotaxic injections are affected by some biological and technical limitations, such as the smaller size of the structures targeted, the lack of ear cuffs suitable for newborn mice, and the employment of hypothermia as anaesthesia method. This requires fast pace while performing the injections (<5min per surgery) thus reducing accuracy. All these factors did not allow to label exclusively M1 and partial contaminations from surrounding areas, such as

M2 and ACC could be observed. For this reason injection areas will be referred as 'anterior-motor' areas.

Across-animals variability could be observed: prior to further analysis, I confirmed the presence of YFP+ fibres in key cortical and subcortical structures, such as L1 and L6 of S1BF, basal ganglia, internal capsule, and sensory-motor thalamus to ensure successful execution of the technique.

While the overall fibre distribution was constant, I observed differences in YFP intensity across layers following 1 or 3 weeks after injections. This suggests either dynamic changes in anterior-motor to S1BF connectivity over development or incremental expression of the virus. I couldn't exclude any of these possibilities: it has been already shown that the variability of ChR2 expression following viral injection is higher than in the *Ai32* reporter line (Zhang et al., 2010). This supports the hypothesis that the layer differences observed could be the result of incremental virus expression. To eliminate this confounding factor, I could have performed injections at different time points and allow the same expression window in all the conditions (see Arruda-Carvalho et al., 2017), however this was not allowed by the lab project licence. L2/3, the layer where the majority of VIP+ INs somata is located and the target of my final experiments, did not show increase in fluorescence signal in L2/3 over time, suggesting maximal expression viral expression already at 7dpi (P7-8).

4.2 – Timeline of input from anterior-motor areas to S1BF

To confirm the functionality of ChR-2 in this preparation, I first set out to test the ChR-2 ability to evoke action potentials at the soma of infected cells in anterior-motor areas upon blue light illumination (470nm).

I performed loose cell-attached recordings in YFP+ cells and varied LED intensities (0.082, 0.38, 0.7, 1.33, 2.83 and 5.52 mW/cm²) and pulse durations (1 vs 10 ms, Figure 4.6) throughout development. I confirmed that ChR-2 expression was sufficient to trigger light-evoked action potentials already at P7 (i.e. 6 dpi) as previously reported (Figure 4.6B) (Arruda-Carvalho et al., 2017). I was able to activate a higher proportion of cells after increasing LED intensity and pulse duration in both young and old animals, but I could not perform statistical analysis due to the low data points recorded (Figure 4.6C and 4.6D). Considering the low number of responsive cells at P7-9 following 1ms light pulse, I only analysed response latency and jitter (defined as standard deviation of response latency across multiple trials, a measure for reliability of the response) in P $\geq$ 13 anterior-motor neurons at different LED intensities (Figure 4.6 E and G). I did not observe any significant reduction in either parameter (see Appendix 2 for detailed statistics).

I then compared response latency and jitter of light-evoked action potentials following 10ms stimulation between the two developmental groups (Figure 4.6F and H). I observed low latency and jitter values in the conditions analysed, suggesting reliable activation of ChR-2 upon 470 nm light stimulation

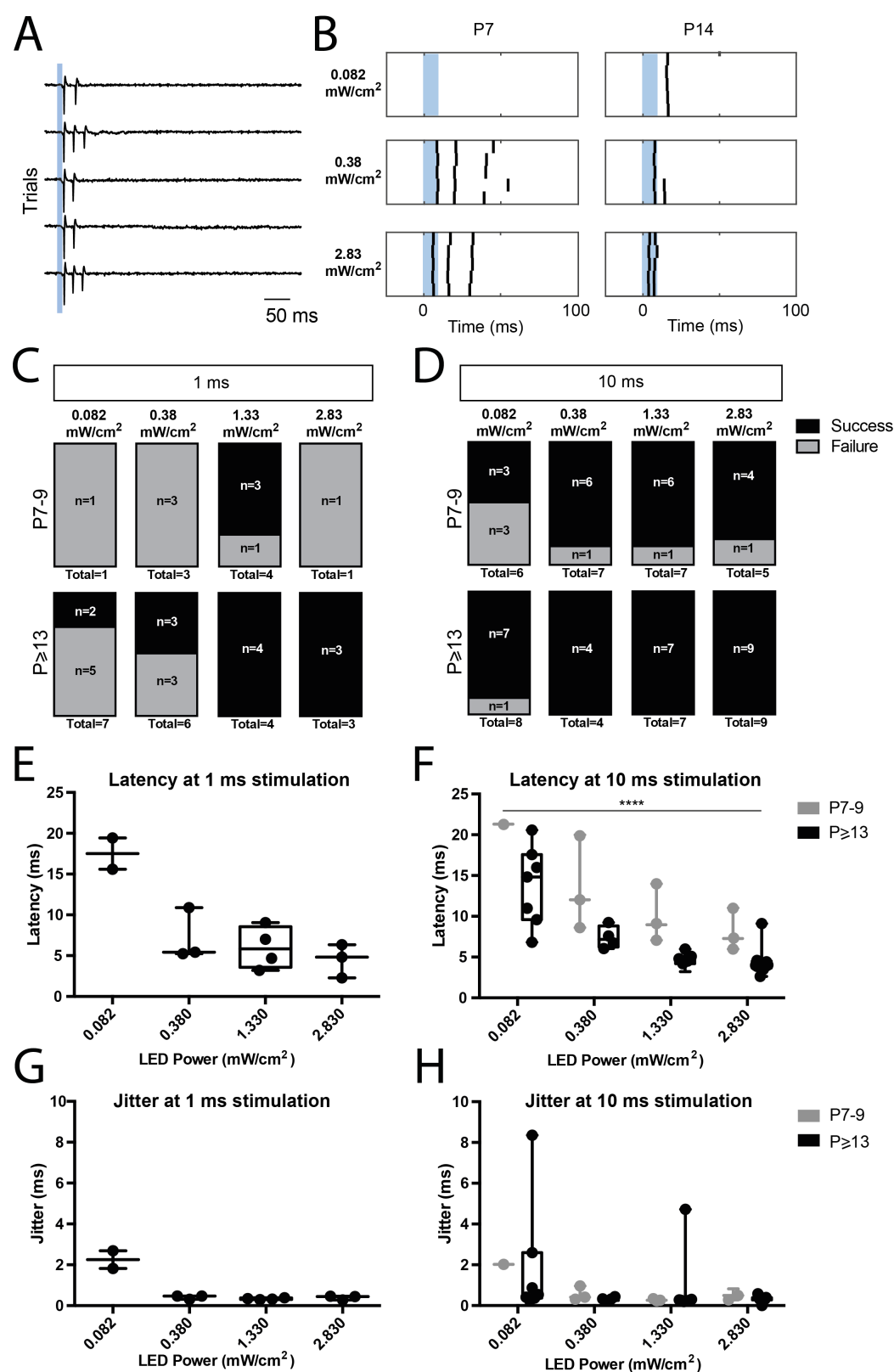


Figure 4.6. ChR-2+ cells in anterior-motor areas respond to light stimulation at 470nm
 (A) Extracellular recordings from an example ChR-2+ cell in anterior-motor areas. Light-evoked action potentials are present in all the trials. Blue: LED pulse (B) Raster plot of a P7 and a P14 ChR-2+ cell at different LED intensity. Lower LED intensity fail to recruit ChR-2+ cells at P7.

Continues from previous page Blue: LED pulse (C) Quantification of the proportion of ChR-2+ cells recruited at different LED intensities with 1ms LED pulse. Top row: P7-9; Bottom row: P $\geq$ 13. A higher proportion of cells are recruited when increasing LED intensity, but only in older animals this is consistent throughout all the conditions tested. (D) Quantification of the proportion of ChR-2+ cells recruited at different LED intensities with 10ms LED pulse. Top row: P7-9; Bottom row: P $\geq$ 13. Increasing LED intensity leads to a higher proportion of recruited cells, at all ages tested (E) Quantification of latency of the first action potential evoked in P $\geq$ 13 responsive cells at different LED intensities with 1ms LED pulse. (F) Quantification of latency of the first action potential evoked in P7-9 and P $\geq$ 13 responsive cells at different LED intensities with 10ms LED pulse. Latency significantly decreases with higher LED intensities. (G) Quantification of jitter across trials of the first action potential evoked in P $\geq$ 13 responsive cells at different LED intensities with 1ms LED pulse. (F) Quantification of jitter across trials of the first action potential evoked in P7-9 and P $\geq$ 13 responsive cells at different LED intensities with 10ms LED pulse. Latency jitter does not change with different LED intensities****: $p \leq 0.0001$

already in the first postnatal week (P7-9: latency= 8.1 ± 2.6 ms; jitter= 0.5 ± 0.3 ms and P $\geq$ 13: latency= 4.5 ± 1.8 ms; jitter= 0.3 ± 0.1 ms, 10ms LED pulse at 2.83 mW/cm²). While I did not observe any significant differences in jitter, I did observe an age and LED intensity effect in latency: increasing LED intensity led to a reduction of the latency of action potentials, although less prominent for P7-9 (see Appendix 2 for detailed statistics). The data so far confirms ChR-2 functionality already in the first postnatal week but suggests age-specific differences in latency. This could be due to differential expression levels in ChR-2 or to maturation of ChR-2 expressing cells, leading to a developmental decrease in membrane time constant.

Having confirmed the ability to generate supra-threshold events in ChR-2+ neurons in anterior-motor areas, I set out to assess long-range inputs onto S1BF VIP+ and pyramidal cells. To confirm the functionality of ChR-2 at the terminals and establish criteria to define monosynaptic light-evoked excitatory post-synaptic currents (EPSCs), I performed whole-cell patch-clamp recordings from S1BF VIP+ INs and pyramidal neurons in L2/3 upon 470nm light stimulation (Figure 4.7). The functionality of channelrhodopsin at the synaptic

terminal was confirmed throughout the time window analysed and light-evoked EPSCs were recorded in a total of 111 L2/3 cells (n=69 P7-9 and n=52 $\geq$ P13).

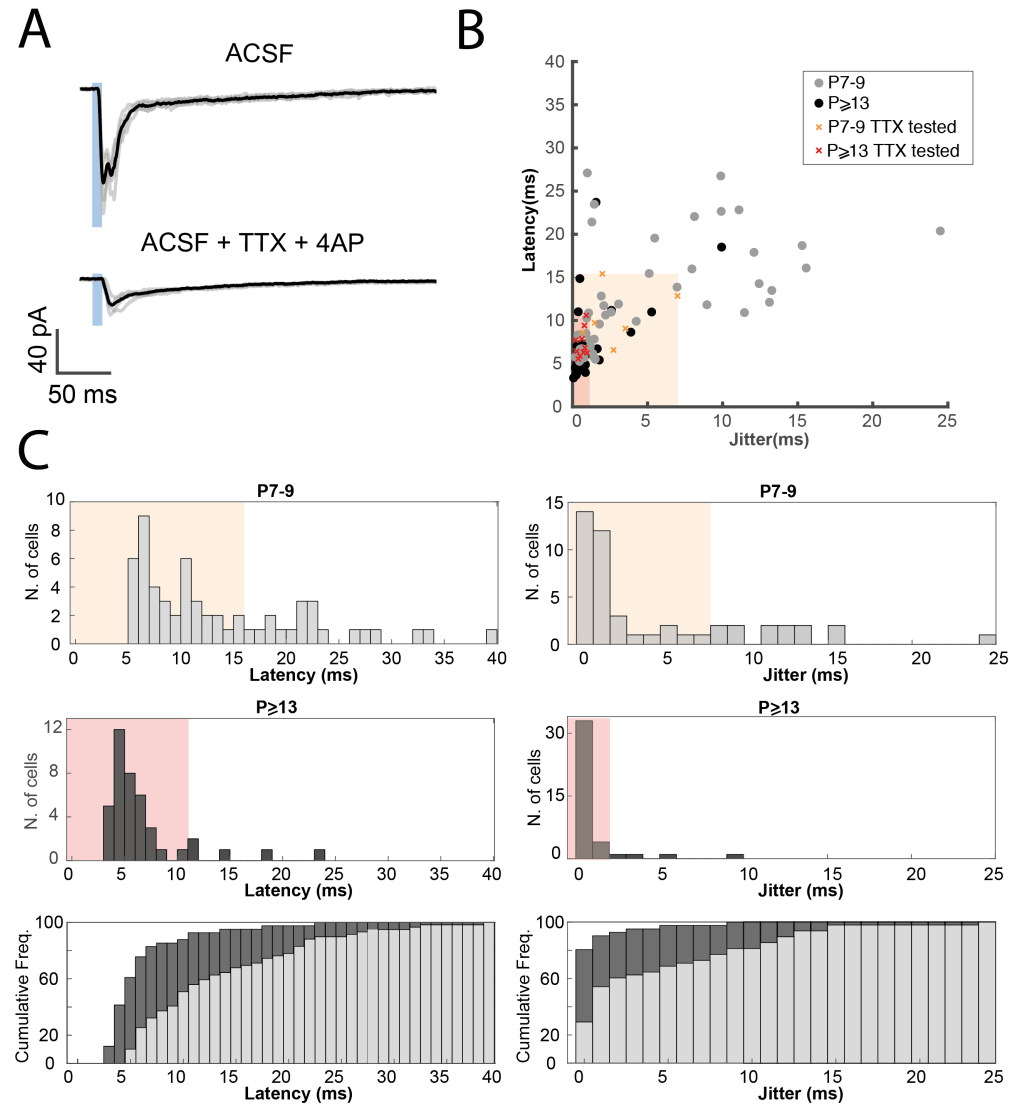


Figure 4.7 Definition of EPSCs monosynaptic window (A) Example of LED-evoked responses in one cell before (top) and after (bottom) addition of TTX and 4-AP to the ACSF. (B) Scatter plot of average latency and jitter of the recorded light-evoked EPSCs at different developmental ages. Stimulation protocol: 10ms pulse at maximal LED intensity, i.e. 5.52mW/cm². Light grey dots: P7-9; Black dots: P $\geq$ 13. Orange cross: P7-9 cells recorded with TTX and 4-AP; Red cross: P $\geq$ 13 cells recorded with TTX and 4-AP. Orange shadow: monosynaptic window for P7-9; Red shadow: monosynaptic window for P $\geq$ 13. Addition of TTX and 4-AP reveals different monosynaptic windows for the time points analysed. (C) Histograms showing the distribution of latency and jitter values showed in (B). The last row represents the cumulative distribution. Light grey bars: P7-9; Dark bars: P $\geq$ 13. Orange shadow: monosynaptic window for P7-9; Red shadow: monosynaptic window for P $\geq$ 13. LED-evoked responses in P7-9 cells present more scattered latency and jitter values.

In a subset of 18 cells (both pyramidal and VIP+ INs; n=8 P7-9 and n=10 $\geq$ P13) light-evoked EPSCs were also tested in the presence of tetrodotoxin (TTX, 1 μ M) and 4-Aminopyridine (4-AP, 100 μ M) in order to isolate monosynaptic events, as previously reported (Petreanu et al., 2009; Suter and Shepherd, 2015; Yamawaki and Shepherd, 2015). At the maximal LED intensity, TTX and 4-AP led to amplitude reduction of light-evoked EPSCs when compared with their relative controls, an indication of successful blocking of polysynaptic transmission (see Figure 4.7A for an example).

I observed a significant age difference in EPSCs features in presence of TTX and 4-AP. In particular, younger monosynaptically connected cells showed a significantly higher latency and jitter when compared to older cells (Latency: P7-9 11.4 ± 3.3 ms vs. $P \geq 13$ 7.3 ± 1.7 ms, Mann-Whitney test $p=0.0062$; Jitter: P7-9 2.9 ± 2.2 ms vs. $P \geq 13$ 0.6 ± 0.3 ms, Mann-Whitney test $p=0.0047$, n=8 and n=10 respectively). Therefore, I established two different criteria for determining the monosynaptic window of light-evoked EPSCs, defined as the maximal values of latency and jitter observed at each time window (i.e. P7-9: Latency: 15.8 ms, Jitter: 7ms; $P \geq 13$: Latency: 10.6 ms, Jitter: 1ms) (Figure 4.7B and C, shading). Thus, cells recorded in the absence of TTX and 4-AP were classified as monosynaptically connected only if their average latency and jitter following the same stimulation protocol as the experiments performed in TTX/4-AP (i.e. 10 ms LED pulse at 5.52mW/cm²) was less or equal to the criteria value.

After confirming whether a cell was monosynaptically connected, I tested different LED intensities and durations (see Materials and Methods) in order to find the minimal LED stimulation able to elicit at least 2 EPSCs over a total of 5 trials. (Figure 4.8A). With this approach I sought to control (at least to certain

extent) for different viral expression levels across time and to minimise the effect of polysynaptic activity, evaluating the minimal input from anterior-motor areas received by VIP+ INs or pyramidal cells across development.

The proportion of VIP+ INs monosynaptically recruited by anterior-motor areas significantly increased over time, from 35.0% (P7-9, n=20) to 81.8% (P $\geq$ 13, n=11) (Fisher's exact test, $p=0.0233$, Figure 4.8B). This was not the case for pyramidal cells (n=12/19 at P7-9 vs n=8/9 at P $\geq$ 13, Fisher's exact test, $p=0.2144$), suggesting their integration with the long-range network remains constant throughout development. No differences were found between proportions of connected VIP+ INs and pyramidal cells at early or late time points (Fisher's exact test, $p=0.1128$ and $p=1$ respectively, Figure 4.8B).

Once the minimal stimulation for each responsive cell was found, I calculated the average amplitude, latency and jitter of its light-induced EPSCs (Figure 4.8C). While no differences were found between amplitude and jitter in VIP+ INs and pyramidal cells at the time points tested (see Appendix 2 for detailed statistics), I observed a significant interaction between cell type and age when analysing the average latency (2 way ANOVA, $F(1, 30) = 10.37$; $p=0.0031$). *Post-hoc* multiple comparisons confirmed a significant difference in latency of VIP+ INs EPSCs at the different time points (P7-9: $15.9\text{ms} \pm 5.4\text{ ms}$; P $\geq$ 13: $8.5\text{ ms} \pm 2.9\text{ ms}$; Sidak's multiple comparisons test, $p \leq 0.01$).

This data suggest that somatosensory VIP+ INs are recruited by anterior-motor cortex connections as early as pyramidal cells, and the proportion of connected VIP+ INs increases over time, reflecting the maturation of their electrophysiological properties and late recruitment by the local glutamatergic network (See Chapter 3). Moreover, it suggests a developmental decrease in

latency in VIP+ INs, and no differences in amplitude or jitter when compared to pyramidal cell controls.

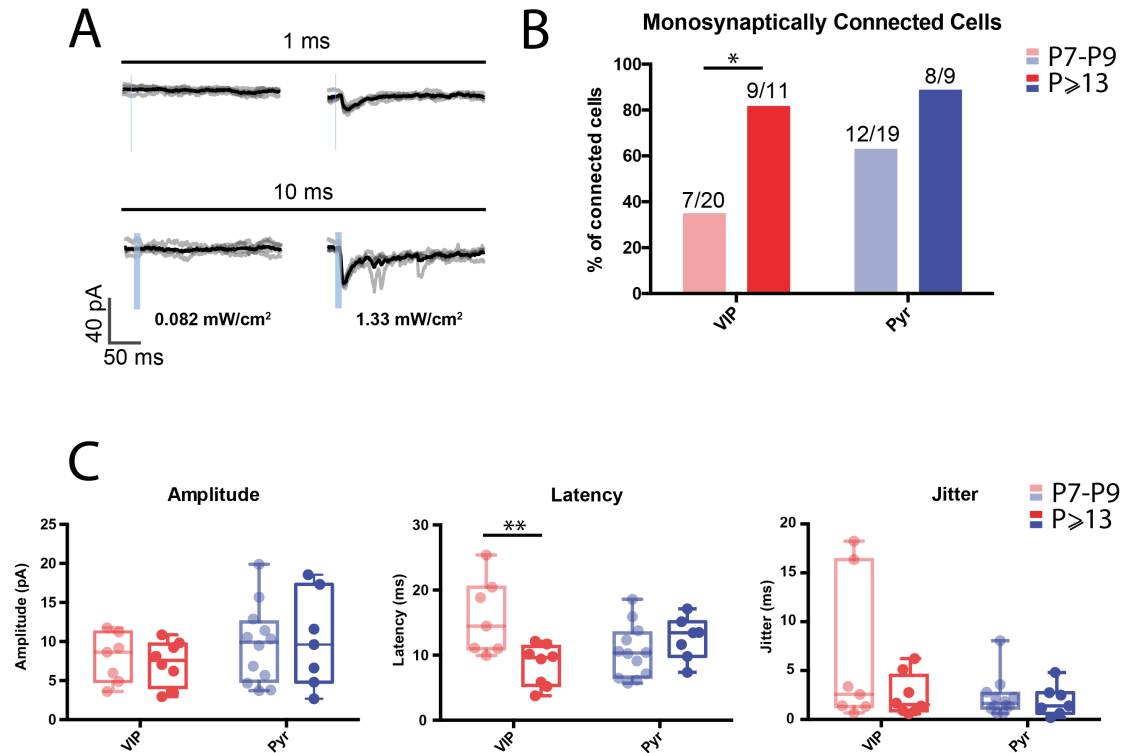


Figure 4.8 Monosynaptic connectivity between anterior-motor areas and S1BF neurons increases over development (A) Example of the experimental approach. (B) Proportions of S1BF VIP+ INs and pyramidal controls that receive monosynaptic connections from anterior-motor areas at different developmental windows. The proportion of VIP+ INs recruited by anterior-motor areas increases over development. (C) Comparison of average amplitude (left), latency (centre) and jitter (right) of minimal stimulation responses in VIP+ INs and pyramidal controls. Latency of evoked responses in VIP+ INs decreases during development, while no cell-specific differences can be found in amplitude or jitter at any time points. *:p≤0.05; **:p≤0.01

4.2.2 – Discussion and limitations

Reciprocal connectivity between motor and somatosensory systems has been previously characterised in the adult brain at a cellular resolution level (Aronoff et al., 2010; Hooks et al., 2013, 2015; Kinnischtzke et al., 2014, 2016; Lee et al., 2013; Mao et al., 2011; Petersen, 2019), but little is known about how this connectivity develops.

To address this, I have used P0-1 ChR-2 injections to probe *in vitro* functional connections from anterior-motor cortex to S1BF during development. I established the functional expression of ChR-2 both at the soma and at the terminals and confirmed the ability to record light-evoked EPSCs across the developmental time windows tested. Using TTX and 4-AP, I have defined the monosynaptic window for EPSCs, that varied throughout development. This could be possibly due to different levels of viral expression or to an increased number of synaptic contacts across development. The values obtained for latency and jitter of light-evoked EPSCs at the latest age ($P \geq 13$) are in line with previous reports (Petreanu et al., 2009).

These data demonstrate that the M1-to-S1BF long-range projections onto VIP+ INs reported in adults (Lee et al., 2013; Wall et al., 2016) are established as early as P7-9, when a third of L2/3 VIP+ INs receive putative monosynaptic input. Following the onset of active whisking ($P \geq 13$), this increases to more than 80% L2/3 VIP+ INs, a value similar to pyramidal cells connectivity.

The end of the second postnatal week is a critical time point in sensorimotor development, characterised by the onset of active whisking (Arakawa and Erzurumlu, 2015; Bureau et al., 2004). S1BF synapses between L4 and L2/3 excitatory neurons are potentiated, while local connections between L2/3 neurons are strengthened (Clem and Barth, 2006; Clem et al., 2008; Itami and Kimura, 2012; Wen and Barth, 2011). L2/3 neurons acquire response selectivity and overall there is an increase of temporal sharpening of sensory-evoked responses (van der Bourg et al., 2016, 2019).

Around the same time, important developmental changes happen in M1, which begins to fully develop its motor functions (Dooley and Blumberg, 2018;

Tiriac and Blumberg, 2016; Tiriac et al., 2014; Young et al., 2012). Even though it is able to generate movements as early as P3-5 (at least to a certain degree, An et al. 2014), still until P8-10 M1 mainly behaves as a somatosensory area, responding to spontaneous twitches (Dooley and Blumberg, 2018; Young et al., 2012). By P12-13 M1 fully responds to awake movements and is able to elicit movements following intracortical microstimulation, supporting the emergence of movement representations maps (i.e. 'motor maps') (Dooley and Blumberg, 2018; Young et al., 2012). Moreover, activity correlation between S1BF and M1 becomes more pronounced around this time (McVea et al., 2017).

This data support a role for VIP+ INs prior to active whisking (Lee et al., 2013; Yu et al., 2019), while they are still developing electrophysiological properties and local glutamatergic connectivity (Chapter 3). Other whisker movements, such as passive whisking and symmetry whisking (i.e. the symmetrical movement of whiskers back and forth) emerge before the second postnatal week (Arakawa and Erzurumlu, 2015), and functional interaction between the somatosensory and motor systems has been reported already in the first postnatal week (An et al, 2014; Luhmann, 2017; McVea et al., 2012, 2017). This could explain why both VIP+ cells and pyramidal neurons are recruited by long-range anterior-motor connections already at P7-9.

When I compared the minimal stimulation across time points and cell types, I observed that VIP+ INs were not preferentially recruited by anterior-motor connections: their average amplitude is the same as pyramidal controls. Although average jitter across cell type and age does not change, I observed that EPSCs in VIP+ INs had a longer latency at P7-9. This might be indicative of a less reliable and more immature input as compared to older VIP+ cells. However,

these conclusions are highly speculative, considering the developmental changes observed in VIP+ INs intrinsic electrophysiological properties (See Chapter 3).

Previous work explored the functional connectivity between M1 and S1BF INs employing optogenetics (Kinnischtzke et al., 2014; Lee et al., 2013). Kinnischtzke et al. (2014) did not record VIP+ INs, but focused only on fast spiking (putative PV+) and SST+ cells, demonstrating that motor cortex strongly contacts the former. Lee et al. (2013) instead performed double patching experiments on pyramidal and VIP+ cells while stimulating ChR-2+ M1 afferents. Using the same stimulation protocol across animals (i.e. 2 mW for 5 ms), they found that EPSCs amplitudes in VIP+ INs are on average more than double than in pyramidal cells.

Double patching allows to directly compare light-evoked EPSCs in different cells, therefore controlling for variability in ChR-2 expression. Given that I used a different approach to control for this (i.e. the analysis of EPSCs at the minimal stimulation), the two dataset cannot be compared directly. Further experiments employing dual-patching combined with long-range optogenetics would allow to compare EPSCs in VIP+ INs and pyramidal cells across development, further clarifying the role of VIP+ INs in early sensorimotor processing.

In this chapter, I developed a reliable method to unravel anterior-motor to S1BF input as early as P7-8 (i.e. 6-7dpi). By modifying the injection site, it could be extended to other cortical and subcortical areas and represent a valuable method for electrophysiological interrogation of long-range developing circuits.

Chapter 5 – Effect of genetic perturbation of VIP+ cells in their integration

In the previous chapters I have demonstrated that VIP+ INs integrate in the local and long-range glutamatergic network within the second postnatal week, yet provide GABAergic input to INs and pyramidal cells at the beginning of the first postnatal week. This suggests an early role for VIP+ INs, which I set out to test using a genetic model to target these cells and assess connectivity and sensory processing defects.

Activity is important for INs maturation, survival and circuit formation (see for example De Marco García et al., 2011, 2015; Denaxa et al., 2018; Karayannis et al., 2012; Marques-Smith et al., 2016; Priya et al., 2018). However, previous work suggested that maturation of VIP+ INs (or at least of the multipolar subtype) is not affected by alteration of neuronal activity (De Marco García et al., 2011, 2015; Karayannis et al., 2012). Moreover, VIP+ INs survival does not depend on activity levels (Denaxa et al., 2018; Priya et al., 2018). This evidence suggests that VIP+ INs (or at least the multipolar subtype) are to a

certain degree 'hardwired' in the cortical network and not affected by activity perturbations.

Therefore, to perturb this IN population and test cell-autonomous effects on glutamatergic connectivity and early sensory processing, I used a genetic approach. The previously described *VIP-Cre;Prox1^{c/c}* mouse line was employed, where VIP+ INs lack the transcription factor *Prox1*, an important player in their synaptic and morphological maturation (Miyoshi et al. 2015).

5.1 – Background on the Prox1^{c/c} model

Prox1 is a homeobox gene expressed in the developing central nervous system and in other structures, recently identified as a lineage tracer for CGE INs (Lavado and Oliver, 2007; Oliver et al., 1993; Rubin and Kessaris, 2013).

Miyoshi et al. (2015) employed the *Dlx6a-Cre* driver (Monory et al., 2006) to remove *Prox1* from all INs at early embryonic stages. The early embryonic loss of function of *Prox1* led to alterations of the expression levels of axon guidance molecules and synaptic proteins, as well as reduction in expression of *Vip*, *Cck* and *Calb2* (*CR*), already in CGE IN progenitors. Postnatally, the authors observed a severe reduction in the numbers of VIP+/CR+ cells in neocortex to almost a sixth of the control numbers, and morphological abnormalities in the remaining VIP+/CR+ population, with loss of their typical bipolar aspect.

To assess the integration of the VIP+/CR- population in the glutamatergic network, the authors performed recordings of spontaneous EPSCs (sEPSCs) and

found a reduction in numbers of excitatory events but not in their amplitude (Miyoshi et al. 2015). While the authors concluded that this result suggested a reduced network integration of VIP+/CR- INs in the *Dlx6a-Cre;Prox1^{c/c}*, sEPSCs analysis has some caveats when used to assess network integration of a post-synaptic targets. For instance, polysynaptic transmission is not blocked (as in the case of miniature EPSCs, where TTX is added in the bath), therefore event frequency and amplitude is a result of both monosynaptic and polysynaptic activity; moreover, pyramidal cells activity may change as a result of reduction of VIP+ INs in the *Dlx6a-Cre;Prox1^{c/c}* line. This could lead to an increased inhibition (and less disinhibition) of pyramidal cells, causing an overall decrease in pyramidal cell activity: therefore measuring less frequent sEPSCs could be just the result of reduction of VIP+ INs mediated inhibition, without affecting *per se* the integration in the network of these cells.

Postnatal loss of function of *Prox1* specifically in VIP+ INs by using the *VIP-Cre;Prox1^{c/c}* line had instead a different effect. In the first postnatal week, it led to a ~30% decrease of VIP/CR co-expression. By P21 there was a ~15% density reduction of VIP+ INs in L2/3, with a further reduction in VIP/CR co-expression and bipolar morphology defects in VIP+ cells (Miyoshi et al. 2015). However, the effect of this perturbation on the integration of VIP+ INs and the consequences for *in vivo* activity are currently unknown.

The results presented so far in this thesis have shown the complexity of early VIP+ IN integration in the local glutamatergic and inhibitory network, as well as in long-range connectivity: does *Prox1* selectively impact one or all of these processes? To unravel local and long-range glutamatergic inputs to VIP+ INs in the *VIP-Cre;Prox1^{c/c}* model, I first performed *in vitro* whole-cell patch

clamp experiments employing LSPS combined with glutamate uncaging and viral channelrhodopsin, respectively. To investigate the overall network effect of *Prox1* depletion in VIP+ INs across development, we then performed *in vivo* electrophysiological recordings combined with whisker stimulation in urethane-anaesthetised animals.

5.2 – Effect of genetic perturbation of VIP+ cells on their local integration

5.2.1 – Basic characterisation of the VIP-Cre;Ai9;Prox1^{c/c} model

To perform *in vitro* and *in vivo* characterisation of VIP+ INs lacking *Prox1*, *VIP-Cre* animals were crossed with the *Prox1^{c/c}* model (Iwano et al., 2012). In this model, the *Prox1* floxed construct is upstream to the enhanced green fluorescent protein gene (*Egfp*, see Figure 5.1A) and upon Cre-dependent *Prox1* excision all the recombined cells express EGFP.

To avoid sampling bias during patch-clamp recordings of both control and mutants VIP+ INs (due to potential differences in fluorescence levels between genotypes and therefore differences in visualisation), *VIP-Cre;Prox1^{c/c}* mice were then crossed with *Ai9;Prox1^{c/+}* to generate *VIP-Cre;Ai9;Prox1^{c/c}* animals (hereafter called Prox1 cKO or cKO) and *VIP-Cre;Ai9;Prox1^{c/+}* control littermates (hereafter called Prox1 cHet or cHet), under the assumption that cHet would recapitulate WT *VIP-Cre;Ai9* animals (however see Discussion in 5.7).

It has been previously shown that the vasoactive intestinal peptide has an endocrine role in maternal–fetal interactions, controlling inflammatory response and embryonic growth (Fraccaroli et al., 2009; Hill et al., 1996a, 1996b; Lim et al., 2008; Ramhorst et al., 2019; Spong et al., 1999). To control for major developmental defects due to perturbation in VIP-secreting cells at the maternal-fetal interface, the weight of the *Prox1* cHet and cKO animals was monitored and compared with previously obtained WT data (Figure 5.1B). No differences between genotypes were found in weight gain over development (see Appendix 3 for detailed statistics).

The presence of the transgene was confirmed via polymerase chain reaction (PCR) and immunofluorescence (Figure 5.1C-D). PCR results showed the products expected (Iwano et al., 2012) (Figure 5.1C). Preliminary immunofluorescence results suggested a slight decrease in the ratio of EGFP+/PROX1+ cells over the total amount of transgene-expressing EGFP+ cells in cKO animals, but overall high variability, probably due to the early time points tested (postnatal day 3; Figure 5.1D, cHet: $17 \pm 3\%$ vs. KO: $13 \pm 10\%$, mean $\pm$ standard deviation, n=2 counts in 1 animal for cHet, n=6 counts in 3 animals for KO). Given the low number of data points available, statistical analysis was not performed.

The data presented so far confirmed the normal weight gain of cHet and cKO animals across development and the presence of the *Prox1* construct in cHet and cKO animals (1 and 2 copies respectively). Further experiments are needed to confirm the decrease in EGFP+/PROX1+ cells in the cKO cortex at early time points.

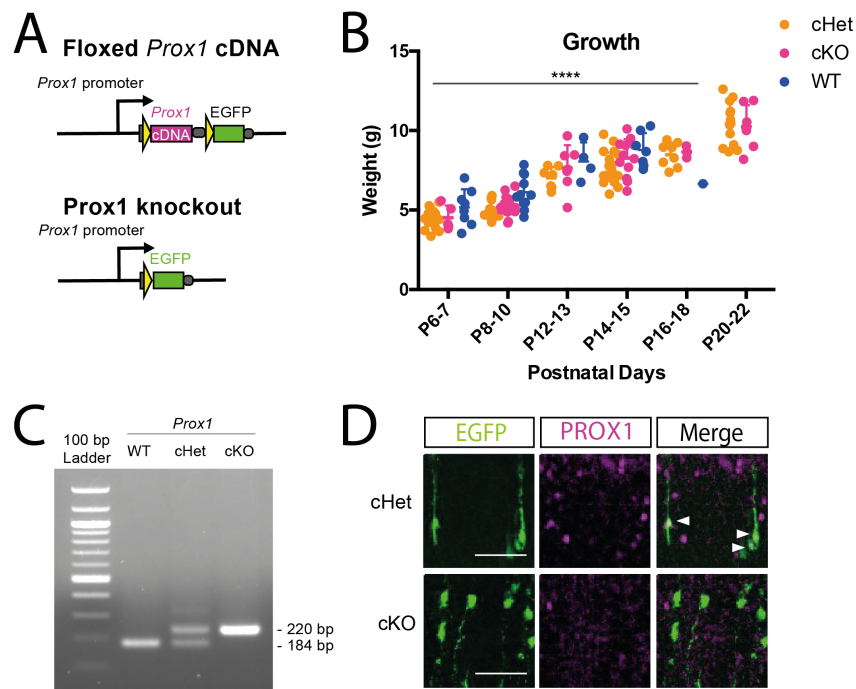


Figure 5.1. Expression of the transgene and protein levels in *VIP-Cre;Ai9;Prox1^{c/c}* animals. (A) Diagram of the *Prox1* transgene, before and after Cre-Lox recombination (top and bottom). Purple box: full-length *Prox1* complementary DNA (cDNA). Triangles: loxP sequences, that are recognised by the Cre and allow recombination. Green box: *Egfp* gene. This structure is integrated in the second exon of the *Prox1* gene. In the knockout, Cre activation replaces *Prox1* with *Egfp* (modified with permission from Iwano et al., 2012 - see Appendix 4). (B) Overall growth of heterozygous (cHet) and knockout (cKO) animals does not differ from Wild-type animals (WT) animals, suggesting the lack of major developmental defect in this line. Dots= single animal weight; line: mean; error bar: standard deviation. (C) PCR products show the presence of the *Prox1* transgene in cHet and cKO – see PCR product at 220 base pair (bp). WT animals do not have the transgene (PCR product at 184 bp). (D). Representative immunofluorescence image of *Prox1* cHet and cKO cortices at P3, suggesting transgene recombination and, in cKO animals, depletion of *Prox1*. Arrowheads=cells expressing EGFP and the PROX1 protein in cHet VIP+ cells. Purple: PROX1; Green: EGFP, that identifies a successfully floxed construct. Scale bar = 50 μ m

5.2.2 – Distribution of VIP+ INs in the *VIP-Cre;Ai9;Prox1^{c/c}* model

Miyoshi et al. (2015) reported that *Prox1* depletion in VIP+ INs leads to a ~15% reduction of L2/3 VIP+ cells in comparison to heterozygous littermate controls

by P21. To confirm this finding in the background I used, I assessed the overall cell density and the normalised layer distribution of VIP+ cells (i.e. tdTomato+) in P21-22 cHet and cKO animals (Figure 5.2 B left and right panel). As additional control, I compared this dataset with the previously characterised WT *VIP-Cre;Ai9* animals (see Chapter 3.1). To make this results directly comparable with what previously reported by Miyoshi et al. (2015), cells were quantified in L1, L2/3, L4, L5 and L6, without subdivision between L5a and L5b (see Chapter 3.1).

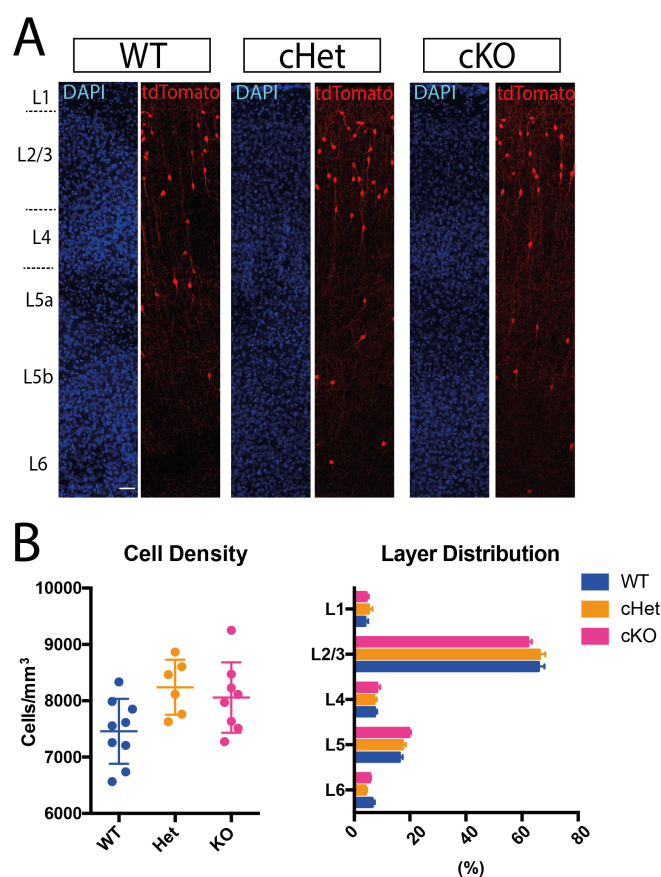


Figure 5.2 Quantification of tdTomato positive cells in the *VIP-Cre;Ai9;Prox1^{c/c}* line. (A) Representative images of cortical columns from the *VIP-Cre;Ai9* and *VIP-Cre;Ai9;Prox1^{c/c}* lines at P21-22. Blue: DAPI, red: tdTomato. Scale bar = 50 μ m. L: Layer. (B) Left: Overall number of dtTomato+ cells across time points indicated in cells/mm³. Lines represent the mean, error bars the standard deviation. Each data point represents a single count. Right: Normalised distribution of dtTomato+ cells across layers. Bars represent the mean, error bars the standard deviation. No differences in density or layer distribution were observed among genotypes. WT: 9 counts from 3 animals; Het: 6 counts from 2 animals; KO: 8 counts from 3 animals.

No difference in cell density was found between cHet and cKO animals, nor between the various backgrounds (WT=7457±577 cell/mm³, n=9 counts from 3 animals; cHet=8240±489 cell/mm³, n=6 counts from 2 animals; KO=8057±624 cell/mm³, n=8 counts from 3 animals; mean ± standard deviation; Mixed effect analysis, p>0.05, see Appendix 3 for details). Similarly, I did not detect any difference in layer distribution of VIP+ cells between cHet and cKO, nor between all genotypes (all F≤2.472, all p≥0.1793, see Appendix 3 for details), in contrast with previous reports (Miyoshi et al. 2015).

Overall, the data presented here do not confirm the previously reported decrease in L2/3 VIP+ INs, although methodological differences may explain the potentially contrasting results (See discussion below).

5.2.3 – Local glutamatergic connectivity onto VIP+ INs in the VIP-Cre;Ai9;Prox1^{c/c} model

To test if the conditional loss of *Prox1* had an impact on VIP+ IN integration into the local glutamatergic network, I performed LSPS combined with glutamate uncaging as previously described (Chapter 3.2; Materials and Methods).

In both cHet (Figure 5.3) and cKO (Figure 5.4), the distribution of laminar glutamatergic input onto VIP+ INs was mainly local across development. Given the low number of data points collected between P5 and P11 (n=3 per genotype), statistical analysis across earlier time points was not performed. However, comparison of the connectivity of cKO and cHet L2/3 VIP+ INs at P12-P16 revealed no difference in the total input (Mann-Whitney test, cHet= 618±350 nC and KO=335±172 nC, n=5 per genotype, p= 0.2222). Likewise, when comparing

distribution of inputs among cortical layers between in cHet and cKO animals (Figure 5.3 and 5.4) genotype effects were not identified, but only a main layer effect was found ($F(3, 24) = 16.45$; $p < 0.0001$). *Post-hoc* multiple comparisons confirmed that the input from L2/3 was significantly higher than any other layer in both cHet and cKO animals (See Appendix 3 for detailed statistics). Normalised input components from each layer were not different between cHet and cKO (See Appendix 3 for detailed statistics). These results suggest a high degree of similarity between local glutamatergic connectivity in P12-16 cHet and cKO VIP+ INs.

Results of LSPS performed on cHet and cKO animals were then compared to wild-type (WT) P12-P16 maps obtained from the dataset previously analysed (Chapter 3.2). Figure 5.5 shows average normalised glutamatergic maps of P12-16 L2/3 VIP+ INs in WT, cHet and Prox1 cKO animals (Figure 5.5A), together with their respective average vertical input profile (Figure 5.5B).

When the total input charge across genotypes was compared, no difference was found (Kruskal-Wallis one-way ANOVA, WT: 313 ± 217 nC, cHet = 618 ± 350 nC and KO = 335 ± 172 nC, mean $\pm$ standard deviation, $n=11$, $n=5$, and $n=5$ respectively, $p=0.1900$) (Figure 5.5C, left panel).

Comparison of distribution of inputs among cortical layers across genotypes revealed a statistically significant interaction between layer and genotype ($F(6, 51) = 2.774$, $P = 0.0207$). *Post hoc* multiple comparisons revealed that L2/3 input in cHet cells was significantly higher than WT (Tukey's multiple comparisons test, WT L2/3 = 160 ± 119 nC, cHet L2/3 = 426 ± 248 nC, $n=11$ and

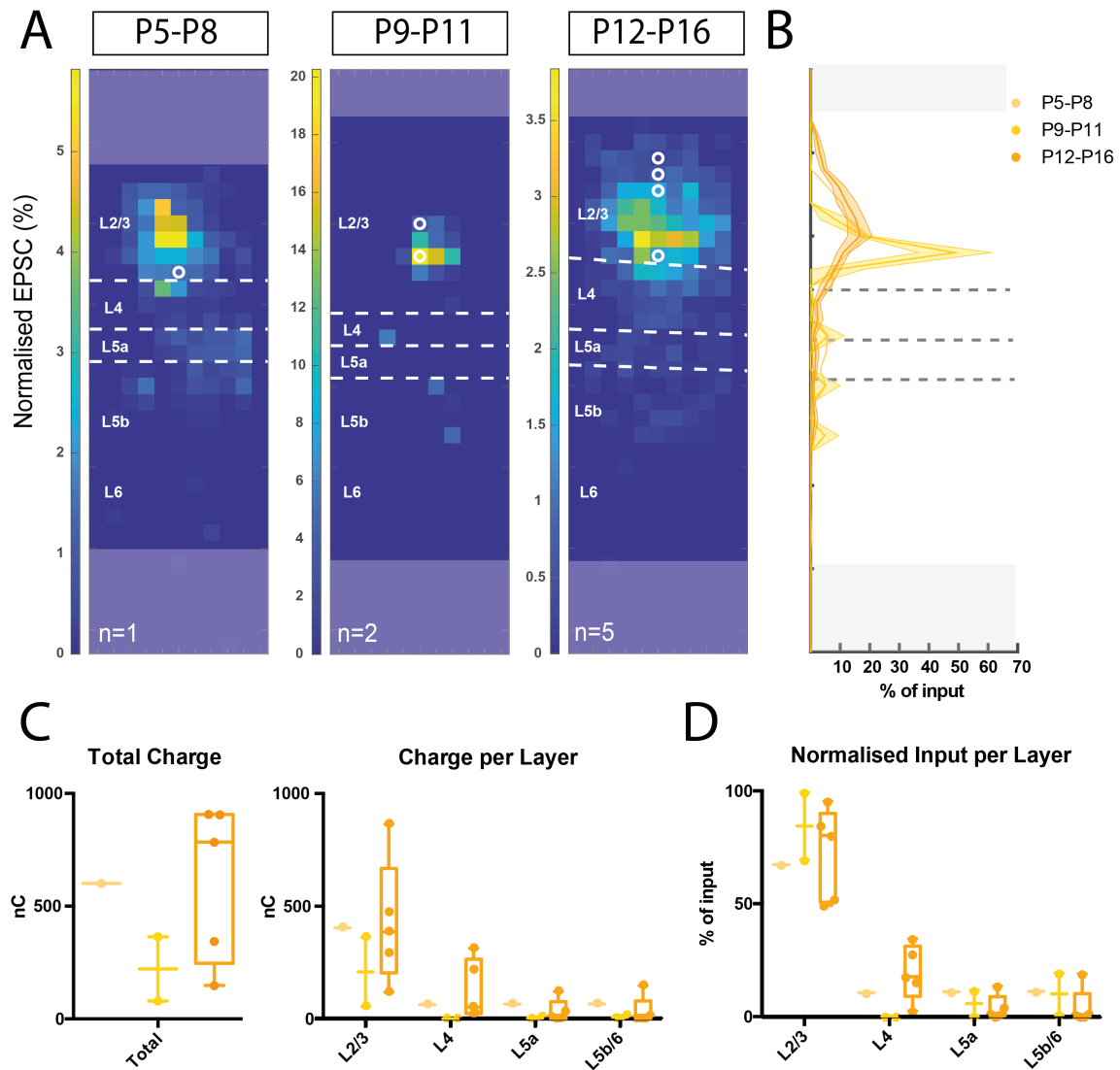


Figure 5.3 LSPS of caged glutamate reveals the laminar organisation of glutamatergic synaptic inputs onto L2/3 VIP+ INs during development in *Prox1* cHet animals. (A) Average normalised glutamatergic inputs onto cHet L2/3 VIP+ INs across the developmental time points analysed. Within each age group, maps of individual cells were aligned by the L4/5a border. White circle: cell position. When two or more cells occupy the same position, only one circle is shown for simplicity. Dashed white lines: average layer boundaries. Transparency: areas outside the cortical depth. (B) Average normalised laminar profiles of cHet L2/3 VIP+ INs across development. (C) Left panel: Total LSPS-evoked glutamatergic synaptic input onto cHet L2/3 VIP+ INs across development. Boxplots: circles=single data points; horizontal line=mean of the data, box= standard deviation; error bars = spread of the data. Right panel: Laminar distribution of glutamatergic synaptic input onto L2/3 VIP+ INs across development. (D) Normalised laminar distribution of glutamatergic inputs onto cHet L2/3 VIP+ INs.

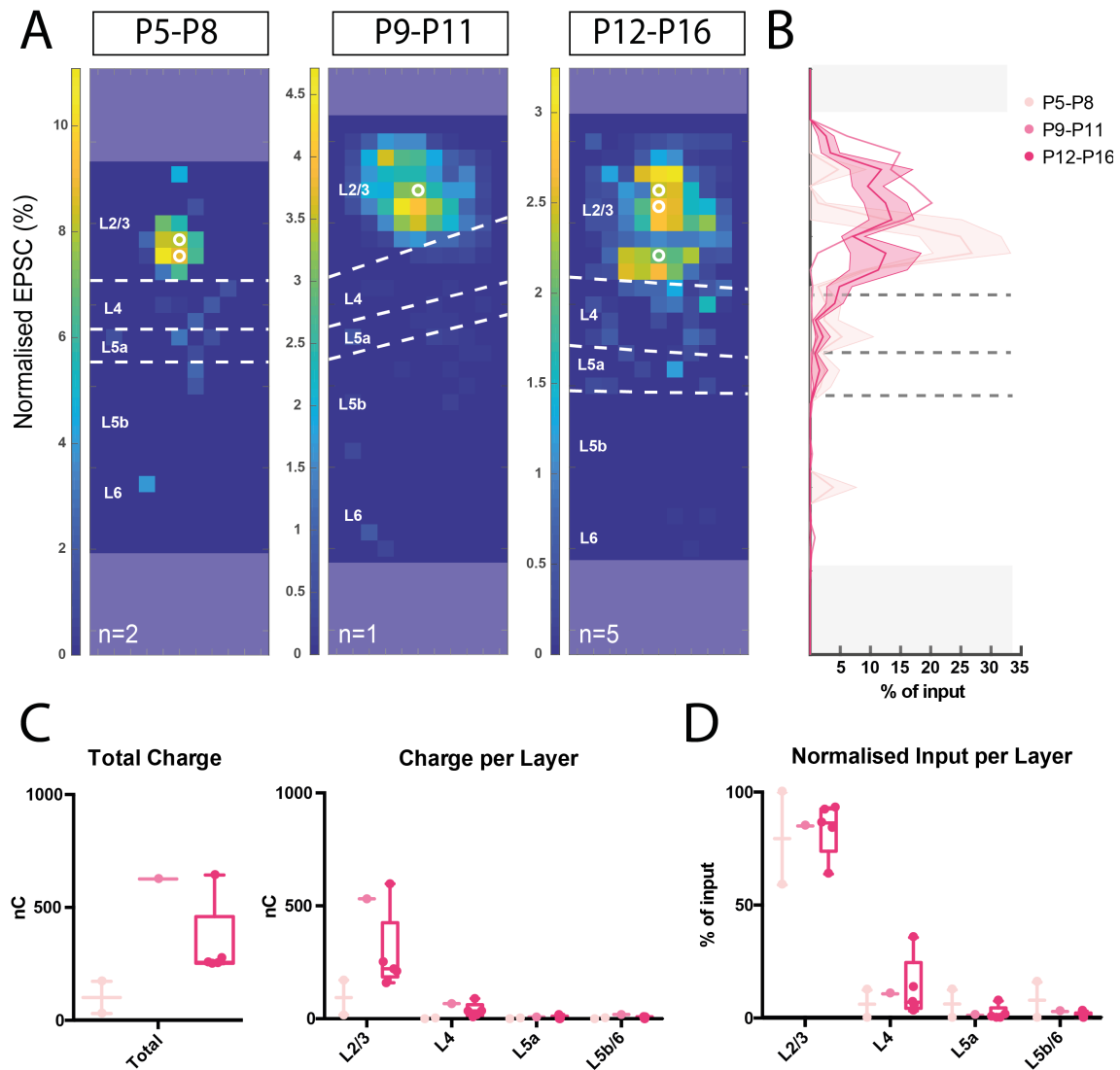


Figure 5.4 LSPS of caged glutamate reveals the laminar organisation of glutamatergic synaptic inputs onto L2/3 VIP+ INs during development in *Prox1* cKO animals. (A) Average normalised glutamatergic inputs onto cKO L2/3 VIP+ INs across the developmental time points analysed. Within each age group, maps of individual cells were aligned by the L4/5a border. White circle: cell position. When two or more cells occupy the same position, only one circle is shown for simplicity. Dashed white lines: average layer boundaries. Transparency: areas outside the cortical depth. (B) Average normalised laminar profiles of cKO L2/3 VIP+ INs across development. (C) Left panel: Total LSPS-evoked glutamatergic synaptic input onto cKO L2/3 VIP+ INs across development. Boxplots: circles=single data points; horizontal line=mean of the data, box= standard deviation; error bars = spread of the data. Right panel: Laminar distribution of glutamatergic synaptic input onto L2/3 VIP+ INs across development. (D) Normalised laminar distribution of glutamatergic inputs onto cKO L2/3 VIP+ INs.

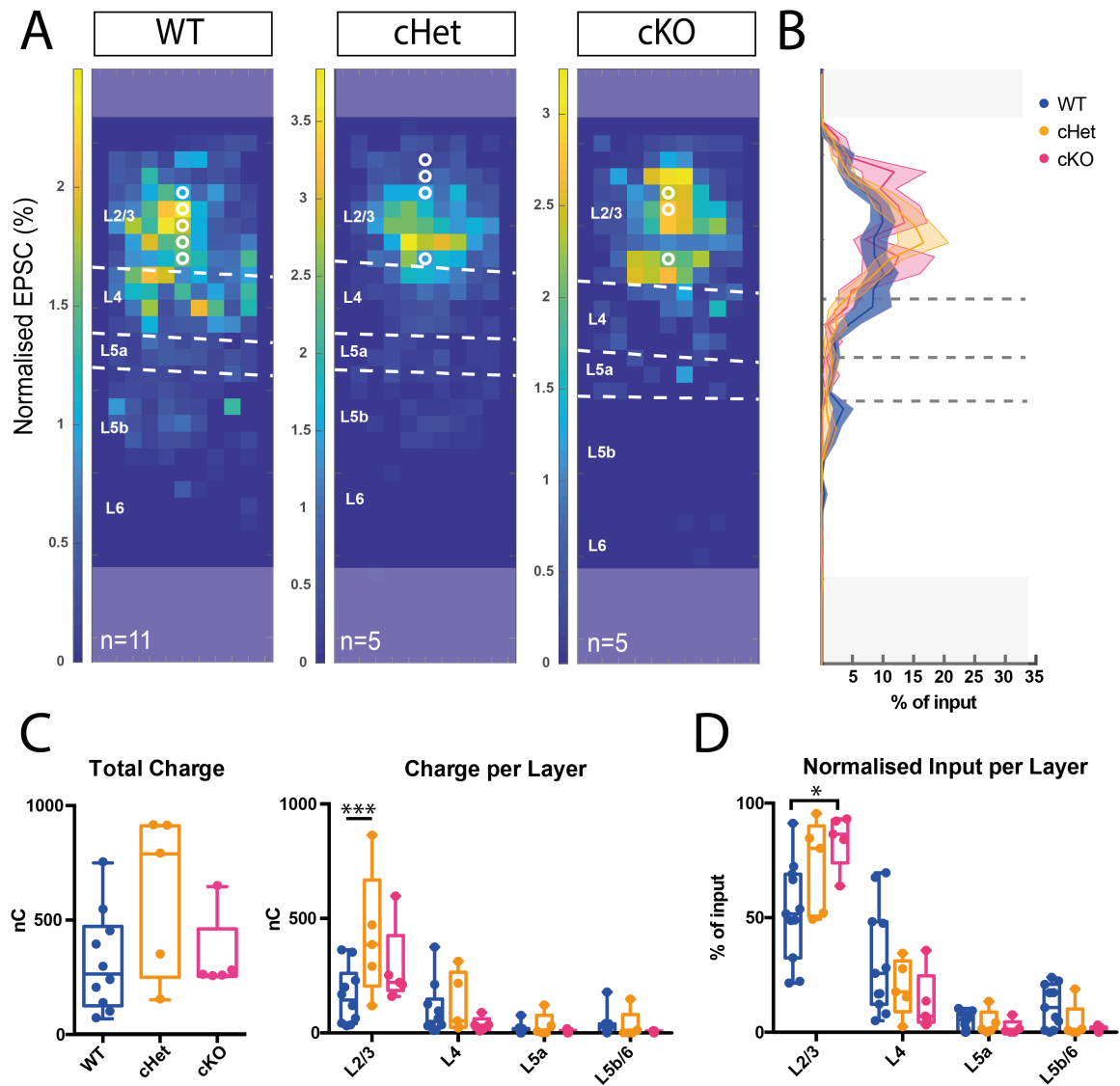


Figure 5.5 Comparison of laminar organisation of glutamatergic synaptic inputs onto L2/3 VIP+ INs at P12-16 across genotypes. (A) Average normalised glutamatergic inputs onto L2/3 VIP+ INs across the developmental time points analysed. Within each age group, maps of individual cells were aligned by the L4/5a border. White circle: cell position. When two or more cells occupy the same position, only one circle is shown for simplicity. Dashed white lines: average layer boundaries. Transparency: areas outside the cortical depth. (B) Average normalised laminar profiles of L2/3 VIP+ INs across development. (C) Left panel: Total LSPS-evoked glutamatergic synaptic input onto L2/3 VIP+ INs across development. Boxplots: circles=single data points; horizontal line=mean of the data, box= standard deviation; error bars = spread of the data. Right panel: Laminar distribution of glutamatergic synaptic input onto L2/3 VIP+ INs across development. (D) Normalised laminar distribution of glutamatergic inputs onto L2/3 VIP+ INs. cHet and cKO VIP+ INs show a higher L2/3 glutamatergic input (raw and normalised, respectively) than WT cells.

n=5 respectively, $p \leq 0.001$) (Figure 5.5C, right panel). Normalised input components from each layer were compared across genotype (Figure 5.5D): a significant difference in proportion of input from L2/3 was found (Kruskal-Wallis one-way ANOVA, $p=0.0216$). In particular, cKO VIP+ cells had a higher proportion of input from L2/3 in comparison to WT cells (Dunn's multiple comparisons test; WT L2/3= $52 \pm 21\%$, cKO L2/3 $84 \pm 11\%$, n=11 and 5 respectively, $p \leq 0.05$) (see Appendix 3 for other details).

These data suggest that VIP+ INs lacking a single copy of *Prox1* do not differ from VIP+ INs lacking both copies of the gene in terms of local glutamatergic connectivity. However, when cHet and cKO cells were compared to WT cells, some differences could be observed: cHet VIP+ INs had a higher local connectivity in terms of raw input when compared with WT. Similarly, *Prox1* mutants showed an increased proportion of excitatory glutamatergic input from the local network in comparison to WT, therefore suggesting a decreased level of excitatory input from other layers. However, these results have to be interpreted carefully, considering the low number of data points available for cHet and cKO VIP+ INs.

5.3 – Effect of genetic perturbation of VIP+ cells on their long-range integration

To investigate whether genetic impairment of VIP+ INs could have a cell autonomous effect on their recruitment from anterior-motor long-range connections, I performed injections of viral channelrhodopsin in *VIP-Cre;Ai9;Prox1^{c/c}* cKO and littermates cHet controls as previously described (see

Chapter 4). *Prox1* depletion in VIP+ INs did not affect gross anatomical projections from anterior-motor areas: key YFP+ structures, such as the internal capsule and S1BF (in particular L1, L6 and septal fibers), were identified in both cHet and cKO animals, confirming the reliability of the methodology previously used (Figure 5.6A).

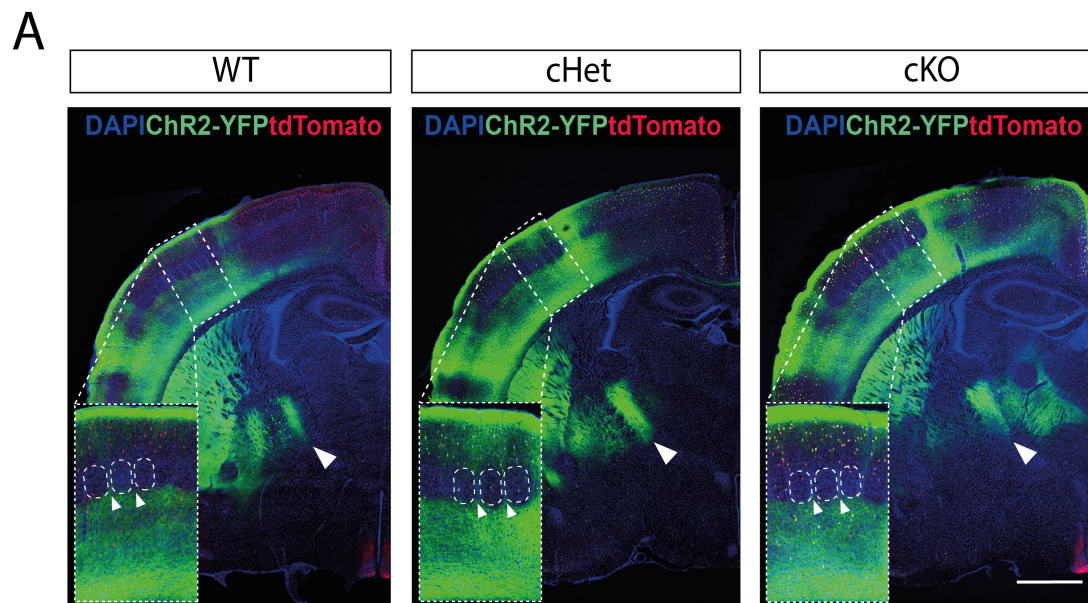


Figure 5.6 YFP+ labelling following viral injections in cHet and cKO animals resemble what observed in WT. (A) Immunofluorescence in WT (left), cHet (centre) and cKO (right) following viral injection. Arrowhead: Thalamus. Magnification of S1BF shows similar connectivity patterns, with a high fluorescence band in L1 and L6 and infrabarrel fibres. Dotted lines in the magnification: barrels; arrowhead in the magnification: septa. Scale bar=1mm. Green:YFP; Red:tdTomato; Blue:DAPI.

Whole-cell patch clamp experiments combined with wide-field blue light illumination were performed on S1BF L2/3 VIP+ INs and pyramidal cells controls across development, using the same experimental paradigm applied

before (Figure 5.7A and B). Pyramidal cells were not included in the analysis, as only a few of them were recorded to confirm connectivity.

Although I could observe an increase in the percentage of VIP+ INs engaged over time in both cHet and cKO animals (cHet: P7-9 25%, n=8 and P $\geq$ 13 57%, n=7, and cKO: P7-9 38%, n=16, and P $\geq$ 13 66%, n=6), this did not reach statistical significance, probably due to the low number of recorded cells (see Appendix 3 for detailed statistics). Genotype-specific differences in the proportion of VIP+ INs monosynaptically engaged by long-range anterior-motor areas were not found at any time points (see Appendix 3 for detailed statistics). Moreover, no differences were found when comparing both these datasets with the previously collected WT data (Chapter 4, see Appendix 3).

Monosynaptic light-evoked EPSCs recorded at the minimal stimulation were then analysed for average amplitude, latency and latency jitter in cHet and cKO animals (Figure 5.7C). Given that only 2 data points per age group were collected at the minimal stimulation in the cHet dataset, this was excluded from further analysis and it was not possible to compare directly it with cKO data.

However, I compared the cKO dataset with WT data and found no difference between WT and cKO animals in terms of average amplitude, latency or latency jitter (Figure 5.7D). The age-related effect found in VIP+ INs average latency was observed only in WT animals. These data suggest that cKO of *Prox1* in VIP+ INs has no effect on the development of anterior-motor synaptic inputs onto VIP+ cells in S1BF.

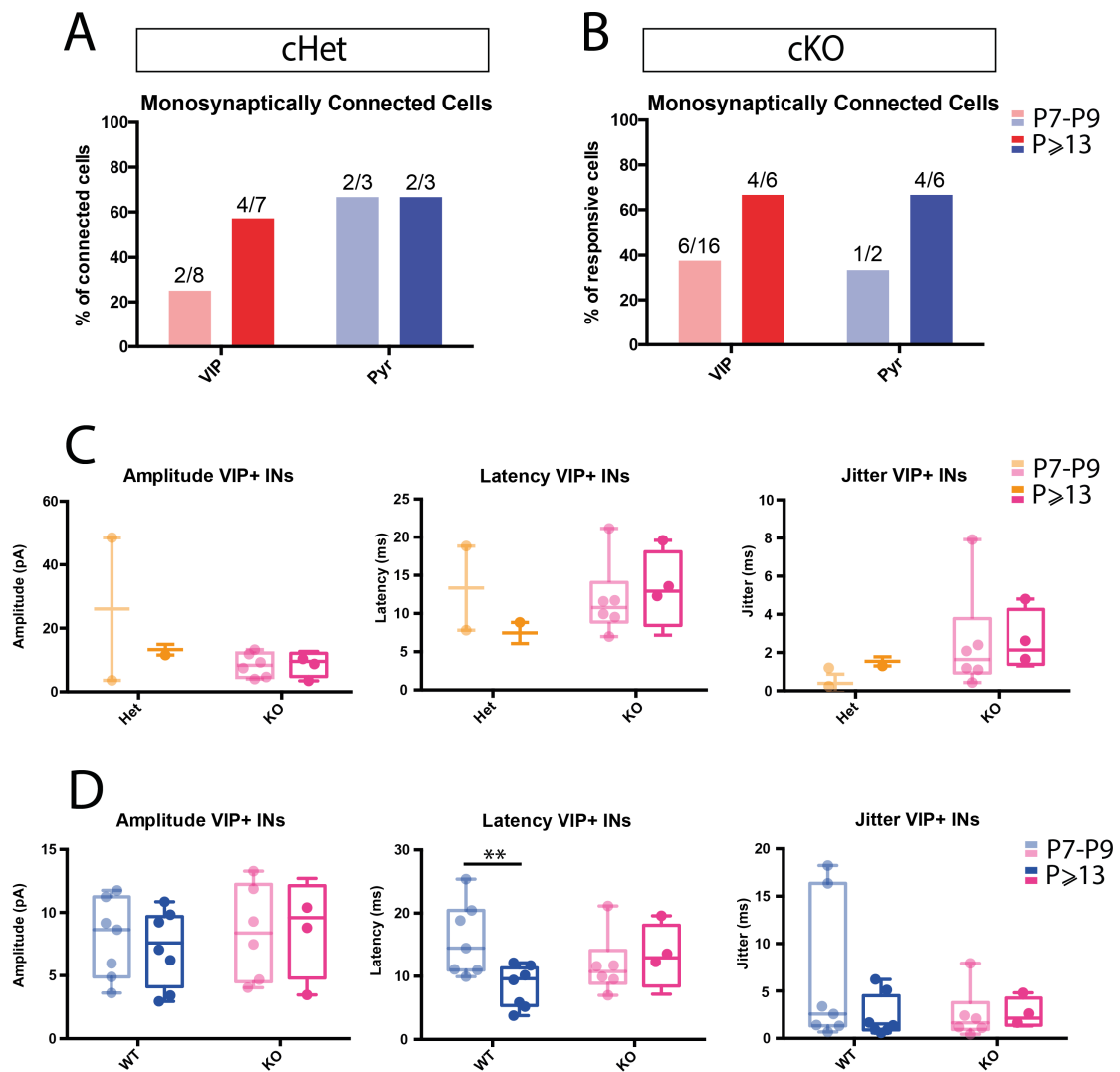


Figure 5.7 Monosynaptic connectivity between anterior-motor areas and S1BF neurons in cHet and cKO animals. (A) Proportions of S1BF VIP+ INs and pyramidal controls that receive monosynaptic connections from anterior-motor areas at different developmental windows in cHet animals. (B) Proportions of S1BF VIP+ INs and pyramidal controls that receive monosynaptic connections from anterior-motor areas at different developmental windows in cKO animals. No differences between genotypes can be identified. (C) Comparison of average amplitude (left), latency (centre) and jitter (right) of minimal stimulation responses in VIP+ INs in the different genotypes across development (D) Comparison of average amplitude (left), latency (centre) and jitter (right) of minimal stimulation responses in VIP+ INs in WT and cKO animals across development. No genotype-specific differences can be found.

5.4 – *In vitro analysis: Discussion and limitations*

The data reported so far confirm the presence of the *Prox1* construct (Iwano et al., 2012) in cHet and cKO animals, and suggest a minor decrease of EGFP+/PROX1+ cells in cKO animals already at P3, even though further experiments would be required to support this statement. No difference between genotypes in overall weight gain across time was found, excluding a potential confounding factor due to the endocrine effect of the vasoactive intestinal peptide on overall foetal development (Passemar et al., 2011).

In contrast to previous reports (Miyoshi et al. 2015), no difference in cell density and layer distribution of VIP+ INs was found between WT, cHet and cKO animals. In particular, Miyoshi et al. (2015) reported a layer-specific decrease in the number of VIP+ INs in the cKO when compared with cHet littermates. The differential normalisation method used may explain different results: Miyoshi et al. (2015) normalised the distribution of cells over the values obtained from control cHet animals, while I normalised over the total number of cells within each field of view. For this reason, it cannot be excluded that the observed differences are simply due to methodologies.

LSPS experiments did not highlight differences in local glutamatergic connectivity between cHet and cKO animals, but suggested differences in cHet and cKO animals when compared to WT. Despite the limited sample size, this preliminary finding raises some interesting points. The current data point to a higher proportion of VIP+ INs with a local connectivity pattern (i.e. Cluster 3 VIP+ INs) in the *Prox1* cKO animals when compared with WT, but further LSPS

experiments are required to test this hypothesis. In particular, it could be possible that I did not patch any L3 cell, and therefore sampling bias caused the phenotype observed.

Analysis of long-range connectivity in cHet and cKO animals showed no genotype-specific difference in the proportion of VIP+ INs recruited over time, nor in EPSC properties measured at minimal stimulation. This suggests that *Prox1* is not required for the development of long-range connections onto VIP+ cells.

5.5. Effect of the genetic perturbation of VIP+ INs in vivo

5.5.1 Baseline activity

To understand the network effect of genetic perturbation of VIP+ INs, we performed *in vivo* extracellular recordings combined with whisker stimulation in urethane-anaesthetised animals at two developmental time points: P14-15 (just after whisking onset) and P19-22 (mature network) (Arakawa and Erzurumlu, 2015; Erzurumlu and Gaspar, 2012).

Firstly, given that cortical LFP activity during urethane-induced anaesthesia is characterised by sleep-like activity patterns (Clement et al., 2008), we analysed the power spectra of LFP oscillations in cHet and cKO animals, focusing on frequencies covering slow oscillations (0.3-1.4 Hz), delta waves (1.5-4 Hz) and sleep spindles (7-15 Hz) (Figure 5.8). As expected, a significant interaction between frequency and age was found (3-way ANOVA, $F(39, 741) =$

14.14; $p < 0.0001$), but no effect of genotype (see Appendix 3 for detailed statistical analysis).

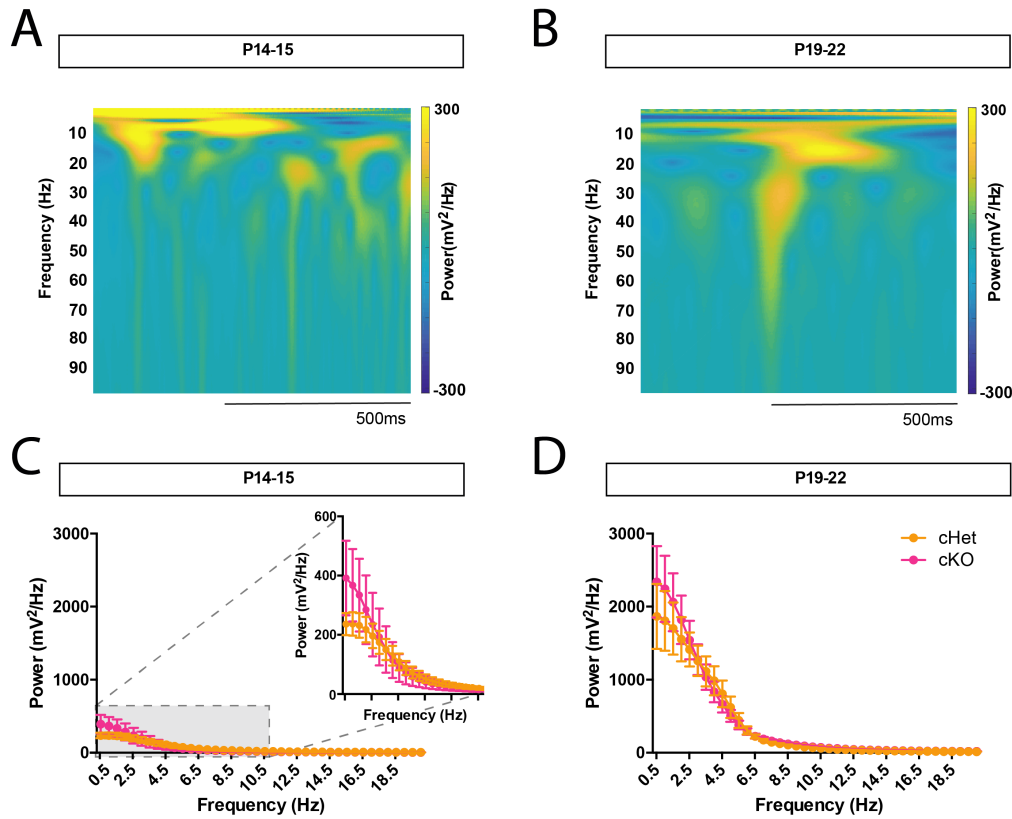


Figure 5.8 LFP Power analysis of baseline activity does not highlight genotype-specific differences in cHet and cKO animals. Exemplary spectrogram in young (P14-15, A) and older animals (P19-22, B) during baseline activity. LFP power analysis in young (P14-15, C) and older animals (P19-22, D) shows age-dependent, but no genotype-dependent differences across the frequencies analysed.

We then assessed delta waves and sleep spindles in *Prox1* cHet and cKO animals in a layer specific manner (Figure 5.9-5.11), using current-source density maps to identify layers (see Materials and Methods).

We first confirmed that all the events extracted in the delta range were indeed in the correct frequency range (1.5-4 Hz, Figure 5.10A). Differences across layers, ages and genotypes could not be found (Mixed-effects linear model analysis, all $F \leq 2.553$, all $p \geq 0.0960$, see Appendix 3 for details). We then

evaluated the fraction of time spent in delta, the average duration of an event, and the interval between events (inter-stimulus interval, ISI) for supragranular, granular and infragranular layers, at the different time points and genotype under investigation (Figure 5.10B-D). There were no significant differences in any of the parameters extracted, suggesting that delta wave activity was not affected by neither development nor layers or genotypes (see Appendix 3 for the results from mixed-effects model analysis).

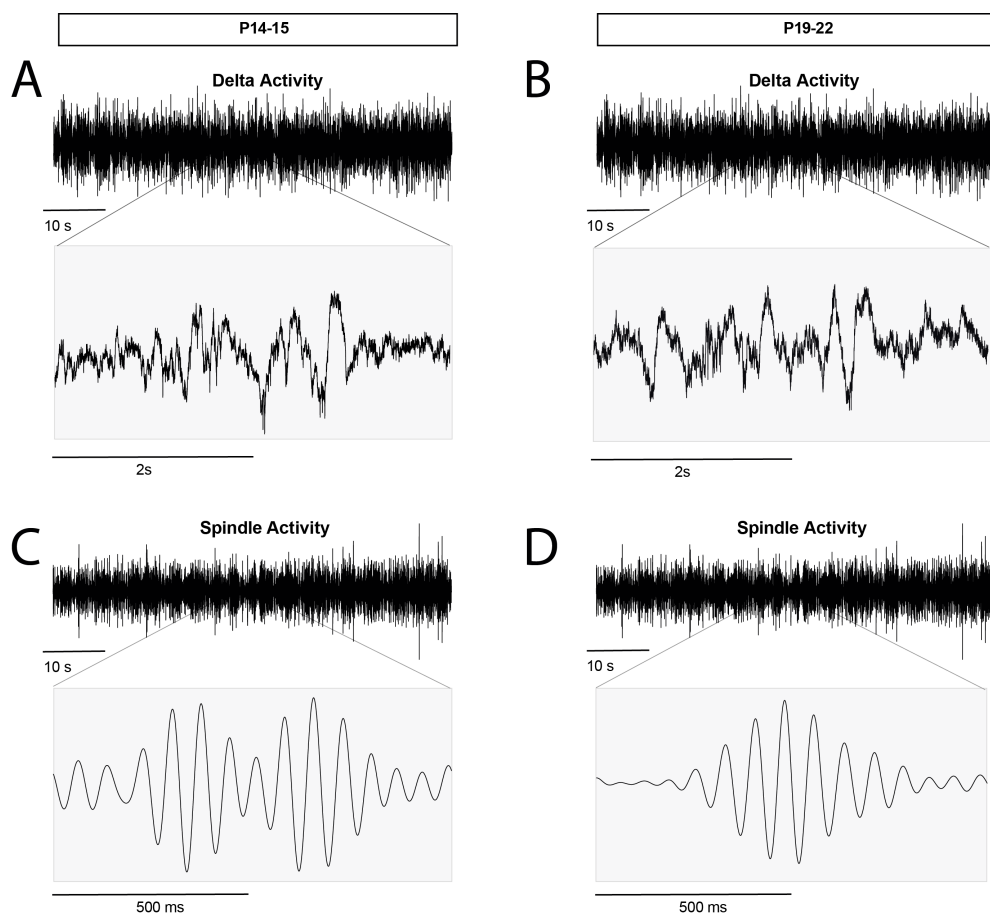


Figure 5.9 Exemplary traces showing delta waves and spindle. Exemplary traces of delta activity in young (P14-15, A) and older animals (P19-22, B). Exemplary traces of spindle activity in young (P14-15, C) and older animals (P19-22, D).

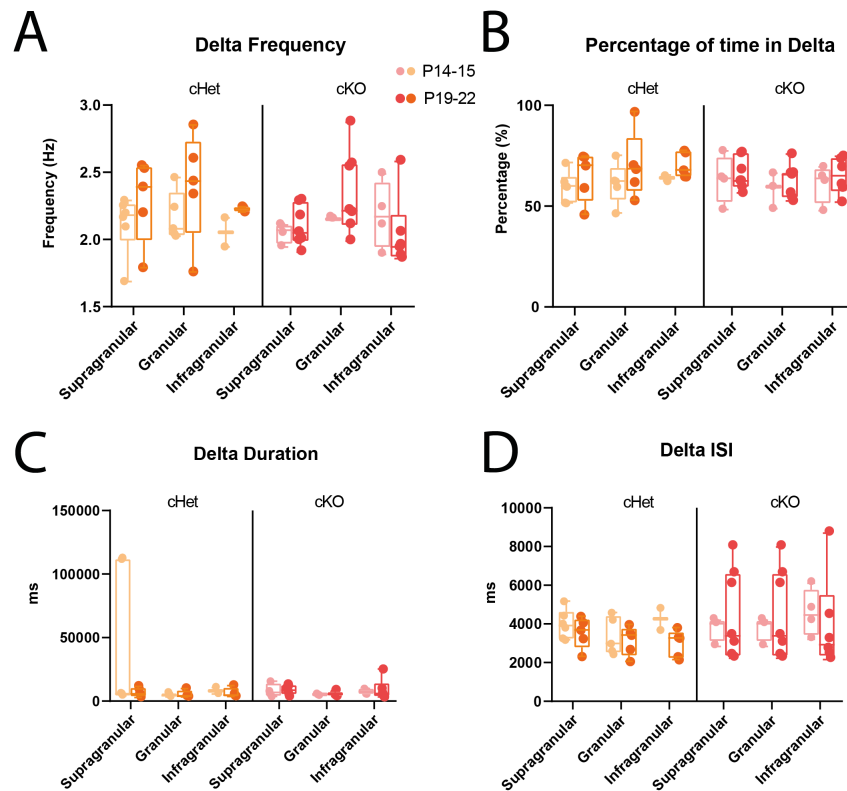


Figure 5.10 Layer specific analysis of delta-waves in *Prox1* cHet and cKO animals during development. (A) Average delta frequency extracted (B) Percentage of time spend in delta (C) Average duration of a delta event (D) Average delta inter-stimulus interval (ISI). No significant effects across layer, age or genotype were found.

A similar analysis was performed on sleep spindles (Figure 5.11A). Surprisingly, mixed-effects model analysis identified a statistically significant age effect on frequency ($F(1, 18) = 7.291$, $p = 0.0146$), although very subtle (P14-15 = 10.7 ± 0.4 Hz and P19-22 = 10.3 ± 0.2 Hz, mean $\pm$ standard deviation, $n = 11$ animals in each developmental group). Given the small effect size of this result (0.4 Hz difference in means), the fact that all the events extracted were in the spindle range (7-15 Hz), and that developmental changes in spindle frequency have not been reported previously (Colonnese et al., 2010), this result was not considered to affect subsequent analysis of the data.

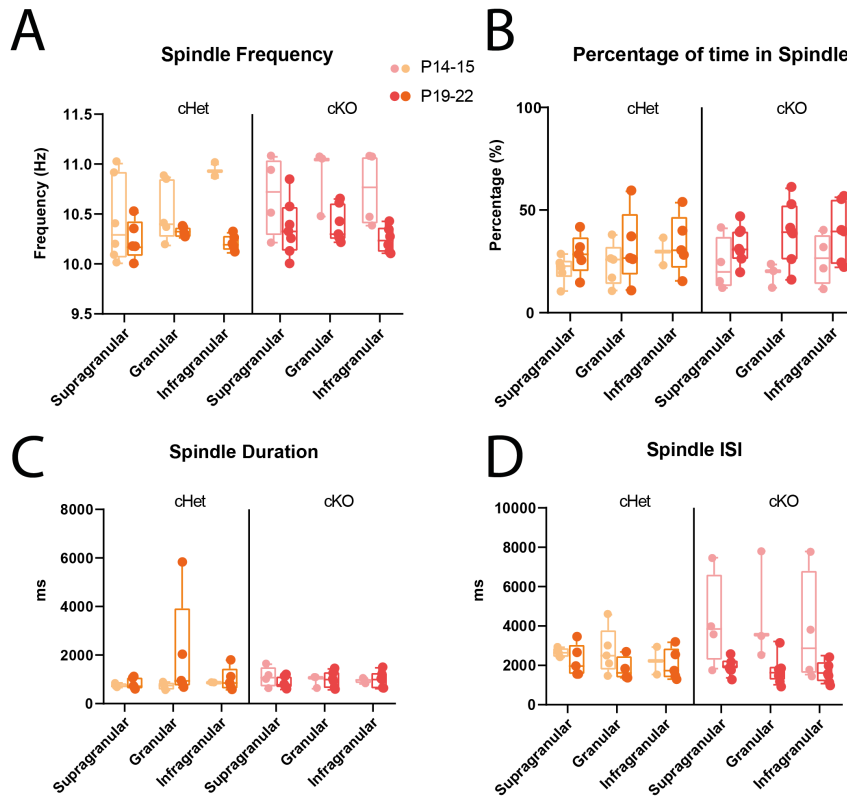


Figure 5.11 Layer specific analysis of spindle activity in *Prox1* cHet and cKO animals during development. (A) Average spindle frequency extracted (B) Percentage of time spend in spindle (C) Average duration of a spindle event (D) Average spindle inter-stimulus interval (ISI). Only layer effects are shown. No genotype-specific effects were found at any time point.

The percentage of time spent in sleep spindle was then analysed (Figure 5.11B). A statistically significant effect of age was found (Mixed-effect linear model analysis, $F(1, 18) = 4.627$, $p = 0.0453$, $P14 = 23.4 \pm 9\%$ and $P21 = 34.8 \pm 13\%$, mean $\pm$ standard deviation, $n = 11$ animals in each developmental group). Figures 5.10C and D respectively show that, while there were no effects on sleep spindle duration (Mixed-effect linear model analysis, all $F \leq 1.531$, all $p \geq 0.222$, see Appendix 3 for details), the ISI between sleep spindle events had a significant age effect (Mixed-effect linear model analysis, $F(1, 18) = 6.455$, $p = 0.0205$, $P14-15 = 3344 \pm 1862$ ms and $P19-22 = 1872. \pm 627$ ms, mean $\pm$ standard deviation, $n = 11$

animals in each developmental group). No effect of genotype was observed in any of the parameters investigated.

The data presented so far suggests that *Prox1* depletion in VIP+ INs does not affect baseline synchronous activity, either in the delta or spindle range. We identified age related effects in the overall percentage of time spent in sleep spindle and in spindle ISI, in line with previous literature (Clawson et al., 2016).

5.5.2 – Whisker stimulation

To test the role of VIP+ INs in whisking sensory processing, we then performed *in vivo* extracellular recordings during whisker stimulation in anaesthetised animals across the selected developmental points (P14-15 and P19-22). To mimic natural whisking, a multi-whisker stimulation protocol was chosen and stimuli were delivered at 10 Hz, approximately the dominant frequency during exploratory whisking behaviour (Cao et al., 2012; Carvell and Simons, 1990; Mégevand et al., 2009; Quairiaux et al., 2011). Local field potential and multi-unit activity were analysed in a layer-specific way, using current-source density maps to identify layers (Figure 5.12 and 5.13).

No significant effects were found in the whisker-evoked LFP amplitude across age groups, layers and genotypes analysed (Figure 5.12A, Mixed-effect linear model analysis, all $F \leq 1.880$, all $p \geq 0.3310$, see Appendix 3).

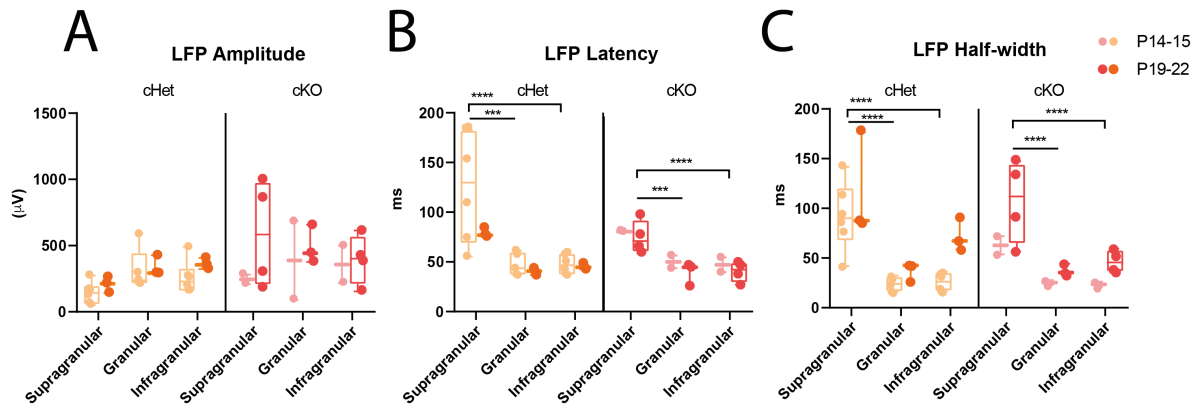


Figure 5.12 Layer specific analysis of local field potential in Prox1 cHet and cKO animals during development. (A) Average LFP amplitude (B) average LFP latency, defined as time to peak from stimulus onset (C) average LFP half-width in cHet and cKO animals. No genotype-specific effects were found at any time point. Only layer effects are shown.

As expected, we did observe a statistically significant layer effect on the response latency regardless of age and genotype (Figure 5.12B, left panel: Mixed-effect linear model analysis, $F(0.9983, 9.983) = 21.33$, $p=0.001$). In particular, supragranular layers responses were significantly delayed compared to granular and infragranular ones (*post-hoc* Tukey's multiple comparisons test, $p=0.0001$ and $p=0.0003$ respectively, supragranular= 98 ± 40 ms; granular= 45 ± 9 ms; and infragranular= 45 ± 8 ms; mean $\pm$ standard deviation, $n=15$; $n=13$ and $n=15$ data points respectively, from a total of 15 animals).

Moreover, a main age and layer effect in the LFP response half-width, without any interaction, was found (Figure 5.12C: Mixed-effect linear model analysis, Age effect: $F(1, 11) = 11.68$, $p=0.0058$; Layer effect: $F(1.139, 11.39) = 24.64$, $p=0.0003$). LFP half-width significantly increased over time (P14-15= 45 ± 35 ms; P19-22= 70 ± 41 ms, $n=23$ and $n=20$ data points per group, from 8 and 7 animals for each developmental group). *Post-hoc* Turkey's multiple comparisons test confirmed that the half-width in supragranular layers was

significantly wider than in granular and infragranular ones (*post-hoc* Tukey's multiple comparisons test, $p < 0.0001$ and $p = 0.0001$ respectively, supragranular = 98 ± 36 ms; granular = 29 ± 8 ms; and infragranular = 40 ± 20 ; $n = 15$; $n = 13$ and $n = 15$ data points respectively, from a total of 15 animals).

In sum, while there were no differences in LFP amplitude and latency in cHet and cKO animals across development, there was a developmental effect on whisker-evoked response dynamics, regardless of the genotype: LFP half-width lasted longer in P19-22 animals than in P14-15 ones. Moreover, layer specific effects were found: granular and infragranular responses were sharper and faster than supragranular ones.

To have a more detailed understanding of the *in vivo* network dynamics during whisker stimulation, we then analysed whisker-evoked MUA, focusing on average firing rate (Figure 5.13A), latency (Figure 5.13B) and the half width of the evoked responses (Figure 5.13C). Mixed-effects linear model analysis of MUA average firing rate revealed a main genotype effect, with cKO animals characterized by higher MUA than cHets (Figure 5.13A: $F(1, 11) = 5.006$, $p = 0.0027$; cHet = 30 ± 22 Hz; cKO = 49 ± 46 , $n = 24$ and $n = 18$ data points respectively, from cHet = 9 and cKO = 6 animals in total), and a significant interaction between layer and age ($F(2, 19) = 5.211$, $p = 0.0157$).

Post-hoc Tukey's multiple comparisons test revealed that supragranular layers at P19-P22 were significantly less active than infragranular ones, regardless of genotype (Supragranular = 17 ± 16 Hz Infragranular = 32 ± 14 Hz, $n = 7$ per group), mirroring the differences in thalamic engagement between those layers and layer-specific coding of sensory-evoked responses (van der Bourg et al., 2017).

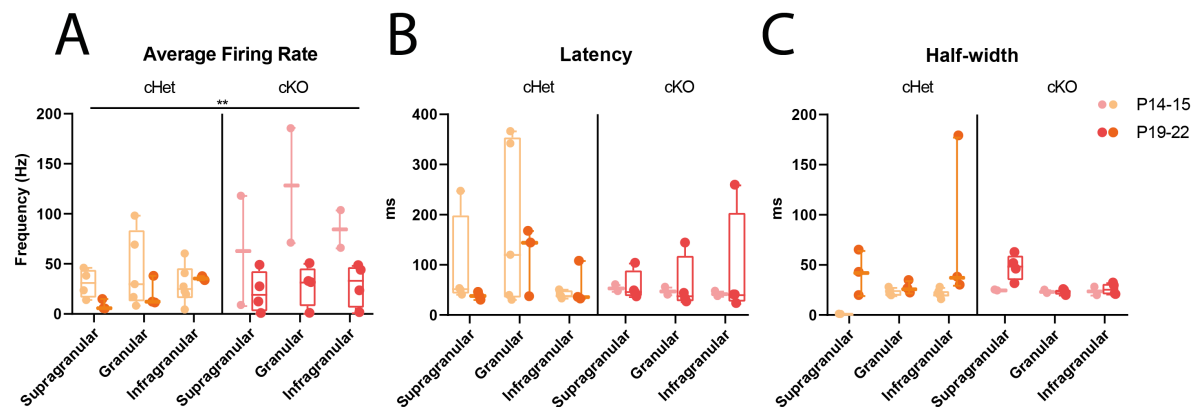


Figure 5.13. Layer-specific analysis of multi-unit activity in Prox1 cHet and cKO animals during development. (A) Average MUA frequency (B) average MUA latency, defined as time to peak from stimulus onset (C) average MUA half-width in Prox1 cHet and cKO. While latency and half-width don't change, cKO animals have a higher average firing rate than cHet following whisker stimulation. Only genotype effects are shown

While no differences were found in MUA latency (Figure 5.13B: Mixed-effect linear model analysis, all $F \leq 1.059$, all $p \geq 0.3262$), we found a significant age effect in the MUA half-width (Figure 5.13C: Mixed-effect linear model analysis, $F(1, 30) = 8.609$; $p = 0.0064$; P14-P15 = 374 ± 181 ms; P19-P22 = 811 ± 673 ms, $n = 21$ data points per group, from respectively 8 and 7 animals within each group). As for the LFP analysed before, the data suggest that responses last longer in the older age group, regardless of genotype.

Finally, in order to investigate the impact of slow-oscillations on cortical integration of whisker inputs (Sachdev et al., 2014), we attempted to analyse the effect of whisker stimulation delivered during up- and down- states. However, we could not perform such analysis, as we did not observe an equal number of up- and down- states across experiments at any time point (P14-15: up-states count in cHet 27 ± 8 and in cKO 24 ± 22 ; down-states count in cHet 28 ± 6 and in

cKO 13 ± 18 . P19-22: up-states count in cHet 32 ± 10 and in cKO 34 ± 6 ; down-state count in cHet 6 ± 5 and in cKO 4 ± 3 . Mean $\pm$ standard deviation).

The data shown so far suggest that cKO animals have higher MUA firing frequency than cHet, irrespectively of the developmental time point analysed. Moreover, supragranular layers are less active than the others at P19-22, in line with coding sparsification happening at later time points (van der Bourg et al., 2017) and MUA half-width increases over time, with responses lasting longer at P19-22.

5.6 – In vivo analysis: Discussion and limitations

5.6.1 – Developmental changes in baseline and sensory-evoked in vivo activity in S1BF

In this result section, we investigated the effect of conditional deletion of *Prox1* in VIP+ INs on network activity *in vivo*. Before focusing on genotype-specific effects, the developmental changes identified will be discussed.

Power spectra analysis revealed an interaction between frequency and age, as previously reported. While we did not find any age-related difference in delta waves, we found an age-dependent increase in overall time spent in sleep spindle and a decrease in sleep spindle ISI, in agreement with previous reports. Over development, sleep spindles are more prominent, and therefore the time between events decreases, while the duration of a single spindle event does not change (Clawson et al., 2016).

As for the whisker-evoked activity, no difference in LFP amplitude across layers was found either at P14-15 or P19-22. We observed longer half-width in supragranular layers and its general increase over development. The latter, in particular, could reflect the maturation of cross-barrel activity: as reported by van der Bourg et al. (2017), cross-columnar activity spread increases over time, especially in supragranular and granular layers.

We also found differences in LFP latencies across layers, and in particular a longer LFP latency in supragranular layers when compared with the others, that can be explained by layer-specific connectivity: granular and infragranular layers are engaged first by the thalamus and therefore show a more closely time-locked response (Feldmeyer et al., 2013).

Looking at the evoked MUA, we found that supragranular layers are less active than others at later time points, confirming the layer-specific sparsification of coding previously reported (van der Bourg et al. 2017). We did not observe any layer or age-dependent change in MUA latencies, but we found an age-related difference in half-width MUA responses, suggesting a more propagated cross-barrel activity at later developmental points (van der Bourg et al. 2017).

Previous work investigating the development of cortical network activity through *in vivo* extracellular recordings mainly relied on single whisker stimulation (van der Bourg et al., 2016, 2017; Khazipov and Milh, 2018; Minlebaev et al., 2011). By performing multi-whisker stimulation, we engaged both the intra-barrel and cross-barrel networks, therefore the activity observed is a combination of these two and direct comparisons with single stimulation protocols cannot be made. Only Quairiaux et al. (2011) analysed whisker-evoked

activity in developing rodents following multi-whisker stimulation; however, their stimulation protocol differs from the one here employed and their latency values do not match the ones here identified. According to findings by other members of the Butt lab, faster stimulation frequencies elicit faster responses and this could explain the discrepancy with what previously reported (Quairiaux et al., 2011).

5.6.2 – Modulation of baseline and sensory-evoked in vivo activity by VIP+ INs in the VIP-Cre;Ai9;Prox1^{c/c} model

The *in vitro* characterisation previously performed (Chapter 5.1-5.3) suggested no differences between cHet and cKO in terms of VIP+ INs number and distribution, nor in connectivity. However, local connectivity differences could be observed between cHet and WT and cKO and WT, suggesting that the lack of a single copy of *Prox1* might already lead to circuit integration impairments.

In this final section, no differences between genotypes in delta waves and sleep spindles were identified. Previous literature showed a role for VIP+ INs in slow wave activity, therefore suggesting a possible role in delta waves as well (Hall et al., 2015): to clarify the potential cHet phenotype, this dataset needs to be compared to WT data.

While no genotype-related differences in whisker-evoked LFP were found, we surprisingly observed a genotype effect in whisker-evoked MUA: cKO animals show an increased stimulus-triggered multi-unit average firing rate than cHet controls, suggesting a role for VIP+ INs in modulation of whisking-evoked somatosensory responses.

It is not surprising that LFP and MUA results are not mirroring each other, as they represent two different measurements. The LFP represents the average synchronised activity of many neurons, related to the input that they receive; the MUA is instead a measurement of non-synchronised neuronal output (i.e. firing of multiple neurons) (Belitski et al., 2008; Rasch et al., 2008).

A higher number of recordings would be beneficial to confirm this finding: several recordings have been excluded from analysis for technical reasons (see Materials and Methods) and all experiments were performed blind to the genotype. For these reasons a consistent number of animals across age groups is missing.

Beside these technical factors, the data reported here indicate a genotype-specific effect on sensory-evoked network activity *in vivo*, despite converging evidence suggesting no differences between cKO and cHet littermates in cell density or local and long-range glutamatergic connectivity.

This could be explained by a differential outputs from VIP+ INs in cHet and cKO animals: for instance, cKO VIP+ INs may inhibit more SST+ cells, therefore leading to higher levels of pyramidal cells disinhibition. Similarly, VIP+ INs in the cKO might directly target a lower number of pyramidal cells, or provide a weaker inhibitory input. This hypothesis could be tested *in vitro* by generating *VIP-Cre;Ai32;Prox^{c/c};Lhx6-EGFP+* and *VIP-Cre;Ai32;Prox^{+/c};Lhx6-EGFP+* mice and performing similar experiments to what showed in Chapter 3.3.

Another interesting avenue to explore to explain this phenotype is the possibility that MGE-derived IN populations are affected in the *Prox1* cKO. A balance between IN populations following subtype specific perturbations has

been previously demonstrated (Azim et al., 2009; Batista-Brito et al., 2009; Butt et al., 2008; Lodato et al., 2011a) and this cannot be excluded in the *Prox1* model.

Overall, our data suggest that developmental perturbations in VIP+ INs have an effect in overall network activity, in line with previously published research (Batista-Brito et al., 2017). Batista-Brito et al. (2017) demonstrated that VIP+ INs lacking the receptor tyrosine kinase ERBB4 led to impairments in sensory processing, cortical state transitions and learning.

Depletion of *ErbB4* and *Prox1* cannot be compared directly, as the former is involved in cell signalling and the latter is a transcription factor (Law et al., 2007; Oliver et al., 1993). Moreover, the authors focused on another primary sensory area, the visual cortex: IN-specific architectural differences in visual and somatosensory cortices have been identified (Kim et al., 2017) and recent evidence reported that molecular marker for glutamatergic cells were area-specific (Tasic et al., 2018), suggesting different cortical circuits in these two areas.

An interesting discovery from Batista-Brito et al. (2017) is the fact that genetic impairment of VIP+ INs leads to defects in cortical state transitions and learning, phenotypes investigated during awake recordings. This raises the question whether we are only able to observe a partial effect of *Prox1* depletion in VIP+ INs during urethane anaesthesia.

Although urethane anaesthesia affects the coordination of cortical activity less than other anaesthesia methods (Hildebrandt et al., 2017), it still suppresses neuronal activity (Huh and Cho, 2013). More importantly, this anaesthetic totally suppresses POm activity (Zhang and Bruno, 2019). It has been shown that VIP+ INs in L2/3 – those affected in the *Prox1* mutant according to Miyoshi et al.

(2015) – are targeted by the POM (Audette et al., 2018; Williams and Holtmaat, 2019), while the VPM is targeting VIP+ INs in L4 (Ji et al., 2016; Staiger et al., 1996). Thus, the MUA effect identified here might be considered as an indication of the state-independent contribution VIP+ INs in cortical circuitry upon depletion of *Prox1*. Perhaps future awake recordings paired with a learning task would reveal a more profound state-dependent effect of the *Prox1* mutation.

Finally, to understand how different levels of *Prox1* in VIP+ INs might affect network activity and have a better understanding of the differences between cHet and cKO, *in vivo* recordings in WT animals need to be performed and compared with the datasets here reported.

5.7 – General considerations regarding the VIP-

Cre;Ai9;Prox1^{c/c} model

In this chapter, the *VIP-Cre;Ai9;Prox1^{c/c}* model was employed to dissect the cell-autonomous effect of *Prox1* depletion in VIP+ INs in their local and long-range connectivity, as well as in sensory processing.

Miyoshi et al. (2015) is currently the only other published work studying the *VIP-Cre;Prox1^{c/c}* model: they identified a significant difference between cHet and cKO animals, with cKO animals showing a ~15% density reduction of VIP+ INs in L2/3, a reduction in VIP/CR co-expression and morphological defects in bipolar cells. While we did not analyse VIP/CR co-expression or morphological defects, we were not able to reproduce their finding about cell density defects.

Importantly, Miyoshi et al. (2015) employed a distinct *Prox1* model than the one used here. They used as experimental animals the offspring from two different strains of *Prox1*^{c/c} animals, published from Harvey et al. (2005) and Iwano et al. (2012) respectively. The constructs to obtain these animals are slightly different: in Harvey et al. (2005), loxP sites were designed to flank exons 2 and 3 of the *Prox1* gene sequence (those encoding the homeodomain and Prox domain), rendering the locus transcriptionally inactive once these 2 exons were removed. In Iwano et al. (2012) (and in this thesis) the authors used a mouse model where the coding sequence in exon 2 was replaced by the floxed *Prox1* cDNA and the EGFP.

The subsequent differences between these two not identical models can be considerable, giving that the mouse *Prox1* gene is located in between two uncharacterised long-non coding sequences, one of which (LOC102632463) is expressed in the embryonic and adult CNS (NCBI Gene Database). Noncoding regulatory DNA have an important role in CNS development (see for a review Wei et al., 2018) and potential minor changes in the noncoding sequence upon insertion of these constructs cannot be excluded. In addition to transgene-specific differences, diversity in the overall genetic background could also explain the potentially contrasting results here reported.

Finally, it is important to consider advantages and disadvantages of the use of cHet mice rather than WT as controls for the *Prox1* cKO mice. Using cHet maximises production of experimental animals, allowing to have 50% cKO and 50% littermate controls (in case of breedings crossing cHet and cKO animals) rather than 25% cKO and 25% WT littermate controls (in case of cHet x cHet crossing). However, haploinsufficiency (i.e. when, in heterozygosity, the

remaining functional copy of the gene is not sufficient to preserve its function) has been reported in *Prox1* heterozygous mice (Harvey et al., 2005). Therefore, careful considerations must be given before using the cHet animals as control, as demonstrated by the local-connectivity analysis here reported (see Figure 5.5).

Chapter 6 – Discussion

6.1 – Summary of contributions

In this thesis I have defined the synaptic integration and putative function of VIP+ INs in neonatal somatosensory cortex, expanding our current understanding of their role in circuit development during the first two postnatal weeks (Batista-Brito et al., 2017; Emson et al., 1979; Goff and Goldberg, 2019; Götz and Bolz, 1989; McDonald et al., 1982; Mossner et al., 2017). I employed acute *in vitro* electrophysiology combined with optical methods (namely LSPS and optogenetics) to probe the early integration of VIP+ INs into the local and long-range circuitry. I have shown that genetic perturbation of VIP+ INs through conditional deletion (cKO) of the transcription factor PROX1 seems to affect their local glutamatergic connectivity, without apparently impairing synaptic input from long-range anterior-motor afferents. *In vivo* electrophysiology confirmed that *Prox1* cKO VIP+ INs have a significant effect at the network level, leading to higher firing frequency in multi-unit activity upon whisker stimulation.

In Chapter 3 I demonstrated that VIP+ INs were located primarily in supragranular layers as early as P1, and that their density and cortical layer distribution did not change from the end of the first postnatal week (P7-8) onwards. The intrinsic electrophysiological properties matured over the course of the first two postnatal weeks, a process that was mirrored by a gradual integration in the local glutamatergic network.

Analysis of the laminar glutamatergic profiles identified three subtypes of layer 2/3 (L2/3) VIP+ INs, characterised by differential laminar glutamatergic components: infragranular, granular and only local supragranular respectively. The infragranular pattern of innervation (cluster 1) was present throughout the developmental window tested (P5-P16). Cluster 2 (granular) pattern of innervation was found preferentially in VIP+ INs located in L3 and only from P10 onward, while cluster 3 (local) cells were preferentially located in superficial L2. This suggests a difference in the function of L2/3 VIP+ INs subtypes, as well as connectivity differences between L2 and L3, in line with previous literature (Bureau et al., 2006; Gur and Snodderly, 2008; Meng et al., 2017; Shepherd and Svoboda, 2005; Staiger et al., 2015).

Analysis of VIP+ INs output using conditional expression of ChR-2 showed that this class of IN formed synaptic connections onto efferent targets from the beginning of the first postnatal week. They targeted both Lhx6-EGFP+ INs and pyramidal cells, but with a preference for the former by P7-8. Moreover, this showed an inside-out pattern of innervation, with infragranular cells (both Lhx6-EGFP+ INs and pyramidal neurons) recruited first and supragranular neurons engaged progressively throughout this time window such that they received robust input by P12-P15, the time of active whisking onset. Altogether, these

data suggest an early role for VIP+ INs in controlling formation of cortical circuits and a gradual recruitment of supragranular cells, in line with their role in active whisking and sensorimotor integration (Barson et al., 2018; Lee et al., 2013; Sachidhanandam et al., 2016; Yu et al., 2019).

In Chapter 4, I employed a viral strategy (Arruda-Carvalho et al., 2017) to target anterior-motor areas at early time points and test the recruitment of S1BF VIP+ INs and pyramidal cells controls. These experiments revealed that VIP+ INs received synaptic connections from anterior-motor areas within the first postnatal week, with the probability of connectivity by these long-range projections increasing in the second postnatal week. Again, this supports an early role for VIP+ INs in mediating sensorimotor integration – albeit at a reduced level when compared to the mature circuit – at a time preceding active whisking.

Finally, in Chapter 5, I addressed what happens to VIP+ INs following conditional deletion of the transcription factor *Prox1*. In contrast to previous reports (Miyoshi et al., 2015), I found that VIP+ INs in both *Prox1* cHet and cKO mice displayed no difference in density or distribution across the depth of the cortical column when compared to wild-types (WT). I did observe a difference in local glutamatergic input onto VIP+ INs in cKO mice when compared to WT animals, but no differences in recruitment from long-range connections.

In vivo electrophysiology combined with multi-whisker stimulation demonstrated no difference in stimulus-triggered LFP, but higher multi-unit activity following whisker stimulation in *Prox1* cKO animals when compared to cHet. This suggests that cell-autonomous changes in VIP+ INs have an effect on

their local integration and result in altered network activity in response to sensory stimulation.

6.2 – Implications

There is increasing evidence supporting the role of VIP+ INs in development and plasticity (Batista-Brito et al., 2017; Fu et al., 2015; Goff and Goldberg, 2019; Mardinly et al., 2016; Mossner et al., 2017; Williams and Holtmaat, 2019). Although the circuit elements underlying the role of VIP+ INs in plasticity are emerging (Fu et al., 2015; Mardinly et al., 2016; Williams and Holtmaat, 2019), how these elements interact in the developing brain remain largely unknown. The results presented here fill this gap, identifying that already within the first postnatal week S1BF VIP+ INs start to integrate in the local and long-range glutamatergic circuitry, and to exert their effect onto local Lhx6-EGFP+ INs and pyramidal cells.

In particular, the early connection (P3-4) to infragranular Lhx6-EGFP+ INs is of interest given the role of early infragranular SST+ INs in the establishment of thalamocortical connectivity (Marques-Smith et al., 2016; Tuncdemir et al., 2016). Moreover, VIP+ INs start to receive synaptic input from long-range anterior-motor connections by P7-9. As such, VIP+ INs are ideally positioned to mediate the development of sensorimotor integration in parallel with the establishment of thalamocortical connectivity.

It is interesting to speculate how the diversity of local connectivity might reflect long-range inputs and the diversity of VIP+ INs previously reported (Cauli

et al., 2014; He et al., 2016; Kubota and Kawaguchi, 1997; Porter et al., 1998; Prönneke et al., 2015, 2018; Rhomberg et al., 2018; Tasic et al., 2016, 2018; Tremblay et al., 2016). Due to the technical limitations of recording from neonatal VIP+ INs, I was unable to combine optogenetic and LSPS experiments. Future analysis could reveal whether or not differences in local VIP+ circuitry based on layer location (i.e. depth from pial surface) reflect long-distance innervation.

The importance of VIP+ INs in development is further demonstrated by their genetic perturbation via conditional deletion of *Prox1*: it leads to cell-autonomous changes in the local glutamatergic circuitry, and has a profound effect in overall network activity upon whisker stimulation. This wide-spread network effect is in line with other studies focusing on genetic perturbation of VIP+ IN integration at early time points (Batista-Brito et al., 2017; Cardin, 2018b).

VIP+ IN-mediated disinhibition is a key mechanism for learning and memory (Letzkus et al., 2015; Pi et al., 2013; Williams and Holtmaat, 2019). Moreover, VIP+ INs have an important role in cortical state transitions and arousal (Batista-Brito et al., 2017, 2018; Cardin, 2018, 2019). Alterations in learning, memory and arousal have been reported in developmental conditions such as autism (Artoni et al., 2019; Ey et al., 2011; Orekhova and Stroganova, 2014) and schizophrenia (Hall et al., 2009; Williams et al., 2004; Yasuda et al., 2017).

INs dysfunction has been associated with these and others developmental disorders (Dienel and Lewis, 2019; Lunden et al., 2019; Nakazawa et al., 2012; Rapanelli et al., 2017). Decreased VIP+ INs density has been reported in human

patients affected by these conditions (Fung et al., 2014; Velmeshev et al., 2019) and VIP+ INs function is impaired in mouse models of autism and schizophrenia (Batista-Brito et al., 2017; Cunniff and Sohal, 2017; Mossner et al., 2017). This evidence, combined with the early integration of VIP+ INs in cortical circuits demonstrated in this thesis, suggests that VIP+ INs have a key role during development that may underlie (at least partially) the impairments observed in developmental conditions such as autism and schizophrenia.

6.3 – Future work

There are a number of avenues for further experimentation. First, considering VIP+ INs diversity (Cauli et al., 2014; He et al., 2016; Kubota and Kawaguchi, 1997; Porter et al., 1998; Prönnke et al., 2015, 2018; Rhomberg et al., 2018; Tasic et al., 2016, 2018; Tremblay et al., 2016), it would be of interest to use take advantage of the recent explosion in transcriptomics to better define the VIP+ IN populations in developing neocortex.

In particular, single-cell RNAseq combined with electrophysiology (i.e. patch-seq) (Cadwell et al., 2016) could be employed to better delineate the various glutamatergic input clusters identified with my LSPS experiments. Patch-seq has been previously used to reveal the molecular profiles that underlie neuronal diversity, in both pyramidal cells and INs (Cadwell et al., 2016; Földy et al., 2016; Muñoz-Manchado et al., 2018; Que et al., 2019).

Furthermore, considering that 2 of the 3 clusters identified correlate with distance from the pial surface, an alternative approach to further understand

VIP+ INs diversity is by employing RNA-scope (Wang et al., 2012), a novel *in situ* RNA analysis method. Transcriptome analysis has recently identified several molecular markers specific for VIP+ INs subtypes (Tasic et al., 2016, 2018), which could be tested to differentiate between L2 and L3 VIP+ cells.

To date, the Lhx6-EGFP+ cells targeted by VIP+ INs in the first postnatal week have not been fully characterised yet. An obvious extension of these experiments is to perform detailed morphological analysis in combination with immunohistochemistry for SST or PV. While PV is not expressed before P10 (de Lecea et al., 1995), this should not be a major issue, as SST is reliably expressed by developing SST+ INs (Anastasiades et al., 2016a; Marques-Smith et al., 2016; Pan et al., 2019) and could help differentiate between the Lhx6-EGFP+/SST+ and Lhx6-EGFP+/SST- (putative PV+) populations.

The work described here has focused on L2/3 VIP+ INs, which represent the majority of the VIP+ population in S1BF (Prönneke et al., 2015). It would be interesting to further investigate whether the same neuronal population provides input to infragranular pyramidal cells and Lhx6-EGFP+ INs. It has been shown that axonal boutons of L2/3 VIP+ INs target infragranular layers, with a preference for L5a (Prönneke et al., 2015). The location of VIP+ input could be addressed by more complex optogenetic experiments combined with LSPS in the *VIP-Cre;Ai32;Lhx6-EGFP* line (Adesnik and Scanziani, 2010; Naka et al., 2019).

I have not explored to-date the full repertoire of potential afferent input onto VIP+ INs at neonatal ages. Three obvious candidates are serotonin, acetylcholine and the thalamus. VIP+ INs express 5HT3aR (Lee et al., 2010; Rudy et al., 2011) and respond to serotonin modulation (Prönneke et al., 2018). Alterations in serotonergic signalling lead to migration defects in this cell

population (Frazer et al., 2015; Murthy et al., 2014). This suggests that early VIP+ INs are sensitive to serotonin, hypothesis that can be tested through *in vitro* experiments.

Pharmacological studies previously addressed the response of juvenile and adult VIP+ INs upon cholinergic stimulation (Cunniff and Sohal, 2017; Goff and Goldberg, 2019; Prönncke et al., 2018). Considering the importance of acetylcholine during development (Liu et al., 2007) and the fact that transcriptome analysis of 5HT3aR+ cells suggested the presence of the cholinergic nicotinic receptor already in late embryonic stages (Frazer et al., 2017), it seems logical to perform patch-cell recordings in neonatal VIP+ INs and test for responses to cholinergic agonists.

Similarly, it has been shown that VIP+ INs are recruited by the thalamus in adult cortex (Audette et al., 2018; Ji et al., 2016; Staiger et al., 1996; Williams and Holtmaat, 2019), with further evidence pointing to thalamic engagement of VIP+ INs early in development (Che et al., 2018). However, the functionality of this input has not been proven yet. To explore this further, I could test for connectivity using electrical stimulation in thalamocortical slices of *VIP-Cre;Ai9* mice (Agmon and Connors, 1991), or take an optogenetic approach using the *Olig3-Cre;Ai32* mouse line to selectively activate thalamocortical presynaptic fibres as previously reported (De Marco García et al., 2015; Stacey, 2018). The latter experiment would require the use of the *5HT3aR-EGFP* line to target L2/3 VIP+ INs (avoiding the L1 Re+ population) (Che et al., 2018; De Marco García et al., 2015).

Furthermore, interesting open questions arise from the analysis of the *Prox1* model. First of all, deletion of *Prox1* in cHet and cKO mice needs to be

confirmed by immunofluorescence at later time points (at least P7), to enable proper comparison with previous data demonstrating the reliable expression of the *Prox1* transgene at P7-8 (Miyoshi et al., 2015). Morphological analysis (e.g. Sholl analysis (Ferreira et al., 2014; Sholl, 1953)) of *Prox1* cHet and cKO VIP+ INs needs to be performed to further compare the model used here with what previously published (Miyoshi et al., 2015). More LSPS experiments are needed to confirm that P12-16 *Prox1* cHet and cKO VIP+ INs have principally local input and the phenotype observed is not due to a sampling bias towards L2 VIP+ cells (i.e. cluster 3 cells, which present only local input).

Finally, *in vivo* MUA needs to be compared to a WT dataset, to confirm the phenotype observed in cKO animals when compared with cHet controls. Data show that cKO animals have higher MUA upon whisker stimulation than cHet. Given that cHet and cKO do not differ for local or long-range glutamatergic connectivity, the current hypothesis is that cHet and cKO have differential output. This could be tested by breeding the *VIP-Cre;Ai32;Lhx6-EGFP+* line onto the *Prox1^{c/c}* background. These experiments will help us test if deletion of *Prox1* leads to specific impairments in local connectivity, while sparing long-range connectivity.

Intriguingly, a recent paper based on single-cell RNA-seq found that *Prox1*+ INs specifically expressed a set of isoforms of a synaptic organizer protein called neurexin (Lukacsovich et al., 2019). Previous work has hypothesised that this protein could act as a ‘code’ for synapses specification, as it can be found in synapses from local, but not long-range, glutamatergic connections, at least in the cerebellum (Pregno et al., 2013). Moreover, transcriptome analysis on IN progenitors depleted of *Prox1* highlighted the

dysregulation of many genes involved in synaptic contact formation or axon guidance, such as *Dtna*, *Robo1*, *Sema4A*, *Sfrp2* (Blake et al., 1996; Leyva-Díaz et al., 2014; Miyoshi et al., 2015; Ranaivoson et al., 2019). Alterations in synaptic contact and axon guidance molecules may underlie the *Prox1* phenotype observed here.

Finally, considering that VIP+ INs-mediated disinhibition is an important mechanism for learning and memory (Letzkus et al., 2015; Pi et al., 2013; Williams and Holtmaat, 2019), it could be of interest to test whether the *Prox1* deletion leads to broader cognitive impairments.

6.4 – Concluding remarks

This thesis shows the integration of VIP+ INs in the sensorimotor circuitry during the first two postnatal developmental weeks, revealing a hitherto unappreciated early role for this IN population, despite its late birthdate (Butt et al., 2005; Miyoshi and Fishell, 2011; Miyoshi et al., 2010). Moreover, it suggests that perturbations in VIP+ INs have effects both at a local and network level. Therefore, although VIP+ INs only represent 1-2% of the overall neuronal population in the cortex (Rudy et al., 2011; Xu et al., 2010), they can have a major impact in cortical circuits development and functioning.

This work takes a developmental approach to system neuroscience, with the hope that similar studies will be pursued in the future to unravel the role of cortical INs in shaping developing circuits and emerging perception.

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Appendix 1 – Statistical analysis in Chapter 3

3.1.2 – Quantification of VIP+ INs distribution across development

Comparisons across bins referring to **Figure 3.2** – Quantification of tdTomato positive cells in the *VIP-Cre;Ai9* line (panel B) Each bin was compared across time points. Note that $\alpha = 0.005$ following Bonferroni correction for multiple comparisons.

	Test?	Details	P values
Bin 1	Mixed effect linear model analysis*	F (4, 9) = 4.280	P=0.0327
Bin 2	Mixed effect linear model analysis*	F (4, 9) = 0.3167	P=0.8599
Bin 3	Mixed effect linear model analysis*	F (4, 9) = 0.3382	P=0.8457
Bin 4	Mixed effect linear model analysis*	F (4, 9) = 2.498;	P=0.1169
Bin 5	Mixed effect linear model analysis*	F (4, 9) = 5.481	P=0.0162
Bin 6	Mixed effect linear model analysis*	F (4, 9) = 2.527	P=0.1142
Bin 7	Mixed effect linear model analysis*	F (4, 9) = 1.130	P=0.4011
Bin 8	Mixed effect linear model analysis*	F (4, 9) = 4.592,	P=0.0270
Bin 9	Mixed effect linear model analysis*	F (4, 23) = 2.85	P=0.0466
Bin 10	Mixed effect linear model analysis*	F (4, 9) = 3.403	P=0.0587

* Fixed effect = Age, Random effect= subject

Tukey's multiple comparisons test referring to **Figure 3.2** – Quantification of tdTomato positive cells in the *VIP-Cre;Ai9* line (panel C). Each cell density value was compared across time points. Mixed effect linear model analysis: F (4, 9) = 26.92, $p < 0.0001$

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
P1 vs. P3	-1172	-3376 to 1031	No	ns	0.3869
P1 vs. P7-8	-5544	-6450 to -4638	Yes	****	<0.0001
P1 vs. P14-15	-5728	-6869 to -4588	Yes	****	<0.0001
P1 vs. P21-22	-5938	-6707 to -5170	Yes	****	<0.0001
P3 vs. P7-8	-4371	-6576 to -2166	Yes	***	0.0009
P3 vs. P14-15	-4556	-6793 to -2319	Yes	***	0.0005
P3 vs. P21-22	-4766	-6965 to -2567	Yes	***	0.0006
P7-8 vs. P14-15	-184.6	-1422 to 1053	No	ns	0.9895
P7-8 vs. P21-22	-394.8	-1348 to 558.2	No	ns	0.7003
P14-15 vs. P21-22	-210.2	-1383 to 962.9	No	ns	0.9782

Comparisons across layers referring to **Figure 3.2** – Quantification of tdTomato positive cells in the *VIP-Cre;Ai9* line (panel D Left) Each normalised layer value was compared across time points. Note that $\alpha = 0.0125$ following Bonferroni correction for multiple comparisons.

	Test?	Details	P values
MZ	Mixed effect linear model analysis*	F (1, 6) = 0.2528	P=0.6330
CP	Mixed effect linear model analysis*	F (1, 3) = 0.5529	P=0.5111
L5	Mixed effect linear model analysis*	F (1, 3) = 1.306	P=0.3360
L6	Mixed effect linear model analysis*	F (1, 3) = 14.06	P=0.0331

* Fixed effect = Age, Random effect= subject

Comparisons across layers referring to **Figure 3.2** – Quantification of tdTomato positive cells in the *VIP-Cre;Ai9* line (panel D right). Each normalised layer value was compared across time points. Note that alpha= 0.0083 following Bonferroni correction for multiple comparisons.

	Test?	Details	P values
L1	Mixed effect linear model analysis*	F (2, 6) = 0.5232	P=0.6174
L2/3	Mixed effect linear model analysis*	F (2, 17) = 1.002	P=0.3879
L4	Mixed effect linear model analysis*	F (2, 17) = 1.744	P=0.2047
L5a	Mixed effect linear model analysis*	F (2, 6) = 1.955	P=0.2220
L5b	Mixed effect linear model analysis*	F (2, 6) = 1.105	P=0.3904
L6	Mixed effect linear model analysis*	F (2, 6) = 0.7858	P=0.4976

* Fixed effect = Age, Random effect= subject

3.1.3 – Characterisation of intrinsic properties in developing VIP+ INs

Tests referring to **Table 3.1** and **Figure 3.3** – Intrinsic properties of VIP+ INs across development.

	Test?	Details	P values
% Adaptation	Kruskal-Wallis ANOVA	K-W statistics=11.55	P=0.0031
10-90% Rising Time	Kruskal-Wallis ANOVA	K-W statistics= 22.34	P< 0.0001
AHP Amplitude	One-way ANOVA	F (2, 39) = 5.390	P = 0.0086
AHP delay	Kruskal-Wallis ANOVA	K-W statistics=5.475	P=0.0647
Firing threshold	Kruskal-Wallis ANOVA	K-W statistics= 5.552	P= 0.0623
Half-width	Kruskal-Wallis ANOVA	K-W statistics=20.83	P< 0.0001
Max Firing Frequency	Kruskal-Wallis ANOVA	K-W statistics=3.243	P=0.1976
Rheobase	Kruskal-Wallis ANOVA	K-W statistics= 12.61	P= 0.0018
R _{IN}	Kruskal-Wallis ANOVA	K-W statistics=15.18	P= 0.0005
RMP	One-way ANOVA	F (2, 49) = 1.958	P = 0.1520
Spike Delay	One-way ANOVA	F (2, 39) = 1.231	P = 0.3032
Spike Height	One-way ANOVA	F (2, 39) = 4.615	P = 0.0159
Tau	One-way ANOVA	F (2, 35) = 10.45	P = 0.0003

% Adaptation – Multiple comparisons

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
P5-P8 vs. P9-P11	-10.92	No	ns
P5-P8 vs. P12+	-15.48	Yes	**
P9-P11 vs. P12+	-4.564	No	ns

10-90% – Multiple comparisons

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
P5-P8 vs. P9-P11	11.04	No	ns
P5-P8 vs. P12+	20.20	Yes	****
P9-P11 vs. P12+	9.159	No	ns

AHP Amplitude – Multiple comparisons

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
P5-P8 vs. P9-P11	0.4409	-4.619 to 5.501	No	ns
P5-P8 vs. P12+	5.588	1.290 to 9.886	Yes	**
P9-P11 vs. P12+	5.147	-0.3254 to 10.62	No	ns

Half-width – Multiple comparisons

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
P5-P8 vs. P9-P11	12.58	Yes	*
P5-P8 vs. P12+	19.18	Yes	****
P9-P11 vs. P12+	6.596	No	ns

Rheobase – Multiple comparisons

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
P5-P8 vs. P9-P11	-10.85	No	ns
P5-P8 vs. P12+	-14.63	Yes	**
P9-P11 vs. P12+	-3.788	No	ns

Spike Height – Multiple comparisons

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
P5-P8 vs. P9-P11	-12.21	-23.49 to -0.9343	Yes	*
P5-P8 vs. P12+	-8.974	-18.55 to 0.6071	No	ns
P9-P11 vs. P12+	3.240	-8.959 to 15.44	No	ns

Tau – Multiple comparisons

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
P5-P8 vs. P9-P11	22.72	2.197 to 43.23	Yes	*
P5-P8 vs. P12+	33.52	15.13 to 51.91	Yes	***

P9-P11 vs. P12+	10.81	-10.55 to 32.16	No	ns
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3.2.1 – Analysis of local glutamatergic connectivity with LSPS

Tests referring to **Figure 3.5** – LSPS of caged glutamate reveals the laminar organisation of glutamatergic synaptic inputs onto L2/3 VIP+ INs during development.

	Test?	Details	P values
Total LSPS input	Kruskal-Wallis ANOVA	K-W statistics=2.887	P=0.2361
LSPS input across layers	Two- way ANOVA	Layer effect: F (3, 84) = 14.17	P< 0.0001
Norm. L2/3 input across ages	One-way ANOVA	F (2, 29) = 3.359	P = 0.0487
Norm. L4 input across ages	Kruskal-Wallis ANOVA	K-W statistics=3.153	P=0.2067
Norm. L5a input across ages	One-way ANOVA	F (2, 29) = 1.366	P = 0.2710
Norm. L5b/6 input across ages	One-way ANOVA	F (2, 29) = 1.523	P = 0.2350

LSPS input across layers – Multiple comparisons

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
L2/3 vs. L4	103332	27279 to 179384	Yes	**
L2/3 vs. L5a	171721	95668 to 247773	Yes	****
L2/3 vs. L5b/6	157820	81768 to 233873	Yes	****
L4 vs. L5a	68389	-7663 to 144442	No	ns
L4 vs. L5b/6	54489	-21564 to 130541	No	ns
L5a vs. L5b/6	-13900	-89953 to 62152	No	ns

Normalised L2/3 input across ages – Multiple comparisons

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
P5-P8 vs. P9-P11	16.74	-4.889 to 38.37	No	ns
P5-P8 vs. P12-P16	22.51	0.4593 to 44.56	Yes	*
P9-P11 vs. P12-P16	5.766	-14.71 to 26.24	No	ns

Tests referring to **Figure 3.8** – Characterisation of clusters of VIP+ INs (panel C to E)

	Test?	Details	P values
Norm. L2/3 input across clusters	Kruskal-Wallis ANOVA	K-W statistics=22.12	P < 0.0001
Norm. L4 input across clusters	Kruskal-Wallis ANOVA	K-W statistics=20.94	P < 0.0001
Norm. L5a input across clusters	Kruskal-Wallis ANOVA	K-W statistics=5.002	P = 0.0820
Norm. L5b/6 input across clusters	Kruskal-Wallis ANOVA	K-W statistics=13.65	P = 0.0011
Age across clusters	One-way ANOVA	F (2, 29) = 3.650	P = 0.0386
Pial distance across clusters	Kruskal-Wallis ANOVA	K-W statistics=8.475	P=0.0144

Normalised L2/3 input across clusters – Multiple comparisons

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
Cluster 1 vs. Cluster 2	8.738	No	ns
Cluster 1 vs. Cluster 3	-12.66	Yes	**
Cluster 2 vs. Cluster 3	-21.40	Yes	****

Normalised L4 input across clusters – Multiple comparisons

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
Cluster 1 vs. Cluster 2	-12.50	Yes	*
Cluster 1 vs. Cluster 3	9.667	Yes	*
Cluster 2 vs. Cluster 3	22.17	Yes	****

Normalised L5b/6 input across clusters – Multiple comparisons

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
Cluster 1 vs. Cluster 2	13.82	Yes	*
Cluster 1 vs. Cluster 3	12.72	Yes	**
Cluster 2 vs. Cluster 3	-1.100	No	ns

Age across clusters – Multiple comparisons

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
Cluster 1 vs. Cluster 2	-2.476	-5.675 to 0.7223	No	ns
Cluster 1 vs. Cluster 3	1.107	-1.472 to 3.686	No	ns
Cluster 2 vs. Cluster 3	3.583	0.3059 to 6.861	Yes	*

Pial distance across clusters – Multiple comparisons

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
Cluster 1 vs. Cluster 2	-5.345	No	ns
Cluster 1 vs. Cluster 3	7.530	No	ns
Cluster 2 vs. Cluster 3	12.88	Yes	*

Tests referring to **Figure 3.8** – Characterisation of clusters of VIP+ INs (panel F).

	Test?	P values
Cluster 1 vs Cluster 2	Fisher's Exact Test	P=1
Cluster 1 vs Cluster 3	Fisher's Exact Test	P= 0.3271
Cluster 2 vs Cluster 3	Fisher's Exact Test	P = 0.2721

3.3.2. Investigating the development of VIP+ cells output using the VIP-Cre;Ai32 mouse line

Tests referring to **Figure 3.11** – Cumulative distribution of responsive cells while increasing LED power and **Figure 3.13** – Cumulative distribution of responsive cells with average latency <17ms and jitter<5ms while increasing LED power.

	Test?	Details	P values
Proportion of responsive cells Lhx6+ vs Pyr	Chi-square	$\chi^2(4) = 4.1387$	P=0.38756
Proportion of responsive cells Lhx6+ vs Pyr (jitter < 5ms and latency <17ms)	Chi-square	$\chi^2(4) = 15.2216$	P= 0.004263

Log-linear analysis results referring to **Figure 3.14** – Developmental increase in the proportion of Lhx6-EGFP+ and Pyramidal cells recruited across the first two postnatal weeks.

The categories were defined as follows: A = Responsive vs Not responsive cell; B = Age group; C = Lhx6-EGFP+ IN vs Pyramidal

Log-linear analysis results are reported as follows:

ABC	Representing the 3-way interaction between A, B, and C.
AB	Representing the 2-way interactions for AB, AC, and BC, respectively.
AC	
BC	
AB(C)	Representing the 2-way interactions for each pair of variables, AB, AC, and BC, when the effects of the third variable are removed from the picture.
AC(B)	
BC(A)	

Adapted from <http://vassarstats.net/abc.html>

Proportion of responsive Lhx6-EGFP+ and pyramidal cells over time				
	G ²	df	P	Significant?
ABC	16.92	7	0.0179	*
AB	8.44	2	0.0147	*
AC	0.5	1	0.4795	ns
BC	3.66	2	0.1604	ns
AB(C)	12.76	4	0.0125	*
AC(B)	4.82	3	0.1855	ns
BC(A)	7.98	4	0.0923	*

Tests referring to **Figure 3.15** – Changes in jitter and latency at minimal and maximal stimulation during development (panels A and B)

	Test?	Details	P values
Jitter at min vs max Lhx6+ P7-8	2-tailed Wilcoxon matched pairs signed rank test		P = 0.1641
Jitter at min vs max Lhx6+ P12-15	2-tailed Wilcoxon matched pairs signed rank test		P=0.2969
Jitter at min vs max Pyr P5-6	2-tailed Wilcoxon matched pairs signed rank test		P=0.2031
Jitter at min vs max Pyr P7-8	2-tailed Wilcoxon matched pairs signed rank test		P=0.5000
Jitter across time, Lhx6+ and Pyr	Three-way ANOVA – LED power effect	F (1, 34) = 7.217	P=0.0111
Jitter across time, Lhx6+ and Pyr	Three-way ANOVA – cell type effect	F (1, 34) = 0.01083	P=0.9177

Tests referring to **Figure 3.15** – Changes in jitter and latency at minimal and maximal stimulation during development (panels C and D)

	Test?	Details	P values
Latency at min vs max Lhx6+ P7-8	2-tailed Wilcoxon matched pairs signed rank test		P=0.0078
Latency at min vs max Lhx6+ P12-15	2-tailed Wilcoxon matched pairs signed rank test		P=0.0156
Latency at min vs max Pyr P5-6	2-tailed Wilcoxon matched pairs signed rank test		P=0.0039
Latency at min vs max Pyr P7-8	2-tailed Wilcoxon matched pairs signed rank test		P=0.2500
Latency at min vs max Pyr P12-15	2-tailed Wilcoxon matched pairs signed rank test		P=0.0010
Latency across time, Lhx6+ and Pyr	Three-way ANOVA	Age effect :F (2, 34) = 4.548	P=0.0178
Latency across time, Lhx6+ and Pyr	Three-way ANOVA	LED Power Effect: F (1, 34) = 31.03	P<0.0001
Latency across time, Lhx6+ and Pyr	Three-way ANOVA	Cell type effect: F (1, 34) = 0.01662	P=0.8982

Log-linear analysis results referring to **Figure 3.16** – VIP+ INs engage differently the Lhx6-EGFP+ and pyramidal networks in infragranular and supragranular layers. The categories were defined as follows: A = Responsive vs Not responsive cell; B = Age group; C = Lhx6-EGFP+ IN vs Pyramidal. Log-linear analysis results are reported as described above.

Proportion of responsive Lhx6-EGFP+ and pyramidal cells over time in supragranular layers				
	G ²	df	P	Significant?
ABC	22.46	7	0.0021	**
AB	16.02	2	0.0003	***
AC	0.1	1	0.7518	ns
BC	0.8	2	0.6703	ns
AB(C)	21.56	4	0.0002	***
AC(B)	5.64	3	0.1305	ns
BC(A)	6.34	4	0.1752	ns

Proportion of responsive Lhx6-EGFP+ and pyramidal cells over time in infragranular layers				
	G ²	df	P	Significant?
ABC	5.6	7	0.5872	ns
AB	0.74	2	0.6907	ns
AC	0.8	1	0.3711	ns
BC	3.5	2	0.1738	ns
AB(C)	1.3	4	0.8614	ns
AC(B)	1.36	3	0.1749	ns
BC(A)	4.06	4	0.3979	ns

In the following log-linear analysis, the categories were defined as follows: A = Responsive vs Not responsive cell; B = Age group; C = Layer.

Proportion of responsive Lhx6-EGFP+ INs over time, supragranular and infragranular layers				
	G ²	df	P	Significant?
ABC	14.74	7	0.0395	*
AB	4.32	2	0.1153	ns
AC	1.34	1	0.247	ns
BC	0.38	2	0.827	ns
AB(C)	13.02	4	0.0112	*
AC(B)	10.04	3	0.0182	*
BC(A)	9.08	4	0.0591	ns

Proportion of responsive Pyramidal cells over time, supragranular and infragranular layers				
	G ²	df	P	Significant?
ABC	14.26	7	0.0451	*
AB	6.28	2	0.0433	*
AC	0.02	1	0.8875	ns
BC	4.52	2	0.1044	ns
AB(C)	9.82	4	0.0436	*
AC(B)	3.56	3	0.3131	ns
BC(A)	8.06	4	0.0894	ns

Tests referring to **Figure 3.18.** – Quantification of transferred charge suggests the differential engagement of VIP+ INs with the supragranular and infragranular networks.

	Test?	Details	P values
VIP Input in Lhx6+ vs Pyr cells, according to layer location and age	Three-way ANOVA	Age and location interaction: F (2, 82) = 5.881	P=0.0041
VIP Input in Lhx6+ vs Pyr cells, according to layer location and age	Three-way ANOVA	Age and cell type interaction: F (2, 82) = 3.128	P=0.0491

Multiple comparisons to investigate the age and cell type interaction

Sidak's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
GFP - Pyr					
P5-6	297.3	-67304 to 67898	No	ns	>0.9999
P7-8	57773	-19325 to 134870	No	ns	0.1997
P12-15	-62081	-137464 to 13303	No	ns	0.1374

Multiple comparisons to investigate the age and location interaction

Tukey's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Significant?	Summary	Adjusted P Value
P5-6:UL vs. P5-6:LL	-43311	-121864 to 35242	No	ns	0.5966
P5-6:UL vs. P7-8:UL	-26604	-111410 to 58202	No	ns	0.9420
P5-6:UL vs. P7-8:LL	-72060	-163224 to 19104	No	ns	0.2040
P5-6:UL vs. P12+:UL	-108665	-196253 to -21078	Yes	**	0.0065
P5-6:UL vs. P12+:LL	-11249	-97364 to 74866	No	ns	0.9989
P5-6:LL vs. P7-8:UL	16707	-58733 to 92146	No	ns	0.9872
P5-6:LL vs. P7-8:LL	-28749	-111271 to 53773	No	ns	0.9118
P5-6:LL vs. P12+:UL	-65355	-143908 to 13198	No	ns	0.1594
P5-6:LL vs. P12+:LL	32062	-44846 to 108970	No	ns	0.8286
P7-8:UL vs. P7-8:LL	-45456	-133951 to 43039	No	ns	0.6674
P7-8:UL vs. P12+:UL	-82061	-166867 to 2745	No	ns	0.0637
P7-8:UL vs. P12+:LL	15355	-67930 to 98640	No	ns	0.9945
P7-8:LL vs. P12+:UL	-36606	-127769 to 54558	No	ns	0.8499
P7-8:LL vs. P12+:LL	60811	-28939 to 150561	No	ns	0.3655
P12+:UL vs. P12+:LL	97416	11301 to 183532	Yes	*	0.0172

Appendix 2 – Statistical analysis in Chapter 4

4.1.3 – Injection of 150nL of virus allows to perform early developmental studies

Results referring to **Figure 4.5** – Analysis of viral spread: cross-animal variability and quantification of YFP fluorescence in S1BF

	Test?	Details	P values
Layer 1	Mixed effect linear model analysis*	F(1, 3) = 95.90	P=0.0023
Layer 2/3	Mixed effect linear model analysis*	F (1, 3) = 0.008420	P=0.9327
Layer 4	Mixed effect linear model analysis*	F(1, 3) = 18.25	P=0.0235
Layer 5a	Mixed effect linear model analysis*	Not converged	Not converged
Layer 5b	Mixed effect linear model analysis*	F (1, 3) = 0.1409	P=0.7323
Layer 6	Mixed effect linear model analysis*	F (1, 3) = 10.99	P=0.0452

* Fixed effect = Age, Random effect= subject

4.2 – Timeline of input from anterior-motor areas to S1BF

Results referring to **Figure 4.6** – ChR-2+ cells in anterior-motor areas respond to light stimulation at 470nm

	Test?	Details	P values
Latency across LED intensity, 1ms	Kruskal-Wallis test	KW statistics=5.654	P=0.1154
Jitter across LED intensity, 1ms	Kruskal-Wallis test	KW statistics=5.878	P=0.0987
Latency across LED intensity, 10ms	Two-way ANOVA	Intensity eff: F (3, 29) =12.19	P < 0.0001
Latency across LED intensity, 10ms	Two-way ANOVA	Age effect: F (1, 29) = 19.73	P = 0.0001
Jitter across LED intensity, 10ms	Two-way ANOVA	Intensity eff: F (3, 29) = 0.9403	P = 0.9375
Jitter across LED intensity, 10ms	Two-way ANOVA	Age eff: F (1, 29) = 0.0006328	P = 0.4339

Results referring to **Figure 4.7** – Definition of EPSCs monosynaptic window

	Test?	Details	P values
Latency of TTX/4-AP events at different ages	Mann-Whitney test	Two-tailed	P=0.0062
Jitter of TTX/4-AP events at different ages	Mann-Whitney test	Two-tailed	P=0.0047

Results referring to **Figure 4.8.** – Monosynaptic connectivity between anterior-motor areas and S1BF neurons increases over development.

	Test?	Details	P values
Connected VIP cells across time	Fisher's Exact Test		P=0.0233
Connected VIP cells vs Pyr P7-9	Fisher's Exact Test		P=0.1128
Connected Pyr cells across time	Fisher's Exact Test		P=0.2144
Connected VIP cells vs Pyr P≥13	Fisher's Exact Test		P=1
Amplitude VIP vs Pyr across time	Two-way ANOVA	Cell type eff: F (1, 30) = 2.133	P = 0.1546
Amplitude VIP vs Pyr across time	Two-way ANOVA	Age eff: F (1, 30) = 0.001050	P = 0.9744
Jitter VIP vs Pyr across time	Two-way ANOVA	Cell type eff: F (1, 30) = 2.835	P = 0.1026
Jitter VIP vs Pyr across time	Two-way ANOVA	Age eff: F (1, 30) = 2.461	P = 0.1272
Latency VIP vs Pyr across time	Two-way ANOVA	Interaction: F (1, 30) = 10.37	P=0.0031

Latency across time – Multiple comparisons

Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
P7-9 vs P≥13				
VIP	0.007371	0.002309 to 0.01243	Yes	**
Pyr	-0.002034	-0.006686 to 0.002618	No	ns

Appendix 3 – Statistical analysis in Chapter 5

5.2.1 – Basic characterisation of the VIP-Cre;Prox1^{c/c} model

Two-way ANOVA results referring to **Figure 5.1** – Expression of the transgene and protein levels in VIP-Cre;Prox1^{c/c} animals.

	Test?	Details	P values
Weight over time in different genotypes	Two-way ANOVA	Interaction:F (8, 121) = 1.297	P = 0.2513
Weight over time in different genotypes	Two-way ANOVA	Age eff:F (4, 121) = 73.97	P < 0.0001
Weight over time in different genotypes	Two-way ANOVA	Genotype eff:F (2, 121) = 2.308	P = 0.1038

5.2.2 – Distribution of VIP+ INs in the VIP-Cre;Prox1^{c/c} model

Results referring to **Figure 5.2** – Quantification of tdTomato positive cells in the VIPCre;Ai9;Prox1^{c/c} line.

cHet vs cKO	Test?	Details	P values
Cell density in different genotypes	Mixed effect linear model analysis*	F (1, 3) = 0.3372	P=0.6022
Normalised L1 distribution	Mixed effect linear model analysis*	F (1, 3) = 0.1303	P=0.7421
Normalised L2/3 distribution	Mixed effect linear model analysis*	F (1, 8) = 2.280	P=0.1695
Normalised L4 distribution	Mixed effect linear model analysis*	F (1, 3) = 0.2613	P=0.6445
Normalised L5 distribution	Mixed effect linear model analysis*	F (1, 8) = 4.006	P=0.0803
Normalised L6 distribution	Mixed effect linear model analysis*	F (1, 3) = 38.30	P=0.0085
Normalised L6 distribution	Mixed effect linear model analysis*	Inter.: F (2, 5) = 43.13	P=0.0007

* Fixed effect = Genotype, Random effect= subject

NOTE: An interaction between Subject and Genotype was found in Normalised L6 distribution (see “Inter.” in table), which led me discard the genotype effect observed.

WT vs cHet vs cKO	Test?	Details	P values
Cell density in different genotypes	Mixed effect linear model analysis*	F (2, 5) = 3.174	P=0.1288
Normalised L1 distribution	Mixed effect linear model analysis*	F (2, 5) = 0.1377	P=0.8745
Normalised L2/3 distribution	Mixed effect linear model analysis*	F (2, 14) = 0.9807	P=0.3994
Normalised L4 distribution	Mixed effect linear model analysis*	F (2, 5) = 0.06839	P=0.9348
Normalised L5 distribution	Mixed effect linear model analysis*	F (2, 5) = 2.472	P=0.1793
Normalised L6 distribution	Mixed effect linear model analysis*	F (2, 5) = 1.879	P=0.2463

* Fixed effect = Genotype, Random effect= subject

5.2.3 – Local glutamatergic connectivity onto VIP+ INs in the VIP-Cre;Ai9;Prox1^{c/c} model

Two-way ANOVA results referring to comparison of the P12-16 data in **Figure 5.3** and **5.4** – LSPS of caged glutamate reveals the laminar organisation of glutamatergic synaptic inputs onto L2/3 VIP+ INs during development in *Prox1* cHet and cKO animals.

P12-16; cHet vs cKO	Test?	Details	P values
Total LSPS input	Mann-Whitney test	Two-tailed p value	P=0.2222
LSPS input across layers	Two-way ANOVA	Interaction: F(3, 24) = 0.4464	P = 0.7221
LSPS input across layers	Two-way ANOVA	Layers eff.: F(3, 24) = 16.45	P < 0.0001
LSPS input across layers	Two-way ANOVA	Genotype eff.: F(1, 8) = 2.633	P = 0.1433

LSPS input across layers – Multiple comparisons

Sidak's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
L2/3 vs. L4	275676	114145 to 437208	Yes	***
L2/3 vs. L5a	338530	176999 to 500061	Yes	****
L2/3 vs. L5b/6	339076	177545 to 500608	Yes	****
L4 vs. L5a	62853	-98678 to 224385	No	ns
L4 vs. L5b/6	63400	-98131 to 224931	No	ns
L5a vs. L5b/6	546.6	-160985 to 162078	No	ns

P12-16; cHet vs cKO	Test?	Details	P values
Norm. L2/3 input	Mann Whitney test	Two-tailed	P =0.4127
Norm. L4 input	Mann Whitney test	Two-tailed	P=0.5317
Norm. L5a input	Mann Whitney test	Two-tailed	P=0.5397
Norm. L5b/6 input	Mann Whitney test	Two-tailed	P > 0.9999

Results referring to **Figure 5.5** – Comparison of laminar organisation of glutamatergic synaptic inputs onto L2/3 VIP+ INs at P12-16 across genotypes.

P12-16; WT vs cHet vs cKO	Test?	Details	P values
Total LSPS input	Kruskal-Wallis ANOVA	K-W statistics=3.363	P=0.1900
LSPS input across layers	Two-way RM ANOVA	Interaction:F(6, 51) = 2.774	P = 0.0207
Norm. L2/3 input	Kruskal-Wallis ANOVA	K-W statistics=7.091	P =0.0216
Norm. L4 input	Kruskal-Wallis ANOVA	K-W statistics=3.065	P =0.2226
Norm. L5a input	Kruskal-Wallis ANOVA	K-W statistics=1.277	P =0.5456
Norm. L5b/6 input	Kruskal-Wallis ANOVA	K-W statistics=4.852	P=0.0842

LSPS input across layers – Multiple comparisons

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
L2/3				
WT vs. cHet	-265557	-409971 to -121143	Yes	***
WT vs. cKO	-127620	-272034 to 16794	No	ns
cHet vs. cKO	137937	-28818 to 304692	No	ns
L4				
WT vs. cHet	-26303	-170717 to 118111	No	ns
WT vs. cKO	62815	-81599 to 207229	No	ns
cHet vs. cKO	89118	-77637 to 255873	No	ns
L5a				
WT vs. cHet	-16565	-160979 to 127849	No	ns
WT vs. cKO	11327	-133087 to 155741	No	ns
cHet vs. cKO	27892	-138863 to 194647	No	ns
L5b/6				
WT vs. cHet	3851	-140563 to 148265	No	ns
WT vs. cKO	31933	-112481 to 176347	No	ns
cHet vs. cKO	28082	-138673 to 194837	No	ns

Normalised L2/3 input – Multiple comparisons

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary
WT vs. cHet	-5.273	No	ns
WT vs. cKO	-8.473	Yes	*
cHet vs. cKO	-3.200	No	ns

5.3 – Effect of genetic perturbation of VIP+ cells on their long-range integration

Analysis referring to **Figure 5.7** – Monosynaptic connectivity between anterior-motor areas and S1BF neurons in cHet and cKO animals.

	Test?	P values
Connected VIP cells across time – cHet	Fisher's Exact Test	P=0.3147
Connected VIP cells across time – cKO	Fisher's Exact Test	P=0.3476
Connected VIP cells P7-9 cHet vs cKO	Fisher's Exact Test	P=0.6674
Connected VIP cells P≥13 cHet vs cKO	Fisher's Exact Test	P=1
Connected VIP cells P7-9 cHet vs WT	Fisher's Exact Test	P=0.6857
Connected VIP cells P≥13 cHet vs WT	Fisher's Exact Test	P=0.326
Connected VIP cells P7-9 cKO vs WT	Fisher's Exact Test	P=1
Connected VIP cells P≥13 cKO vs WT	Fisher's Exact Test	P=0.584

ANOVAs to compare EPSCs features

	Test?	Details	P values
Amplitude across time – cHet vs cKO	Two-way ANOVA	Genotype eff: F (1, 10) = 3.034	P = 0.1122
Amplitude across time – cHet vs cKO	Two-way ANOVA	Age eff: F (1, 10) = 0.9639	P = 0.3494
Latency across time – cHet vs cKO	Two-way ANOVA	Genotype eff.: F (1, 10) = 0.4722	P = 0.5076
Latency across time – cHet vs cKO	Two-way ANOVA	Age eff.: F (1, 10) = 0.5539	P = 0.4739
Jitter across time – cHet vs cKO	Two-way ANOVA	Genotype eff. F (1, 10) = 1.264	P = 0.2872
Jitter across time – cHet vs cKO	Two-way ANOVA	Age eff.: F (1, 10) = 0.1236	P = 0.7325
Amplitude across time – WT vs cKO	Two-way ANOVA	Genotype eff.: F (1, 21) = 0.6165	P = 0.4411
Amplitude across time – WT vs cKO	Two-way ANOVA	Age eff.: F (1, 21) = 0.01108	P = 0.9171
Latency across time – WT vs cKO	Two-way ANOVA	Interaction: F (1, 21) = 4.941	P = 0.0373
Jitter across time – WT vs cKO	Two-way ANOVA	Genotype eff.: F (1, 21) = 0.9394	P = 0.3435
Jitter across time – WT vs cKO	Two-way ANOVA	Age eff.: F (1, 21) = 1.029	P = 0.3220

5.5.1 – Baseline activity

Analysis referring to **Figure 5.8** – LFP Power analysis of baseline activity does not highlight genotype-specific differences in cHet and cKO animals.

Power spectrum, 3 way ANOVA		
ANOVA table	P value	F (DFn, DFd)
Frequency	P<0.0001	F (1.083, 20.58) = 25.28
Age	P=0.0003	F (1, 19) = 18.90
Genotype	P=0.7925	F (1, 19) = 0.07121
Frequency x Age	P<0.0001	F (39, 741) = 14.14
Frequency x Genotype	P>0.9999	F (39, 741) = 0.3397
Age x Genotype	P=0.8706	F (1, 19) = 0.02727

Analysis referring to **Figure 5.10** – Layer specific analysis of delta-waves in *Prox1* cHet and cKO animals during development.

Delta frequency, Mixed-effects model Analysis		
Fixed effects	P value	F (DFn, DFd)
Layer	0.0960	F(1.996, 27.94) = 2.553
Genotype	0.4456	F (1, 18) = 0.6082
Age	0.2286	F (1, 18) = 1.554

Percentage of time in Delta, Mixed-effects model Analysis		
Fixed effects	P value	F (DFn, DFd)
Layer	0.6819	F (1.287, 18.66) = 0.2508
Genotype	0.5180	F (1, 18) = 0.4348
Age	0.1436	F (1, 18) = 2.339

Delta Duration, Mixed-effects model Analysis		
Fixed effects	P value	F (DFn, DFd)
Layer	0.2252	F (1.044, 13.57) = 1.623
Genotype	0.3086	F (1, 18) = 1.098
Age	0.2487	F (1, 18) = 1.421

Delta ISI, Mixed-effects model Analysis		
Fixed effects	P value	F (DFn, DFd)
Layer	0.6421	F (1.151, 17.27) = 0.271
Genotype	0.1382	F (1, 18) = 2.407
Age	0.8716	F (1, 18) = 0.02688

Analysis referring to **Figure 5.11** – Layer specific analysis of spindle activity in *Prox1* cHet and cKO animals during development.

Spindle Duration , Mixed-effects model Analysis		
Fixed effects	P value	F (DFn, DFd)
Layer	0.3623	F (1.076, 24.75) = 0.8896
Genotype	0.7849	F (1, 46) = 0.07537
Age	0.2222	F(1, 46)=1.531

5.5.2– Whisker stimulation

Analysis referring to **Figure 5.12** – Layer-specific analysis of local field potential in *Prox1* cHet and cKO animals during development.

LFP amplitude , Mixed-effects model Analysis		
Fixed effects	P value	F (DFn, DFd)
Layer	0.3310	F (1.213, 12.13) = 1.094
Genotype	0.1977	F (1, 11) = 1.880
Age	0.3202	F (1, 11) = 1.084

Analysis referring to **Figure 5.13** – Analysis of multi-unit activity in *Prox1* cHet and cKO animals in a layer specific way during development.

MUA latency , Mixed-effects model Analysis		
Fixed effects	P value	F (DFn, DFd)
Layer	0.3521	F (1.457, 13.11) = 1.059
Genotype	0.3262	F (1, 11) = 1.056
Age	0.8018	F (1, 11) = 0.06614

Appendix 4 – Permissions

Figure 1.1 Diversity of cortical GABAergic INs, Adapted from Tremblay et al (2016).

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Figure 1.2 Transient circuits involving infragranular SST+ INs in the early postnatal S1BF, Adapted from Marques-Smith et al. (2016), Creative Commons Attribution License (CC BY).

Figure 1.3 Do VIP+ INs have a role in developing circuits? The timeline from panel (A) was adapted from Butt et al., (2017). I am an author of the article therefore permission is not required.

Figure 5.1. Expression of the transgene and protein levels in *VIP-Cre;Ai9;Prox1^{c/c}* animals.

Panel A was adapted from Iwano et al (2012).

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