Thesis for doctoral degree (Ph.D.)
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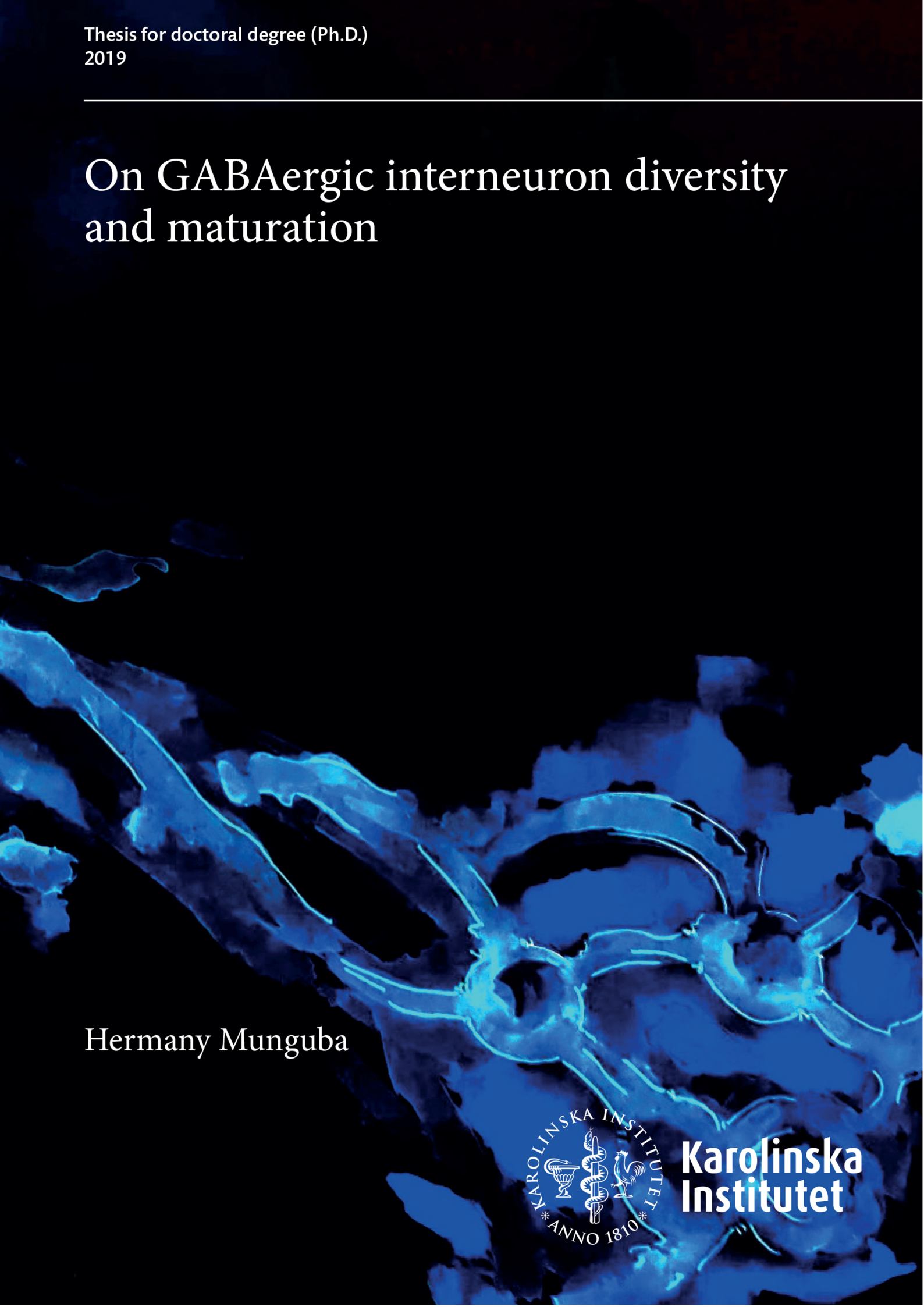
On GABAergic interneuron diversity and maturation



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Cover image: original watercolor drawing by **João Marcos Munguba Vieira** portraying neurons in two timepoints, connected in time by a double helix representing their DNA state.

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Stockholm 2019

To the randomness of life, which gave me amazing parents.

"Who in the rainbow can draw the line where the violet tint ends and the orange tint begins? Distinctly we see the difference of the colors, but where exactly does the one first blendingly enter into the other? So with sanity and insanity."

Herman Melville

ABSTRACT

GABAergic interneurons provide finely, distinct styles of inhibition granted by their unique targeting preferences, molecular profiles, and morphological silhouettes. A central quest of my thesis was to explore what constitutes such diversity, touching upon how this diversity is reached and preserved during development and what maintains distinct functional features later on. This is a compilation of my overview on a vibrant, fast-paced research field that still holds several unresolved questions.

In **Paper I**, we used large-scale single-cell RNA sequencing to dissect the cellular composition of the mouse somatosensory cortex and hippocampal CA1 region by identifying the distinct molecular subclasses of cells forming these brain regions. In particular, we unveiled a previously undescribed inhibitory interneuron labeled by transcription factor Pax6, which was further confirmed with immunohistochemistry, electrophysiology and morphological reconstructions.

In **Paper II**, we characterized all striatal neuronal populations and compared them to their cortical counterparts using single-cell RNA sequencing. An important finding was that the typical parvalbumin-expressing neurons are part of a larger group of neurons expressing a novel marker, Pthlh, and that they exhibit a continuum of electrophysiological properties correlated with the expression of parvalbumin. Furthermore, cortical and striatal parvalbumin-expressing neurons show significant transcriptomic and electrophysiological differences.

In **Paper III**, we show that cortical somatostatin-expressing interneurons need the transcription factor Sox6 to maintain their subtype identity, specifically during migration. Using a combination of mouse genetics, single-cell RNA-sequencing, and electrophysiology we show that, while in controls the somatostatin-expressing *class* comprises nine molecularly distinct neuronal *subtypes*, the Sox6-mutant cortex contained only three molecular *subtypes*, without any significant somatostatin-cell loss.

In **Paper IV**, we utilized conditional knockout strategies to remove Sox6 in parvalbumin-expressing interneurons at different postnatal stages. Our data revealed that *class* of interneurons relies on postnatal expression of Sox6 for the growth and maintenance of their axonal boutons and synaptic function until adulthood.

Altogether, the studies included in this thesis shine light on what GABAergic interneuron diversity encompasses, highlighting the particular role a transcription factor in maintenance of subtype identity (in somatostatin neurons) or particular functional features (in parvalbumin neurons).

LIST OF SCIENTIFIC PAPERS

I. Amit Zeisel, Ana B. Muñoz-Manchado, Simone Codeluppi, Peter Lonnerberg, Gioele La Manno, Anna Juréus, Sueli Marques, **Hermany Munguba**, Liqun He, Christer Betsholtz, Charlotte Rolny, Gonçalo Castelo-Branco, Jens Hjerling-Leffler, Sten Linnarsson. Cell types in the mouse cortex and hippocampus revealed by single-cell RNA-seq. Science, 2015, volume 347, 1139-1142.

II. Ana B. Muñoz-Manchado, Carolina Bengtsson Gonzales, Amit Zeisel, **Hermany Munguba**, Bo Bekkouche, Nathan G. Skene, Peter Lonnerberg, Jesper Ryge, Kenneth D. Harris, Sten Linnarsson, Jens Hjerling-Leffler. Diversity of Interneurons in the Dorsal Striatum Revealed by Single-Cell RNA Sequencing and PatchSeq. Cell Reports, 2018, vol. 24, 2179-90

III. **Hermany Munguba**, Kasra Nikouei, Hannah Hochgerner, Polina Oberst, Alexandra Kouznetsova, Jesper Ryge, Renata Batista-Brito, Ana B. Muñoz-Manchado, Jennie Close, Sten Linnarsson, Jens Hjerling-Leffler. Transcriptional maintenance of cortical somatostatin interneuron subtype identity during migration. *Submitted manuscript*

IV. **Hermany Munguba**, Bidisha Chattopadhyaya, Stephan Nilsson, Josianne N. Carriço, Polina Oberst, Ilya Kruglikov, Renata Batista-Brito, Ana Belen Muñoz-Manchado, Michael Wegner, Bernardo Rudy, Gordon Fishell, Graziella di Cristo, Jens Hjerling-Leffler. Sox6 regulates cortical parvalbumin-expressing neurons' synaptic function until adulthood. *Manuscript*

Scientific papers not included in this thesis

- Sueli Marques, David van Bruggen, Darya P.Vanichkina, Elisa M.Floriddia, **Hermany Munguba**, Leif Väremo, Stefania Giacomello, Ana M. Falcão, Mandy Meijer, Åsa K. Björklund, Jens Hjerling-Leffler, Ryan J. Taft, Gonçalo Castelo-Branco. Transcriptional Convergence of Oligodendrocyte Lineage Progenitors during Development. Developmental Cell, 2018, 504-517

- Christina Göngrich, Favio A. Krapacher[*], **Hermany Munguba**[*] Annika Andersson, Jens Hjerling-Leffler, Carlos F. Ibáñez.
  Activin receptor ALK4 coordinates extracellular signals and intrinsic transcriptional programs to regulate development of cortical somatostatin interneurons.
  Submitted manuscript

LIST OF ABBREVIATIONS

5HT3Ra – 5-HT (serotonin) receptor 3a

CGE – Caudal ganglionic eminence

E – Embryonic day

FS – Fast-spiking

GABA – Gamma-aminobutyric acid

GFP – Green fluorescent protein

HMG – High-mobility group

L – Layer

LGE – Lateral ganglionic eminence

MGE – Medial ganglionic eminence

MZ – Marginal zone

NPY – Neuropeptide Y

PNNs – Perineuronal nets

P – Postnatal day

Pvalb – Parvalbumin

S1 – Primary somatosensory cortex

scRNAseq – single-cell RNA sequencing

SOX – Sex-determining region Y (Sry)-related HMG box

Sst – Somatostatin

SVZ – Subventricular zone

T – Tamoxifen

VIP – Vasoactive intestinal peptide

VZ – Ventricular zone

CONTENTS

1 INTRODUCTION

The formation of the human brain involves a finely guided wiring of more than 80 billion neurons (Azevedo et al. 2009), in which numerous neuronal classes intricately connect, (de)synchronize their activity and yield a striking range of functions, such as integration of sensory information, decision-making, and language. As neurons interact in a combinatorial fashion, the large neuronal diversity present in mammalian brains provides increasing combinations of processing units and, therefore, of computational abilities (Roth and Dicke 2012). Although neuronal heterogeneity is probably one of the most outstanding features of the brain, it still is a grand bottleneck within neuroscience.

Another fascinating feature of the human brain is that it requires over two decades after birth to fully mature. In particular, the prefrontal cortex is only completely developed after approximately 30 years of life (Crews, He, and Hodge 2007). Throughout this process, intrinsic genetic programs set the pace and directions of human brain development (Colantuoni et al. 2011; Skene, Roy, and Grant 2017), following an overall gene expression trajectory that seems to outdo individual variability (Colantuoni et al. 2011). Beyond engaging a universal genetic framework, a brain that slowly matures provides humans with an outstanding versatility to adapt to the environment they experience, such as parental care or its absence, nurture or scarcity, stress or stimulus deprivation, among others. The price for such adaptability is, however, the great vulnerability that this developmental window imposes to circuit formation, when compared to adulthood. Accordingly, a combination of an intrinsic genetic clock and environmental stressors renders early life the period in which most psychiatric disorders have their onset (Kessler et al. 2007).

Therefore, understanding the neuronal composition and their maturational trajectory are key to revealing what factors can fail and lead to brain dysfunction. By using the mouse brain as a model, the work included in this thesis aims to expand our understanding on neuronal diversity and genetic programs involved in these cells' maturation hoping to provide a stronger foundation for future research on brain development, maturation and their implications to the manifestation of psychiatric diseases.

1.1 General remarks about the cortex

The mammalian neocortex is composed of numerous types of cells, where intricate and diverse neuronal circuits interact and rely on astrocytes, microglia, oligodendrocytes, as well as vasculature cells (Zeisel et al. 2015). The neocortex displays a layer-organization that incorporates defined patterns of cellular distribution, subcortical and contralateral cortical afferents, as well as subtype specific local connectivity and outward projections.

The broadest classification of cortical neurons splits them in two large groups: the excitatory pyramidal neurons and the inhibitory interneurons. In fact, an intricate balance between excitation and inhibition is indispensable for the proper formation and function of neuronal circuits (Rossignol 2011; Lewis 2011; Volk, Edelson, and Lewis 2014; Lin and Sibille 2013). Pyramidal neurons are glutamatergic, myelinated, long-projecting cells that correspond to 80% of all cortical neurons. During development, pyramidal cells acquire their identity and regional position depending on the location of their progenitors, while their layer position is defined by

the time of their birth, in an inside-out sequence (Kriegstein, Noctor, and Martínez-cerdeño 2006). Even though they share general features, pyramidal neurons show a great deal of heterogeneity in terms of morphology, projections, electrophysiological and molecular properties (Huang 2014). In terms of their morphology and firing properties, a recent study suggested the existence of 35 morphologically and 17 electrophysiologically distinct cell types in the mouse primary visual cortex, with some relation with molecular cell types (Gouwens et al. 2018). In particular, the advent of single-cell RNA sequencing (scRNAseq) has raised awareness on the molecular heterogeneity of cortical excitatory neurons (Zeisel et al. 2018; Tasic et al. 2018). In spite of broad generalizations suggesting that cortical columns are repetitive building blocks across different cortical areas, important differences among cortical areas have been proposed (Luebke 2017; Tasic et al. 2018; Scala et al. 2019).

Across different species and cortical areas, inhibitory interneurons correspond to approximately 20% of all cortical neurons and constitute a highly heterogeneous neuronal population (Hendry et al. 1987). They release GABA and, in contrast to excitatory neurons, interneurons expand their axons mostly locally, making them highly specialized in silencing neighboring neurons. Moreover, contrasting what was once believed, some GABAergic neurons in the cortex and hippocampus are long-projecting neurons (Tomioka et al. 2005; Tamamaki and Tomioka 2010; Melzer et al. 2017) and some are highly myelinated (Micheva et al. 2016; Stedehouder et al. 2017).

Although **Paper I** incorporates both cortical and hippocampal neurons, and **Paper II** focuses on striatal and cortical interneurons, further on in this thesis the focus will be on the current literature in terms of diversity and maturation of cortical interneurons, in particular somatostatin (Sst)- and parvalbumin (Pvalb)-expressing neurons, which were the classes further investigated in **Papers III** and **IV**.

1.2 Cortical interneuron diversity

GABAergic interneurons are remarkably diverse. The main features exposing such exuberant diversity include unique molecular markers, dendritic and axonal morphology, synaptic characteristics, embryonic origins, intrinsic and firing properties, and most recently gene expression profile enabled through whole-transcriptome analysis (Ascoli et al. 2008; Rudy et al. 2011; Kepecs and Fishell 2014; Zeisel et al. 2015; Tasic et al. 2018).

A well-established way to classify cortical interneurons is to consider hierarchical blocks that can be subsequently subdivided. One widely accepted initial categorizing step is to group interneurons according to markers uniquely expressed (Figure 1). In fact, the vast majority of cortical interneurons can be grouped according to the expression of three virtually non-overlapping molecular markers: parvalbumin (Pvalb), somatostatin (Sst) and the ionotropic serotonin receptor-3a (5HT3aR). Nevertheless, each large interneuron class still owns a great deal of heterogeneity in terms of morphological, firing and molecular properties. Such sequential level of complexity and specificity can be illustrated with the following example: (i) the large group of GABAergic interneurons can be divided in (ii) discrete interneuron *classes*, e.g. $Pvalb^+$, Sst^+ or $5HT3aR^+$ interneurons. Subsequently, in the case of the Sst^+ class, they can be further subdivided in (iii) *subtypes* of interneurons with distinct features, such as

Martinotti cells, which are Sst$^+$ neurons that target distal dendrites of pyramidal neurons, *versus* X94 cells, Sst$^+$ neurons that target Pvalb$^+$ neurons in layer (L)IV (Figure 1 and 2).

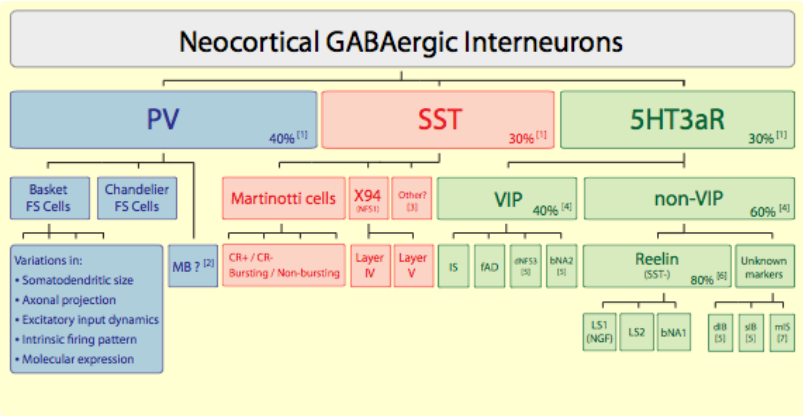

Figure 1. Traditional classification of cortical interneurons based on three mostly non-overlapping markers, morphology and firing properties. 5HT3aR: serotonin receptor 3a; bNA: bursting non-accommodating; CR: calretinin; FS: fast-spiking; IB: intrinsic bursting; IS: irregular spiking; LS: late-spiking; PV: parvalbumin; SST: somatostatin; VIP: vasoactive intestinal peptide. Adapted from: Rudy et al 2011.

Therefore, since neuronal diversity encompasses multiple dimensions of variability, a reliable classification system must integrate a combination of criteria in order to build comprehensive definitions of neuronal identity (Kepecs and Fishell 2014). And although different classification schemes have been suggested; yet, no consensus has been reached (Ascoli et al. 2008; Rudy et al. 2011; Tasic et al. 2018). While certain classification systems would suggest a great deal of diversity considering 15-20 different cortical GABAergic neurons (Ascoli et al. 2008; Zeisel et al. 2015), recent scRNAseq data suggest approximately sixty distinct molecular subtypes (Tasic et al. 2018). A current direction in the field is to develop approached that extract as many aspects from a single neuron as possible, especially in terms of their morphological, electrophysiological and molecular identity. Most recently, a few studies have combined firing properties with scRNAseq (Patch-seq) and morphology (Fuzik et al. 2015; Cadwell et al. 2015; Muñoz-Manchado et al. 2018; Gouwens et al. 2018). However, uncertainty in identifying the same neuronal types in different experimental settings and with distinct techniques is nonetheless a hindering issue.

1.2.1 Cortical somatostatin-expressing interneurons

About one third of all cortical inhibitory neurons express somatostatin, forming the second largest class of cortical interneurons (Lee et al. 2010). Such diversity can be appreciated in terms of their axonal targeting, connectivity patterns, firing properties, transcriptomic signatures and *in vivo* functions (Ma et al. 2006; Tasic et al. 2018; Silberberg and Markram 2007; Nigro, Hashikawa, and Rudy 2018; Fino and Yuste 2011; Muñoz et al. 2017). While considering all Sst$^+$ neurons as a single entity has led to the suggestion that their connectivity to surrounding pyramidal neurons is nonselective (Fino and Yuste 2011), there is robust indication that distinct Sst$^+$ subtypes play complementary roles in cortical circuits (Naka and Adesnik 2016; Naka et al. 2019; Nigro, Hashikawa, and Rudy 2018; Adesnik et al. 2012; Gentet et al. 2012; Hilscher et al. 2017).

The most well-characterized cortical Sst$^+$ interneuron subtype is known as Martinotti cell (Figure 2). These neurons are found in cortical layers II-VI, from which they send their axons all the way to LI where they inhibit distal dendrites of pyramidal neurons, thereby

regulating the integration of "top-down" inputs (Wang et al. 2004; Silberberg and Markram 2007; Urban-Ciecko and Barth 2016). Furthermore, LV Martinotti cells mediate disynaptic inhibition between pyramidal cells triggered by high-frequency stimulation and are also capable of synchronizing the activity of pyramidal neurons after brief bursts (Silberberg and Markram 2007; Berger et al. 2010; Hilscher et al. 2017). Despite shared morphological characteristics, Martinotti cells are known to exhibit a great deal of variability with respect to markers (e.g. reelin, calretinin, calbindin, etc.), as well as firing patterns. In those terms, electrophysiological recordings performed in these cells shows at least three firing profiles: intrinsic bursting (when they burst after a depolarizing step), rebound bursting (when after hyperpolarizing steps), and accommodating spiking.

Little has been known whether these firing qualities reflect within-group variability or authentic subtype diversity (i.e. the existence of distinct molecular, functional subtypes). Recent data suggest that rebound bursting Sst^+ neurons constitute a distinct functional subtype, $Sst^+Chrna2^+$ (Hilscher et al. 2017; Tasic et al. 2018). Nevertheless, there is still a long journey until all the knowledge gathered before the scRNAseq era matches the molecular subtypes currently proposed.

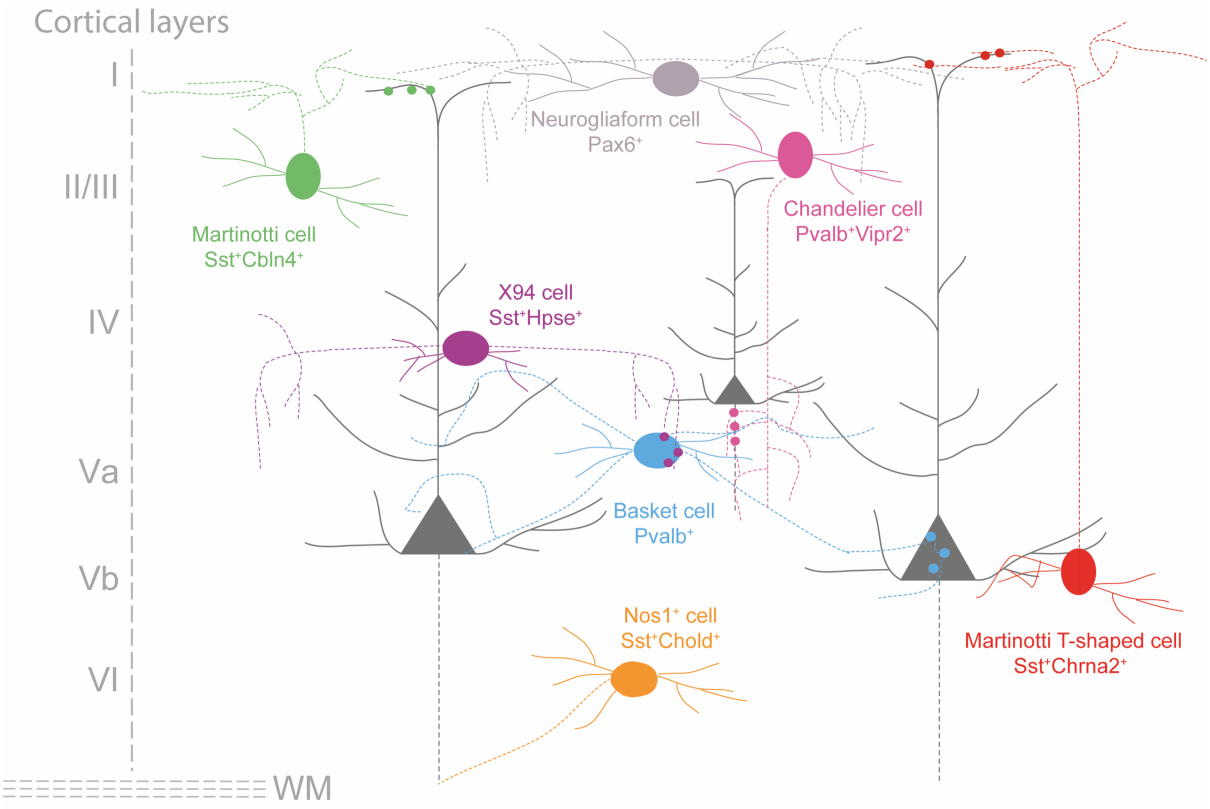

Figure 2. Schematic representation of the main subtypes of cortical GABAergic neurons investigated in this thesis. Cells bodies are allocated to the layers in which each subtype is enriched, full lines represent dendrites and dashed lines represent axons, with synaptic boutons exemplifying the main target (compartment and/or cell class). WM: white matter.

The second most well-characterized Sst^+ interneuron subtype is known as X94 (Figure 2). Found only in L4-5 (Xu et al. 2013; Naka et al. 2019), these interneurons arborize their

axons profusely into L4, targeting mainly Pvalb$^+$ interneurons and pyramidal neurons also in that layer (Ma et al. 2006; Naka et al. 2019). This establishes a disinhibitory circuit where X94 cells silence Pvalb$^+$ cells and allow surrounding pyramidal cells to fire (Xu et al. 2013). Compared to intrinsic properties of Martinotti cells, X94 cells display lower input resistance, fire in higher frequencies (sometimes resembling Pvalb$^+$ cells), and often stutter. Nevertheless, an important feature shared between these two cell types is that they also show frequency adaptation, while Pvalb$^+$ neurons adapt much less. Another feature shared amongst Sst$^+$ interneurons is that they receive strongly facilitating excitatory synapses, whereas Pvalb$^+$ neurons receive depressing synapses (Silberberg and Markram 2007). Previously a molecularly obscure cell type, X94 cells were recently shown to differentially express Hpse (Naka et al. 2019).

In addition, in spite of oftentimes being referred to as Nos1$^+$ interneurons (Figure 2), Sst$^+$Nos1$^+$ neurons are long-range projecting GABAergic neurons, known to be active during sleep (Tomioka et al. 2005; Kilduff, Cauli, and Gerashchenko 2011; Dittrich et al. 2014) and represent a consistently clear molecular cluster in scRNAseq studies, characterized by the expression of *Sst*, *Chodl*, *Nos1*, *Tacr1*, *Calb2* (Zeisel et al. 2015; Tasic et al. 2016; Tasic et al. 2018).

Recent scRNAseq studies from dissociated cortical tissue suggest between 10-20 different molecular Sst-expressing interneurons (Tasic et al. 2018; Naka et al. 2019). And although some of the widely known subtypes started to be matched to specific molecular identities (Hilscher et al. 2017; Naka et al. 2019), little is known about layer distribution, morphology, firing patterns and function of these new molecular subtypes. This is further addressed in **Paper III**, in which we confirm previously suggested molecular Sst$^+$ subtypes, such as Chrna2$^+$, Hpse$^+$ and Chold$^+$Nos1$^+$ neurons.

1.2.2 Cortical parvalbumin-expressing interneurons

About 40% of cortical interneurons express the calcium binding protein parvalbumin (Pvalb) and occupy all cortical layers, except for LI. Compared to the Sst-class, Pvalb-neurons are electrophysiologically much more homogenous, largely characterized by fast action potentials and the ability to fire in sustained high frequency with little adaptation (Markram et al. 2004).

Yet, detailed morphological characterization of individual Pvalb-cells has led to further subdivisions of this class into three different morphological and functional subgroups (Figure 2): basket cells, which have a multipolar morphology and target the soma and proximal dendrites of pyramidal neurons (Hu, Gan, and Jonas 2014); chandelier cells, which target the axon initial segment of pyramidal cells and are mostly present at the border between LI-II, and in LVI (Taniguchi, Lu, and Huang 2013; Inda, DeFelipe, and Muñoz 2009); and lastly, translaminar Pvalb-neurons constitute a small population in LV-VI, whose axonal arbor spreads throughout all cortical layers (Bortone, Olsen, and Scanziani 2014).

A majority of Pvalb$^+$ cells are basket cells and have a unique role in cortical circuitry: by contacting pyramidal neurons mainly onto their soma, they provide precise, fast and reliable post-synaptic inhibitory control of pyramidal cells' output. Therefore, Pvalb$^+$ cells regulate synaptic activity and the dynamics of cortical circuits with high sensitivity and temporal

fidelity (Swadlow 2003). Furthermore, basket cells are the major contributors of feed forward inhibition (FFI) of thalamocortical inputs, crucial in regulating the duration and integration window of excitatory inputs from the thalamus (Cruikshank, Lewis, and Connors 2007). scRNAseq studies suggest that up to ten different molecular subtypes of Pvalb-expressing neurons might exist (Tasic et al. 2018). Up-to-date, while chandelier cells comprise a unique molecular subtype that expresses Pthlh and Vipr2 and can now be labeled with advance mouse genetics (He et al. 2016; Steinecke et al. 2017; Fish et al. 2013; Tasic et al. 2018), the laminar distribution and function of the other molecular subtypes is less clear.

1.2.3 Cortical 5HT3Ra-expressing interneurons

The remaining 30% of cortical interneurons are labeled by the ionotropic serotonin receptor 5HT3aR (Figure 1). Being probably the most heterogeneous population of interneurons, this diverse group mostly occupies upper cortical layers (LI-III), although are found throughout all layers. This broad population includes several interneuron subtypes, characterized by the expression of specific markers, such as vasoactive intestinal peptide (VIP), cholecystokinin and reelin (Lee et al. 2010). VIP^+ neurons are bipolar cells, often express calretinin and mediate cortical disinhibition by targeting Sst^+ and $Pvalb^+$ interneurons (Lee et al. 2013; Pi et al. 2013). Neurogliaform cells, on the other hand, are late-spiking neurons, characterized by extensive horizontal axonal branching and often express reelin and NPY. Although no single marker has been found to optimally label all neurogliaform cells, recent molecular approaches have advanced the field closer to reaching that goal (Abs et al. 2018; Niquille et al. 2018).

1.3 Striatal interneurons

The striatum constitutes one of the main input areas of the basal ganglia. In contrast to the neocortex, which is composed primarily of excitatory neurons, the striatum is formed by 95% of GABAergic spiny projection neurons and 5% of locally-connected interneurons, most of which are also GABAergic and provide local inhibition, while one population is cholinergic. Multiple studies have addressed the cellular diversity of striatal GABAergic interneurons combining expression of key markers paralleled with firing properties (Kawaguchi et al. 1995; Gittis et al. 2010). This revealed the following interneuronal types: $Pvalb^+$ fast-spiking cells, Sst^+ low-threshold-spiking cells, late-spiking NPY^+Sst^- neurogliaform cells, and Th^+ cells with mixed intrinsic firing properties (Ibanez-Sandoval et al. 2010; English et al. 2012; Ibáñez-Sandoval et al. 2011).

Early studies on the neuronal complexity of the striatum suggested that interneurons constitute 5% of the neurons in this structure (Graveland and Difiglia 1985). Nevertheless, only recently has this percentage been reproduced when utilizing key markers covering the molecular diversity in this region (Muñoz-Manchado et al. 2016). The 5HT3aEGFP mouse was shown to label a considerable portion of GABAergic interneurons which, summed up with the key markers (populations) aforementioned, reach a total of 5% of striatal neurons (Muñoz-Manchado et al. 2016). In **Paper II**, we utilized scRNAseq combined with electrophysiology (and Patch-seq) in order to provide a sounder characterization of the striatal interneuronal heterogeneity.

1.4 Development of cortical interneuron diversity

1.4.1 Birth of interneurons

Cortical interneurons are generated in the ventral part of the telencephalon (subpallium), a transient proliferation niche existent only during embryonic development (Anderson et al. 2001). In the mouse, the subpallium can be subdivided into three transient regions that become first apparent at embryonic day 11: the medial (MGE), caudal (CGE) and lateral (LGE) ganglionic eminences. The MGE and the CGE, together with the MGE-adjacent preoptic area, are the main sources of cortical GABAergic interneurons (Batista-Brito and Fishell 2009). While Pvalb- and Sst-expressing neurons are born in the MGE (Butt et al. 2005), the CGE is the major source of 5HT3aR-expressing interneurons (Lee et al. 2010). Most recently, neurogliaform neurons were shown to be generated mostly the preoptic area (Niquille et al. 2018).

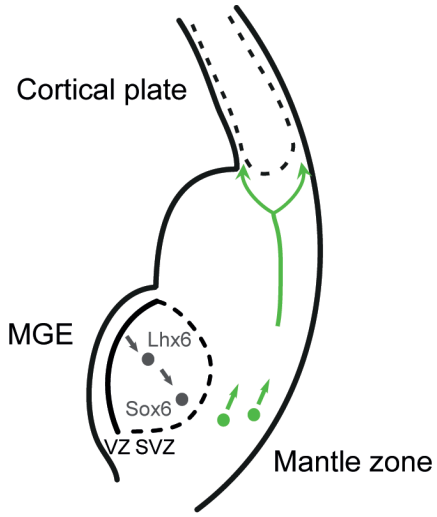

Figure 3. Scheme of newly-born MGE-derived interneurons tangentially migrating towards the cortex. Two alternative migratory streams represented in green arrows. MGE: medial ganglionic eminence. SVZ: subventricular zone. VZ: ventricular zone.

Many key transcription factors have been linked to the specification of the major interneuron classes (Lim, Mi, et al. 2018). With regards to the MGE, which generates the two interneuron classes focused in this thesis (Pvalb$^+$ and Sst$^+$ cells), the transcription factor Nkx2-1 establishes and maintains MGE progenitors and plays a major role in specification of these neurons (Butt et al. 2008). Loss of Nkx2-1 results in a complete switch from MGE-derived fates (Pvalb$^+$ or Sst$^+$) to CGE/LGE derived fate (VIP$^+$ or reelin$^+$, or striatal medium spiny neurons) (Butt et al. 2008). Downstream of Nkx2-1, the transcription factor Lhx6 (Du et al. 2008) is necessary for normal migration and maturation of all MGE-derived cortical interneurons (Liodis et al. 2007). Lhx6 deficient mice show normal numbers of GABAergic interneurons but have a drastic decrease in Pvalb$^+$ and Sst$^+$ interneuron numbers. Thus, while Lhx6 is not required for the GABAergic specification of MGE-derived interneurons, it is required for the specification of Pvalb$^+$ and Sst$^+$ subtypes and their correct migration. Downstream of both Nkx2.1 and Lhx6 acts the transcription factor Sox6 (Figure 3), which is expressed in all MGE-derived cortical interneuron progenitors until postnatal mature stages (Batista-Brito et al. 2009). Loss of Sox6 in these interneurons arrests Pvalb cell in an immature state and leads to deficits in tangential migration (Batista-Brito et al. 2009). (*Box 1 provides further details on the transcription factor Sox6, in page 8*).

Fate-mapping studies (for example, reporter lines under Nkx2-1 or Lhx6 promoters to target MGE-derived neurons) have helped to elucidate several spatial, temporal and genetic aspects of the generation of the different *classes* of interneurons (e.g. Pvalb$^+$, Sst$^+$, VIP$^+$ classes) (Lim, Mi, et al. 2018). Nonetheless, much less is understood about exclusive genetic programs for specific interneuron *subtypes*, such as unique genetic factors to generate Sst$^+$Chrna2$^+$ Martinotti cells *versus* Sst$^+$Hpse$^+$ X94 cells.

Box 1. Transcription factor Sox6

The large family of transcription factor SOX (sex-determining region Y [SRY]-type high-mobility group [HMG] box) has been extensively implicated in the regulation of cell fate determination and differentiation in a variety of systems (Wegner 1999). Different from most transcription factors, which bind to the major groove of DNA, HMG-containing proteins bind to the minor groove of the DNA helix, leading to a drastic bend in the DNA (Connor et al. 1994). The minor groove binding and the ability to bend DNA raised the hypothesis that SOX proteins work in close proximity to other transcription factors and act as architectural proteins by assembling other DNA-binding proteins (Wegner 1999).

In vertebrates, this family can be divided into eight groups according to the amino acid identity of the HMG domain (A–H) (Schepers, Teasdale, and Koopman 2002). Sox6 is allocated in the subgroup SOXD (with Sox5 and Sox13). These proteins possess an exclusive coiled-coil domain that mediates homo- and heterodimarization within SOXD group members (Lefebvre, Li, and De Crombrugghe 1998). Importantly, although SOXD proteins are believed to be unable to independently regulate transcription (since they have no identified transactivation or transrepression domains), they do play a role in gene regulation (Hagiwara 2011) by interacting with different partner proteins (Kamachi, Uchikawa, and Kondoh 2000; Lee et al. 2014). Such profile provides incredible versatility to the functions of Sox6, allowing it to regulate a variety of systems at different developmental stages, with multiple effects. Sox6 is strongly involved with cell-type specification in different systems (Hagiwara 2011). During embryonic development, muscle fibers that lack Sox6 fail to undergo the expected transition from slow to fast twitching muscles. Accordingly, the expression of slow isoforms remains overexpressed compared to wild type (Hagiwara, Ma, and Ly 2005; Hagiwara, Yeh, and Liu 2007). This up-regulation of slow fiber–specific genes suggests that Sox6 functions as a transcriptional suppressor of slow fiber-specific genes (Hagiwara, Ma, and Ly 2005; Hagiwara, Yeh, and Liu 2007). Although that does not prove a subtype specification, the expression of the commonly slow fiber protein myosin heavy chain beta (MyHC-beta, slow isoform) in fast twitching muscle fibers suggests a role in cell type determination.

In the Central Nervous System (CNS), Sox6 was first associated to oligodendrocyte progenitor cells' (OPC) differentiation. Working together with Sox5, Sox6 restrains OPCs from exiting cell cycle, confirmed when removal of both transcription factors anticipates differentiation (Stolt et al. 2006). Most recently, Sox6 was also shown to be key during the development of dopaminergic neurons in the substancia nigra (Panman et al. 2013). With regards to interneuron development, Sox6 has been previously shown to play a vital role in early maturation of interneurons during embryonic stages (Batista-Brito et al. 2009). Removal of Sox6 specifically in MGE-derived precursors caused a drastic decrease of Pvalb (77%) and a noticeable decrease of SST (30%) interneurons, without affecting the total number of MGE-derived interneurons. Furthermore, there is prominent deficit in radial migration as MGE-derived interneurons are found accumulated in the superficial and deeper cortical layers (Batista-Brito et al. 2009). Consequently, Sox6 mutant mice develop seizures and eventually die between P17-P19 (Batista-Brito et al. 2009). Due to the relatively early death of these mice, it has been difficult to fully characterize the role of Sox6 in the postnatal maturation of MGE-derived interneurons.

Although very little is known regarding the role of this transcription factor in the maturation of Sst cells, as well as its postnatal function in both interneuron subtypes, its sustained expression during adolescence and adulthood suggests a potential role on late maturation, synaptic function and perhaps plasticity.

This has been the case at least partially because our classification systems take into consideration predominantly features that only emerge postnatally (e.g. mature molecular markers, firing properties and morphology). Therefore, the fact that it is only during the first postnatal weeks that interneurons start displaying their well-defined, distinguishable characteristics has challenged the notion of whether interneuron *subtype* diversity arises prior to arriving to the cortex or once there, after receiving local cues or activity-dependent events (Kepecs and Fishell 2014; Wamsley and Fishell 2017). Currently, although it is still not entirely known how interneuron subtype diversity is generated, especially with regards to subtype specific genetic programs, recent data strongly suggest that interneurons are prespecified before arriving to the cortex and have distinctive transcriptomic signatures beyond that expected for broad interneuron classes (Mi et al. 2018; Mayer et al. 2018).

If that scenario is correct, the secret for generating interneuron diversity probably lies on asymmetric divisions. During development, from insects to mammals, the origin of diversity relies at least partly on asymmetric cell divisions. In such divisions, daughter-cells segregate what is called fate determinants, which are most likely clusters of transcription factors (or specific epigenetic landscapes). Even though these processes carry intrinsic "rules" to follow (e.g. genes that are downstream from specific transcription factors), the initial induction of such cascades also depends on local extracellular cues that follow spatial and temporal patterns. In *Drosophila*, for example, neural progenitor cells allocate different transcription factors into daughter cells, providing them with specific differentiation potentials (Li et al. 2016). Additionally, sequential expression of transcription factors in neural progenitors suggest a gradual patterning to generate neuronal diversity (Bayraktar and Doe 2013). Moreover, epigenetic modulations can be established by transcription factors before cell division and such chromatin accessibility states will determine the expression of other fate determinants (Endo et al. 2011). While asymmetric divisions have also been shown to play a key role in cortical neurogenesis of excitatory neurons (Noctor et al. 2004), it remains to be further elucidated how diversity arises in the proliferative zones in the subpallium of mammals.

1.4.2 Migration and integration into cortical circuits

After interneurons are born in the ganglionic eminences, they begin to tangentially migrate towards the developing cortex (Figure 3). Immature cortical interneurons travel long distances before reaching the cortical place, where they shift to radial migration to reach their final destination along the cortical layers (Bartolini, Ciceri, and Marín 2013; Anderson et al. 2001). In mice, migration of cortical interneurons begins at embryonic day (E)12.5 and is completed by birth, when integration into circuits begins (Anderson et al. 2001).

During tangential migration, cortical interneurons can enter two main migratory streams into the forming cortex (Figure 3): the mantle zone (MZ) and the subventricular zone (SVZ). Gene expression analysis of interneurons taking each of the streams shows transcriptional differences, suggesting that different types of interneurons would favor different routes (Antypa et al. 2011; Lim, Pakan, et al. 2018). In accordance, different subtypes of Sst^+ interneurons were recently shown to prefer different migratory routes, in particular Sst^+ Martinotti cells favoring the MZ route (Lim, Pakan, et al. 2018). Thus, specific subtypes favoring particular migratory streams corroborates the suggestion that interneurons are prespecified before arriving to the forming cortex.

During this migratory phase interneurons must allocate a considerable part of their transcriptional program in order to sustain the molecular machinery necessary for migration (Peyre, Silva, and Nguyen 2015; Cobos, Borello, and Rubenstein 2007). Yet, the information encoding for their identity must remain intact until they reach their final destination. This time window was the focus addressed in **Paper III**, where we investigated the role of the transcription factor Sox6 in maintaining the identity of migrating cortical Sst^+ interneurons.

1.5 Postnatal maturation of cortical interneurons

After entering the cortex, interneurons spread and settle down at their allocated cortical layer, when they begin to receive and form synapses (Favuzzi et al. 2019). Interestingly enough, by the end of the third postnatal week, around 30-40% of all cortical interneurons undergo programmed cell death (Southwell et al. 2012), extending to all classes of interneurons (Priya et al. 2018), while distinct primary sensorial areas have been shown to vary in the degree of neuronal apoptosis in a layer specific manner (Blanquie et al. 2017). Therefore, a finer execution of programmed cell death could be another potential element occurring during development to optimize the outcome of such cellular diversity, according to the cortical area and layer in question.

GABAergic synaptic formation accelerates at the end of postnatal week one, expanding during the entire first postnatal month (Chattopadhyaya 2004; Micheva and Beaulieu 1996; Pangratz-Fuehrer and Hestrin 2011; Favuzzi et al. 2019). Accordingly, by the end of postnatal week three, the pattern of GABA immunoreactivity is comparable to that of the adult brain (Del Rio, Sorriano and Ferrer 1992).

Overall, after settling down interneurons experience robust maturation towards their adult molecular, electrophysiological and morphological profile. Nevertheless, as expected, there are some indications that different interneuron classes undergo different maturational trajectories. $Pvalb^+$ and Sst^+ show important differences in their electrophysiological development (Lazarus and Huang 2011). Sst-expressing interneurons achieve their mature intrinsic membrane properties after the second postnatal week, a process that appears to depend on excitatory inputs during first postnatal days (Pan et al. 2016; Pan et al. 2018). In particular, the manifestation of fast-spiking properties of Pvalb-expressing neurons displays a clear-cut developmental switch (Goldberg et al. 2011), consequent of a transcriptonal shift they undergo at the end of postnatal week one (Okaty et al. 2009). With regards to CGE-derived interneurons, early postnatal neuronal activity is required for the development of $reelin^+$ interneurons, (neurogliaform cells) while dispensable for VIP^+ interneurons' dendritic and axonal growth (De Marco García, Karayannis, and Fishell 2011).

1.5.1 Postnatal maturation of parvalbumin-expressing neurons

At the end of the first postnatal week, Pvalb^{+} cells experience a transcriptional shift, through which hundreds of genes are up- or down-regulated (Okaty et al. 2009). From postnatal day (P)7 to P40, nearly 2000 genes are differently expressed in Pvalb-expressing interneurons, including a prominent up-regulation of Na^{+}, K^{+} and Ca^{2+} channels, required for their mature fast-spiking phenotype, as well as transcription factors, cell adhesion molecules and synaptic-associated genes. The expression of Pvalb itself begins in somatosensory cortex around P7-P10, reaching its highest levels by the end of postnatal week four.

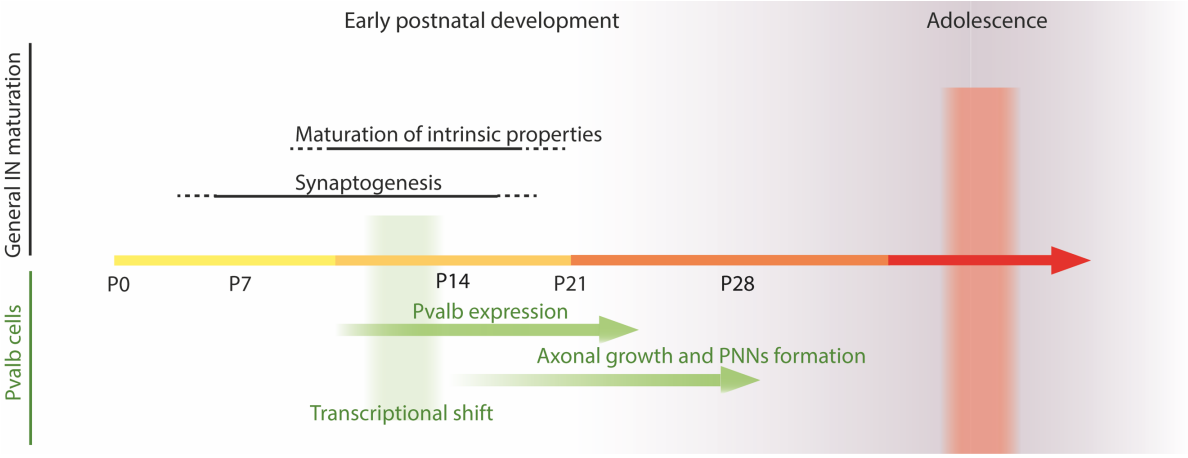

Figure 4. Representation of key maturational elements on Pvalb-expressing cells' postnatal development. *P: postnatal day. IN: interneurons.*

Through these maturational processes, Pvalb-expressing neurons ultimately acquire their hallmark characteristics, such as high frequency action potentials (APs), Pvalb expression, robust somatic innervation of neighboring pyramidal neurons, as well as a specialized extracellular matrix, known as perineuronal nets (PNNs) (Hu, et al., 2014). PNNs are produced by both Pvalb neurons themselves and glial cells, which release a variety of components in the extracellular matrix, such as proteoglycans (Dityatev, Schachner, and Sonderegger 2010). These extracellular matrix specializations play a role in stabilization of synapses arriving onto Pvalb cells and therefore affect plasticity levels of cortical circuits in general (Wang and Fawcett 2012). Furthermore, today we also know that PNNs are malleable structures that adapt to incorporate "new information" into cortical circuits after learning sessions, such as fear conditioning (Banerjee et al. 2017).

To study the postnatal maturation of Pvalb-expressing interneurons, different approaches can be used to address distinct developmental processes, such as marker expression, firing properties, synaptic function. Nonetheless, the regulatory programs underlying the postnatal maturation and maintenance of Pvalb-neurons' functions have up-to-date been scarcely studied (Dehorter et al. 2017), as well as the interdependence among these features. In **Paper IV**, we investigate the postnatal role the transcription factor Sox6 in Pvalb-neurons' maturation and synaptic function.

1.6 Cortical interneurons in humans

Compared to mice, much less is known about the development and mature neuronal composition of the neocortex in humans. Nonetheless, rodents' and primates' interneuron development and diversity appear to share a great deal of key elements: human cortical interneurons are born in subpallium ganglionic eminences; cells born in the human MGE express the same key markers as in mice, such as Nkx2-1 and Sox6; they migrate long distances towards the forming cortex, and once mature express the same cell type markers to that of mouse interneuron classes, e.g. $Pvalb^+$, Sst^+ and $nNos^+$ cells all co-express Sox6. (Ma et al. 2013; Hansen et al. 2013), among many other shared molecular markers. Moreover, human cortical $Pvalb^+$ and Sst^+ GABAergic neurons (as well as Sst^+nNos^+ cells) have similar layer distribution and express Sox6 throughout embryonic development and postnatal maturation.

Although important differences exist (Boldog et al. 2018), recent studies investigating synaptic connectivity in human cortical slices have revealed conserved microcircuit rules (Szegedi et al. 2017; Obermayer et al. 2018), such as disynaptic inhibition of neighboring pyramidal neurons, mediated by Martinotti cells (Obermayer et al. 2018). Thus, evidence from studies in human samples are providing encouraging insights on the similarity and, hence, relevance of the extensive and laborious effort of dissecting the cellular and genetic composition of the mouse neocortex.

2 AIMS

Broadly, the studies in this thesis aimed at shining light on the cellular composition of the mammalian cerebral cortex (and striatum), focusing particularly on genetic programs involved in GABAergic interneurons diversity and maturation.

- In **Paper I** we dissected the cellular heterogeneity of the mouse somatosensory cortex and hippocampus using scRNAseq.
- In **Paper II**, using scRNAseq, we determined the interneuron composition of the mouse dorsal striatum in two postnatal timepoints and ultimately compared it to that in the cortex.
- In **Paper III** we investigated the role of the transcription factor Sox6 on cortical Sst^{+} interneurons' subtype identity maintenance.
- Finally, in **Paper IV**, with a temporal perspective on gene expression, we looked at the role of the transcription factor Sox6 in distinct developmental timepoints of cortical $Pvalb^{+}$ interneurons.

3 RESULTS AND DISCUSSION

This section includes discussion points that were not be included in the original manuscripts and consist of aspects from my work that I think are particularly interesting. With regards to **Paper I**, I briefly discuss the progress and expansion of the taxonomy of the cortical cells proposed by studies published the years following our first study. From **Paper II**, I comment on the implications of generalizations when it comes to naming two similar cell types from two different brain areas, "putatively the same". From **Paper III**, I write about the journey of interpreting our results as the field simultaneously progressed. Finally, regarding **Paper IV** I discuss the concept of the same protein having variable functions depending on the developmental point of that same cell type.

3.1 Does it hold true?

Paper I was the first large-scale application of scRNAseq to classify cells in the mouse somatosensory cortex and hippocampal CA1 region. We found 47 molecularly distinct subtypes, comprising all known major cell types in the cortex, including new cell types (new molecular signature). To validate some of the findings, we performed immunohistochemistry, single-molecule RNA FISH (fluorescence *in situ* hybridization), electrophysiology and morphological reconstructions, according to numerous marker genes identified.

In particular, we reported a new type of inhibitory interneuron labeled by the transcription factor Pax6 - a marker previously only described in excitatory cells. One important validation of our scRNAseq findings was to demonstrate in tissue the presence of interneurons that express Pax6, using electrophysiology, protein expression and morphological characterizations (Figure 5). We, therefore, show that this class of GABAergic interneurons is enriched in LI and that most $Pax6^+$ recorded neurons resemble neurogliaform cells, morphologically and electrophysiologically, although some heterogeneity was apparent. In fact, our electrophysiological data from $Pax6^+$ neurons (by post-hoc staining) suggested two different electrophysiological profiles. In accordance, subsequent large-scale scRNAseq studies suggested the existence of two $Pax6^+$ subtypes of interneurons enriched in LI-III (Tasic et al. 2018).

Several studies following **Paper I** addressed the cellular complexity of the cortex (Tasic et al. 2016, 2018; Zeisel et al. 2018). In comparison, while our study provided a generally good description of non-neuronal cell clusters, subsequent studies with samples enriched for specific cell classes revealed a greater level of neuronal complexity (Tasic et al. 2016, 2018), as well as for other cell classes, such as oligodendrocytes and their precursor cells (Marques et al. 2018, 2016). The high rate of publications and fast advance of scRNAseq technologies leads to a regular "update" on the proposed number of cells types in the cortex.

Importantly, robust tissue validations and reproducibility are needed in order to determine how many neuronal types there are (as well as a consensus on what constitutes a neuronal type). One important factor in this saga is that we are reaching a plateaued number of neuronal classes regardless of larger numbers of sequenced cells. When comparing the two largest studies on cortical neuronal diversity so far (Tasic et al. 2016, 2018), while Tasic and collaborators (2018) analyzed ten times more cells than in their previous study (2016), this resulted in only a twofold increase in suggested number of neuronal subtypes. This suggests, to me, that we are

on the right direction to reaching a common-ground classification system for neuronal cell types, relying on their transcriptome as a platform for further validations and exploration (Poulin et al. 2016; Tasic 2018).

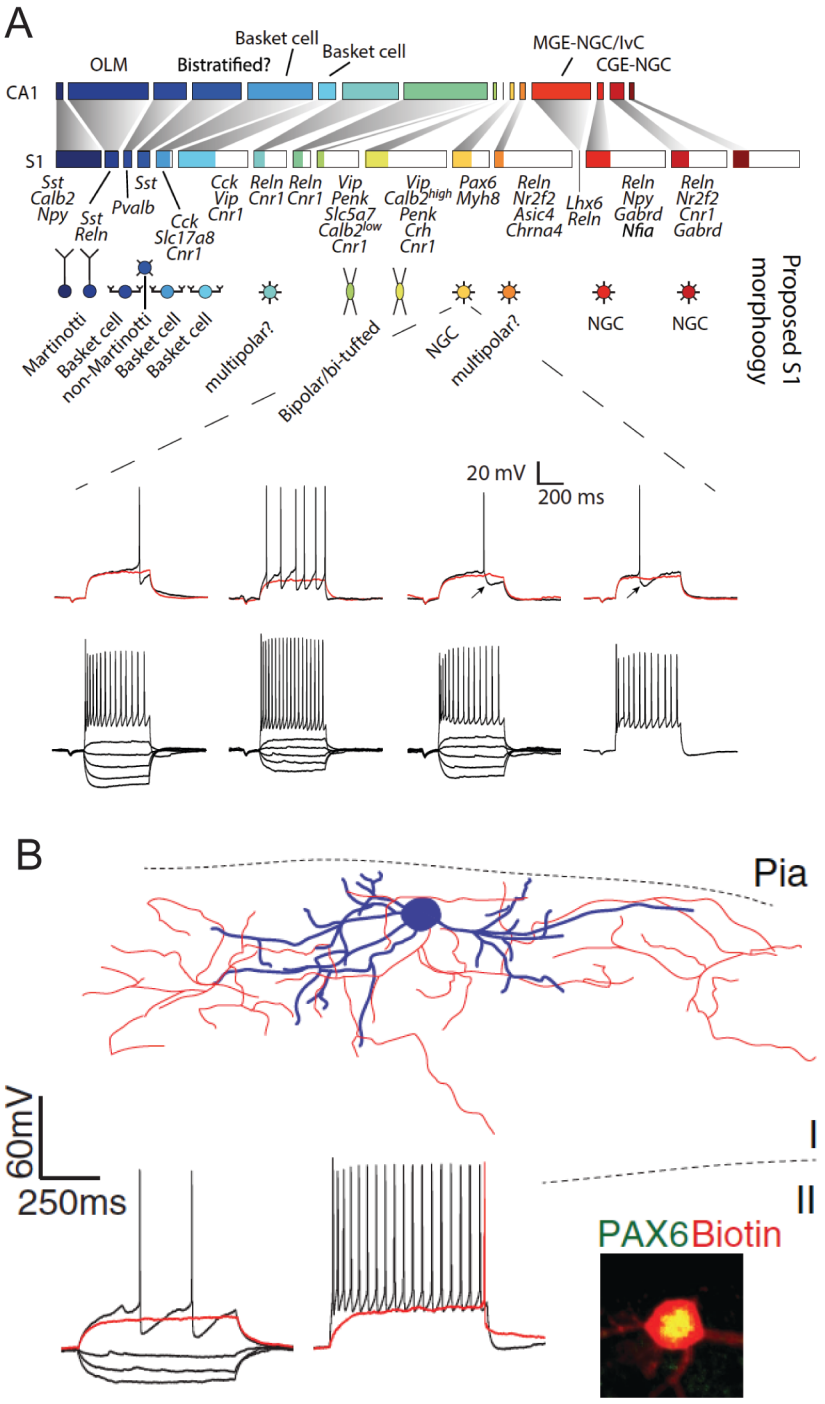

Figure 5. Pax6⁺ interneuron characterization. (A) Names of selected known markers expressed in each subgroup are shown together with the proposed morphology in S1. Bellow, traces of Pax6⁺ showing late-spiking or biphasic after-hyperpolarization (arrows). (B) Example traces of Pax6+ neurons recorded in layer 1 showing a late-spiking firing and morphology resembling neurogliaform cells. Adapted from Zeisel et al., 2015.

One particularity of the latest work by Tasic and collaborators was that they compared samples from two functionally distinct cortical regions, primary visual and anterior later motor cortex. One interesting discovery was that, while nearly all interneuron clusters were found in both cortical areas, clusters of excitatory neurons were oftentimes segregated by area. Further analysis with differentially expressed genes for the best-matched clusters from the two areas confirmed a high degree of differential expression, compared to the very small differences within interneurons from the same cluster, but different areas. A possible interpretation of these data is that, if assuming that most cortical areas would developmentally start sharing the same core cellular types, these results suggest that pyramidal neurons' expression profiles can specialize depending on which cortical area they are allocated to and, therefore, the local and long-distance connections they give and receive (Tasic et al. 2018). Whether these differences are due to variations in network enrollments or not, it underlines that the scRNAseq data consists of a snapshot a cell's lifetime and it does not necessarily include previously acquired assets, such as morphological features set-up early on. Therefore, it remains to be confirmed where such regional differences originate from and if such differences correspond to functional roles in their respective cortical network.

In spite of the findings suggesting interneurons to not vary so much among different cortical areas, a recent study proposed that the layer composition of interneurons and excitatory neurons differ between different primary cortices (somatosensory *versus* visual). Particularly excitatory and Sst^{+} interneurons found in LIV are morphologically and electrophysiologically immensely distinct (Scala et al. 2019). This highlights the importance of also addressing the spatial distribution of the cell types (Lein, Borm, and Linnarsson 2017; Codeluppi et al. 2018).

3.2 Same cell type in different structures?

One of the main goals when searching for unique features that characterize specific cell types is to be able to locate those cells in future experiments and, in the best-case scenario, also using different approaches. A good example would be fast-spiking Pvalb-expressing basket cells. Generally speaking, a whole-cell current-clamp recorded cortical neuron that responds to injected currents with brief spikes and reaches high-frequency with low adaptation is very likely to be a basket cell. If post-hoc staining shows that they also express Pvalb, the likelihood is even greater. Despite the higher probability, one has to be cautious on such extrapolation. It is known today that Pvalb-expressing cells comprise different types of basket cells, this class includes also chandelier neurons, and there are interneurons that spike at high-frequencies but are not Pvalb cells.

While such extrapolations and/or assumptions are important insights for a more agile progress of neuroscience research, in **Paper II** our data raises two important concerns in that matter. We characterized all striatal neuronal populations using scRNAseq, from which we described seven striatal interneuron subtypes, six of which GABAergic. The first important finding was that the typical $Pvalb^{+}$ cells do not constitute a discrete cluster. Instead, they are part of a larger group of cells expressing Pthlh, that exhibit a continuum of electrophysiological properties correlated with Pvalb expression. Consequently, assuming Pvalb-expression to unquestionably label this neuronal class has in fact been inadequate. The second important finding turned up when comparing scRNAseq data from striatal interneurons to their cortical counterparts (Figure 6). Even though these two large classes are developmentally related (Du et al.

2008; Butt et al. 2008; Sussel et al. 1999) and share a great deal of molecular and firing features (Muñoz-Manchado et al. 2018), our detailed electrophysiological and molecular data suggest these two cells types to differ a great deal [this was previously suggested only based on electrophysiological properties (Kaiser et al. 2016)]. In particular, hierarchical clustering of these cells revealed their molecular profiles to be surprisingly distant (Figure 6B) and differential expression analysis showed that over 500 genes are significantly higher or lower in either cell class. This shows that despite developmentally related and functionally still sharing many attributes, cortical and striatal fast-spiking Pvalb-labeled (Pthlh) interneurons display important differences, both regarding their transcriptome and physiology.

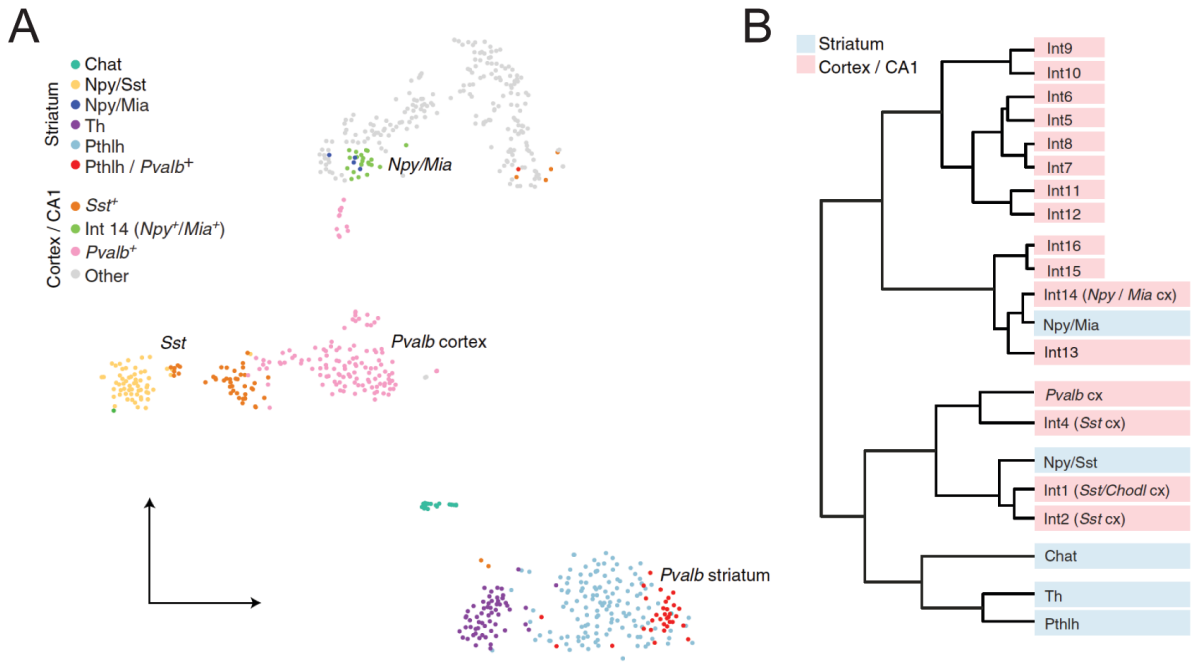

Figure 6. Comparison of single-cell transcriptomes of interneurons from the striatum and cortex and hippocampus. (A) t-SNE of most differentially expressed genes of striatal and cortical-hippocampal interneurons. (B) Hierarchical clustering of the same cell populations shown in (A). Adapted from Munoz-Manchado et al., 2018.

Both cortical *and* striatal Pvalb$^+$ neurons are born in the MGE (Du et al. 2008; Butt et al. 2008; Sussel et al. 1999). However, while Pvalb$^+$ cells destined to enter the cortex stop expressing the transcription factor Nkx2-1, Pvalb$^+$ cells directed to entering the striatum maintain its expression (Nóbrega-Pereira et al. 2008). Could it be that they were initially nearly the same subtype but considerable differences in circuit integration would lead to such transcriptomic differences (striatum is mostly an inhibitory structure while cortex is excitatory and more heterogeneous)? Or that prolonged expression of Nkx2-1 only is enough to initiate such differences (Sandberg et al. 2016)? To my knowledge, it remains unknown if there are other major transcriptional differences (besides Nkx2-1,) already at the arrival to their allocated structure.

3.3 Interpreting data as the field develops.

In **Paper III** we investigated the role of the transcription factor Sox6 in developing GABAergic Sst-expressing interneurons. This consists of a diverse *class* of neurons composed of various unique *subtypes* of neurons. We show that Sox6 maintains the subtype identity of these neurons during their migration to the developing cortex. While in controls the Sst-class comprised nine molecularly distinct neuronal types, in the Sox6-mutant cortex Sst^{+} subtypes consisted of only three molecular subtypes: two of which belonged to the nine naturally occurring Sst-expressing populations, and the third subtype comprised a molecular hybrid of the same two naturally occurring subtypes. We confirmed the loss of specific cell populations using both electrophysiology and *in situ* hybridization. Additionally, we show that the Chodl-Nos1-expressing type does not require Sox6 after the first postnatal week, a point when they have reached their final destination and begun to integrate into the network.

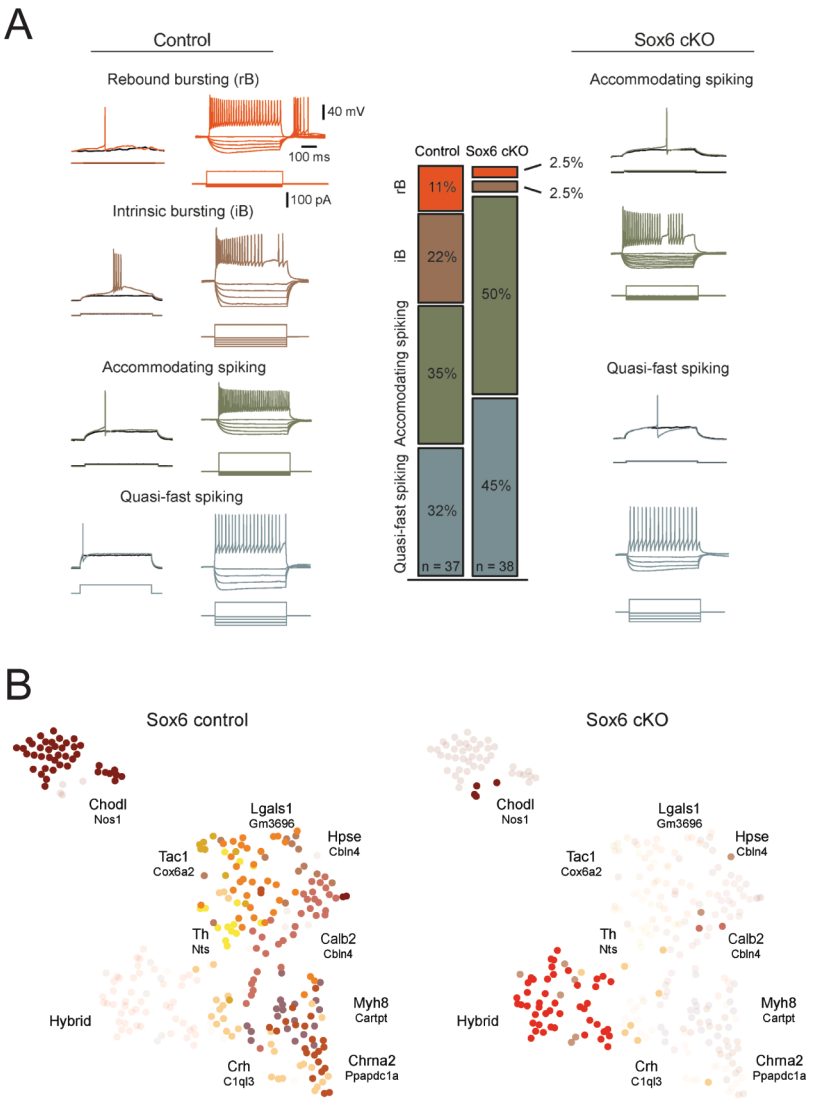

Figure 7. Loss of diversity among Sst-expressing neurons after Sox6 removal. *(A)* Whole-cell patch-clamp recordings of LV Sst-expressing neurons reveal that rebound and intrinsic bursting neurons are nearly absent from the Sox6-mutant, while their intrinsic properties remain comparable to control cells. (B) Left t-SNE highlights control cells; right t-SNE highlights Sox6-cKO cells. Adapted from Paper III.

Interpreting the results of this project involved the entire duration of my PhD. We started off the project with a striking phenotype in which Sox6-mutant neurons would never display bursts, which is an important firing property showed by neurons belonging to the Sst-class. Such "loss of bursting neurons" was *not* accompanied by a reduction in Sst-neurons. Therefore, our initial hypothesis was that these neurons were virtually the same as controls, though unable to burst. We searched for possible maturation delays or ion channels that underlie bursting properties, however no difference would justify the phenotype.

One important observation from our data was that, besides loss of bursting, Sox6-mutant neurons would in fact spike at higher frequencies, resembling fast-spiking neurons. During this period, the first systematic study described the Sst^+ non-Martinotti cells, labeled in X94-GFP mouse, were enriched in LIV-V and displayed fast-spiking-like firing properties (Xu et al. 2013). Could it be that loss of Sox6 could affect Sst^+ neurons' identity? Two years in the project, we performed scRNAseq to test the hypothesis that Sox6 loss leads to more extensive perturbations, to the level of their subtype identity. Nevertheless, even after data collection was accomplished, making sense out of the it took much longer. Back then, the existing understanding of Sst-class diversity suggested three or four subtypes of Sst^+ interneurons (Ma et al. 2006). Preliminary clustering approaches were taken, which clearly showed that most Sox6-mutant cells did not cluster with control cells, but how many subtypes of Sst^+ interneurons there are in the control was still uncertain.

Two milestone studies served as a guide on our understanding of our data (Tasic et al. 2016; Tasic et al. 2018). Our control data reproduced their suggestion of Sst clusters, therefore we now knew with more detail which Sst neurons were absent after loss of Sox6. At this time point, it was unknown whether interneurons were prespecified or not before reaching the cortex. And because in our model Sox6 is removed during migration, our understanding then was that Sox6 was acting on diversification of Sst^+ interneurons. However, at last one later study pivoted the final aspects of our understanding (Mi et al. 2018). They showed that at the time Sox6 removal occurs, these neurons have already acquired their adult molecular signature. Thus, the role of Sox6 is more likely to relate to interneuron identity maintenance, rather than interneuron diversification. Up to date, those conclusions of early subtype identity establishment have only been shown using scRNAseq of embryonic cells matched to mature ones. Therefore, further *in vivo* validations must be shown to confirm that at the time of their last division, their fate (subtype identity) is fully or potentially established, and to what extent it is independent on extrinsic factors encountered on their way or once arrived at the forming cortex (Wamsley and Fishell 2017).

3.4 It depends on when.

In **Paper IV** we investigated the role of the transcription factor Sox6 in the postnatal maturation and function of cortical Pvalb-expressing interneurons. Cortical interneurons undergo extensive synaptogenesis and maturation of intrinsic properties during the first weeks after birth. In particular, Pvalb-expressing interneurons go through a shift in their transcriptional profile indispensable for acquiring their mature identity. Because Sox6 is expressed in these cells until adulthood, we investigated its role in the postnatal maturation of Pvalb-expressing cells as well as their synaptic function. For this, we utilized conditional knockout strategies to specifically remove Sox6 in interneurons at different postnatal stages. Our data

revealed that, although Pvalb-interneurons do not rely on Sox6 to undergo their transcriptomic switch (shown by normal maturation of intrinsic properties and expression of perineuronal nets), loss of Sox6 in individual Pvalb-interneurons led to a robust growth stagnation and shrinkage of their axonal boutons contacting pyramidal neuron cell bodies. Furthermore, Pvalb-interneurons lacking Sox6 displayed reduced TrkB-full-length (FL) expression, which, when overexpressed in cells lacking Sox6, was sufficient to rescue the axonal phenotype. Most strikingly, when Sox6 was removed in adult Pvalb-interneurons (as labeled in a Gad67-EGFP (G42) line) the synaptic phenotype was also observed. Therefore, our data points out to a possible constitutive role for Sox6 in regulation synaptic dynamics of Pvalb-interneurons until late adulthood.

Our data suggest that, while postnatal expression of Sox6 is dispensable for several features of Pvalb-neurons' maturation, it is required for early axonal growth and synaptic dynamics throughout life, therefore indicating that distinct developmental processes can be independently controlled by different genetic programs. Importantly, although SOXD proteins, such as Sox6, are believed to be unable to independently regulate transcription (for they have no identified transactivation or transrepression domains), they do play a role in gene regulation by interacting with different partner proteins (Hagiwara 2011; Kamachi, Uchikawa, and Kondoh 2000; K. E. Lee et al. 2014). Such profile provides versatility to the functions of Sox6, enabling it to modulate a range of systems at different developmental stages, with multiple effects.

Therefore, in search of the role of specific genes in neuronal development and function, one has to always bear in mind that a certain protein might be involved in regulating various different cellular processes depending on the time point in consideration. Recent studies have shined light on this matter, where the expression of different genes is enriched in different time points of mouse and human brain development and that, therefore, cell-type specific genetic programs seem to follow a lifespan timeline (Skene, Roy, and Grant 2017).

4 CONCLUSIONS AND PERSPECTIVES

The work included in this thesis shed new light on the neuronal complexity of the mammalian neocortex and striatum, as well as on transcriptional programs required for maintenance of subtype identity and synaptic function of cortical interneurons.

In reality, I look forward to witnessing the near future in cortical diversity research. Particularly when it comes to the establishment of a neuronal subtype classification system that incorporates scRNAseq, morphology, firing patterns, together with local and long-range connectivity signatures. In my view, this classification system will be feasible by the development of new transgenic mouse lines and viral tools based on cell specific gene candidates obtained from high-throughput scRNAseq data, as well as new approaches that combine multiple elements of a neuron's identity (such as Patch-seq). Only by achieving sufficient specificity to target and, therefore, describe such "nuanced" subtypes, this system would be reproduceable across different laboratories, "accessible" using different methods and analytical tools, and ideally consistent throughout development and adulthood.

When classifying cell types, one must incorporate as many dimensions as possible, including crucial and challenging elements, such as circuit function and recruitment during specific behaviors. After recognizing functional properties that seem unique to a previously hypothesized subtype, I believe we can pinpoint and identify with greater certainty which neuronal subtypes form neocortical circuits. For example: say, in a large-scale transcriptomic dataset two largely similar neuronal groups are suggested to constitute two different neuronal subtypes. After robust characterizations, these groups are shown to share the same developmental origin, layer distribution, comparable morphology, local connectivity and firing patterns. If these two groups are, however, shown to be recruited in non-overlapping behavioral scenarios (due to for instance, one subtype responding to cholinergic and the other to serotonergic afferents), in my view these would represent different *functional* subtypes since their engagement and contribution to the circuit are not the same. Importantly, one must bear in mind that this interpretation is based on an initial hypothesis based on the multi-dimensionality of scRNAseq analysis that revealed subtle but significant molecular differences. Perhaps distinct expression of receptors or else the outcome of long-term microcircuit recruitment patterns could be underlying the split suggested from the transcriptomic data.

For now though, I do not believe our field is overly hindered by not having all "neuronal types" sorted out. Whether neuroscience research is at the molecular, cellular, systems or behavioral realm, generalizations are still pertinent and justifiable and will advance our understanding of the central nervous system. In this thesis for example, in **Paper IV** we investigated Pvalb-expressing neurons as an entire interneuron class (although diverse), because these neurons still carry several common features. While we show that Sox6 plays a role in their synaptic function, one cannot exclude that this effect might vary within different types of basket cells and/or chandelier cells. On the contrary, in **Paper III**, knowing the cellular complexity of the population studied was vital to understanding the effects resulting from loss of Sox6 in Sst-expressing neurons. Not knowing how heterogeneous this neuronal class is would have hindered the interpretation of this study.

Beyond understanding how diverse the mammalian cortex is, it is paramount to progress towards elucidating how this complexity is formed. In particular, rather than a "yes or no" view on gene expression, understanding the temporal aspects of gene expression will help to elucidate critical periods of brain development in a cell-type specific manner. We will thereafter recognize that mutations in certain genes affect specific cell types, conceivably in specific temporal windows, when particular developmental processes occur. Ultimately, a better understanding of the building blocks of neuronal circuits and the genetic programs that regulate their formation and maturation will serve as a steadier standpoint from where to address what underlies dysfunctional brain circuits, with a temporal and cell-type specific approach.

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