Parvalbumin interneuron subpopulations: genetic characterization and hippocampal connectivity

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Preface

One of the main open questions in the field of learning and memory is how a neuronal network balances the stability of memory representations with the flexibility necessary to adapt to changing environmental circumstances. While it became clear very early on that the brain supports different types of memories by implementing parallel channels that are highly integrated but can operate independently, the same notion is, to these days, less recognized at the microcircuit level. The hippocampus has a pivotal role in the encoding of episodic memories within a large spectrum (goal-directed, temporal associative, contextual and spatial) and, although recent studies have suggested that learning and memory are carried out by discrete groups of neurons (Holtmaat and Caroni, 2016) until recently, principal excitatory cells in each hippocampal areas have been treated by experimentalists and theoreticians as homogeneous networks of randomly connected neurons performing essentially similar operations (Kesner and Rolls, 2015; Treves and Rolls, 1994). In this framework of random synaptic connectivity among homogeneous populations of excitatory neurons it's not understood which are the mechanisms that allocate specific memories to particular ensembles of neurons, and consequentially how it might be possible to recall, update and extinguish behaviorally relevant memories. Seminal studies in this field have shown that hippocampal neurons born during similar developmental windows are preferentially connected to each other and share similar gene expression profiles (Deguchi et al., 2011) and that a great amount of cell to cell heterogeneity is present in many axes of the hippocampus (Donato et al., 2015; Soltesz and Losonczy, 2018). These and other findings have uncovered a previously underappreciated degree of cellular heterogeneity, which we have only begun to describe. A plethora of technological approaches have come together to classify this heterogeneity which unfolds along different axes that include morphology and connectivity, intrinsic physiological properties and molecular identity. In particular, advances in single-cell genomics and genome editing have recently revolutionized this pursuit, allowing for characterization and genetic access of newly identified cell types.

In this thesis, I focus on the heterogeneity of hippocampal CA1, uncovering molecular diversity in developmentally defined groups of parvalbumin expressing basket cells. This molecular diversity is then integrated, through findings on differential connectivity, with previously described populations of CA1 principal cells and with cell-type specific manipulations during behavioral testing.

1.Introduction

The ability of the brain to retain information about the past is not the product of a single sequence of events that culminates in a permanent long-term memory. Rather, it is the dynamic consequence of numerous related processes working in parallel such as: acquisition or encoding of new information, short-term memory, consolidation of long-term memory, destabilization and updating in the course of memory retrieval, and integration or merging of different memories. These processes encompass the entire multi-scale organization of the brain, from individual synapses, to ensemble of cells, to entire neuronal networks whose interactions ultimately result in behaviour. But how are memories stored in the brain? And where are these representations physically allocated? At the micro- and sub-micro scale, the substrates of memories are phylogenetically conserved molecular mechanisms in individual neurons of both invertebrates and vertebrates. More complex brains, however, have evolved entire neuronal networks devoted to the encoding of information. In mammals, the hippocampus has been identified 60 years ago as a critical player for the acquisition and retention of autobiographic memories (Scoville and Milner, 1957). In this section I will first describe the basic hippocampal neuroanatomy and its cell type composition. Following this, I will describe the function of the hippocampus in the encoding of episodic memory, and later I will focus on the phylogenetically-conserved molecular mechanisms that have been proven necessary for memory formation across species.

1.1 Hippocampal Neuroanatomy

In rodents, the hippocampus is a large cashew-shaped structure bilaterally positioned just beneath the posterior half of the cerebral cortex. The hippocampus is functionally connected to the subiculum, presubiculum, parasubiculum and entorhinal cortices (EC). All together these structures comprise the hippocampal formation. The hippocampus proper has two main divisions: the dentate gyrus (DG) and the *cornu ammonis* (CA) which is further subdivided in CA1, CA2 and CA3 fields. Although the hippocampus matures from an invagination of the telencephalic vesicles, thus sharing a developmental origin with the cortex, the architecture and laminar organization of these regions are far from similar (Khalaf-Nazzal and Francis, 2013). Also, unlike cortical circuits, where there's a high degree of reciprocal connectivity within and between areas, each division of the hippocampus is unidirectionally connected. Cells in the superficial layers of the entorhinal cortex give rise to axons that project to granule cells (GCs) in the DG. GCs send their mossy fiber axons to CA3 pyramidal cells which in turn project, through the Schaffer collateral, to CA1 pyramidal cells. CA1 pyramidal cells, in turn, connect to the subiculum and to the entorhinal cortex layer V, constituting a recursive,

unidirectional information flow known as the hippocampus-EC loop. Even though the hippocampus-EC loop "begins" in the superficial layers of the entorhinal cortex and "ends" in its deep layers, it must be noted that the EC also directly projects to CA3 pyramidal cells (via the perforant path) and CA1 (via the temporoammonic path) probably conveying different types of information (Andersen et al., 2007).

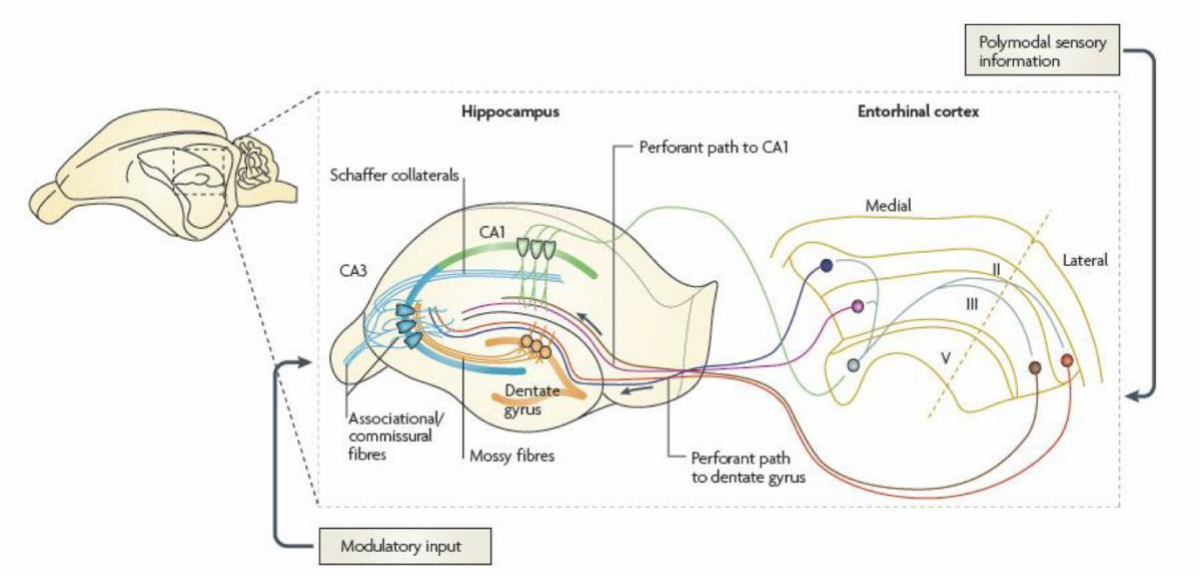


Figure 1.1: anatomy of the hippocampus

From Neves *et al*., 2008. Unidirectional information flow in the hippocampus. The main information input to the hippocampus is represented by the entorhinal cortex that projects to the dentate gyrus and the CA1 through the perforant pathway (in red and blue) and to the CA1 via the temporoammonic path (in brown and magenta). From the dentate gyrus, granule cells send information via their axons, the mossy fibers, unidirectionally to the CA3 which in turn, through the Schaffer collateral, project to the CA1.

Different processing roles have been assigned to different subdivisions of the hippocampus. The DG transforms dense cortical input into a sparse hippocampal code and it's therefore though as a pattern separator, converting relatively similar input patterns into substantially different output patterns. This is reflected in its firing scheme, in which only a small number of cells are active at the same time (Jonas and Lisman, 2014) and in its connectivity, with a very low level of recurrent connectivity among granule cells (Acsády and Káli, 2007). Dentate GCs provide CA3 with sparse but powerful mossy fiber synapses which exhibit a dynamic range of presynaptic plasticity. Contrary to the DG, local recurrent connectivity among CA3 pyramidal neurons is extensive and for these reason CA3 is often referred to as an auto-associative network (Rebola et al., 2017) even though some recent evidence suggest a more sparse code (Guzman et al., 2016). This characteristic is believed to allow for patter completion which is the ability to reinstate entire representations given only a partial input (Guzman et al., 2016).

Interestingly, although the close proximity to CA3 region, CA1 principal cells only form negligible connection with each other. The cellular composition and functional representation implemented by CA1 neurons will be discussed in depth below.

1.2 Hippocampal Function

Over the years the hippocampus has attracted the interest of neuroscientists, and as of today still remains one of the most studied neuronal network in the brain. One of the reasons that attracted neuroscientist's attention was the early observation that, following surgical procedures involving hippocampal formation to relieve a severe case of epilepsy, memory processes were profoundly affected. The removal of the medial temporal lobe from the now-famous patient Henry Molaison (known until his death in 2008 as "Patient H.M.") resulted in a profound case of anterograde amnesia as well as a partial temporally-graded retrograde amnesia. Following the intervention, H.M. was unable to encode new episodic memories, but recollections of events that occurred years earlier as well as childhood memories were spared. However, it was later shown that H.M. could still memorize new hand-eye motor skills (drawing looking through a mirror) over a period of days (Milner et al., 1968) and this singular observation paved the way for a series of experiments in humans and lesion studies in rodents that made clear that memory is not an unitary ability of one particular brain region but it is instead implemented through the parallel deployment of numerous memory systems (Squire, 2004).

Over the following decades, memory systems have been classified into explicit (or declarative), further subdivided into episodic- and semantic-memories (which consist in consciously accessible recollection of factual information) and implicit memories, comprising of phenomena such as perceptual-motor learning, priming and classical conditioning, that are considered as memories without conscious remembering (Aggleton and Morris, 2018).

In this framework, the hippocampus plays a key role in the formation of one particular type of memory, called episodic memory, which has been defined as the recollection of past personal experiences that occurred at a particular time and place (Tulving, 1972). Indeed, the robust representation of both space and time in the hippocampus is indicative of fundamental mechanisms for categorizing the subjective components of experience (the what, who, where, when) into coherent memories.

The encoding of space in the hippocampus was first recognized through electrophysiological recording of single hippocampal neurons in freely moving rats. Many cells in the hippocampus, defined as "place cells", were found to fire action potentials when the animal crosses a precise

portion of the environment, defined by distal landmarks, with different cells firing at different locations (O'Keefe and Conway, 1978; O'Keefe and Dostrovsky, 1971). These findings were later integrated in a comprehensive theory named "the Cognitive Map Theory" (O'Keefe and Nadel, 1978), supported by numerous indications that lesions of the hippocampus in rodents consistently result in impairments on spatial learning tasks (Hartley et al., 2014; Moser et al., 2008), as well as evidences that other brain areas connected to the hippocampus also host neurons with peculiar spatial tuning: head direction cells, grid cells, border cells, and others (Hafting et al., 2005; O'Keefe and Conway, 1978; Taube et al., 1990). At the population level, hippocampal place cells will generally tile the entire environment or, in other words, it is possible to find a place cell for every location of the environment in which the mouse is navigating. Thus, collectively, the active ensemble of place cells is thought to represents a spatial map of the animal's current experience (cognitive map) that can be used for spatial navigation via path integration (McNaughton et al., 2006).

Recent experimental and theoretical work on the hippocampus has been trying to reconcile the above-mentioned two fundamental discoveries of the hippocampal function: the amnestic consequences of the removal of the temporal lobe from the patient H.M. and the findings that activity in hippocampal neurons is related to the location of the rat in the three-dimensional space. A highly regarded opinion in the field is that mechanisms of memory and planning have evolved from mechanisms of navigation in the physical world and that the neuronal computations underlying navigation in real and mental space are fundamentally the same (Buzsáki and Moser, 2013). According to this view, the same mechanisms that support the encoding of unique locations and their association in a coherent map (Allocentric navigation) are being used to describe or denote actions, objects and other individuals (Semantic memory) (Hampson et al., 1996; Squire, 1992; Suzuki et al., 1997; Wood et al., 2000). Similarly, hippocampal mechanisms that support serf-referenced (Egocentric) navigation are deployed when remembering of the past (Episodic memory) or planning of future events (Buckner and Carroll, 2007; Burgess et al., 2002; Maguire and Hassabis, 2011).

In this context, semantic knowledge might be learned gradually as similar episodes are encoded repeatedly by the self-referenced (egocentric) episodic memory system, so that, analogously to the formation of allocentric maps based on the recurrent self-referenced exploration of the environment, context-dependent memories are turned into context-independent knowledge (Buzsáki, 2005; Eichenbaum et al., 1999; Lever et al., 2002).

1.3 Hippocampal Cell Diversity

Neuronal diversity encompasses different axes that can be taken into account when classifying cells into types. Most of the variability can be appreciated when considering three main axes: the morphology, the intrinsic physiological properties and the molecular identity. Morphological differences include the 3D structure of the cells but also the local connectivity (particular relevance has been assigned to the location of their synapses onto subcellular compartments of neighboring cell types). Intrinsic properties include the different electrophysiological firing pattern that neurons are able to produce (classically neurons have been categorized into fast and regularly spiking neurons). The molecular diversity of excitatory and inhibitory neurons has been particularly important for the understanding of their function in neuronal circuits as it allowed for genetic access, and therefore, *in vivo* manipulation and tracing, of different classes of neurons.

Taking a reductionist approach we can divide the diversity of cell types in the hippocampus in just two groups: excitatory, long-range projecting principal cells (PC), which represents ~80% of the total number of cells, and inhibitory local interneurons (IN), which accounts for the remaining ~20% (Meinecke and Peters, 1987). These two broad classes of cell types are further subdivided into numerous subpopulations of neurons whose functional classification is still largely undiscovered and that I will describe more in details in the following section.

1.3.1 Inhibitory Cell Diversity and Function

Inhibitory local interneurons are thought to possess the widest diversity in terms of morphology, physiology, connectivity and molecular identity. The pronounced molecular heterogeneity, in particular, has been exploited to generate Cre lines widely used for the functional manipulation and the physiological characterization of different cell types. Although often treated like a continuum, studies from the past decades have consolidated the view that a handful of molecular markers, typically calcium binding proteins (Parvalbumin, Calretinin or Calbinding), neuropeptides (Somatostatin, Cholecystokinine, or Vasoactive Intestinal Peptide) or specific receptors (CB1 or 5HT3A) could be used to identify large, non-overlapping populations of interneurons sharing common circuit functions (Kepecs and Fishell, 2014). Yet, when also the morphological and physiological axes are taken into consideration, more than twenty classes of interneurons have been described only in the CA1 region (Fig 1.2), and it is likely that an even larger diversity can be dissected in the cortex (Klausberger and Somogyi, 2008).

How is interneuronal diversity generated? Various studies have suggested that diversity is produced by the interplay between transcriptional programs established in progenitor cells and subsequent post-mitotic interactions taking place during neuronal migration and in the final local environment (Gelman et al., 2011; Xu et al., 2004). Interneurons of the forebrain are generated during a limited developmental window in transitory structures of the subpallium called ganglionic eminences. The Medial Ganglionic Eminence (MGE) give rise to parvalbumin (PV)-expressing fast spiking interneurons and the somatostatin (SST)-expressing population. The Caudal Ganglionic Eminence (CGE) gives rise to Vasoactive Intestinal Peptide (VIP)-expressing interneurons, as well as other less numerous populations (Kepecs and Fishell, 2014). Once postmitotic, newly generated interneurons migrate along multiple tangential streams to settle into cortical and subcortical networks (Silva et al., 2018).

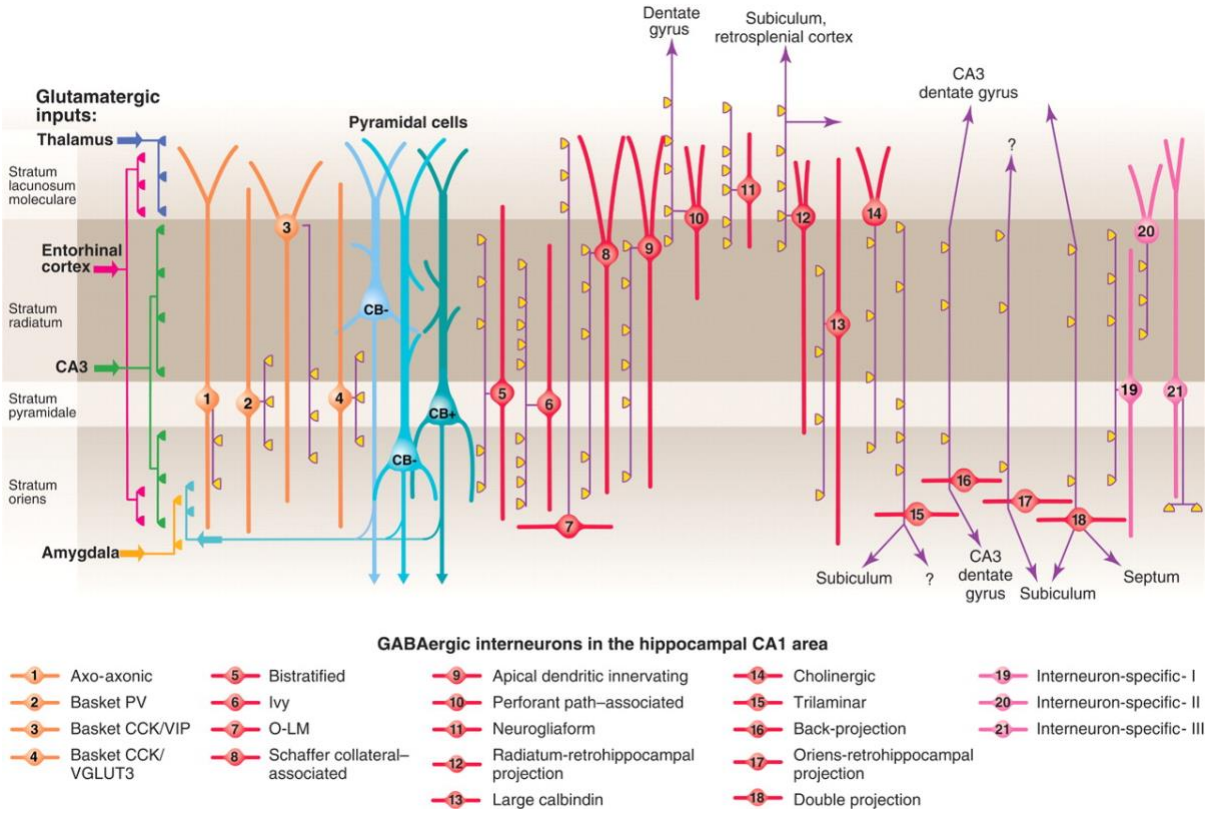


Figure 1.2: Interneuronal diversity in hippocampal CA1

From Klausberger and Somogyi, 2008. Cell type classification based on morphological diferences. 21 different classes of inhibitory interneurons have been recognized providing inhibition to a restrict number of principal cells types (in blue). The locationof the main synaptic terminals is represented in yellow.

Several lines of research have explored the relations between the developmental origins of postmitotic interneurons and the final allocation of clonally related cells in adult neuronal circuits. Two recent studies have found that clonally related interneurons are preferentially

confined in particular cortical layers or columns, suggesting the existence of dedicated progenitor lineages generating clones predestined to particular brain locations (Brown et al., 2011; Ciceri et al., 2013). However recently, using a very similar approach, two groups have independently come to the opposite conclusion that lineage is a poor predictor of interneuron positioning within the forebrain, leaving the question unsolved (Harwell et al., 2015; Mayer et al., 2015).

Understanding the generation and the degree of interneuronal diversity is pivotal for the understanding of the different computations implemented in neuronal networks. The computational processing of interneurons is vast; some authors have divided these computations into either arithmetic or timing. Different classes of interneurons are thought to perform different arithmetic operations, such as subtraction or divisions, of the synaptic input-output firing relationship which in turn allows for more complex canonical computations such as gain control and normalization (Carandini and Heeger, 2011; Silver, 2010). Timing computations are, on the other hand, responsible for the orchestration of neuronal oscillations: rhythmic and synchronous oscillations of the membrane potential of populations of neurons, characteristic of cortical and hippocampal activity that are so prominent to be detectable with scalp electrodes as features of the electroencephalogram.

In the next paragraph I will describe in detail a particular class of interneurons which is relevant for this thesis: fast-spiking parvalbumin expressing interneurons.

1.3.2 Parvalbumin Interneuron Plasticity for Consolidation of Reinforced Learning

Published review:

Matteo Tripodi, Komal Bhandari, Ananya Chowdhury, Arghya Mukherjee, and Pico Caroni
Cold Spring Harb. Symp. Quant. Biol. 2019.

Parvalbumin (PV) basket cells are widely distributed and abundant GABAergic inhibitory interneurons that provide powerful local feedforward and feedback inhibition onto principal excitatory neurons (Bartos et al., 2007; Hu et al., 2014). In addition, PV basket cells inhibit each other reciprocally through perisomatic innervation and are dynamically coupled electrically through gap junctions (Bartos et al., 2007; Hu et al., 2014). PV basket cells filter activation of principal neurons, and networks of PV basket cells have major roles in regulating local ensemble activities, including θ and γ oscillations (Buzsáki and Wang, 2012; Cardin et al., 2009; Fuchs et al., 2007a; Hu et al., 2014; Isaacson and Scanziani, 2011; Kuhlman et al., 2010; Lee et al., 2012; Sohal et al., 2009). Synaptic regulation of PV basket cells has been implicated in adult learning, and the maturation state of PV basket cells has been implicated in critical period-type plasticity (Di Cristo et al., 2007; Hensch, 2005; Hensch et al., 1998; Kuhlman et al., 2013; Southwell et al., 2010). We recently discovered that PV basket cells

show robust plasticity upon reinforced forms of learning in the adult (Donato et al., 2013), and that two subpopulations of PV basket cells generated during the first/second half of neurogenesis in the median ganglionic eminence show plasticity upon definite (early-born)/provisional (late-born) learning (Donato et al., 2015). PV neuron plasticity only becomes detectable with a delay of ~6 h after the initial learning event, and persists for 2–3 d (Donato et al., 2015; Karunakaran et al., 2016). In this review, we focus on the function of this unusual form of cellular plasticity and on what it reveals about circuit and systems mechanisms of long-term memory consolidation. Analysis of learning-related plasticity markers such as pERK, cFos, and Arc expression in the hippocampus after fear conditioning revealed a second peak of expression from +9 to +15 h after acquisition (Bekinschtein et al., 2007; Caroni et al., 2014; Karunakaran et al., 2016; Katche et al., 2013, 2010; Trifilieff et al., 2007, 2006). Interfering with dopamine D1 receptor (D1R) signaling or organized network activity at +12 h after fear conditioning suppressed long-term consolidation of fear memory, providing evidence for the existence of a late time window at +12h critically important for long-term memory consolidation (Carr et al., 2011; Girardeau et al., 2009; Jadhav et al., 2012; McNamara et al., 2014; Rossato et al., 2009). As discussed below, learning-related PV neuron plasticity appears to be specifically important to support offline network activity essential for long-term memory consolidation upon reinforced learning during the late time window at +12–15 h.

PV neuron plasticity of opposite signs upon provisional and definite reinforced learning

Reinforced trial-and-error learning tasks provide attractive experimental paradigms to investigate how plasticity is flexibly adjusted during learning. Thus, effective acquisition and flexible combination of potentially task-relevant information is essential during early phases of trial and error learning, whereas reliable application of validated routines dominates toward learning curve completion (Kaelbling et al., 1996). We found that at the circuit level, these contrasting requirements are correlated to shifts in the configuration of local PV-expressing basket cell networks, which show pronounced plasticity induced by certain forms of learning (Fig. 1; Donato et al. 2013). Thus, PV network configurations enriched in neurons expressing low levels of PV and GAD67 and showing relatively high inhibitory connectivity onto them are induced locally early during trial-and-error incremental learning, when they are required for effective learning (Fig. 1A–E). Such “low-PV configurations” promote acquisition and retention of new memories and structural synaptic plasticity (Donato et al., 2013). In contrast, configurations enriched in neurons expressing high levels of PV and GAD67 and showing high excitatory connectivity onto them are induced upon Pavlovian conditioning and toward completion of trial-and-error learning protocols. Such “high-PV configurations” interfere with the acquisition of new incidental memories (Familiar Object Recognition test) and structural

synaptic plasticity (Fig. 1D; Donato et al. 2013). The PV plasticity was not detected in tasks involving memory formation in the absence of reinforcement, suggesting that it might be

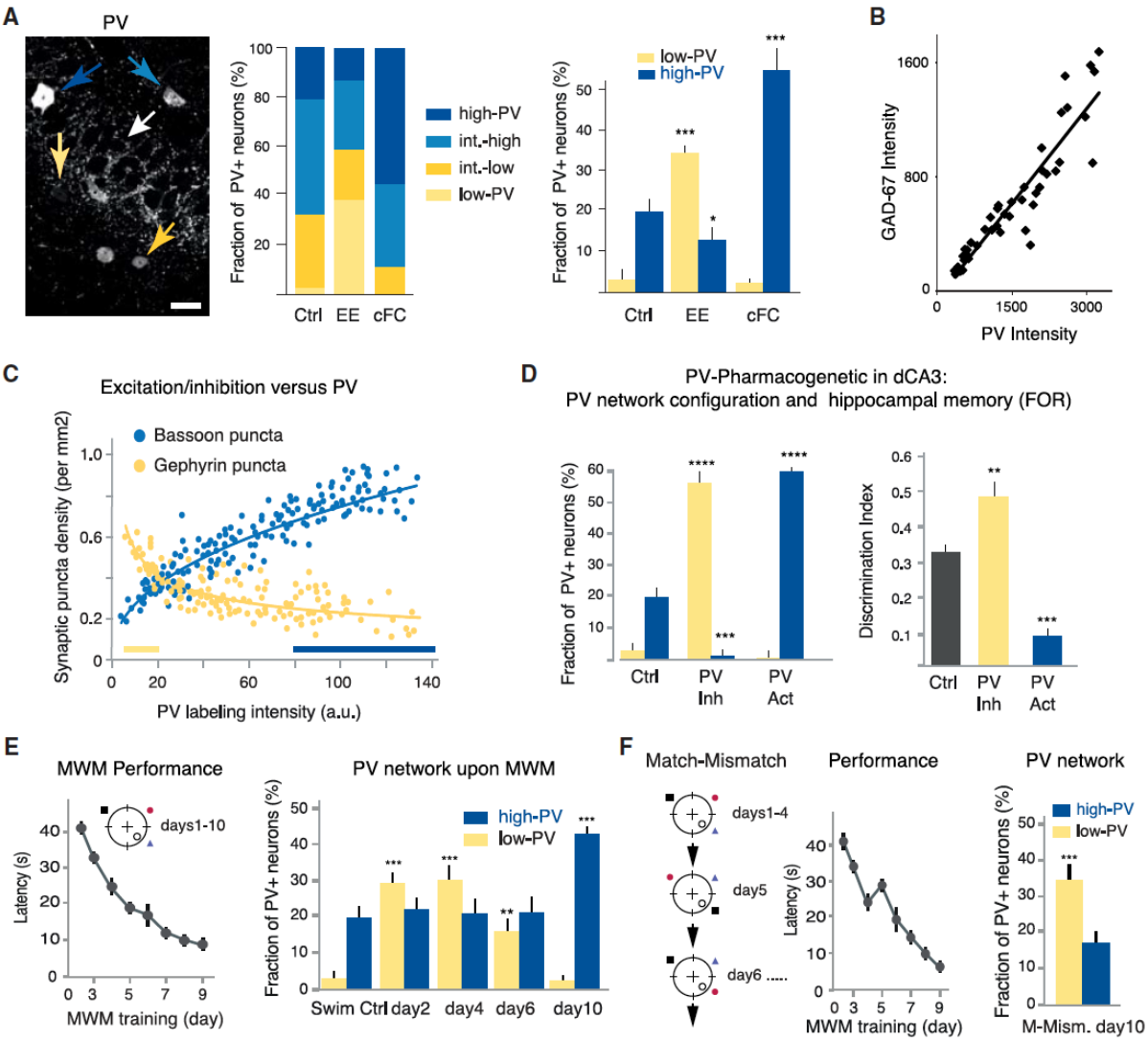


Figure 1.3 Learning-induced plasticity of PV basket cells. (A) Experience-related plasticity of PV neurons. (Arrows) High- (dark blue), intermediate high- (blue), intermediate low- (gold), and low-PV (yellow) neurons. Relative contents of PV neurons in control (Ctrl), enriched (EE, 3 wk), and fear-conditioned (cFC, day 1) mice. (B) PV/GAD67 signal relationship in individual PV neurons from Ctrl, EE, and cFC mice. (C) Local densities of excitatory and inhibitory synaptic puncta as a function of PV immunoreactivity signal. (D) PV neuron plasticity induced upon pharmacogenetic activation or inhibition of dCA3 PV neurons (values 31 h after ligand delivery) and impact on hippocampal memory. (E) Morris water maze learning curve (left) and PV network configuration in CA3b during MWM learning (right). (F) Persistence of low-PV configuration in spite of spatial learning when external cues are rearranged daily, from training day 5 on. Schematic of match–mismatch protocol (left), corresponding learning curve (center), and PV network configuration on day 10 for match–mismatch protocol (right). (Adapted from Donato et al. 2013.)

specifically induced upon reinforced learning. Furthermore, the plasticity was specifically detected in brain areas known to have a role in the particular form of learning (e.g., hippocampus for the water maze but not for a motor skill task such as staying on an accelerating rotating rod, and primary motor cortex but not hippocampus for the motor skill task). Two additional observations warrant mentioning here. First, when a variation of the standard water maze protocol was introduced so that, from training day 5 on, mice had to adjust each day to a new position of the hidden platform relative to allocentric cues on the surrounding walls, mice rapidly adjusted to this more challenging protocol, but the high-PV plasticity consistently observed once mice had reliably learned the spatial map did not occur (Fig. 1F;Donato et al. 2013). This observation suggests that a shift to a high-PV network configuration might specifically be induced when mice effectively manage a task in a way that does not anymore involve unexpected features (such as the relationship between allocentric cues and the position of the hidden platform). Rather than reflecting performance, high-PV plasticity might therefore reflect the absence of deviations and unexpected value-related findings in a trial and error task. The second informative observation is that the learning-related plasticity does not seem to induce any distinct new phenotype within the population of local PV neurons. Instead, the plasticity appears to shift the relative prevalence of PV neurons with a given distribution of PV/GAD67 expression of excitatory versus inhibitory synaptic puncta onto them (Donato et al., 2015, 2013). This suggests the presence of consistent, predictable and reversible learning-induced shifts in the relative prevalence of local PV neurons with relatively high- or low-PV/GAD67 expression levels. Given the high degree of connectivity between individual local PV neurons and principal neurons and among the PV neurons themselves, these observations suggest that the plasticity induced upon certain types and stages of learning might influence the functioning of local principal and inhibitory neuron networks.

Distinct PV neuron subpopulations implement plasticity upon provisional and definite learning

The observed opposite learning-induced shifts toward either more or less PV/GAD67 expression and excitatory/ inhibitory synaptic puncta distributions within the ensemble of local PV neurons might reflect concerted global network shifts possibly involving most PV neurons. Alternatively, there might be PV neurons susceptible to low-PV plasticity during provisional learning, and other PV neurons susceptible to high-PV plasticity upon definite learning. Indeed, using BrdU-mediated birth-dating during medial ganglionic eminence development, we found that PV basket cells consist of two previously unrecognized subpopulations, which are specified during the first and second half of neurogenesis in mouse subpallium and differ

in their connectivities and plasticity regulation (Fig.2A–D; Donato et al. 2015). Early-born PV basket cells generated during the first half of neurogenesis show comparatively high-PV/GAD67 levels and excitation/inhibition connectivity ratios under baseline conditions, which in most cases further increase upon learning-induced plasticity (Fig. 2A–C). Furthermore, early-born PV neurons show plasticity upon cFC and at the end of maze learning, but not upon

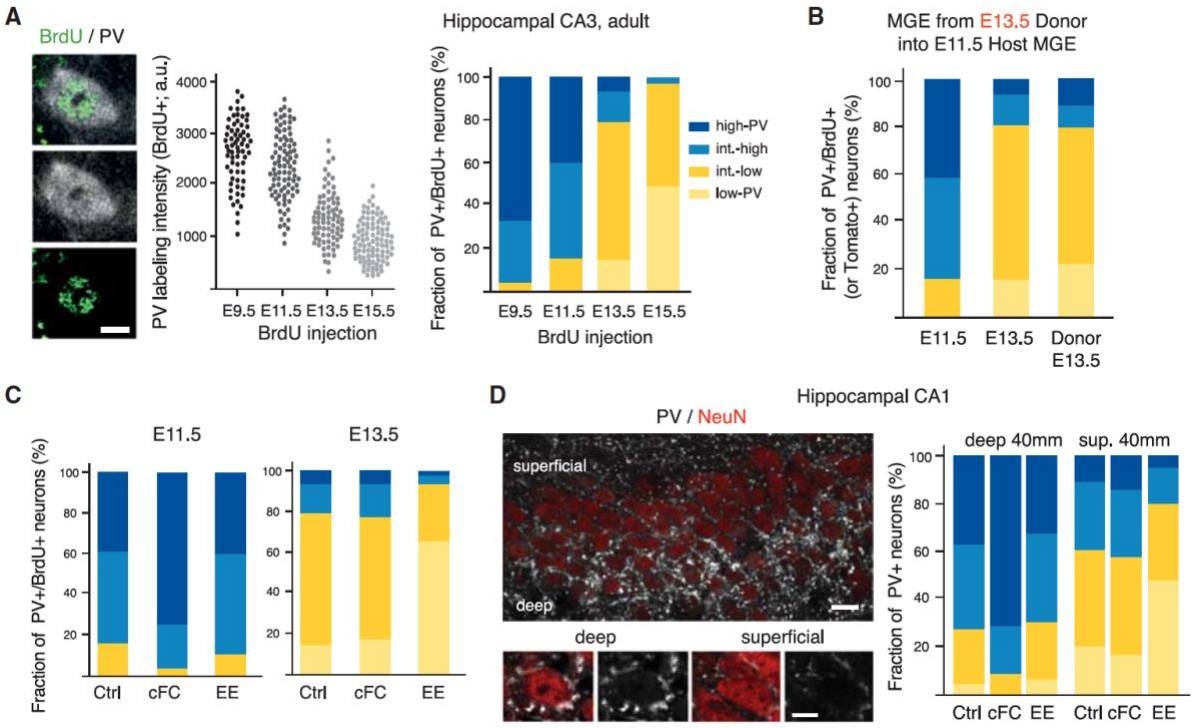


Figure 1.4 Developmentally specified subpopulations of PV basket cells showing plasticity upon definite or provisional learning. (A) Relationship between schedule of neurogenesis and PV/GAD67 levels in adult hippocampal CA3b. (Left) Representative example of PV neuron in adult hippocampal CA3b labeled with BrdU, and scatter plot of PV labeling values for cells (individual dots) labeled at E9.5, E11.5, E13.5, and E15.5. (Right) Labeling value distributions as a function of birth date. (B) Grafting E13.5 MGE cells into E11.5 host. Adult distribution of PV labeling intensities in donor neurons closely matched to late-born PV neurons of host. (C) Time schedule of neurogenesis versus regulation of PV levels upon cFC or EE. (D) PV signals of perisomatic boutons at deep- and superficial-layer CA1 pyramidal cells. (Left) Representative examples of bouton signal distributions at deep- and superficial-layer pyramidal neurons. Scale bars, 15 μm (top) and 5 μm(bottom). (Right) PV-level distributions of perisomatic boutons at deep- and superficial-layer CA1 pyramidal cells upon cFC or EE. (Adapted from Donato et al. 2015.)

environmental enrichment (EE) or during maze learning (Fig. 2C). Early-born PV basket cells appear to account for characteristic features of mature fast spiking PV neuron networks such as narrow synchronization windows and learning-related θ–γ entrainment within and among brain systems (Bartos et al., 2007). Thus, high-PV and GAD67 levels enhance fast and high-frequency firing properties of PV neuron networks important for γ band network activity (Cardin

et al., 2009; Fuchs et al., 2007a; Kuhlman et al., 2010). In contrast, late-born PV basket cells specified during the second half of neurogenesis show low-PV/GAD67 levels and excitation/inhibition connectivity ratios under baseline conditions, which in most cases further decrease upon learning-induced plasticity. Furthermore, late-born PV neurons show plasticity upon EE, during maze learning and when critical period type plasticity is induced (Fig. 2A–C). Notably, early-born PV neuron cell plasticity is specifically regulated by excitation, whereas late-born PV neuron cell plasticity is specifically regulated by PV neuron inhibition (Donato et al., 2015). Furthermore, cell plasticity in early-born neurons mainly involved alterations in the densities of excitatory synaptic puncta, whereas cell plasticity in late-born neurons mainly involved alterations in inhibitory synaptic puncta densities onto PV neuron dendrites. These matched specificities of regulation were particularly striking in comparisons of how cFC or EE induced PV cell plasticity in CA3/CA1 versus DG. Thus, although cFC consistently involved early-born neuron plasticity and EE consistently involved late-born neuron plasticity, the signs of PV (and Mef2a) changes were opposite in the hippocampal subdivisions, and this was reflected in opposite signs of excitatory (and inhibitory) connectivity regulation in the two subpopulations (Donato et al., 2015). The consistent experience-related plasticity regulation of the two subpopulations of PV basket cells raised the issue of whether these might also show distinct output targets related to behavioral function (Kepecs and Fishell, 2014; Lee et al., 2014). Indeed, we found that early-born PV neurons preferentially target deep cells in the pyramidal layer of hippocampal CA1, whereas late-born PV neurons preferentially target superficial cells (Fig. 2D). The selective connectivity between early-/late-born PV neurons and early-/late-born pyramidal cells is reminiscent of selective connectivity among principal neuron subpopulations in hippocampal dentate gyrus, CA3 and CA1, suggesting that it might reflect circuit assembly principles based on relative schedules of neurogenesis and neuronal maturation (Deguchi et al., 2011). Although the functional implications of the selective output connectivity of PV neuron subpopulations remain to be determined, our findings would be consistent with the notion that the distinct excitation/inhibition input ratios onto early- and late-born PV neurons and the specific regulation upon learning might reflect information flow through functionally distinct microcircuits (Fig. 3; Cembrowski et al., 2016; English et al., 2017; Knierim et al., 2014; Lagler et al., 2016; Lapray et al., 2012; Mizuseki et al., 2011; Soltesz and Losonczy, 2018).

Learning-induced PV neuron plasticity: delayed onset and persistence for days

To investigate which aspects of learning and memory might be influenced by learning-induced plasticity in local PV neurons, we monitored the time course of high-PV plasticity induced in

mouse hippocampal CA3 upon cFC. In ventral (vH) and dorsal hippocampus (dH), PV neuron labeling distributions were not detectably altered up to +4.5 h (i.e., 4.5 h after acquisition) and had reached peak high-PV values at +6 h. These were maintained until +30 h, had decreased to half-maximal values around +36 h, and reached again baseline values at +48 h after acquisition (Fig. 4A). Low-PV plasticity induced upon 1 d of MWM training also only became detectable at +6 h. Notably, and like for high- or low-PV plasticity induced upon learning, pharmacogenetically induced PV plasticity only became detectable at +6 h (and not yet at +4.5 h) upon induction (Karunakaran et al., 2016). Unlike plasticity induced upon learning,

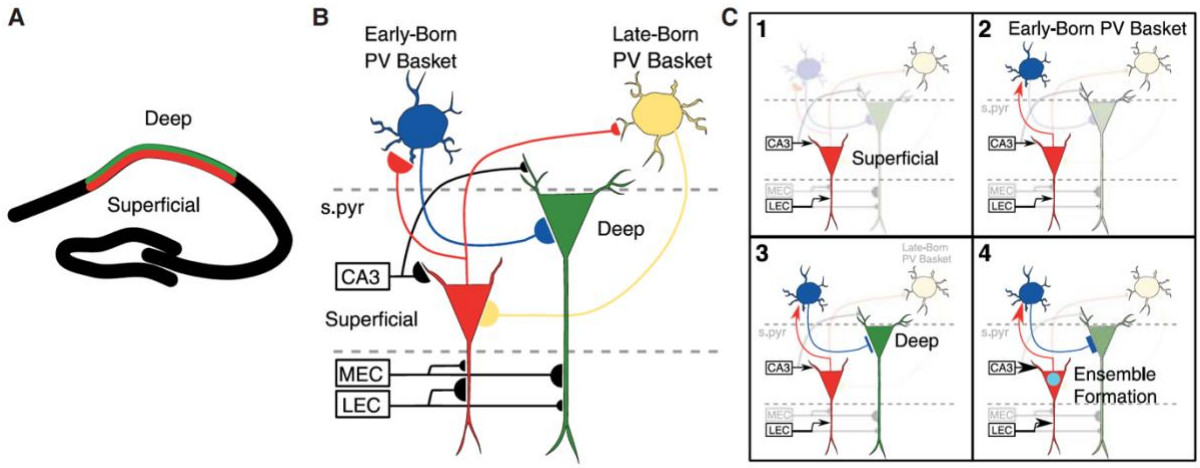


Figure 1.5 Distinct local microcircuit connectivities for early- and late-born PV neurons in hippocampal CA1. (A) Position of deep (green) and superficial (red) pyramidal neurons in CA1. (B) Schematic representation of connectivity involving deep/superficial and early-/late-born PV neurons. (C) Proposed sequence of recruitments (1–4) for a hypothetical superficial (1) to early-born PV neuron (2) to deep pyramidal neuron (3) local circuit. Panel 4 indicates how the microcircuit might support the formation of neuronal ensembles in superficial pyramidal neurons.

elevated contents of high- or low-PV neurons begun to decline from +10 to 11 h, and had returned to baseline values at +15 h (Fig. 4B). These observations suggested that long-lasting PV plasticity possibly required for long-term memory consolidation might depend on additional signals delivered locally. Given the role of late dopamine (DA) signaling in fear memory consolidation, we hypothesized that this might involve local DA signaling acting through D1/5 receptors. Indeed, local delivery of a specific D1 receptor agonist to vH at +12 h rescued and extended both the high- and the low-PV shifts up to at least +24 h (Fig. 4B; Karunakaran et al. 2016). Notably, local delivery of D1 agonist to vH of naive mice induced dramatic increases in the fraction of pDARPP-32+ and pERK+ PV neurons, whereas corresponding changes in non-PV neurons were much more modest (pDARPP-32) or absent (pERK), suggesting that D1R might signal in PV neurons to modulate PV neuron plasticity (Karunakaran et al. 2016).

Given the dependence on D1R signaling by the PV neuron plasticity induced upon pharmacogenetic activation or inhibition of PV neurons, we hypothesized that PV neuron plasticity induced upon learning might be sustained through endogenous DA signaling onto D1R. Indeed, local delivery of D1R antagonist any time between +10 and +16 h after cFC or water maze learning led to a complete loss of any detectable local PV plasticity within 10–15 min. Furthermore, local delivery of D1R antagonist to vH at +12 h upon cFC suppressed any subsequent freezing to context. This finding led us to determine when local delivery of D1R antagonist to vH influences freezing to context upon cFC. These experiments revealed the existence of two sensitive time windows to influence consolidation of learning, one up to +5–

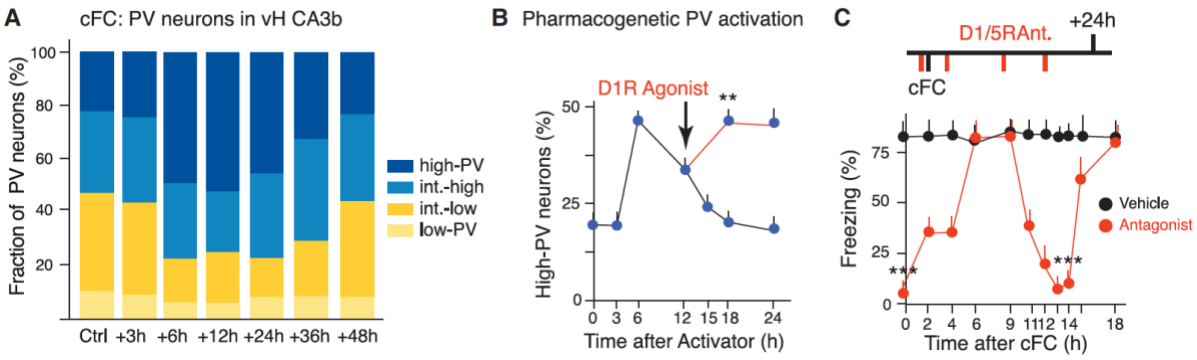


Figure 1.6 Regulation of PV neuron plasticity by D1R signaling. (A) Time course of high-PV neuron plasticity induced upon cFC in vH CA3. (B) Time course of high-PV neuron plasticity induced upon pharmacogenetic activation of PV neurons in CA3 and prolongation of the plasticity upon local delivery of D1R agonist. (C) Two time windows after acquisition of cFC when local delivery of D1/5R antagonist to vH interferes with long-term consolidation of fear memory (tested at +24 h). (Adapted in part from Karunakaran et al. 2016.)

6 h after acquisition, and the second one between +10 and +15 h (Karunakaran et al. 2016). The first time window was closely correlated to the delay period preceding the appearance of detectable PV neuron plasticity, whereas the second one was reminiscent of a previously described time window involving immediate early gene expression and offline network activity essential for long-term memory consolidation (Fig. 4C). The unusual features of delayed onset, and prolonged persistence of the PV plasticity raise the question of what might be its roles in learning and memory.

Six-hour time window upon acquisition to define what will be consolidated

The 5- to 6-h time window after learning, when PV plasticity is not yet detectable, coincides with a time when memories of related experiences are merged into shared neuronal assemblies (Cai et al., 2016; Rashid et al., 2016). As recently reported, this corresponds to a

time window when information acquired separately, but linked through shared elements such as context or task relevant objects, is combined to determine whether and what is being learned (time units for learning) (Chowdhury and Caroni, 2018). The duration of the time units depends on cFos protein expression and function in local neuronal assemblies, which becomes detectable 45 min after acquisition, and is maintained against proteasome mediated degradation during about 4–5 h through processes depending on offline network activity within brain systems involved in that particular learning (Chowdhury and Caroni, 2018). The 5–6 h time window that precedes detectable PV plasticity might therefore reflect a time when the magnitude, and possibly also the sign (toward high- vs. low-PV), of the PV neuron plasticity might be influenced by experience. To determine whether the status of D1/5 receptor activation up to 5 h after acquisition might modulate the strength of PV plasticity and learning,

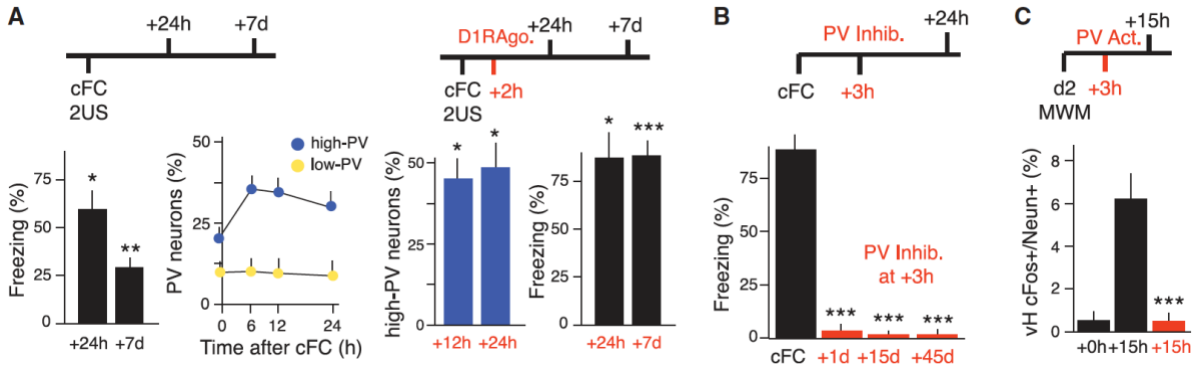


Figure 1.7 Modulation of PV plasticity and long-term memory during early time window up to 5–6 h after acquisition of cFC. (A) Strengthening of weak 2US PV plasticity and fear memory (left) upon delivery of D1R agonist to vH at +2 h (right). (B) Suppression of long-term fear memory upon local delivery of D1/5R antagonist to vH at +3 h after acquisition of cFC. Control bar: test at +24 h. (C) Suppression of vH cFos induction at +15 h induced upon water maze learning by local induction of opposite-sign PV neuron plasticity (high-PV) in vH at +3 h. (Adapted from Karunakaran et al. 2016.)

we performed experiments in mice that underwent a comparatively weak cFC protocol. The unconditioned stimulus (US) (here foot shock) is indicated in the following as US. A 2Å~US (instead of 5Å~US) protocol resulted in a comparatively modest high-PV shift in vH at +12 and +24 h after acquisition. In parallel, when compared to a 5Å~US protocol, the 2Å~US protocol produced a reduced freezing response at +24 h, and strongly reduced freezing at +7 d. Local application of D1 receptor agonist to vH at +2 h after acquisition of 2Å~US fear conditioning produced a sustained enhancement of vH high-PV contents, and freezing to context at +24 h and at +7 d was now undistinguishable to fear memory upon a 5Å~US protocol (Fig. 5A; Karunakaran et al. 2016). These and related results provided evidence that D1/5 receptor signaling during the +0–5 h time windowafter acquisition modulates the strength of subsequent

PV plasticity and fear memories. In a second approach, we pharmacogenetically imposed a low-PV shift (i.e., plasticity of opposite sign to that induced by learning) at +3 h after cFC specifically in vH PV neurons. Although memory recalled at +6.5 h was not affected, the procedure led to suppression of freezing to context (i.e., of any behaviorally detectable memory) at +24 h, and up to at least 7 wk. Likewise, when high-PV plasticity was imposed pharmacogenetically at +3 h after water maze learning (a learning processes inducing low-PV plasticity), no learning-related cFos expression was detected at +15 h, and no learning was detected behaviorally on the subsequent day (Fig. 5B,C). Therefore, although alterations in PV levels only become detectable 6 h after acquisition, PV plasticity is already modulated during the first 5 h after acquisition through endogenous D1/5 receptor signaling, providing a potential mechanism through which events occurring subsequent to the initial acquisition process might influence long-term memory consolidation processes at +12–14 h. Notably, interventions that specifically suppressed detectable learning-induced PV plasticity from +6 h on did not affect intermediate memory tested, for example, at +6.5 h. These results suggest that requirements for consolidation of short- and intermediate-term memories differ from those for long-term memories.

Twelve-hour time window for PV plasticity-dependent memory consolidation

In good agreement with reports that D1R signaling 12 h after acquisition is important for long-term consolidation and strengthening of fear memories, we found that PV neuron plasticity sustained during a +11–15 h time window is critically important for enhanced ripple densities, for the cFos peak at +15 h, and for long-term memory consolidation, causally tying the PV plasticity to the occurrence of key offline network and cell assembly processes involved in memory consolidation (Fig. 6; Karunakaran et al. 2016). We further found that local D1/5 receptor signaling at +12–14 h is specifically required in PV neurons to ensure maintenance of learning-induced PV plasticity critically important for long-term memory consolidation (Fig. 6A). Incidental learning (e.g., object recognition) not involving reinforcement but involving a second peak of cFos expression at +12 h did not show PV neuron plasticity and did not depend on D1R signaling at +12–14 h for long-term memory consolidation, suggesting that a requirement for local D1/5 receptor signaling at +12–14 h is a shared feature of reinforced learning involving PV plasticity. Mechanistically, PV basket cell recruitment enhances network activity through synchronization of principal neurons to support coordinated fast network activities such as ripples, spindles, and γ-range oscillations (Stark et al., 2014), which are critically important for long-term memory consolidation (Buzsáki, 2015). The circuit mechanisms through which opposite-sign low- and high-PV plasticity specifically ensure long-term consolidation of provisional versus definite memories remain to be determined, but one

plausible hypothesis involves distinct local networks and related neuronal assemblies in learning involving low- or high-PV plasticity (Fig. 3).

Why might long-term memory consolidation depend on organized network activity and cellular plasticity induced about 12 h after acquisition, long after known biochemical and cellular processes involved in memory consolidation (Redondo and Morris, 2011) have subsided? It is possible that slow biochemical processes affecting synaptic rearrangement processes involved in memory might require such a long time, but such a process would seem to put long-term memories at risk of interference through unrelated network activity. A further possibility could have been that the late time window might coincide with network processes during sleep, a brain state known to have an important role in memory consolidation, but direct testing of this possibility suggested that the time window occurred at the same delay from the original learning process, irrespective of the time of the day or night at which it had occurred

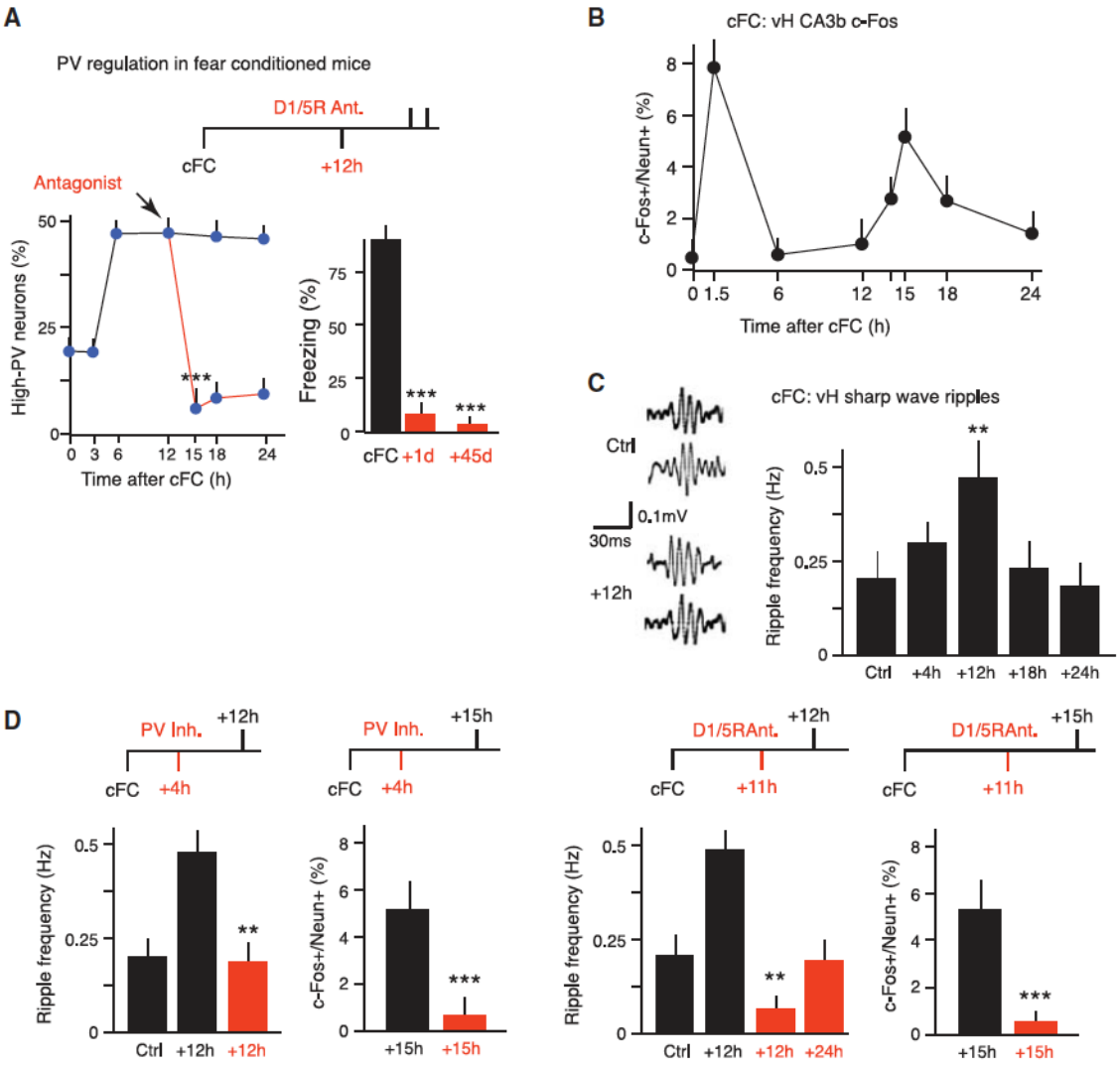


Figure 1.8 Modulation of PV plasticity, ripple activity, cFos expression, and long-term memory during late time window at +11–15 h after acquisition of cFC. (A) Rapid loss of high-PV neuron plasticity and loss of fear memory upon local delivery of D1/5R antagonist to vH 12 h after cFC. PV neuron analysis in vH at different time points upon cFC. (B,C) Time course of cFos expression (B) and sharp wave ripple induction (C) in vH upon cFC. (D) Enhanced ripple density at +12 h after cFC and cFos expression at +15 h after cFC are suppressed upon pharmacogenetic induction of low-PV plasticity (i.e., opposite sign to cFC) at +4 h (two left panels) or local delivery of D1/5R antagonist at +11 h (two right panels). All treatments and analyses were performed in vH. (Adapted from Karunakaran et al. 2016.)

(Karunakaran et al., 2016). Instead, we propose that the late time window might serve to separate learning processes occurring on any given day from the long-lasting consequences of the learning for the animal's behavior (which includes integration with previous learning and memory processes). Such a view is consistent with our recent findings that the essential role of infralimbic cortex in subsequent alternative learning (e.g., fear extinction learning) is set up during a +11–14 h time window after fear learning through circuit and systems interactions

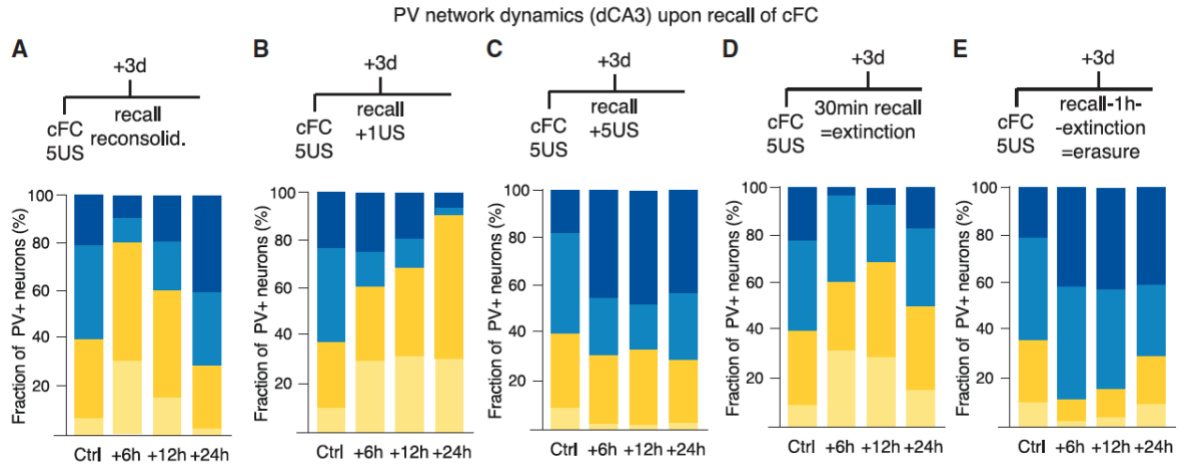


Figure 1.9 Dynamics of dH PV plasticity upon recall of cFC. All data: recall 3 d after 5US cFC; analysis in CA3b. (A–E) Analysis at +6, +12, and +24 h after recall (A), recall + 1US (B), recall + 5US (C), 30-min recall (extinction) (D), and 5-min recall followed 1 h later by 30-min recall (erasure) (E).

involving specific reciprocal connectivity between prelimbic and infralimbic cortex (Mukherjee and Caroni, 2018). Additional findings from our laboratory focusing on the dynamics of PV neuron plasticity in area CA3 of dH upon recall of fear memory under different experimental conditions provide further support for a role of the +11–14 h long-term consolidation window in consolidating behaviorally adaptive memories. Recall of fear memory (i.e., exposure to conditioned context in the absence of foot shocks) 3 d after cFC induced low-PV plasticity at +6 h, which changed to baseline-like values at +12 h, and to high-PV plasticity at +24 h (Fig. 7A). Interfering with the PV plasticity at +12 h prevented detectable high-PV plasticity at +24

h and produced a permanent loss of the fear memory, suggesting that the late shift to high-PV plasticity was necessary for reconsolidation of the fear memory. When recall was paired to 1US instead of the 5US during fear conditioning, the low-PV plasticity persisted through the 12-h time window and had become more robust at +24 h (Fig. 7B). In contrast, if recall was paired to 5US, high-PV plasticity was induced at +6 h, and the plasticity lasted at least until +24 h (Fig. 7C). These and additional findings suggest that experience deviating from expectations (no US or only 1US instead of 5US) initially induces low-PV plasticity, which is followed by a reassessment of the new learning to either reconsolidate the original fear learning (no US), or to prepare for possible further learning (1US). In further related experiments, and extinction protocol (30 min of recall in the absence of US) induced low-PV plasticity at +6 h, which persisted through the +12-h time window (Fig. 7D). In stark contrast, an erasure protocol (5 min recall followed 1 h later by a 30-min extinction protocol) induced high-PV plasticity at +6 h, which persisted up to and beyond +24 h (Fig. 7E).Within the context of learning related PV plasticity, extinction represents a transient adjustment to absence of US (i.e., provisional learning), whereas erasure leads to a permanent loss of the association between context and fear (i.e., definite learning). Taken together, these findings provide evidence that PV neuron plasticity induced upon experience, and detected at +6 h, can be subsequently modified without further behavioral experience during the +11–15-h time window of long-term memory consolidation, leading to PV neuron plasticity that reflects the long-term behavioral consequences of the experience. With other words, and consistent with our hypothesis, the late time window for memory consolidation appears to reflect a time when local plasticity is readjusted through offline network activity to match the corresponding long-term memory.

Conclusion

PV basket cells, the most abundant local inhibitory interneurons in the brain, show dramatic cellular and structural synaptic plasticity upon reinforced learning. The plasticity comes in two flavors: one (“high-PV”) that leads to increased expression of PV neuron functional markers such as PV and GAD67 (Lazarus et al., 2015; Volman et al., 2011) and increased densities of excitatory synaptic puncta onto PV neurons, and one (“low-PV”) that leads to decreased expression of PV/GAD67 and increased densities of inhibitory synaptic puncta onto PV neurons. High-PV plasticity is implemented by early-born PV neurons, whose plasticity is induced by changes in PV neuron excitation, whereas low-PV plasticity is implemented by late-born PV neurons, whose plasticity is induced by changes in PV neuron inhibition. Low-PV plasticity is specifically induced upon provisional reinforced learning, whereas high-PV plasticity is specifically induced upon definite reinforced learning. PV neuron plasticity

becomes detectable at the end of time units for learning, once experience and signaling collected during 5–6 h upon initial acquisition determine whether and what will be learned. Notably, the PV plasticity is critically important for long term consolidation of reinforced memories during a time window +11–15 h after acquisition. The mechanisms through which PV plasticity ensure long-term memory consolidation appear to involve support of offline network activity specifically required for enhanced ripple activity and immediate early gene expression in neuronal assemblies thought to encode the corresponding memories (Ognjanovski et al., 2017; Rothschild et al., 2017; Singer and Frank, 2009; van de Ven et al., 2016; Xia et al., 2017). Long term consolidation of nonreinforced (incidental) memories also depends on a time window around +12 h, when cFos in corresponding assemblies needs to be re-expressed, but the memories do not depend on local D1R signaling and PV neuron plasticity for long-term consolidation. These findings suggest the existence of fundamental differences between reinforced and nonreinforced memories and their consolidation, possibly reflecting the more consequential impact of reinforced memories for behavior. Enhanced functionality of early-born PV neuron networks upon definite learning might promote consolidation of strong memories within and between brain systems. Such a mechanism might account for the enhanced γ-phase coupling between entorhinal cortex and hippocampal CA1 that was detected during late phases of hippocampal learning (Igarashi et al., 2014). Selective connectivity to and from specific functional subpopulations of principal neurons might ensure specific stabilization of neuronal assemblies encoding definite learning memories. Likewise, reduced functionality of late-born PV neuron networks might be permissive to bind assemblies of more flexible and possibly less strongly interconnected neuronal assemblies. This form of plasticity appears to be the counterpart of critical period plasticity and of how EE supports learning and brain function (Pizzorusso et al., 2002). Like for definite learning assemblies, provisional memory assemblies likely involve dedicated subpopulations of principal neurons and specific connectivity to and from early-born PV neurons. These findings raise a number of questions for further research. One of them involves the likely prospect that provisional and definite learning might be implemented through partially separate circuits within and possibly also between brain areas. Identifying the subpopulations of neurons and the connectivity principles involved in such functional subcircuits will likely provide fundamental insights into how the brain deals with coexisting variability and stability in learning and memory (Clopath et al., 2017; Grosmark and Buzsáki, 2016). A second important question involves the systems mechanisms for memory consolidation during the late time window (+11–15 h) after acquisition. Elucidating principles of how previous knowledge might be integrated with recent memories during offline network activity to support adaptive learning and behavior will not only provide important advances into mechanisms of learning and memory, but might also provide valuable entry points to begin to investigate mechanisms of long-term systems consolidation

in the brain. Finally, it might be useful to consider the possibility that long-term cellular plasticity involved in learning and memory might not be exclusive to PV basket cells.

1.3.3 Role of Fast-Spiking PV Interneurons in Network Activity

Networks of PV basket cells contribute in regulating local ensemble and network activity primarily by orchestrating oscillations. Two essential properties of PV interneurons are critical for the generation of oscillations: first, basket cells make numerous reciprocal inhibitory connections onto each other and with a large fraction of the neighboring principal cells (Bartos et al., 2007). Second, PV cells are electrically coupled via gap junctions, thus allowing them to be synchronized with millisecond accuracy (Hestrin and Galarreta, 2005). Excitatory inputs depolarize downstream regions more effectively when they are active synchronously, therefore oscillations allow neurons to cooperatively depolarize common downstream targets, thus resulting in more effective signal transduction. Moreover, the cells in distal brain regions that are synchronously firing together are more prone to undergo classical Hebbian plasticity mechanisms that will strengthen their synapses, making them more likely to fire together in the future (explained in more details below). Various oscillation frequencies in the hippocampus have been associated with particular brain states or behavioral commitments and PV interneurons have been shown to be critically important for the generation of hippocampal oscillations (Roux and Buzsáki, 2015). Of particular relevance for memory processes in the hippocampus are theta (4-12 Hz) and gamma oscillations, these are further divided into low-gamma (30-48 Hz) and high gamma (52-90 Hz) oscillations. Simultaneous activity at different frequencies can be coincident in the hippocampus and is generally believed that lower (theta) oscillation can modulate the power of higher (gamma) frequency oscillations (Hartley et al., 2014). Such modulation correlate with memory performance in both rats (Tort et al., 2009) and humans (Canolty et al., 2006). Theta oscillations have long been considered the "on line" state of the hippocampus and in CA1 are generated by multiple mechanisms that include inputs from the medial and lateral septum, the entorhinal cortex and various circuit and intrinsic properties of classes of neurons (Buzsáki, 2002). Theta is particularly prominent during active exploration and memory guided behaviour and has been proposed to orchestrate the clustering of principal cell spiking into functional ensemble by creating discrete time windows during which incoming information is processed (Buzsáki and Moser, 2013; Lopes-dos-Santos et al., 2018). PV positive basket cells have been shown to coordinate the spike timing of hippocampal pyramidal neurons in the theta band both *in vitro* (Amilhon et al., 2015) and *in vivo* (Cobb et al., 1995) . In particular, it has been shown that optogenetic stimulation of PV+ cells at theta frequency causes theta resonance in CA1 pyramidal cells (Stark et al., 2013). Furthermore, removing $GABA_A$ receptors from PV neurons, therefore disrupting the

entire PV network which relies on recurrent inhibition from neighboring PV cells and from inhibitory septal projections, strongly reduces the theta rhythm and its coupling to the gamma frequencies (Wulff et al., 2009). Field potential oscillations in the gamma frequency, on the other hand, are believed to play important roles in information transfer between synaptically-connected distal brain regions. For example, it has been shown that hippocampal CA1 can be selectively coupled to signals from the CA3 or the entorhinal cortex by switching to gamma oscillation at different frequencies existing in these structures (Colgin et al., 2009). Through similar mechanisms, gamma oscillations are suggested to orchestrate the integration of information processed in distinct brain regions, by "binding" neuronal ensembles that oscillate in phase (Lasztóczi and Klausberger, 2014). Like for the theta rhythm, also gamma is highly dependent on PV interneuron function. It has been shown that entrainment of PV neurons at gamma frequency can generate gamma oscillation in somatosensory and visual cortex. Conversely, optogenetic inhibition has been shown to reduce gamma oscillation (Cardin et al., 2009; Iaccarino et al., 2016; Sohal et al., 2009).

1.3.4 Principal Cell Diversity in Hippocampal CA1

Hippocampal CA1 is the only output of the hippocampus proper, it receives its main input projections from the entorhinal cortex and from the CA3 region and sends output connectivity to the subiculum and the entorhinal cortex, although additional projections to nucleus accumbens, amygdala and medial prefontal cortex arise from its ventral subdivision (Ciocchi et al., 2015). Some of the functions that have been proposed for CA1 include novelty detection, context decoding and the redistribution of information from the hippocampus proper to a greater number of cortical regions; in this framework CA1 is thought to be pairing the heavily decorrelated ensembles stored in CA3 with information-rich memory representations, prompting the hippocampal circuit during memory recall (Allen et al., 2016; Kaifosh and Losonczy, 2016; Leutgeb et al., 2004; Treves and Rolls, 1994). Recent technological advances have uncovered the existence of a high degree of heterogeneity in pyramidal cells of CA1 both in the proximo-distal and in the radial axes (Cembrowski and Spruston, 2019). In particular, morphological heterogeneity in the radial axes was already observed more than eighty years ago (Lorente De Nó, 1934) and as of today is the best understood example of excitatory cell diversity within a hippocampal sub region (Soltesz and Losonczy, 2018). Cells in the radial axes of hippocampal CA1 are named deep and superficial principal cells (PCs) because of differences in the timing of neurogenesis and the fact that the hippocampus develops as an invagination of the developing cortex that follows an inside-out pattern of development. Therefore, deep cells correspond to the deep cortical layers, develop earlier

during embryogenesis (E14) and are located closer to the cortex, whereas superficial cells correspond to superficial layers of the cortex, arise later during development (E18) and are located closer to the dentate gyrus (Slomianka et al., 2011). Associated with the difference in the schedule of neurogenesis are also molecular, morphological, physiological and differences in connectivity. Molecular markers such as calbindin have been used to identify superficial PCs (Baimbridge et al., 1991) but numerous differentially expressed genes have also been identified in the two layers (Cembrowski et al., 2016). Also, the two subpopulations display slightly different morphology, with deep cells having a larger soma and more complex basal dendritic ramifications than superficial PCs, and superficial PCs exhibiting a more depolarized somatic resting membrane potential (Lee et al., 2014). Recent studies have focused on the functional role that deep and superficial PCs play in supporting behaviour. Ca^{2+} imaging in head fixed mice running on an enriched treadmill has been used to observe the place dynamics of the two subpopulations during exploration and learning over a span of days. During exploration, superficial PCs formed more stable spatial maps of the environment over multiple time scales, whereas the deep PCs, even though generally active in forming place-coding responses than the superficial PCs, were more prone to remapping and less stable when features of the context (auditory, tactile, visual and olfactory) were changed (Danielson et al., 2016). In a conceptually similar experiment, using simultaneous electrophysiological recording from the two layers in head-fixed mice running on a landmark-rich treadmill, it has been shown that deep PCs have firing fields highly correlated with landmarks position. Indeed, rearrangements in the position of the landmarks caused an instantaneous remapping in deep PCs that were reported to be tightly linked to their saliency and identity, whereas superficial PCs have been shown to have single, stable firing fields that were highly context specific and only rarely reacted with slow dynamics to the relocation of landmarks (Geiller et al., 2017). Importantly, only deep PCs were modulated during a goal-directed learning paradigm (learning of a narrow reward zone on the enriched treadmill). The presence of the reward zone tended to stabilize the place maps in deep cells to the point that the representation of the reward zone by deep cells became predictive of task performance to a much greater extent than superficial PCs (Danielson et al., 2016). Also, it has been recently shown that calbindin-expressing superficial PCs exhibit selective spiking responses to odor cues and support olfactory associative learning (Li et al., 2017). In conclusion, emerging from these recent finding and others is the view that superficial PCs are more modulated by sensory cues (odors) during the initial phases of learning and are therefore important to discriminate - and store memories of - the context. Nested inside the global context are landmarks and locations that are more dynamically and efficiently encoded by the activity of deep PCs that, in this view, support a more stable representation during the later phases of learning and can adapt to changes in the context. Together, these studies provide strong evidence for the existence of

distinct microcircuits supporting in parallel distinct types of memories, which can be the substrate for the ability of the hippocampus to balance the stability and the plasticity required to adapt to changes in the environment (Overington and Jeffery, 2016).

How do these excitatory sub circuits integrate with the hippocampal GABAergic microcircuit? Recent work has explored in detail the weight of perisomatic inhibition originating from CA1 PV basket cells, uncovering a biased inhibitory influence on the two excitatory sub circuits. In particular, by injecting currents in PV cells and simultaneously recording from deep and superficial PCs, it has been shown that PV basket cells in hippocampal CA1 generate inhibitory postsynaptic currents (IPSCs) three times greater in deep PCs. Even though immunohistochemistry approaches revealed a higher number of PV+ boutons innervating the soma of deep PCs compared to superficial, the electrophysiological connection probability between pairs of PV neurons and PCs was not biased for one of the two layers. Importantly tough, this bias was found when analyzing connectivity in the reverse order: the connection probability between superficial PCs and PV neurons was shown to be three times higher compared to deep PCs to PV neurons, demonstrating that superficial PCs provide more excitatory connections to PV BC compared to deep PCs. In summary, the PCs population that received the weakest inhibition from PV cells provided more excitation to these neurons, suggesting the existence of a local microcircuit that might specifically direct PV-mediated inhibition from superficial PCs to deep PCs (Lee et al., 2014).

1.4 Memory - A Cellular Perspective

1.4.1 Synaptic Plasticity and Gene Expression

How does the above described cellular heterogeneity of the hippocampal system contribute to the ability to encode and store memories of past events?

The current understanding is that neurons have evolved multiple coordinated mechanisms that allow them to concomitantly respond to changes in the environment dynamically and to store memories permanently. At the molecular level, the fundamental adaptation that enabled to retain information on longer time scales has been the coupling of the synaptic signaling to the nucleus. I will first describe the synaptic mechanisms responsible for the retention of information for short periods of time (up to hours) and then I will describe how the expression of so called activity-dependent genes is related to long-term memory storage. Interestingly, most of the molecular pathways that are activated in response to neuronal activity and that act as substrates for memory processes seems to be shared among different neuronal cell types immediately following stimulation (within 60 min) but at later time points (>120 min) become increasingly divergent in different classes, pointing to the importance of cell type

classification for the understanding of memory processes (Sanz et al., 2009; Spiegel et al., 2014; Yap and Greenberg, 2018).

1.4.2 LTP - The Synapse

One of the most important discoveries in the field of learning and memory, and in neuroscience at large, are the mechanisms that allow for Long Term Potentiation (LTP) of synapses, first described in the rabbit dentate gyrus (Bliss and Collingridge, 1993; Bliss and Lomo, 1973). LTP consist in the persistent strengthening of synapses which is dependent on previous patterning of activity not only of the presynaptic but also of the postsynaptic neuron. Different forms of LTP can be distinguished on the basis of the different signaling pathways they operate on and different brain circuits vary in the mechanisms implemented for such synaptic potentiation (Malenka and Bear, 2004). Hippocampal LTP is strongly dependent on NMDARs, a specific type of ionotropic glutamate receptor with the unique peculiarity of being both ligand and voltage gated. In NMDA-receptor dependent LTP, presynaptic glutamate release activate at first AMPA receptors. NMDARs are located in close proximity to AMPA receptors, but are not activated by low levels of glutamate which is instead sufficient to activate AMPA receptors. This is because the ion pore of NMDARs is blocked by a single magnesium ion at resting membrane potential. In the case of recurrent action potentials causing greater activation of AMPA receptors, the resulting postsynaptic neuron depolarization will eventually cause the voltage-dependent magnesium blockage to be removed by electrostatic interactions, allowing Na^{+}, but most importantly Ca^{2+} to flow through the NMDA receptor. This influx of Ca^{2+} initiate a long chain of signaling mechanisms that begins with more AMPA receptors being inserted in the postsynaptic membrane of the neuron, making it more likely to be activated by a subsequent and equal presynaptic release of glutamate (Kandel et al., 2013). As a consequence, only when the activation of the presynaptic neuron will coincide with the depolarization of the postsynaptic neurons, LTP will increase the probability and the strength of future synaptic connection between the two neurons.

This sophisticated mechanism provides the physical substrate in support of the popular Hebb's postulate of synaptic plasticity, summarized by the maxim that "cells that fire together wire together", and also suggests that memories might be allocated to specific ensembles neurons that were coactive at the time of memory encoding (“Hebb, D. O. Organization of behavior. New York,” 1950).

Early pharmacological blockade experiments of NMDA receptors in the hippocampus supported the notion that LTP is essential for spatial learning (Morris et al., 1986). A more targeted approach later identified the NMDARs in the CA1 subfield of the hippocampus as crucial for associative long-term spatial memory and showed that the genetic removal of the NR1 subunit from CA1 principal cells results in disrupted place fields and poor spatial memory

performances (Martin et al., 2000; McHugh et al., 1996). More recent optogenetic approaches have provided more significant causal link between LTP and memory by showing that rats conditioned to associate a foot shock with optogenetic stimulation of auditory inputs to the amygdala could have this memory bidirectionally activated or inactivated by high- or low-frequency optogenetic stimulation of the same auditory input (Nabavi et al., 2014). These and other experimental evidences support the notion that long-lasting synaptic changes are not only a by-product of neuronal activity, but rather serve as physical traces of a memory in neuronal networks (McNaughton and Morris, 1987; Neves et al., 2008).

1.4.3 IEG - The Nucleus

An additional consequence of NMDA receptor activation, and the subsequent increase in Ca^{2+} concentration in the synapse, is a cascade of second messengers that, beside upregulating the incorporation of AMPA glutamate receptors in the membrane (Hayashi et al., 2000; Ramachandran and Frey, 2009), initiate a wide range of signaling pathways, also dependent on increased Ca^{2+} concentration such as PKC, PKA, MAPK and CaMKII that play an important role in the stabilization of this early-phase LTP (E-LTP) (Lisman et al., 2012; Malenka and Bear, 2004). A subsequent late-phase (L-LTP) can be distinguished based on its dependency on protein synthesis. L-LTP is the results of the activation of transcription factors, chromatin remodelers and synaptic proteins as well as structural changes such as elimination of old and addition of new spines (Abel et al., 1997; Caroni et al., 2012; English and Sweatt, 1997; Silva et al., 1992). Collectively, these mechanisms are at the foundation of a phenomenon called memory consolidation that is responsible for the transition from short-term to long-term memories. Among the classes of genes that are transcribed within minutes from stimulation are Immediate Early Genes (IEGs) which are a class of genes that responds rapidly and transiently to a variety of cellular stimuli with more than 100 classified members, although only a small group is found in neurons (Minatohara et al., 2015).

cFos and Arc are the most well studied IEGs found in the brain, but many other have been recently investigated (DeNardo and Luo, 2017). Many IEGs encode DNA-binding proteins that function as transcription factors (TFs) and regulate a subsequent wave of late response gene expression, which is highly specific for the particular cell type in the activated neuronal network. Essentially, IEGs and the multitude of interacting molecules with which they form complexes act as combinatorial integrators of different molecular cascade inside individual neurons. I will now describe more in detail *cFos*, which provided the first evidence that mammalian cells could adapt their transcriptional profile to adapt to changes in the environment within minutes.

1.4.4 cFos

The canonical immediate early gene cFos has a rapid and transient induction of transcription that follows stimulation of many cell types (Greenberg and Ziff, 1984). As mention above also cFos induction, like all known IEGs, is dependent upon a neurotransmitter-mediated influx of extracellular Ca^{2+} which triggers a cascade of signaling events, including the activation of Ras-mitogen-associated protein kinases MAPK, calcium/calmodulin dependent protein kinases CaMKs and calcineurin-dependent signaling pathways (Bito et al., 1996; Hardingham et al., 1997). These pathways, in turns, can result in the activation of constitutively expressed transcription factors such as the cyclic adenosine monophosphate (cAMP)-responsive element binding protein (CREB) and serum response factor (SRF) which in turn control cFos transcription (Norman et al., 1988; Sheng et al., 1988). The cFos mRNA encode a nuclear protein (Curran et al., 1984) that together with its partner Jun forms the major heterodimer of the activating protein complex 1 (AP-1), able to bind DNA at an estimated 10^4 binding sites and regulates an estimated 3-500 late response genes that typically encode proteins that play roles in dendritic growth, spine maturation, synapses rearrangements as well as the expression of membrane channels and receptors (Benito and Barco, 2015; Mardinly et al., 2016; West et al., 2001; Yap and Greenberg, 2018). Contrary to what initially proposed Fos/Jun heterodimer do not bind to the promoter of their target genes but instead to the distal enhancer elements (Malik et al., 2014), being therefore well positioned to control gene expression in a cell-type specific manner (Heintzman et al., 2009).

Many lines of evidence point to the fact that cFos expression is not merely correlated with neuronal activation but instead plays a key role in memory consolidation. Intrahippocampal administration of cFos antisense nucleotides has been shown to impair memory consolidation without affecting short-term memory (Guzowski, 2002). Moreover the CNS-specific KO of the cFos gene similarly results in impairments of hippocampal-dependent spatial and fear memory and reductions in LTP (Fleischmann et al., 2003).

The discovery of the above-described molecular and cellular mechanisms that underlie learning and memory have been a major goal of the past decades and much progress has been made in the understanding of the contribution of synaptic plasticity and rearrangements of genome organization in supporting long lasting memories. In the next session I will briefly describe a different scale of biological organization: local ensembles of cells and multiregional networks that together represents the physical substrate of memories, the so-called memory engram.

1.5 Memory - A Network Perspective

Memory Allocation

As initially postulated by Hebb and later supported by several lines of evidence, a fraction of the neurons that are recruited together and fire synchronously during the exposition to a salient experience tend to form the neuronal assembly that encodes that particular memory (Josselyn et al., 2015; Tonegawa et al., 2015). This, together with the observation that the above-described mechanisms for LTP and the expression of IEGs necessary for memory consolidation are induced only in a fraction of the neurons in a given neuronal circuit led to a very recent line of research that is aiming at further clarifying the role of the ensemble of neurons defined by the expression of IEGs as repository of memory. Recent technological approaches have allowed experimental access to neuronal assembly, mainly by exploiting the promoter of different IEGs (mostly Arc and cFos). These approaches rely on the generation of transgenic mice in which IEG coding sequences have been replaced by a variety of "effector proteins" whose activity allows for the "tagging" (i.e. they confer permanent genetic access) of the neurons of a particular ensemble under inducible timing control (DeNardo and Luo, 2017; DeNardo et al., 2019; Guenthner et al., 2013). It is however important to bear in mind that there might not be a perfect correspondence between the expression of a particular IEG through which ensemble neurons are interrogated in the vast majority of experimental approaches and the entire population of neurons representing a memory ensemble and elucidating this relationship is still a major unsolved question. It is also important to mention that recent evidences have shown that expression of cFos and Arc does not simply reflect previous firing activity but instead is informative of the induction of plasticity mechanisms in a subset of active neurons (Holtmaat and Caroni, 2016).

The evidences that provide characteristics of both necessity and sufficiency to the hypothesis of neuronal ensembles as carriers of memories come from loss and gain of function studies respectively: it has been shown that tagging the hippocampal neuronal population that express the IEG Arc during contextual fear conditioning and subsequently inhibiting these cells during recall using optogenetics leads to impairments of fear memory recall (Denny et al., 2014). A similar memory impairment has been observed in another study in which the hippocampal ensemble was tagged at acquisition of contextual fear conditioning and silenced during recall. Notably, this study also shows that silencing the hippocampal tagged neurons prevents the reactivation of cortical regions that are known to be important for context memory, suggesting that locals ensembles are able to influence each other through long range connectivity (Tanaka et al., 2014). Conversely, reactivating tagged hippocampal contextual fear conditioning ensembles with ChR2 can elicit memory recall in neutral contexts upon light stimulation (Liu et al., 2012) and, similarly, pairing contextual fear conditioning with the

reactivation of neurons that had previously been tagged during the exploration of a neutral environment leads to the formation of a "false" memory association, with mice displaying freezing in the previously neutral context (Ramirez et al., 2013).

But how are these neurons selected from the pool of active neurons at the time of the experience? The answer to this question involves the understanding of the activity dynamics (pre, during and post learning) that ultimately lead to the incorporation of selected neurons in specific ensembles: a process referred known as memory allocation (Holtmaat and Caroni, 2016; Lisman et al., 2018).

It has been shown that increasing the excitability of a small fraction of randomly targeted neurons by chemogenetics activation or by overexpression of CREB before learning increases the probability that those cells will be incorporated into the relative memory ensemble (Dong et al., 2006; Garner et al., 2012; Yiu et al., 2014; Zhou et al., 2009). Importantly, the size of the experimentally induced ensembles are comparable to the physiologically induced ones, suggesting the existence of lateral inhibition mechanisms that keep engrams at a relative stable sizes. However, the circuit mechanisms required to link learning-related neuronal activity to cFos and other IEGs expression remain to be identified, although some recent studies provided some indication about possible mechanisms (Tanaka et al., 2018).

A deeper understanding of the network dynamics and the identity of the circuit elements that ultimately support the expression of IEGs in the correct subset of neurons are major future goals that will allow for a better understanding of learning and memory processes.

1.6 Aim and Rationale of the Thesis

Evidence from our lab and others have pointed to the existence of parallel excitatory microcircuits operating in the hippocampus composed of cells with similar schedules of neurogenesis and preferential synaptic contact (Deguchi et al., 2011). One particularly well-defined example can be appreciated on the radial axis of CA1 and is composed by deep and superficial principal cells (Soltesz and Losonczy, 2018). These two layers have been characterized at the molecular, morphological and functional level and support functionally distinct representation of the environment during spatial navigation and learning (Danielson et al., 2016). Importantly, inhibition provided by parvalbumin basket cells is not homogeneously distributed in the deep and superficial layer, with deep cells receiving inhibitory post synaptic currents three times wider compared to superficial cells (Lee et al., 2014). This difference could derive from the preferential targeting of the deep layer from a homogeneous population of PV neurons or, alternatively, from the existence of dedicated, heterogenous subpopulations of PV cells possessing different levels of inhibitory capability and sublayer selective connectivity. Indeed, the existence of subpopulations of parvalbumin positive basket cells with different regulation and roles in learning as well as biased connectivity to hippocampal CA1 sublayers has recently been described by our Lab, suggesting the existence of dedicated microcircuits supporting distinct aspects of learning and memory (Donato et al., 2015). So far, the in vivo manipulation of these microcircuits and the further characterization of the subpopulation-specific contribution to the High and Low PV network configurations has not been possible due to the lack of consistent genetic access to the subpopulations of parvalbumin interneurons. In this thesis, I set to address the following questions: do early and late born PV neurons have different molecular profiles? Is it possible to identify subpopulation-specific marker genes that allow for selective genetic access in the hippocampal system? Do early- and late-born PV neurons have differential input and output connectivity and how do they integrate with the existing heterogeneity of hippocampal PCs? What are the effects of the subpopulation-specific activity perturbation during memory acquisition on the emergence of local memory ensemble and memory performance?

To answer these questions I combined in utero retroviral targeting, RNA-seq, generation of Cre lines and functional chemogenetic manipulation during different types of learning. I identified **a)** families of differentially expressed genes that suggest functional segregation **b)** differential output connectivity to subpopulations of CA1 PCs and **c)** marker genes for the selective manipulation of the early-born PV basket cells. Early-born selective manipulation during memory acquisition caused unexpected effects on the size of memory ensemble, suggesting complex interplay between early- and late-born PV neurons, and was able to

bidirectionally shift the network configuration in the hippocampus. Overall my results further supports previous findings on the subpopulation-specific roles of early- and late-born PV cells during definite and provisional learning respectively.

2. Results

2.1 In-Utero Retroviral Targeting of Early- and Late-born PV Basket Cells

In order to identify molecular markers that could allow for genetic access to the two subpopulations of PV expressing basket cells I performed a screen of early- and late-born PV neuron transcriptomes. To accomplish this end, I first had to devise a strategy to tag the two subpopulations in adult animals. Early- and late-born PV neurons have previously been targeted by injecting synthetic nucleosides analogous of thymidine, such as Bromodeoxyuridin (BrdU), intraperitoneally into pregnant mothers during two separate windows of embryonic development: E9-E11 for early born neurons and E13-E15 for late born neurons (Donato et al., 2015). These nucleosides analogous are found in the circulatory system for a few hours after the intraperitoneal injection and during this time they can be stably incorporated into newly synthesized DNA of actively mitotic neuronal precursors that give rise to postmitotic neurons where they persist for the entire life of the animal. The detection of these molecules, however, requires denaturation of the DNA, usually achieved through exposure to acid or heat for prolonged periods of time, that would cause degradation of RNA, making it impossible to be used for the identification of markers. Retroviruses on the other hand, although less efficient in the yield of labelled cells, have been widely deployed for the targeting of actively mitotic cells (Ciceri et al., 2013; Harwell et al., 2015; Mayer et al., 2015). Differently from HIV-1 and other lentiviruses that can infect both dividing and non-dividing cells, Moloney murine leukemia virus (MoMLV) are the only retroviral class that requires the nuclear membrane to break down during cell division in order to access the nucleus, where they are retrotranscribed and stably integrated into the host genome (Suzuki and Craigie, 2007). I therefore decided to combine MoMLV vectors with mouse genetics in order to reliably target early and late-born PV neurons differentially. A MoMLV retroviral vector containing a Cre recombinase-dependent expression system carrying a reversed and double-floxed sequence coding for a membrane-bound mCherry under the control of the strong chicken β-actin promoter CAG **(Fig 2.1A)** was used to produce retroviral vectors (rv:CAG-FLEX-mCherry). To restrict the expression of the fluorescent marker in PV+ cells, injections were made in PV-Cre positive embryos, therefore limiting the fluorescent labelling to the portion of PV neurons that was still mitotically active at the time of the injection, but not postmitotic PV neurons generated before or after. PV neurons arise by pools of progenitor cells located in a transitory structure of the subpallium called Medial Ganglionic Eminence (MGE) during a protracted window of neurogenesis that starts around E9 and ends around E15. From there, post-mitotic neurons migrate into cortical and subcortical regions were they integrate in local circuits (Kepecs and Fishell, 2014). Ultrasound-guided intraventricular injections of high-titer retroviral particles were performed in parallel in E9.5 and E13.5 embryos and led to a robust

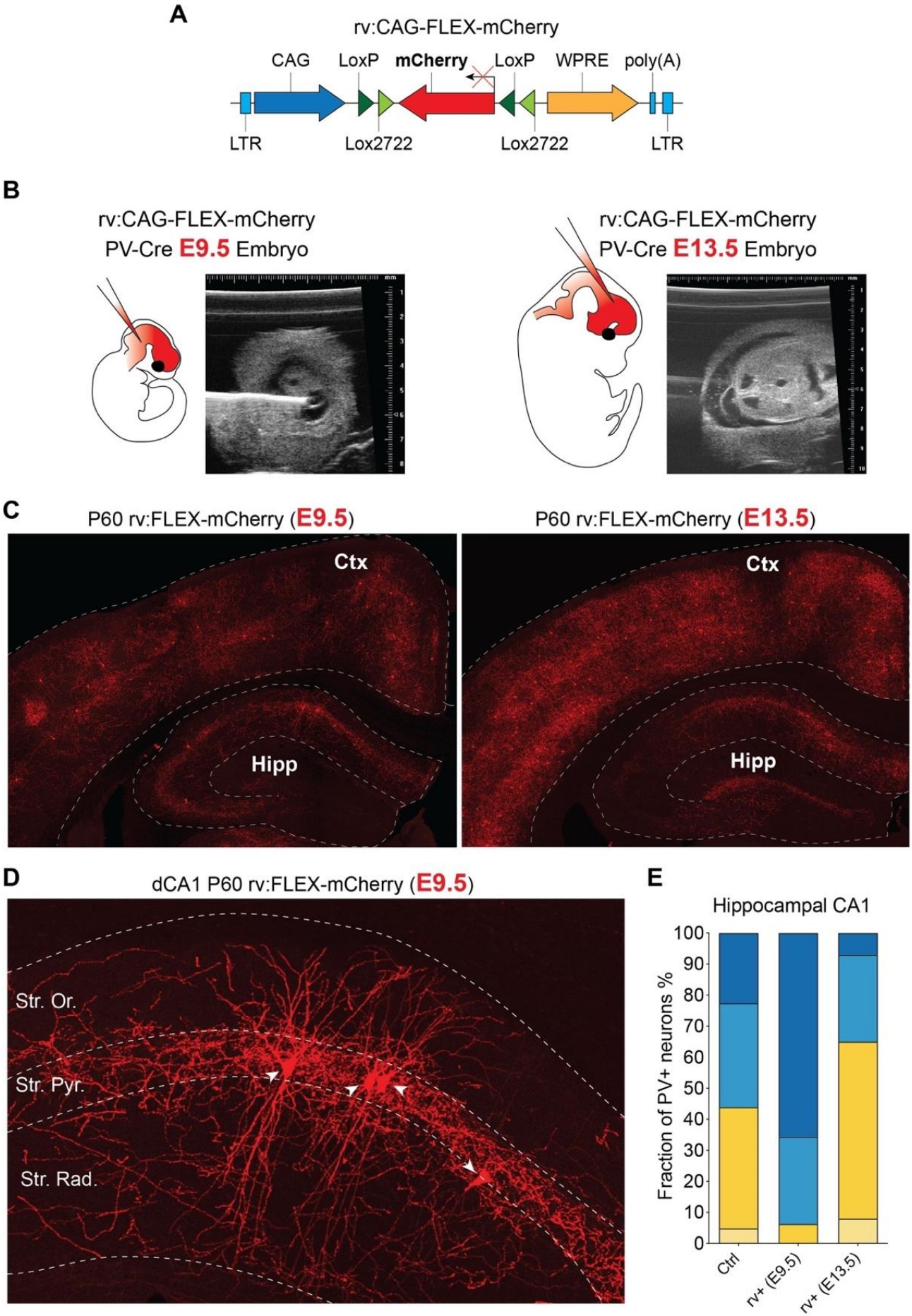

A
rv:CAG-FLEX-mCherry
CAG
LoxP
mCherry
LoxP
WPRE
poly(A)
LTR
Lox2722
Lox2722
LTR
B
rv:CAG-FLEX-mCherry
PV-Cre E9.5 Embryo
rv:CAG-FLEX-mCherry
PV-Cre E13.5 Embryo
C
P60 rv:FLEX-mCherry (E9.5)
Ctx
Hipp
P60 rv:FLEX-mCherry (E13.5)
Ctx
Hipp
D
dCA1 P60 rv:FLEX-mCherry (E9.5)
Str. Or.
Str. Pyr.
Str. Rad.
E
Hippocampal CA1
Fraction of PV+ neurons %
100
90
80
70
60
50
40
30
20
10
0
Ctrl
rv+ (E9.5)
rv+ (E13.5)

Figure 2.1: In utero retroviral targeting of early and late born PV cells. **A)** Schematic showing the structure of the retroviral vector used to produce rv:CAG-FLEX-mCherry particles. **B)** Experimental model and example images from ultrasound imaging of parallel injection into E9.5 and E13.5 PV-Cre embryos. **C)** Coronal sections of the telencephalon of a P60 PV-Cre mouse infected with high-titer Cre-dependent retroviruses at E9.5 and E13.5. **D)** High magnification of four retrovirally labeled PV neurons in Hippocampal CA1 (white arrowheads) with dentrides spanning in the stratum oriens and radiatum and charachteristic basket-like axonal projection making perisomatic conctact in the stratum pyramidale, where the targeted Principal Neurons resides. **E)** Quantification of PV immunoreactivity of control mice (Ctrl; n=4, 100 neurons per mouse) or retrovirally labelled mice injected during embryonic development at E9.5 or E13.5 (n=3, 25 neurons per mouse) Dark Blue: high-PV, Light Blue: Intermediate high-PV, Dark Yellow: Intermediate low-PV, Light Yellow: low-PV .

and highly specific labelling of PV neurons of adult mice **(Fig 2.1B, C, D)**. As a control, wild-type embryos were injected at similar embryonic stages and, as expected, did not contain retrovirally labelled cells at P60 (not shown). Retrovirally-labelled PV neurons in hippocampal CA1 exhibited the classical basket cell morphology with characteristic axonal arborizations in the stratum pyramidale and multiple dendrites crossing layers **(Fig. 2.1D)** (Tukker et al., 2013). Consistent with previous findings from our lab (Donato et al., 2015), the quantification of the PV immunoreactivity in retrovirally-labelled cells (rv+) indicated that the population of neurons labelled at E9.5 was consistently enriched in high-PV expressing cells whereas, the population labelled at E13.5 was enriched in low-PV expressing cells **(Fig. 2.1E)**. This result indicates that the devised retroviral approach can reliably label early and late born PV neurons, also suggesting the existence of dedicated pools of progenitors that develops at different time points during development.

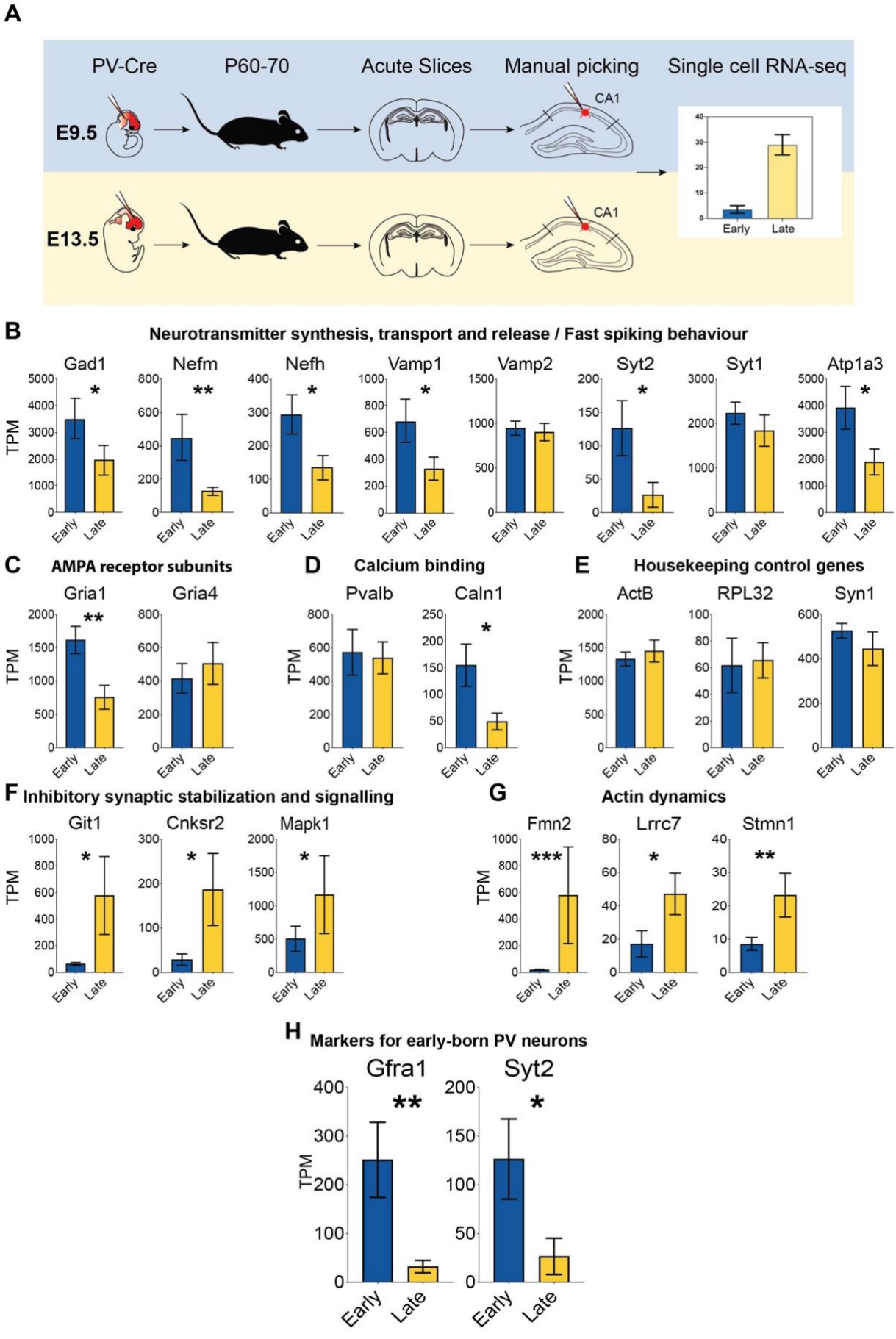
2.2 Single-cell RNA-seq of Early- and Late-born PV Neurons

Once labelled with retroviruses, early and late born PV cells permanently express a fluorescent marker compatible with RNA extraction procedures. I therefore set out to explore the transcriptional profile of the two subpopulation in order to identify suitable molecular markers for the in-vivo manipulation in behaving mice. Due to the relative scarcity of the labelled cells pool in the CA1 region of the hippocampus, the high yield of conventional approaches for the dissection and isolation of identified cell types such as FACS sorting or microfluidic devices was not balanced by the caveats represented by the harsh treatments necessary for the tissue dissection and the long time required for the cell harvest. I therefore decided to manually isolate single cells using a glass microcapillary directly from acute brain slices in order to better preserve the physiological brain environment and, consequently, the fidelity of the transcriptomes **(Fig 2.2A)**. A total of 22 single, retrovirally-labelled cells (11 from

E9.5 injected animals and 11 from E13.5) were manually aspirated from hippocampal CA1 and processed for RNA-seq using the Smart-seq2 protocol (Picelli et al., 2014). Some of the most interesting differentially expressed genes are illustrated in **Fig 2.2**. Consistent with previous results obtained in our laboratory (Donato et al., 2013), early-born PV neurons expressed higher levels of *Gad1* (**Fig 2.2B**), the transcript coding for the GAD67 protein, responsible for the synthesis of the neurotransmitter GABA. On the contrary, the mRNA coding for the calcium binding protein PV (**Fig 2.2D**), that is expressed at higher level in early-born PV neurons, was present in both populations at the same levels, suggesting that the high and low PV states might be dependent upon post-transcriptional mechanisms that could also account for the concurrent shifts in both the soma and the synapses in relatively short time intervals (Karunakaran et al., 2016). Other calcium-binding proteins coding mRNA such as *Caln1* (**Fig 2.2D**) on the other hand, were found to be overexpressed in early-born PV neurons together with specific members of the neurofilament family such as *Nefm* and *Nefh* (**Fig 2.2B**) whose expression level have recently been correlated with the fast spiking capabilities of PV neurons (Harris et al., 2018; Saunders et al., 2018). Notably, members of the v-SNARE complex present in the synaptic vesicles such as *Vamp1* and *Syt2* (but not *Vamp2* or *Syt1*) (**Fig 2.2B**) were also found to be enriched in early-born PV neurons. Very interestingly, Syt2 has recently been reported as the synaptotagmin family member with the fastest Ca^{2+} binding kinetics required for synchronous release and it's known to be expressed by a fraction of hippocampal PV neurons (Hu et al., 2014; Kerr et al., 2008; Sommeijer and Levelt, 2012). Syt2 and Syt1 are co-expressed by presynaptic terminals of PV neurons and believed to be working in concert (Mittelsteadt et al., 2009), however, recent evidence from cerebellar PV neurons has shown that the genetic deletion of Syt2, but not Syt1, is sufficient to abolish fast transmitter release, and that Syt2 generate faster release kinetics and faster vesicular pool refilling than Syt1, consistent with high frequency spiking behaviour (Chen et al., 2017). Moreover, early-born PV neurons also expressed higher levels of *Atp1a3* (**Fig 2.2B**), an ATP-dependent transmembrane sodium pump necessary to re-establish the membrane potential after bursts of activity (Vaillend et al., 2002) and repeatedly associated with childhood-onset schizophrenia (Chaumette et al., 2018; Enwright Iii et al., 2018; Smedemark-Margulies et al., 2016). This collection of genes is consistent with the idea that early-born PV neurons resemble more closely the prototypical fast-spiking PV cells, possessing a more potent and fast inhibitory capability that results from a higher expression of a class of genes necessary for the synthesis, the transport and the fast, synchronous release of GABA. Moreover, consistent with previous observations that early-born neurons are enriched in excitatory synaptic puncta whereas late-born neurons are enriched in inhibitory synaptic puncta (Donato et al., 2015), I find an over representation of transcripts coding for the AMPA receptor 1 *Gria1* coding for the GluA1, that together with *Gria4* (expressed at similar levels in both subpopulations) are the

most represented AMPA subunit in PV neurons (Matta et al., 2013) (**Fig 2.2C**). Late born PV-cells, on the other hand, did not possess a specular increase in the expression of GABA receptor subunits, however, a class of gene necessary for $GABA_A$ receptor stabilization and phosphorylation of synaptic protein was found to be overexpressed in the late PV fraction. Member of this class are *Git1* and *Cnksr2*, which are part of the same molecular complex and act as a scaffold for the stimulation of the mitogen-activated protein kinase, *MAPK1*, also overexpressed in late-born PV neurons (Kim et al., 2017; Lim et al., 2014) (**Fig 2.2F**). Furthermore, *Git1* has recently been shown to form complexes with $GABA_A$Rs promoting the stability and strength of inhibitory synapses by promoting the stabilization of F-actin filaments (Smith et al., 2014). Consistent with this, other genes enriched in the late-born fraction are known to play a role in actin stabilization such as *Stmn1* (Harris et al., 2018), *Fmn2* (Peleg et al., 2010) and *Lrrc7* (Kappe, 2014) (**Fig 2.2G**). As expected, a number of housekeeping control genes, which have been recently shown to be stably express also at the single-cell level (Lin et al., 2018), did not show any variation (**Fig 2.2E**).

Figure 2.2: Single cell RNA-seq from retrovirally labelled early- and late-born PV neurons. A) Schematic showing the experimental model **B)** Genes overexpressed in early-born PV neurons. Many of the genes overexpressed in early-born PV neurons suche as *Gad1*, *Vamp1*, *Atp1a3* and *Syt2* suggest a high spiking behaviour. **C)** Early-born PV cells express higher level of the subunit 1 but not 4 of AMPA glutamate receptors. **D)** Although the protein levels are higher in early-born PV cells, the trascript coding for parvalbumin is expressed at similar levels in the two subpopulation, other calcium binding protein such as *Caln1* are however expressed more in the early-born fraction. **E)** Housekeeping genes, which have been validated at the single cell level, are stably expressed in early and late born at similar levels. **F)** Genes overexpressed in late-born PV neurons. *Git1* and *Cnksr2* are part of the same molecular complex, necessary for the stabilization of $GABA_A$Rs at inhibitory synapses. **G)** The stabilization of $GABA_A$Rs requires the involvement of F-actin filaments that could be achieved by higher expression of specialized actin-binding proteins overexpressed in late-born PV neurons. **H)** Proposed markers for the early-born fraction, based on expression levels and selective expression by PV cells. Statistical significance is tested using a Mann-Whitney unpaired t-test ($P<0.05$).


A
PV-Cre
P60-70
Acute Slices
Manual picking
Single cell RNA-seq
E9.5
E13.5
CA1
CA1
Early
Late
B
Neurotransmitter synthesis, transport and release / Fast spiking behaviour
Gad1
Nefm
Nefh
Vamp1
Vamp2
Syt2
Syt1
Atp1a3
TPM
Early
Late
C
AMPA receptor subunits
Gria1
Gria4
D
Calcium binding
Pvalb
Caln1
E
Housekeeping control genes
ActB
RPL32
Syn1
F
Inhibitory synaptic stabilization and signalling
Git1
Cnksr2
Mapk1
G
Actin dynamics
Fmn2
Lrrc7
Stmn1
H
Markers for early-born PV neurons
Gfra1
Syt2

2.3 Validation of Early-born PV Neurons Markers

Validation of *Gfra1* overexpression in early-born PV neurons

The main scope of the screening was to identify molecular markers for the two subpopulations of PV neurons in order to gain genetic access and therefore manipulation potential to further understand their role in learning and memory. To this end, I focused on genes that were reported to be selectively expressed in PV neurons so that the manipulation conducted during behaviour would only target the desired fraction of cell without impinging on other nodes of the hippocampal network. All the genes differentially expressed in late-born PV neurons were also ubiquitously expressed in excitatory neurons or other classes of inhibitory interneurons, being therefore unsuitable selective markers. The early-born fraction, conversely, encompassed transcripts of high physiological relevance that were also reported to be selectively expressed by PV neurons. I therefore proceeded with the validation of two of the genes: *Gfra1* and *Syt2* (**Fig 2.2H**). Gfra1 is a membrane protein that acts as receptor for the glial cell line derived neurotrophic factor (GDNF) and has been shown to be expressed by a fraction of GAD expressing neurons in hippocampal CA1 (Sarabi et al., 2000). Also, mutant mice lacking this gene show a regionalized loss of parvalbumin interneurons from cortical regions that can be counteracted with neuronal activity (Canty et al., 2009), indicating that

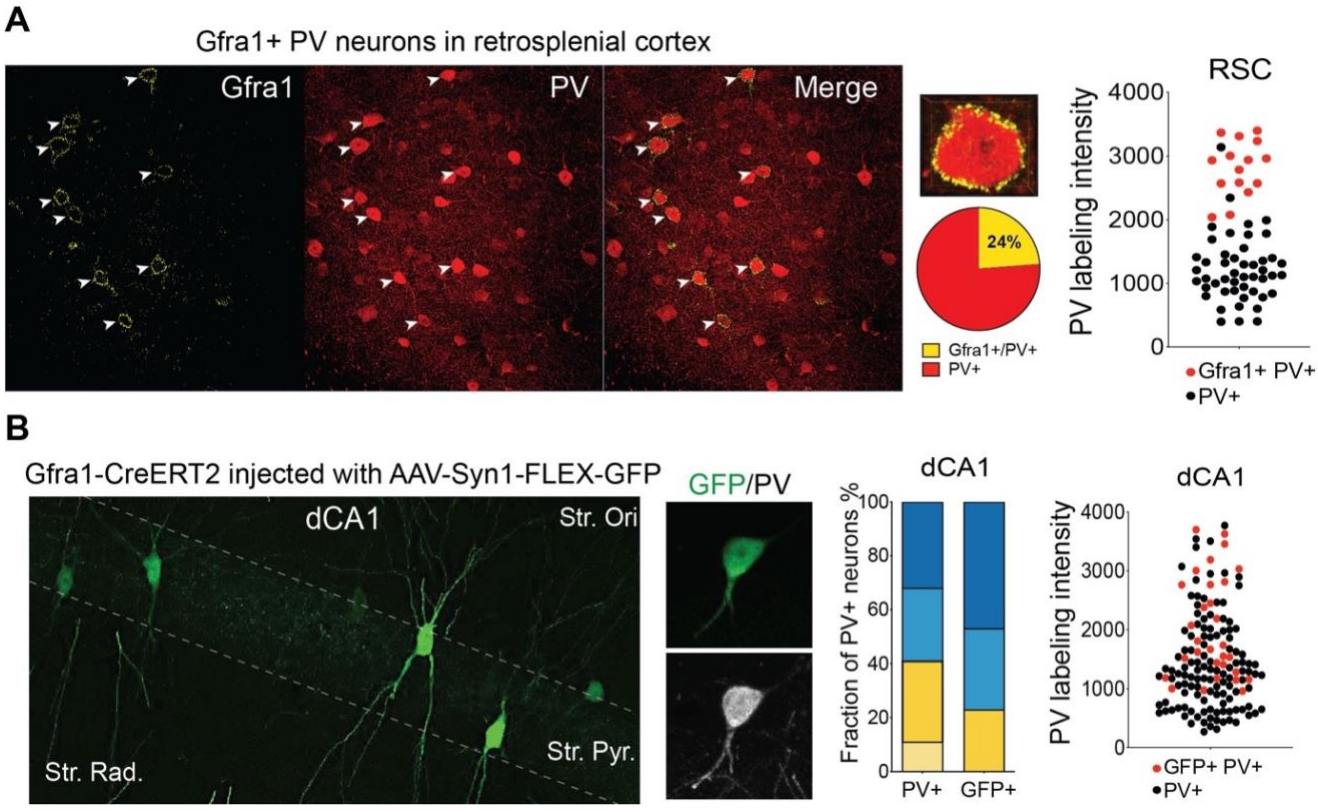


Figure 2.3: Validation of Gfra1 as a marker for high PV neurons. A) Staining for Gfra1 and PV in retrosplenial cortex reveals colocalization of Gfra1 with a fraction of high-PV expressing cells. **B)** AAV deliverying a Cre-dependent GFP were injected in the dCA1 of Gfra1-CreERT2 mice. Upon tamoxifen delivery all the GFP positive cells were also PV positive and expressed high levels of PV. n=3 mice, 50 cells per mouse.

this gene might be particularly important for the survival of a fraction of PV+ cells. I therefore set out to confirm Gfra1 as a marker for early-born/high PV expressing neurons by staining for both PV and Gfra1 and quantified the intensity of the PV positive colocalizing cells (**Fig2.3A**). The immunostaining for Gfra1 labelled the membrane of a fraction of PV neurons in the retrosplenial cortex that, notably, were the cells with the highest PV immunoreactivity (**Fig 2.3A**). No signal was however detected in the hippocampus or in other cortical regions. To further validate the finding that Gfra1 is expressed by a fraction of the high PV cells with an independent approach I turned to a knock-in mouse line in which a tamoxifen-dependent Cre is expressed under the control of the Gfra1 promoter (Keefe Davis et al., 2013). AAV9 encoding a Cre-dependent GFP were bilaterally injected in the dCA1 of Gfra1-CreERT2 mice. Upon tamoxifen delivery, the GFP signal was detected only in PV expressing neurons, that, also in this condition, were a sub fraction of the high expressing PV cells. Overall this suggests that Gfra1 is selectively express in a fraction of early-born PV neurons both in the cortex and in the hippocampus and can be considered a reliable marker for early-born PV cells.

Validation of Syt2 overexpression in early-born PV neurons

In order to validate the selective expression of the synaptic protein Syt2 in the early born fraction I took once more a retroviral approach. The above described rv:CAG-FLEX-mCherry retrovirus encodes a membrane-bound mCherry that fully labels axonal processes and synapses, allowing for unequivocal colocalization of presynaptic proteins that would otherwise be difficult to be traced back to their originating cell soma. To stain for Syt2 I used a Znp1 antibody, which has been shown to selectively bind to Syt2 but not Syt1 in mice (Fox and Sanes, 2007). Ultrasound-guided intraventricular injections of high-titer retroviral particles were performed in parallel in E9.5 and E13.5 and, again, led to a robust and highly specific labelling of PV neurons in adult mice (**Fig 2.3A**). Importantly, mice injected with retroviruses at E9.5 showed an extensive degree of colocalization in PV boutons in the stratum pyramidale, radiatum and oriens (**Fig 2.3B**), indicating that the vast majority of the cells that are born at early time points express Syt2. Strikingly, when the retroviral injection was performed a E13.5 the colocalization of mCherry and Syt2 was totally absent (**Fig 2.3C**), indicating that, on the contrary, PV neurons that are born later during embryonic development do not express Syt2 in the adult. In both conditions the synapses of the retrovirally labelled PV cells were found

mostly in the stratum pyramidale, indicating that the fraction of PV labelled cells is representative of basket cells.

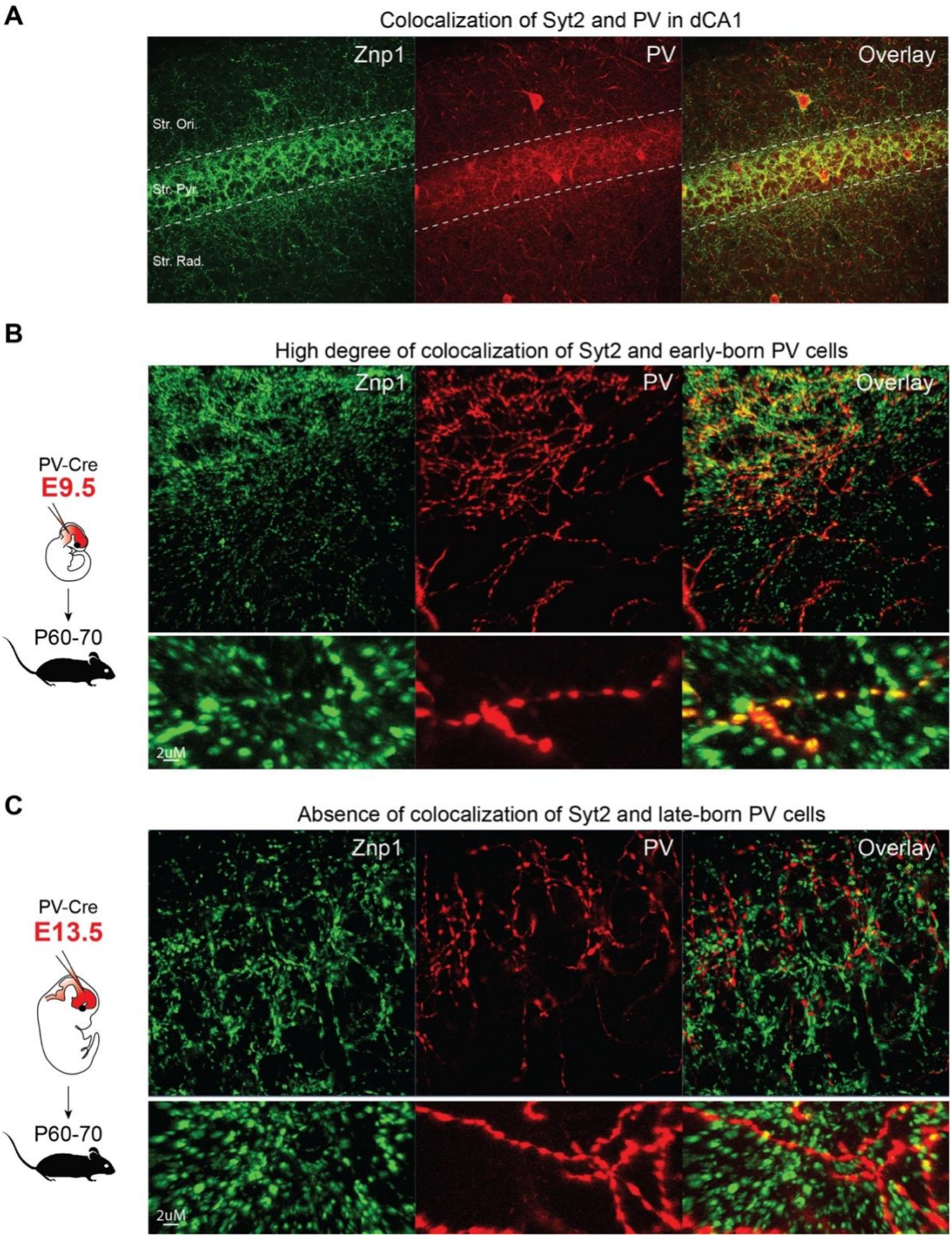


Figure 2.4: Validation of Syt2 as a marker for high PV neurons. A) Staining for Znp1 and PV reveals a high degree of colocalization in dCA1. **B)** Colocalization between Znp1 and early-born PV neurons. rv:CAG-FLEX-mCherry was injected at E9.5 in PV-Cre positive embryos. Colocalization was assessed in dCA1 of P60 animals. **C)** Absence of colocalization between Znp1 and late born PV neurons. rv:CAG-FLEX-mCherry was injected at E13.5 in PV-Cre positive embryos. Absence of colocalization was assessed in dCA1 of P60 animals.

2.5 Generation of Syt2-Cre Mouse Line

Given the - above described - important physiological role of Syt2 and its selective expression in early-born PV neurons I proceeded with the generation of a knock in mouse line where the Cre recombinase is expressed under the control of the synaptotagmin 2 promoter. In order to do so, I conceived a CRISPR editing strategy targeting the 3'UTR of the Syt2 locus and designed an homology cassette containing an IRES-Cre recombinase that, upon delivery to the zygote pronucleus, integrated successfully in the desired genomic location (**Fig 2.4A**). Newly generated Syt2-IRES-Cre mice were subsequently injected with Cre-dependent GFP-expressing AAVs in dCA1. This resulted in restricted GFP expression into PV positive cells that displayed high levels of PV immunoreactivity (**Fig 2.4B**). Around the injection site, only around 40% of the total PV+ population was labelled by GFP, indicating that, as observed for the Syt2 protein, also the Cre expression was restricted to a subpopulation of PV cells. To further validate Syt2 as a selective marker for early-born PV neurons I performed birthdating experiments using BrdU. Pregnant Syt2-Cre positive mothers were injected with BrdU at E11.5 to label the early born fraction and, in parallel at E13.5 and E14.4 to label the late-born fraction. The quantification of Cre-BrdU double positive revealed that the portion of cells born at early time points (E11.5) that also expressed the Cre under the control of the Syt2 promoter excided the portion born at E13.5 around 2.5 times and around 6 times that born at E14.5 (**Fig 2.4C**). In order to label the low-PV expressing, late-born PV fraction I used an intersectional approach (Fenno et al., 2014). I crossed PV-FlpO mice (Madisen et al., 2010) with Syt2-Cre generating PV-FlpO/Syt2-Cre double knock-in mice. A Coff/Fon dependent AVV (in which the expression of GFP is dependent on the presence of FlpO but is subsequently repressed in the presence of the Cre) was injected in the dCA1 of PV-FlpO/Syt2-Cre, resulting in the GFP labelling of the PV positive cells that did not expressed Syt2 that. Importantly the labeled fraction had low immunoreactivity for PV (**Fig 2.4D**). This results confirm that the Syt2-Cre line is a consistent tool to manipulate and trace the connectivity of early-born, high PV expressing PV neurons and that using AAV-based intersectional approaches can reliably target the late-born, low PV expressing PV subpopulation.

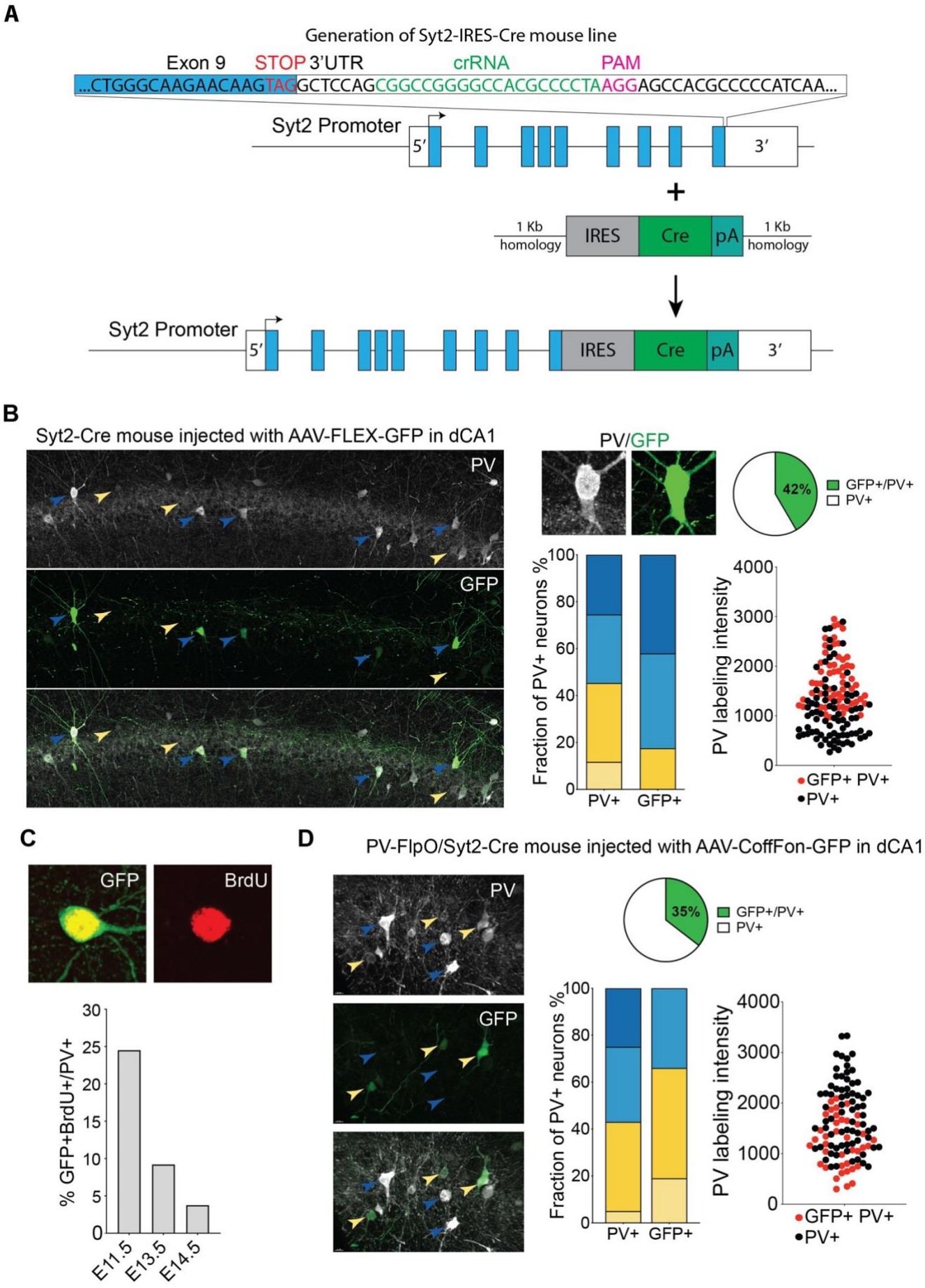

A
Generation of Syt2-IRES-Cre mouse line
Exon 9
STOP
3'UTR
crRNA
PAM
...CTGGGCAAGAACAAGTAGGCTCCAGCGGCCGGGGCCACGCCCCTAAGGAGCCACGCCCCCATCAA...
Syt2 Promoter
5'
3'
1 Kb homology
IRES
Cre
pA
1 Kb homology
Syt2 Promoter
5'
IRES
Cre
pA
3'
B
Syt2-Cre mouse injected with AAV-FLEX-GFP in dCA1
PV
GFP
PV/GFP
42%
GFP+/PV+
PV+
Fraction of PV+ neurons %
100
80
60
40
20
0
PV+
GFP+
PV labeling intensity
4000
3000
2000
1000
0
GFP+ PV+
PV+
C
GFP
BrdU
% GFP+BrdU+/PV+
30
25
20
15
10
5
0
E11.5
E13.5
E14.5
D
PV-FlpO/Syt2-Cre mouse injected with AAV-CoffFon-GFP in dCA1
PV
GFP
35%
GFP+/PV+
PV+
Fraction of PV+ neurons %
100
80
60
40
20
0
PV+
GFP+
PV labeling intensity
4000
3000
2000
1000
0
GFP+ PV+
PV+

Figure 2.5: Generation of Syt2-Cre mouse line. A) Devised strategy for the generation of Syt2-Cre knock in mouse line. CRISPR-Cas9 mediated DNA cutting was directed to the 3'UTR. A crRNA (in green) was designe flanking a PAM sequence (in magenta). A 1.8 Kb cassette containing an internal ribosome entry site and a Cre recombinase followed by a polyadenilation was flanked by 1Kb of homology to the Syt2 locus in order to promote homology-directed repair **B)** Cre-dependent GFP expresing AAV injected in the dCA1 of Syt2-Cre mice. The high-PV fraction is selectively labelled. Around the injection site around 40% of the neurons are labelled n=3 mice, 50 neurons per mouse. **C)** BrdU validation of Syt2 as a marker of early-born PV neurons. PV cells born at E11.5 are 2.5 times more likely to express Syt2 than cells born at E13.5 and 6 times more likely than PV cells born at E14.5. **D)** PV-FlpO/Syt2-Cre double transgenic mice injected with Coff/Fon AAVs labelling cells PV+Syt2-. The GFP+ cells exress lower levels of PV and were around 35% of the total PV pupolation. n=2 mice, 50 cells per animal.

2.6 Local Connectivity of Early- and Late-born PV Neurons

In the hippocampus, single PV+ basket cells innervate only a small fraction of the cells within their axonal cloud (Bezaire and Soltesz, 2013), suggesting that rather than provide a so called "blanket inhibition", often reported in neocortical circuits (Karnani et al., 2014), they might select their synaptic partner forming inhibitory microcircuits that integrate with the already described excitatory heterogeneity. Previous results in our lab have shown that the PV-level distributions of perisomatic boutons onto deep and superficial CA1 pyramidal cells layers suggest that early- and late-born PV neurons preferentially target deep and superficial compartments respectively (Donato et al., 2015). I therefore set out to explore, using a transgenic approach, the local connectivity pattern of genetically identified early- and late-born PV cells onto dCA1 deep and superficial sublayers. To this end, I tagged the synapses of the entire PV population and of early-born PV neurons by selective expression of a fusion protein between the pan neuronal synaptic protein synaptophysin and enhanced green fluorescent protein delivered by stereotaxic injection of a Cre-dependent AAV (AAV-FLEX-SynGFP) in PV-Cre or Syt2-Cre mice (Esposito et al., 2014) (**Fig 2.6A,C,D**). In order to tag synapses of late-born neurons, on the other hand, I applied an intersectional approach, injecting a CreOFF/FlpON vector that expresses SynGFP in PV+ neurons that do not express Syt2 (AAV-CoffFon-SynGFP) in Syt2-Cre/PV-FlpO double transgenic mice (**Fig 2.6E**). The injection of AAV-FLEX-SynGFP in dCA1 of PV-Cre animals resulted in the labelling of the synapses of the entire PV population (belonging to both high and low PV neurons) that were found to be equally distributed in both layers, with only a slightly bias toward the deep layer (**Fig 2.6B**). In contrast, using the Syt2-Cre mouse line to label only the boutons of early born PV neurons resulted in the emergence of a selective connectivity pattern, with almost twice as many projection targeting the deep PCs layer compared to the superficial. The projection selectivity

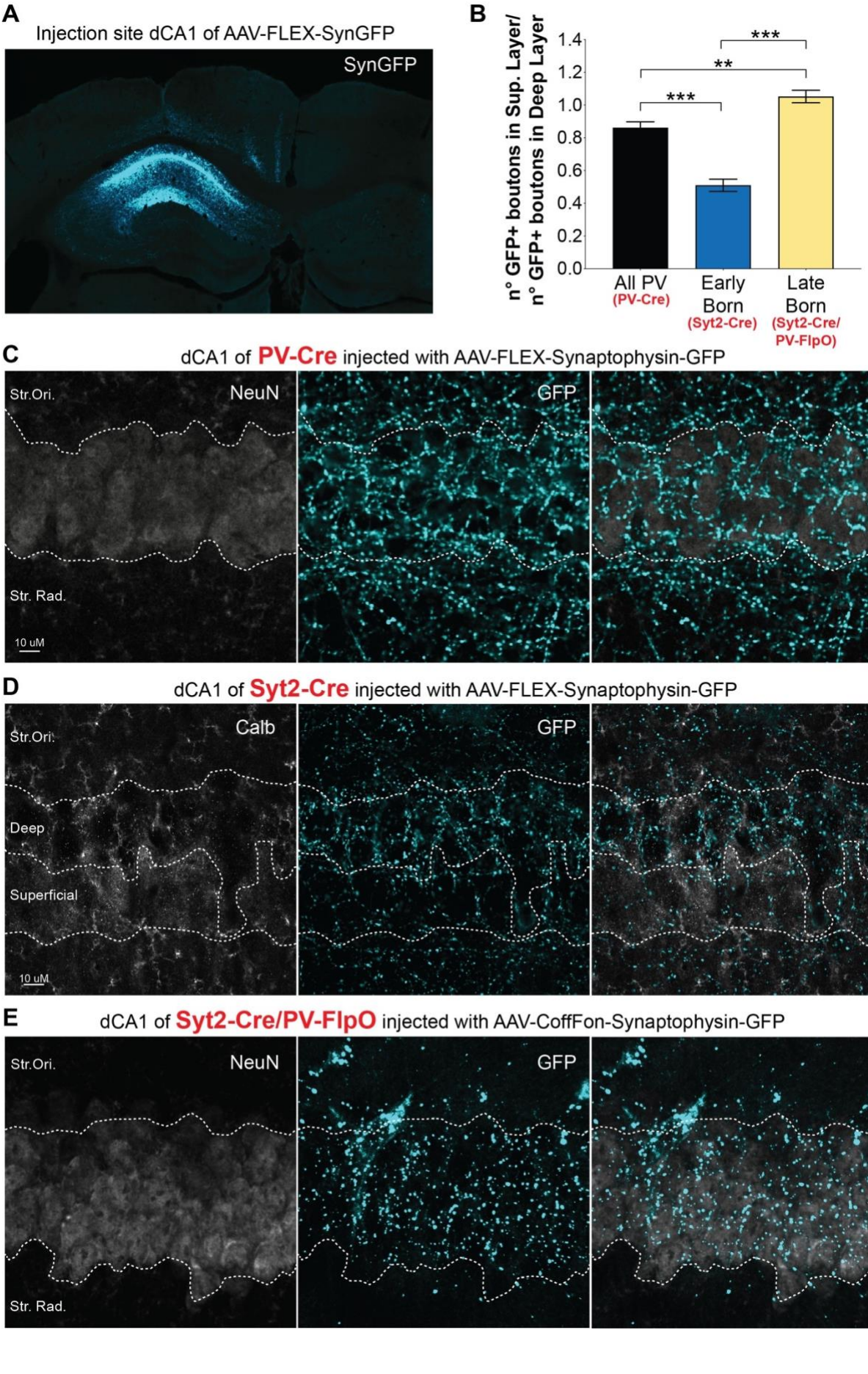

A
Injection site dCA1 of AAV-FLEX-SynGFP
SynGFP
B
n° GFP+ boutons in Sup. Layer/
n° GFP+ boutons in Deep Layer
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
***
**
***
All PV
(PV-Cre)
Early
Born
(Syt2-Cre)
Late
Born
(Syt2-Cre/
PV-FlpO)
C
dCA1 of PV-Cre injected with AAV-FLEX-Synaptophysin-GFP
Str.Ori.
NeuN
GFP
Str. Rad.
10 uM
D
dCA1 of Syt2-Cre injected with AAV-FLEX-Synaptophysin-GFP
Str.Ori.
Calb
GFP
Deep
Superficial
10 uM
E
dCA1 of Syt2-Cre/PV-FlpO injected with AAV-CoffFon-Synaptophysin-GFP
Str.Ori.
NeuN
GFP
Str. Rad.

Figure 2.6: Early Born Syt2+ PV neurons make preferential contact with deep CA1 PCs.
A) Injection site in dCA1. Representative image of AAV-FLEX-SynGFP injected in PV-Cre mice. **B)** Quantification of the synaptic contact in the deep and superficial compartment. Two regions of equal surface area were selected dividing the pyramidal layer in two equally sized compartments and the number of labelled synapase present in each was quantified in order to obtain a relative ratio **C)** Representative image of dCA1 of PV-Cre mouse injected with AAV-FLEX-SynGFP. **D)** Representative image of dCA1 of Syt2-Cre mouse injected with AAV-FLEX-SynGFP. The deep layer of PCs is identified by the absence of Calb staining. **E)** Representative image of dCA1 of PV-FlpO/Syt2-Cre mouse injected with AAV-CoffFon-SynGFP.

of late-born neurons, on the other hand, was found to be mostly homogeneous, with only a modest increase in the superficial. This results shows that, using genetically identified circuit elements, is possible to uncover the fine circuits architecture of hippocampal CA1 PCs and PV basket cells subpopulations. Early-born Syt2+ PV neurons selectively target the deep layer of hippocampal CA1 whereas, late-born Syt2- PV cells provides largely unbiased inhibition to both the sublayers.

2.7 Long Range Connectivity onto Early-born PV Cells

Although PV neurons are predominantly local interneurons gaiting feed-forward and feedback connectivity within the hippocampal system, they also receive direct input connectivity from distally projecting areas that, among other function, synchronizes their firing orchestrating local oscillations (Unal et al., 2015). Therefore, the subpopulation-specific targeting of CA1 sublayers could reflect a wider embedding in parallel networks, involving distal brain areas, differentially connecting to early- and late-born PV neurons. In order to investigate the existence of subpopulation-specific long range connectivity onto early- and late-born PV neurons I used monosynaptic rabies technology (Wickersham et al., 2010, 2007). I first initiated the monosynaptic virus spread from both early- and late-born PV neurons of dCA1 by injecting PV-Cre mice sequentially with helper AAVs delivering the avian sarcoma and leukosis virus subtype A receptor (AAV-FLEX-TVA-IRES-GFP) and the rabies glycoprotein (AAV-FLEX-Gly) followed by avian sarcoma leukosis virus envelope protein (EnvA)-coated rabies particles. This revealed a pattern of connectivity which included the ipsilateral and contralateral CA1 and CA3, the Medial Entorhinal Cortex (MEC), the dorsal subiculum and a very sparse projection from the raphe nucleus. The most conspicuous projection was however detected coming from the basal forebrain, in particular, the medial septum and the horizontal limb of the diagonal band (HDB) (**Fig 2.7A**). I then conducted a parallel experiment initiating the rabies tracing from the early-born fraction of PV cells. To this end, Gfra1-CreERT2 mice were sequentially injected with helper AAVs and with the EnvA coated rabies revealing a

pattern of connectivity which was very comparable to that of the entire PV population and which included the medial entorhinal cortex, the contralateral and ipsilateral CA3 and, and the medial septal nuclei, which were also for the early-born fraction the most prominent input connectivity (**Fig 2.7B**).

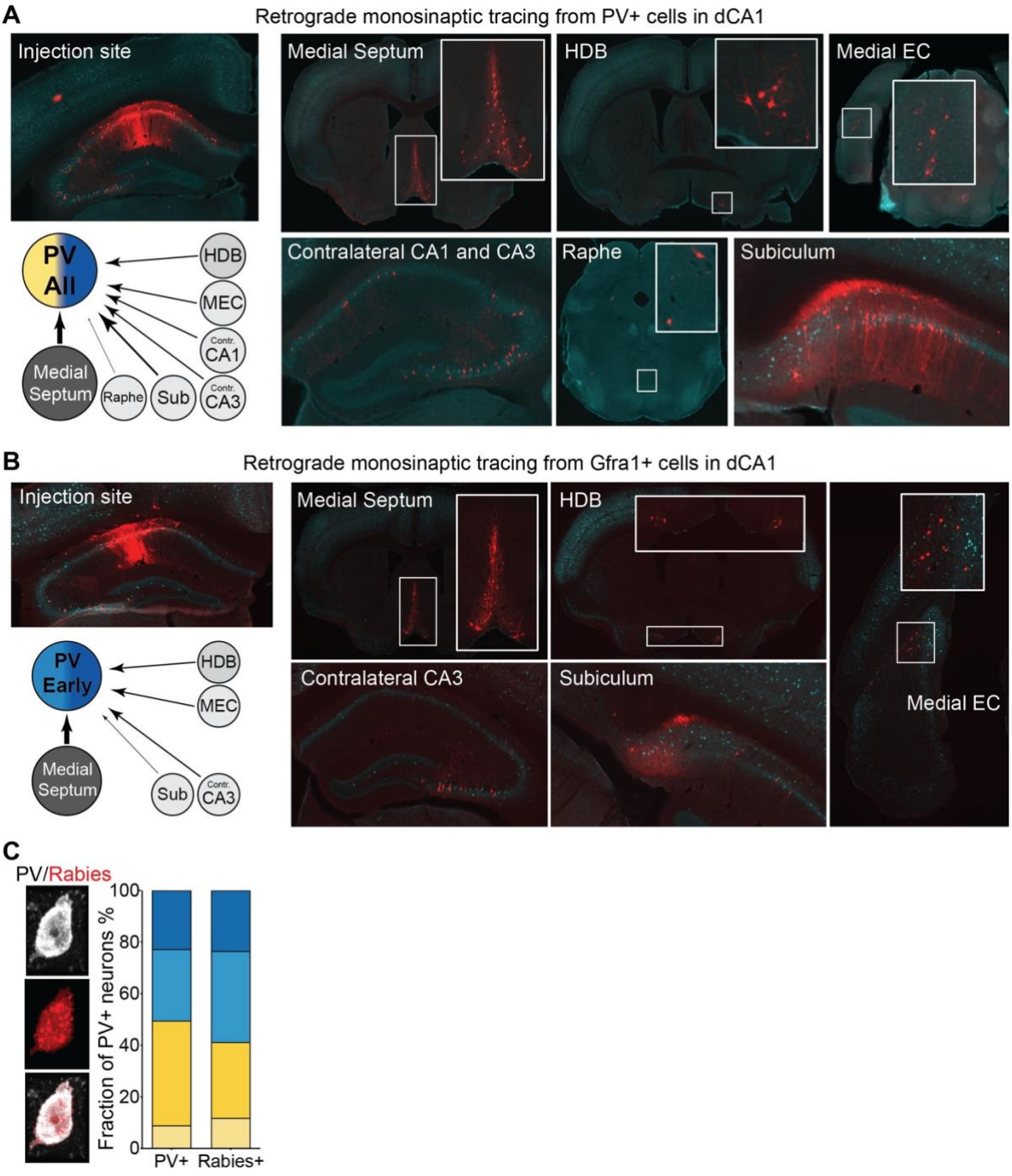


Figure 2.7: Retrograde monosynaptic connectivity of Early-born PV cells. A) Retrograde monosynaptic rabies tracing from dCA1 restricted to PV expressing interneurons (PV-Cre mice). HDB: horizontal

limb of the diagonal band. Schema indicating the principal projections. **B)** Retrograde monosynaptic rabies tracing from dCA1 restricted to early-born PV neurons (Gfra1-CreERT2 mice). **C)** PV distributions in labelled cells of Gfra1-CreERT2 indicating that the late-born low-PV fraction is sending projections to the early-born fraction.

Parvalbumin expressing basket cells make numerous reciprocal inhibitory connections onto each other (Bartos et al., 2007; Pfeffer et al., 2013), creating a distributed network of reciprocally connected cells that orchestrate synchronous activity (Hu et al., 2014). The existence of subpopulations of parvalbumin basket cells raises the question of whether these might form parallel independent subnetworks or, conversely, they might operate in the same, monosynaptically connected, inhibitory microcircuit. To test for this I've examined the PV expression levels of labelled cells in the dCA1 of Gfra1-CreERT2 mice injected with the rabies virus. Starter cells, identified by the presence of GFP in the helper virus were excluded from the quantification (**Fig 2.7C**). Analysis of the distribution of PV immunoreactivity reveals similar amounts of the low-PV fractions in Rabies+ PV cells compared to the general PV population, suggesting that late-born PV low-PV cells make direct synaptic contact onto early-born PV neurons, and that together the two populations might function as an integrated inhibitory circuit.

The medial septum and diagonal band complex are a mosaic of long-range projecting cholinergic and GABAergic cells known to innervate the hippocampus, contributing to the coordination of neuronal activity and the generation of theta oscillations (Unal et al., 2015). In particular, PV positive GABAergic cells in the medial septum have been shown to selectively target PV neurons in the hippocampus (Sun et al., 2014; Unal et al., 2018) and related cortical areas. I therefore decided to test whether GABAergic cells might preferentially target Early-born PV neurons in dorsal CA1, since the retrograde rabies experiment previously described did not exclude this possibility. To do so, I injected a AAV-FLEX-SynGFP in the medial septum of PV-Cre mice, labelling the synapses of the long-range GABAergic projection neurons that, in the previous experiment, were found to project to both the entire PV population and the early-born fraction (**Fig 2.8A**). Projections to the hippocampus were found predominantly in the CA3 region but also in the CA1 and particularly innervating the soma of PV+ cells. The number of perisomatic septal PV boutons onto PV cells in the CA3 and CA1 regions was similar for both low-PV late born cells and for high-PV early born cells, suggesting that the medial septum sends similar amount of inhibitory projections to the two subpopulations of PV neurons.

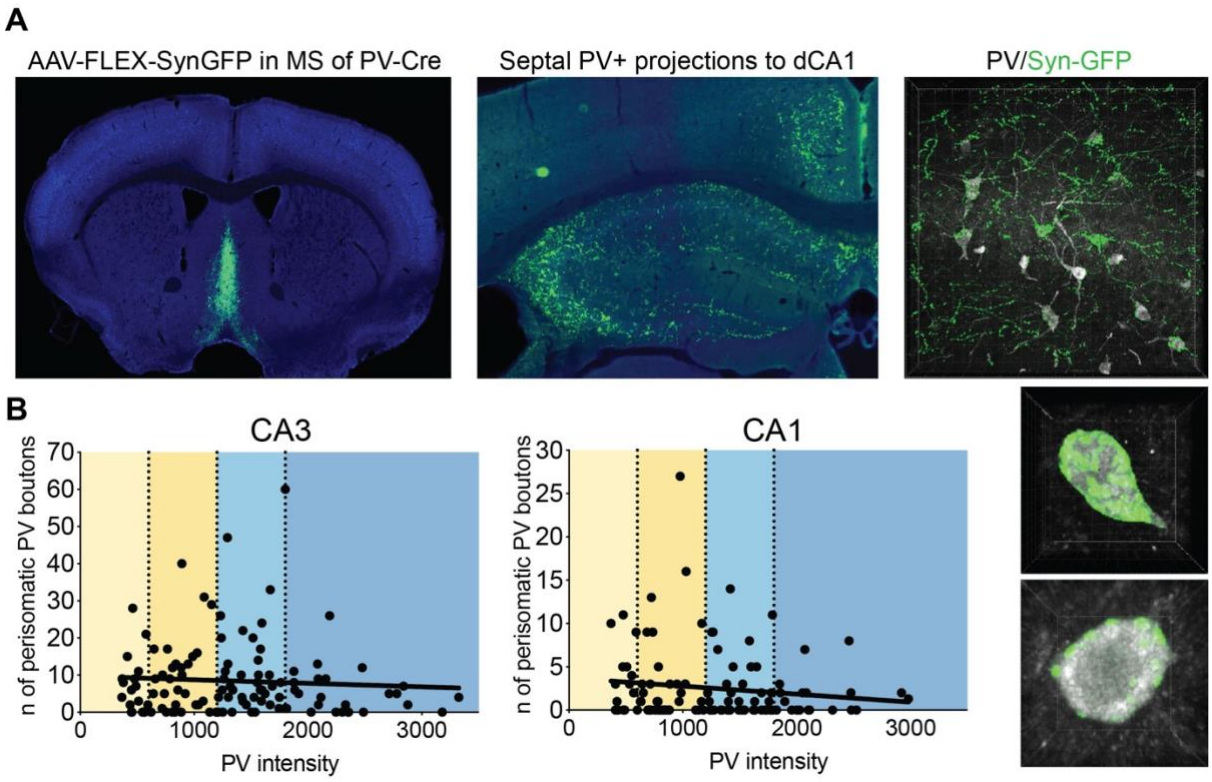


Figure 2.8: Anterograde connectivity from medial septal nuclei. A) Injection site (on the left) and hippocampal projections of PV-positive long-range projection neurons of the medial septum (on the right). PV staining reveals that hipocampal PV positive cells are the main synaptic target of septal PV neurons that make predominatly perisomatic contact with most of the labelled cells. **B)** Quantification of septal PV+ projections onto Early-born high-PV neurons and Late-born Low-PV neurons of dCA3 and dCA1. The septal GABAergic projection is mainly targeting CA3 PV cells, but both the PV BC subpopulations receive similar amount of perisomatic contact.

Together, this and previous results suggest that the differential modulation and the different roles that the two subpopulation have during the course of learning are not the results of a different long range input connectivity but rather the consequence of local dynamics within the hippocampal microcircuit.

2.9 Manipulation of Early-born PV Neurons Activity During Learning

As previously demonstrated in our Lab, early-born PV neurons show plasticity upon cFC and at the end of maze learning (definite learning), but not during maze learning. Conversely, late-born PV neurons show plasticity upon environmental enrichment, during maze learning and when critical-period type plasticity is induced (provisional learning) (Donato et al., 2015). To

assess the differential role of PV neuron subpopulations during the acquisition of definite and provisional learning, I carried out *in-vivo* manipulation of early-born cells during the acquisition of contextual fear conditioning (cFC) and during the learning of the Morris Water Maze (MWM). Adult (P60-P90) mice (WT, PV-Cre and Syt2-Cre) were stereotaxically injected in dCA1 bilaterally with an AAV9 carrying a Cre-dependent chemogenetic activator channel construct (Activator virus, described in Magnus et al., 2011). Two weeks after virus injections the chemogenetic channels were activated from 15 min before behavioral testing by systemic delivery of ligand PSEM; under this In vivo conditions, PSAM channel activation can be detected within 2min and lasts for ~60min (Magnus et al., 2011). Mice were subsequently fear conditioned with 5 foot shocks, and re-exposed to the same conditioning context the following day through a 5 minute time interval during which freezing responses were measured. The activation of both Syt2+ and PV+ cells in dCA1 during the acquisition of fear memory resulted in a decrease of the freezing response during memory recall, consistent with previous findings that the early-born population regulates definite learning (**Fig 2.9A**). To determine whether the early-born fraction is also required during provisional learning I focused on the Morris Water Maze. During the first 4-5 days of MWM learning, mice naturally shift towards low-PV configurations. Importantly, only the low-PV expressing late-born PV neurons induce plasticity in this conditions by shifting towards even lower levels of PV expression (Donato et al., 2015). Mice were injected bilaterally with the Activator Virus and, subsequently, trained on a standard MWM protocol which include a first day in which the platform is visible, followed by standard training days during which the animals underwent 4 trials with an invisible platform. Every day for the first 4 days of invisible platform, the ligand PSEM was administered intraperitoneally 15 minutes before the session in order to depolarize either all the infected PV neurons (when using PV-Cre) or only the early-born fraction (when using Syt2-Cre). Activation of the entire PV population resulted in spatial learning impairments with longer latencies compared to controls (**Fig 2.9B**). Conversely, activating only the early-born fraction did not interfere with maze learning as the latencies of injected Syt2-Cre mice were comparable to those of control mice (**Fig 2.9B**). To further validate the involvement of early-born PV neurons in definite learning I focused on the late phase of the MWM learning, when animals have to adhere to validated spatial rules and the global PV network shifts to a high-PV configuration. This usually occurs starting from day 5 and persist till completion of the MWM and can be prevented by continuously changing the position of the hidden platform (Donato et al., 2013). Taking a similar approach, I injected the ligand PSEM, depolarizing the infected PV+ or Syt2+ neurons, starting from day 5 each day till day 8, 15 minutes before each learning session. The spatial memory, assessed by latency quantification, was consistently and similarly impaired in both PV-Cre and Syt2-Cre mice on day 8 (**Fig 2.9C**). Furthermore, I assessed the time spent in each quadrant during a single trial on the 9th day after having removed the platform (probe

day). On probe day the PSEM was not injected in order to test the authentic spatial memory without the confounding of the ongoing neuronal manipulation. In this conditions, Syt2-Cre and PV-Cre mice had similar impairments of the spatial memory and, when compared to controls, spent less time in the correct quadrant indicating that early-born cells play a fundamental role in the acquisition of definite learning.

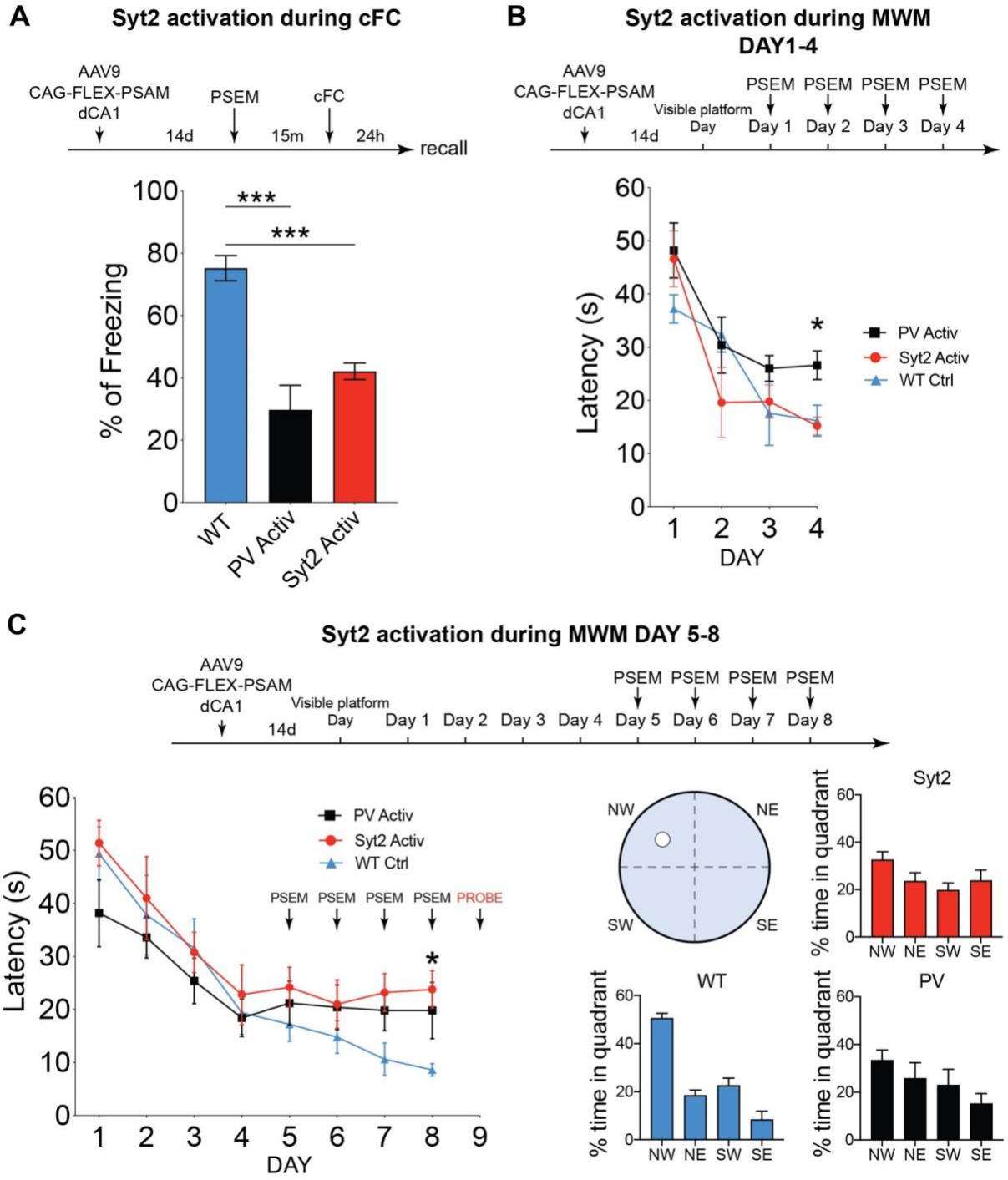


Figure 2.9: Behavioral manipulation of early-born PV neurons **A)** Quantification of freezing responses following cFC and during PV and Syt2+ cells activation. Interfering with either the entire (PV-Cre) or

only the early-born (Syt2-Cre) PV population during memory acquisition leads to decreased freezing responses during memory recall. N=5, one-way ANOVA, WT vs PV act P<0.0001, WT vs Syt2 Act P=0.0001. **B)** Quantification of latencies (in seconds) during the first 4 days of MWM (provisional learning). Interfering with the entire PV population leads to deficits in spatial memory. The selective manipualtion of early-born PV neurons (Syt2-Cre) has no effects on the latencies. N=5, one-way ANOVA, WTday4 vs. PVday4 P=0.03, PVday4 vs. Syt2day4 P=0.0185. **C) Left:** Manipulation of PV+ or Syt2+ cells during the late phase of MWM learning (day5-day8, definite learning) both lead to impairments in memory performance N=5, one-way ANOVA, WTday8 vs. PVday8 P=0.1282, WTday8 vs. Syt2day8 P=0.0349. **Right:** Results of probe test conducted on Day 9 without any pharmacological intervention. WT mice developed a spatial map as indicated by the greater proportion of time spent in the NW quadrant: NW vs.NE P<0.0001, NW vs. SW P<0.0001, NW vs. SE P<0.0001. In Syt2-Cre and PV-Cre animals the time spent in the NW quadrant was not significantly higher than the time spent in the rest of the quadrands.

2.10 Cellular Counterparts of Early-born PV Neurons Activation

In order to investigate the cellular counterparts that the PV+ and Syt2+ neurons manipulation caused in the CA1 network, I analyzed ensembles of cFos+ neurons associated with the acquisition of contextual fear conditioning. Recent evidence has uncovered a wide functional diversification of the two CA1 radial subdivisions in representation of environmental elements and in the course of learning (Soltesz and Losonczy, 2018). A particular attention was therefore devoted to the formation of sublayer-specific ensembles in the deep and in the superficial layers as respectively identified by the presence and the absence of the molecular marker Calbindin (Cembrowski et al., 2016). Similarly to previous experiments adult (P60-P90) mice (WT, PV-Cre and Syt2-Cre) were stereotaxically injected in dCA1, bilaterally, with an AAV9 carrying a Cre-dependent chemogenetic activator channel construct (PSAM). The virally transduced chemogenetic channel was allowed a two weeks' time-interval to be fully expressed and subsequently activated from 15 min before behavioral testing by systemic delivery of ligand PSEM. Experimental protocols and conditions were carefully standardized in order to replicate the same conditions that led to fear memory impairments during memory recall in the previous experiment (**Fig 2.9A**). Mice were perfused after 90 minutes from learning, when the cFos induction is at its peak, and the fraction of neurons expressing cFos (cFos+/NeuN+) was measured. Activation of Syt2+ early-born PV cells caused an unexpected increase in the fraction of cFos+ cells that was specifically induced in the deep layer of dCA1 that, importantly, is the layer that deep cells preferentially connect to. Conversely, although the behavioral impairment produced by the activation of the entire PV population and the early-born restricted Syt2+ had similar magnitude, the amount of cFos+ cells in both the layers of CA1 was comparable to that of controls.

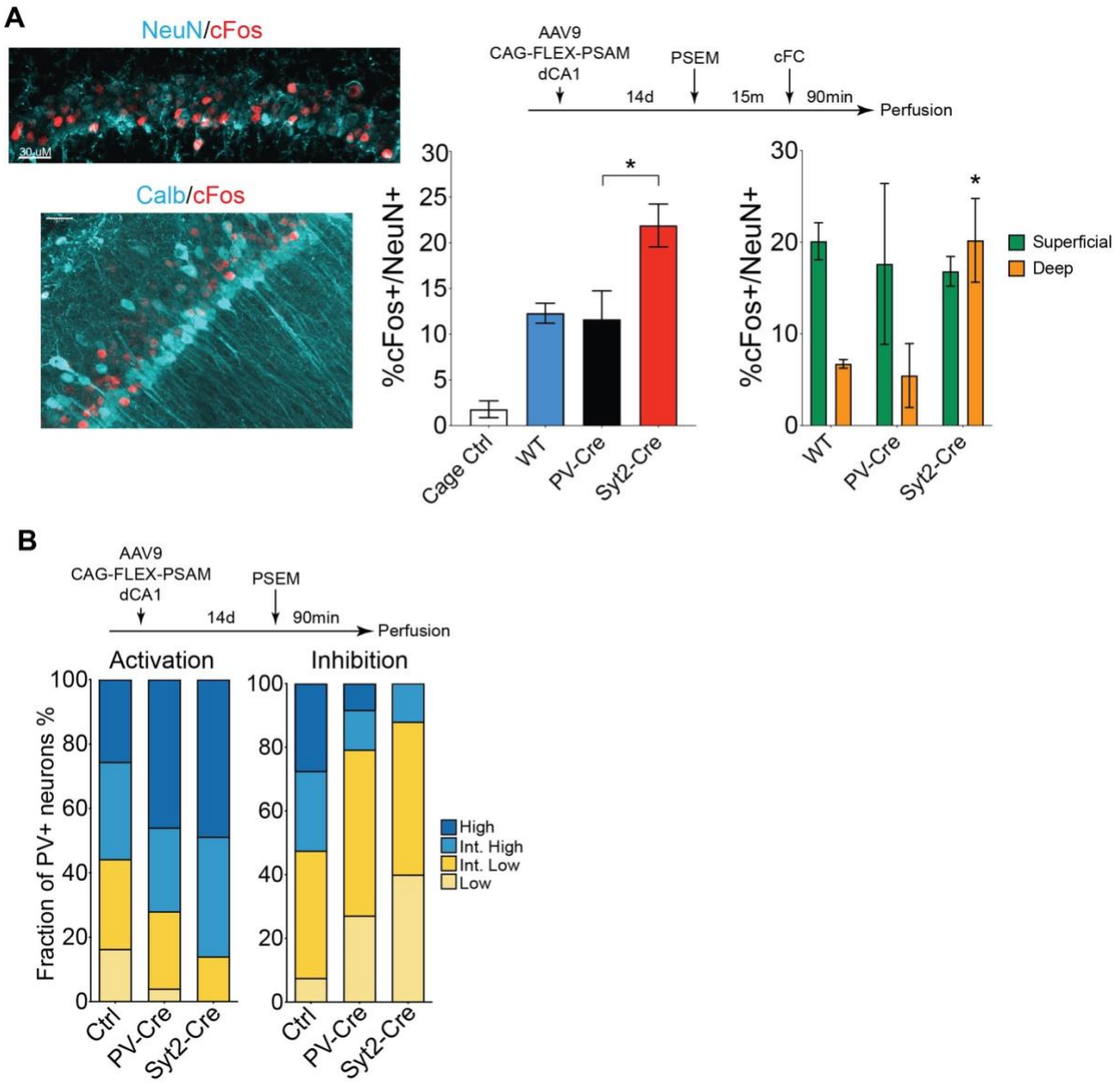


Figure 2.10: Cellular counterparts of PV+ and Syt2+ neuron manipulation in dCA1. A) **Left:** Representative image of staining for NeuN, Calb and cFos+ neurons in dCA1. **Right:** Syt2+ cell activation generates an increase in the number of cFos+ neurons in the deep PCs layer which is not observed when the entire PV population is activated. N=4 one-way ANOVA WT vs. Syt2-Cre P=0.0120, PV-Cre vs. Syt2-Cre P=0.0075. WT deep vs.Syt2-Cre deep P= 0.0449, PV-Cre deep vs. Syt2-Cre deep P=0.0295. **B)** Early-born PV neurons chemogenetic activation and inhibition is sufficient to cause a global high- or low-PV network reconfiguration respectively.

It has been previously shown that chemogenetic activation or inhibition of PV neurons induce opposite high or low PV plasticity respectively. While the low-PV plasticity enhanced memory consolidation and retrieval and augmented structural plasticity, the opposite was achieved by the induction of a high-PV network configuration (Donato et al., 2013). Importantly, an increase in the content of high PV neurons was detected in both early- and late-born PV neurons when a chemogenetic DREADD activator was delivered the entire hippocampal PV population,

suggesting that this artificial activation is able to drive into a high-PV state also late-born PV cells (Karunakaran et al., 2016). I therefore explored the possibility that the perturbation of early-born PV cells alone might be sufficient to induce this opposite network configurations. I therefore injected in the dCA1 of two separate cohorts of mice AAVs delivering Cre dependent chemogenetically-diriven depolarizing (Activaton) or hyperpolarizing (Inhibition) channels. Interestingly, both the Activation and the Inhibition of Syt2+ PV neuron were able to induce modifications in the PV network in the expected direction, but manipulating early-born PV cells caused a shift of higher magnitude to that of the entire PV network manipulation, suggesting that the network plasticity might be predominantly under the control of the early-born fraction.

3. Discussion

3.1 Retroviral Targeting and Transcriptional Profiling of PV Subpopulations

Neurons have different molecular, connectional and functional properties. Although still elusive, the concept of "cell type" is extremely useful in order to understand how these properties relate to each other and to describe the complex dynamics underlying biological processes. Since cell diversity is often characterized by the maintenance of developmental relationships in both the molecular and in the connectivity architecture, lineage and molecular composition are potent organizing principles for the study of cell types (Deguchi et al., 2011). In the first part of this thesis I therefore combined the ability of retroviruses to label clonally-related actively-mitotic neurons with recent technological advances in transcriptomics, in order to directly identify marker genes that confers genetic access to developmentally related subpopulation of parvalbumin positive basket cells.
The retroviral approach used in this thesis has been widely deployed recently to investigate the spatial distribution that clonally related interneurons, arising in the medial ganglionic eminence during development, reach in adult animals. It's therefore a well proven approach to parallelly label clonally related early- and late-born PV neurons that arise from progenitor cells. (Brown et al., 2011; Ciceri et al., 2013; Harwell et al., 2015; Mayer et al., 2015). In my experiments, consistently with previous results obtained in the lab, early-born retrovirally-labelled PV cells were found to be high-PV expressing cells and late-born retrovirally-labelled cells were low-PV expressing basket cells (Donato et al., 2015).

Many recent studies have explored, at an unprecedented scale, the molecular composition of neuronal cells both across brain areas and within the hippocampal network (Harris et al., 2018; Saunders et al., 2018; Tasic et al., 2018; Zeisel et al., 2018, 2015). In these studies, glutamatergic neurons where found to express regionally confined molecular markers, suggesting that most excitatory cell-types are area-specific, whereas nearly all types of GABAergic interneurons where found to be shared across cortical areas, suggesting that the vast majority of interneuronal cell-type specification is generated early during cell differentiation, possibly already in neuronal precursor cells, rather than environmentally acquired in the local circuit where postmitotic interneurons are allocated (Tasic et al., 2018). Indeed, previous results obtained in our lab from transplantation experiments pointed to the cell-intrinsic specification of the functional characteristics of early- and late-born PV neurons (Donato et al., 2015). In line with these observation, I find groups of functionally related genes differentially expressed in the two subpopulations. In particular, early-born basket cells were enriched in a collection of genes including *Gad1*, *Syt2*, *Vamp1*, *Atp1a3*, *Nefm* and *Nefm* necessary to sustain or associated with fast spiking behaviour (Harris et al., 2018; Saunders et al., 2018). This observations, in particular the selective expression of *Syt2* in early-born PV

neurons, suggest that early-born PV neurons represent the prototypical fast-spiking PV neurons, essential for effective network synchronization. Indeed, Syt2 is the member of the synaptotagmin family (which counts 15 members) with the fastest Ca^{2+} binding kinetics (Xu et al., 2007) and is therefore a key presynaptic Ca^{2+} sensor that, when nanodomain-coupled with Ca^{2+} channel activation, allows for rapid signalling during fast network events (Bucurenciu et al., 2008; Eggermann et al., 2012; Hu et al., 2014). Conversely, late-born cells were found expressing lower levels of these genes suggesting that they might be sustaining lower spiking rates, but were instead enriched in gene families involved in the regulation of actin filaments (*Fmn2*, *Lrrc7*, *Stmn1*) and in the stabilization of inhibitory synapses (*Git1*, *Cnksr2*).

It has recently been shown that hippocampal PV basket cells segregate of into two functionally distinct groups, one of which can reach higher firing frequencies during fast network events (Varga et al., 2014). This and other recent results indicate that, although often considered as a homogeneous population, the true scope of hippocampal PV basket cells heterogeneity is still underappreciated and further physiological characterization of early- and late-born PV basket cells populations is required to understand how their molecular specialization is translated into differential physiological properties (Hu et al., 2014).

Importantly, my gene expression analysis also supports previous observations of increased excitatory connectivity onto early-born cells (Donato et al., 2015). Interestingly, it has recently been shown that the selective ablation of the glutamate AMPA receptor GluR-1 (encoded by the Gria1 gene) from PV positive neurons in the hippocampus causes reductions in pyramidal cell-mediated synaptic drive to PV-positive cells that results in reductions in the power of fast gamma oscillations (Fuchs et al., 2007b). Therefore, the increased expression of *Gria1* in early born PV neurons is yet another evidence in support of the notion that this subpopulation plays a predominant role during fast spiking events.

Two of the genes differentially expressed in early-born PV neurons, *Gfra1* and *Syt2*, were of particular interest both for their known physiological relevance and because previous studies in the literature (including ISH experiments from the Allen Brain Institute) reported them following a sparse expression pattern similar to that of inhibitory interneurons (Kerr et al., 2008; Sarabi et al., 2000), suggesting that their expression might be restricted to the early-born subpopulation of PV basket cells. Since such a restricted pattern of expression would allow for selective in vivo manipulation I proceeded with the validation of these marker genes, that were both found to colocalize with the early-born High-PV fraction, and, for Syt2 with neurons tagged by retroviruses at early-time points. The newly generated Syt2-Cre mouse line, consistently, allowed for genetic access to the high-PV early-born fraction, as measured with

independent birthdating experiments involving BrdU. The generation of the Syt2-Cre mouse line, therefore, allows for the preferential manipulation of the early-born PV fraction.

3.2 Local Connectivity of PV Neurons

In neocortical circuits, inhibitory interneurons were thought to integrate the inputs of neighbouring excitatory cells and return them with non-specific inhibition related to the activity of the local network (Packer and Yuste, 2011; Scholl et al., 2015). This view has, however, evolved to include the formation of subgroups of more strongly interconnected principal and PV neurons tuned to the same features as a consequence of learning (Znamenskiy et al., 2018). In the hippocampus, dedicated inhibitory microcircuits targeting the two radial layers of CA1 were recently uncovered, thereby providing a selective and targeted inhibition to one particular subpopulation of PCs (Mizuseki et al., 2011; Soltesz and Losonczy, 2018). The main evidence in support of this view has been the finding that PV+ basket cells evoked three times larger Inhibitory Post-Synaptic Currents (IPSCs) in deep PCs compared to superficial PCs (Lee et al., 2014). The difference in IPSCs generated in deep vs superficial PCs could arise from an increased targeting of PV neurons synapses onto deep PCs, or, alternatively, from the existence of dedicated subpopulations of PV basket cells, with different physiological properties, that innervate the two layers differentially. Remarkably, two recent studies have reported an increased number of perisomatic PV+ boutons surrounding deep PCs compared to superficial PCs, leading the authors to conclude that PV basket cells are preferentially targeting the deep radial layer (Lee et al., 2014; Valero et al., 2015). In these approaches the quantification relied on immunohistochemical identification of PV+ synapses, that were estimated to be around 2 times more numerous in the deep layer, providing a possible mechanism for the difference in the evoked IPSCs generated in this layer. However, in one of this studies, when the quantification was performed using genetically encoded indicators, that don't rely on comparable levels of PV immunoreactivity in the synapses contacting the two sublayers, the number of PV boutons was estimated to be only around 25% higher in the deep layer (Lee et al., 2014). Indeed, we know from previous results obtained in the lab that PV-level distributions of perisomatic boutons onto deep and superficial CA1 pyramidal cells layers vary, with early- and late-born PV neurons preferentially targeting deep and superficial compartments respectively (Donato et al., 2015). Here, I developed and applied genetic approaches to label the synapses of subpopulations of PV neurons to elucidate the microcircuit architecture of CA1 PCs and PV basket cell subpopulations.

When the synapses from the entire PV population were labelled using AAVs delivering Cre-dependent synapse-tagging constructs in PV-Cre mice, I found that only a small amount of

preferential postsynaptic innervation was present in the deep layer. This approach, which relies on equal expression of tagging proteins from constitutive AAV promoters rather than from variable endogenous levels of PV immunoreactivity, eliminates biases introduced in the analysis, especially for low-expressing PV neurons synapses that might not have been considered otherwise. This might explain the discrepancies between this and other studies. Conversely, when injections were made in Syt2-Cre animals, early-born PV neurons were found to innervate the deep layer with twice as many synapses compared to the superficial layer. The genetic profiling of the two subpopulations revealed that the early-born PV cells possess the molecular machinery necessary for the delivery of more powerful inhibition and therefore, through specific connectivity, might be responsible for the increased IPSCs in the deep layer. The late-born PV fraction, on the other hand, had a much lower selectivity and projected almost equally to both the sublayers. Late-born PV cells have been shown to induce low-PV plasticity early during water-maze learning (Donato et al., 2015). During this initial phase, learning is characterized by a trade-off between the deployment of innate behaviours or recently-acquired knowledge and the need for exploration of new strategies and gathering of new information. The exploration of new strategies is mirrored, at the structural level, by an early formation of new synapses that are later eliminated when the reinforced ones are selected (Hofer et al., 2009; Yang et al., 2009). In this view, a subpopulation of late-born PV cells with non-specific inhibitory connectivity, low hyperpolarizing potential and that is also sending inhibitory connectivity to the early-born PV fraction, might be important to allow the formation of new synapses during the early-phases of learning. Recent studies have shown that the superficial PCs have a more stable representation of the global context in which the animal is embedded, whereas the deep PCs exhibit more dynamic firing patterns that encode for the position of landmarks and that are more modulated by goal-directed learning (Danielson et al., 2016; Geiller et al., 2017; Soltesz and Losonczy, 2018). Also, it has been shown that superficial PCs send much stronger excitatory projections onto PV basket cells compared to deep PCs (Lee et al., 2014). This, together with previous knowledge of the behavioural roles of early- and late-born PV cells, suggests that ensemble formation in deep and superficial PCs might be initially directed by lower-spiking late-born PV neurons that allows the exploration of novel strategies during the learning phase of the maze, when the animal has to generate a more general representation of the task and the context. Ensembles of superficial PCs that are producing successful representations of the context might then be orchestrating, through early-born PV neurons-mediated lateral inhibition, ensemble formation in the deep cells, linking the presence of landmarks to a more global representation of the context. A network architecture with these characteristics might be well-suited to support the flexibility necessary during the course of learning to maintain stable representations of the

context when landmarks are altered partially, in order to dynamically adapt to changes in the environment.

3.3 Retrograde Monosynaptic Connectivity onto PV Cells

In this thesis, I also investigated the global source of inputs to PV cells in dCA1, as well as to the early-born fraction using monosynaptic rabies technology. Regarding the entire PV population, my results are in agreement with previous reports that identified similar input connectivity (Sun et al., 2014), and suggest that the medial septum is the main extra-hippocampal source of projections to PV neurons. The "textbook" circuit functions ascribed to cortical inhibitory interneurons are, classically, to provide feedforward and feedback inhibition to local excitatory neurons, maintaining the excitation-inhibition balance required during network events (Hu et al., 2014). The extremely selective targeting of hippocampal PV neurons from septal long-range projecting PV neurons offers an interesting exception to this rule, representing an input channel that is not sending projections to local excitatory PCs but predominantly to PV cells that, in this case, do not function as feedforward inhibitors but as the main gate for the information carried by medial septal nuclei. The septal GABAergic neurons are thought to be major coordinators of hippocampal theta oscillations, and their inactivation results in the complete ablation of theta oscillatory activity in the hippocampus, which is accompanied by impairments in memory formation (Salib et al., 2019). Interestingly, these perisomatic-targeting projections were detected around the soma of both high- and low-expressing PV neurons, suggesting that the intrinsic properties of PV neurons and the PV-plasticity associated with learning might be differentially integrating this organizing input. Similarly, the monosynaptic retrograde inputs to the Gfra1 expressing early-born fraction are very comparable to those of the entire population, suggesting again that the two subpopulations are not part of distinct long-range brain networks. Importantly, low-PV expressing late-born cells were labelled by the retrograde tracer in Gfra1-Cre mice, indicating that they send a direct input to the early-born fraction and that the two subpopulations directly interact with each other. Further experiments will be required to understand the physiological role of these connection during network events.

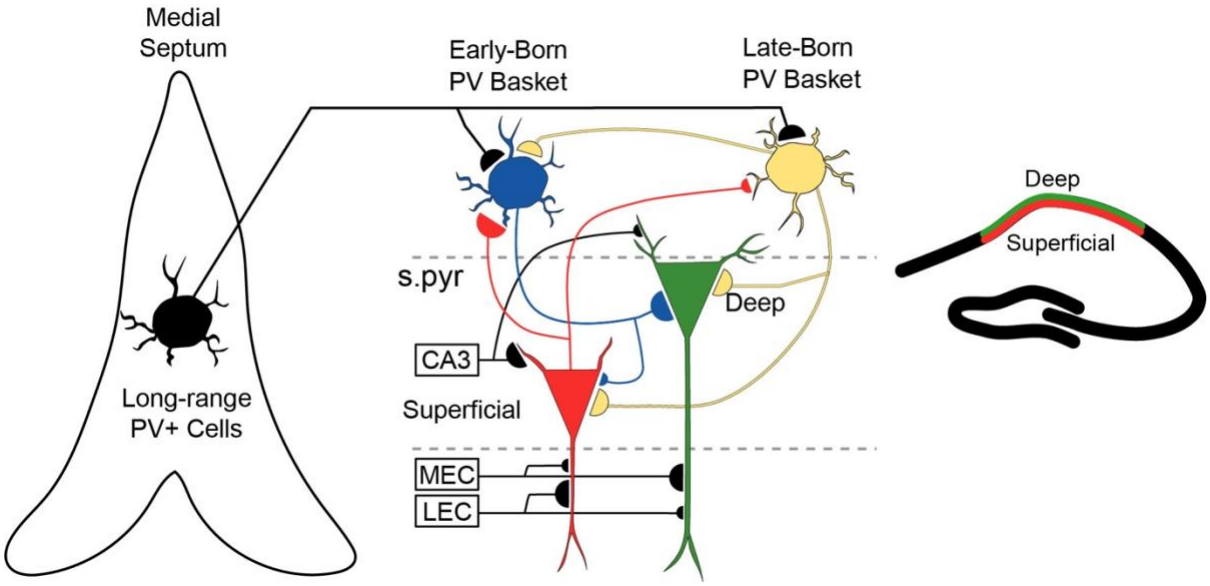


Figure 3.1: Schematics of connectivity findings

3.4 Activation of dCA1 Early-born PV Neurons During Learning, and Consequences on Memory and Local Ensemble Formation

In agreement with previous findings in the lab, the results presented here provide evidence that Parvalbumin expressing basket cells consist of two subpopulations that can be segregated based on their schedules of neurogenesis, that possess distinct molecular profiles, and that have different roles in definite and provisional learning (Caroni, 2015a, 2015b). The Cre-dependent manipulation conducted during the acquisition of cFC and at the end of the water maze learning (during definite learning) resulted in similar memory deficits in both PV-Cre and Syt2-Cre animals. This suggests that activity in early-born Syt2+ PV cells is required during memory acquisition, and that its perturbation is sufficient to recapitulate the manipulation of the entire PV population. Conversely, while the manipulation of the entire PV population carried out during the first phases of the MWM learning (during provisional learning) had an impact on the latencies, interfering selectively with the activity of Syt2+ cells did not produce any noticeable deficit, indicating that the activity of early-born PV cells can be perturbed during this phase without affecting the precision of newly acquired memories (**Fig. 2.9 B)**.

Consistent with findings of selective connectivity of early-born PV neurons to the deep CA1 PCs layer, the manipulation of Syt2+ early born cells was accompanied by a selective increase of cFos+ cells in this compartment, suggesting that increasing the overall excitability of early born PV neurons might cause an aberrant ensemble formation that is correlated with impaired memory performance. Interestingly, the manipulation of the entire PV population did not

produce an increase of the ensemble size, but was nonetheless accompanied by behavioural deficits during both provisional and definite learning. This suggests that there might be compensatory feedback loops that, through reciprocal connectivity of PV basket cell subpopulations, might keep in a balanced state the size of the emerging engram. Therefore, acting on both subpopulations at the same time might interfere with functional memory allocation mechanisms leading to memory deficits, while keeping the ensemble size at the physiological size while manipulating the activity of only one subpopulation might disrupt the balance leading to the emergence of an anomalous engram size. A possible mechanism for the maintenance of physiological ensemble size during entire PV neuron activation might be the observed lateral inhibition that late-born basket cells PV neurons exert on early-born PV cells or, possibly, the contribution of axo-axonic (chandelier) and/or bistratified cells that also express PV.

Chemogenetic activation or inhibition of PV neurons leads to sustained and reversible shifts in the network configuration that are detectable starting from 6h after the learning episode and regulate further acquisition of information (Donato et al., 2013). Remarkably, here I showed that enhancing or reducing the activity of early-born Syt2+ cells is sufficient to cause network shifts in both directions, suggesting that, even though during physiological events low-PV plasticity is specifically associated with the late-born fraction, early-born cells hyperpolarization is sufficient to drive a global shift towards the low configuration. Also remarkable is the magnitude of the observed shift, that appeared to be even more marked when the activity of early-born cells alone was manipulated compared to the activity of the entire PV population, as indicated by the complete disappearance of low- , respectively high-PV expressing cells. This suggests that the activity of early-born PV cells at acquisition might be more strongly correlated with the appearance of PV plasticity, and might therefore play a predominant role during memory consolidation.

3.5 Technical Considerations and Possible Mechanisms

Importantly, the manipulation applied here consisted in the activation of ligand-gated ion channels that were overexpressed through Cre-dependent AAVs on the membrane of infected neurons. These recombinant ion channels are activated by selective effector molecules within minutes upon systemic delivery, causing the depolarization of the neuronal membrane leading to increased firing rates *in vivo* (Magnus et al., 2011).
It has recently been shown, both *in vitro* and *in vivo,* that synchronizing the activity of populations of PV neurons through optogenetic activation at theta frequencies causes the

entrainment of the hippocampal network, regulating the power and rhythmicity of intrinsically generated oscillations (Amilhon et al., 2015; Stark et al., 2013). Furthermore, in analogous experiments using silencing opsins, it has been shown that also the rhythmic hyperpolarization of PV cells has effects comparable to the depolarization, entraining the network at 6 to 10 Hz, indicating that theta is the optimal frequency driven by hippocampal PV neurons. In support of this are also evidences for the increment in the baseline frequency of the network, that reached 8 Hz during a continuous and prolonged (tonic) light-induced activation of PV neurons delivered over a period of 10 seconds. This result suggests that mimicking overall increases in PV neuronal activity, possibly in a similar way to that of pharmacogenetic activation, leads to the synchronization of the PV network and causes the hippocampal PCs to be entrained in theta oscillations, increasing their total activity rather than decreasing it (Amilhon et al., 2015). The hippocampus is thought to support navigation and episodic memory by generating dynamic ensembles of cells that can support the underlying necessary operations even in the absence of environmental stimulation (Itskov et al., 2011; Pastalkova et al., 2008). Recent evidence is supporting the notion that PV neurons are more strongly connected to excitatory neurons with similar environmental responses and that, throughout the course of learning their firing might become more strongly correlated to that of the ensemble they're part of (Agetsuma et al., 2018; Znamenskiy et al., 2018). It has been recently proposed that the spikes of PV interneurons which are part of the active CA1 ensemble might suppress competing ensembles but, at the same time, may induce rebound spikes at the opposite phase of the theta cycle in the most strongly inhibited/competing neurons which, in turn, can become a seed of activation for the upcoming ensemble (Stark et al., 2013). Artificial activation of large populations of PV neurons, interfering with this lateral inhibition mechanism, might prevent the emergence of sequences of ensembles that are physiologically required to acquire episodic memories and therefore result in behavioral impairments closely comparable to those of network inhibition (achieved through muscimol administration).

But what might be the link between the role of PV neurons in network activity and IEGs expression and memory allocation? Although this remains a fundamental open question in the field of learning and memory, in a recent study the authors provided a first characterization of the activity patterns that ultimately lead to the expression of cFos in hippocampal PCs. Using tetrode recordings of optogenetically-tagged cells that expressed cFos following memory acquisition, they could show that the cells that were later incorporated in the memory engram exhibited an increased number of spike bursts strongly paced at theta frequencies and that, rather than simply higher activity, these theta bursts might trigger cFos expression and the consequent incorporation of neurons into memory engrams (Tanaka et al., 2018; Tanaka and McHugh, 2018). Since PV neurons play an important role in the generation of theta

oscillations, future experiments should elucidate the differential contribution of genetically identified early- and late-born PV cells during these network events.

3.6 Conclusions

Understanding how the brain turns sensory information into stable representations that can be retrieved when needed is a central question in neuroscience. Neuronal networks have evolved highly structured architectures to optimize this processes, relying on selective connectivity of genetically specified circuit elements. In this thesis, I focused on parvalbumin expressing interneurons, exploring the molecular identity and the connectivity of two functionally distinct subpopulations that were previously described in our lab (Donato et al., 2015). The approach used here, based on lineage-dependent interrogation of molecular identity and generation of Cre lines for the in vivo study of connectivity, can be translated to other populations of cortical interneurons and, when coupled with electrophysiological characterization and behavioural phenotyping, has the potential to greatly improve our understanding of the assembly and function of neuronal networks (Zeng and Sanes, 2017).

4.Methods

Mice

PV-Cre mice (129P2-Pvalbtm1(Cre)Arbr/J) were from Jackson laboratories. PV-FlpO mice were a kind gift from Professor Silvia Arber (Friedrich Miescher Institute). C57Bl6/J mice were from Janvier labs. Gfra1-CreERT2 mice were a kind gift from Professor Sanjay Jain (Washington University in St. Louis)

Viral Vectors

The Cre-dependent retroviral construct containing a membrane bound mCherry was a kind gift from Oscar Marin (King's College London). This vector contains an internal CAG chicken β-actin promoter and the woodchuck hepatitis post-transcriptional regulatory element (WPRE) and was used for the production of Moloney murine leukemia viruses (MoMLV) by transient transfection of HEK293T cells along with CMV-vsvg and CMV-gagpol. Supernatant was collected, concentrated by ultracentrifugation and resuspended in PBS. Titration of the virus was performed as described in (Tashiro et al., 2006).

The following AAVs were injected: AAV9-Syn1-FLEX-GFP (addgene 100043), AAV9-FLEX-SynGFP, AAV9-FLEX-Gly, AAV9-FLEX-TVA-IRES-H2BGFP were a kind gift of Prof. Silvia Arber. Pharmacogenetics AAVs were as follows: Activation (depolarization inducing channel): AAV9-CAG-FLEX-PSAMLeu41Phe,Tyr116Phe5HT3-WPRE. Inhibition (hyperpolarization inducing channel) AAV9-CAG-FLEX-PSAMLeu141Phe,Tyr116PheGlyR-WPRE. For the intersectional targeting of late-born PV neurons the following AAV was used: AAVdj-hSyn-Coff/FonEYFP-WPRE (UNC vector core). The Env-A coated rabies virus was a kind gift from Prof. Silvia Arber.

In utero injections

Pregnant females were deeply anesthetized with isoflurane. Depending on the experiment, E9 or E13 embryos were exposed and stabilized in a custom-made petri dish filled with sterile, warm PBS. The retrovirus was injected through a beveled glass micropipette in the embryonic ventricles. The injection area was visualized using a real-time high-resolution ultrasound scanner (VisualSonics Vevo 770). In birthdating experiments, pregnant females received intraperitoneal injections with saturating pulses of BrdU (1mg BrdU/10gm mother, 3 times every 4h) as described in (Butt et al., 2005)

Single cell RNA-seq from acute brain slices

Retrovirus-injected mice (P60) were deeply anaesthetized with thiopental and transcardially perfused with 5mL of ice cold bubbled ACSF. Brains were extracted and cut using a vibratome in 350 um slices in ice cold cutting solution. Slices were briefly rinsed in bubbled room temperature ACSF and then incubated in bubbled ACSF at 34 C for 30 minutes. Slices were

transferred to a microscope and perfused with bubbled ACSF at RT. A pipette (3-4 um diameter, 1-2 MOhm) filled with internal solution was used to collect cells. Once harvested, the tip of the pipet was withdraw, broke against the bottom of a PCR tube containing the Smart-seq2 lysis buffer, and immediately frozen on dry ice. Cutting solution was as follows: Choline chloride 110mM, $MgCl_2$ 7mM, sodium ascorbate 11.6mM, sodium pyruvate 3.1mM, KCl 2.5mM, NaH_2PO_4 1.25mM, $CaCl_2$ 0.5mM, $NaHCO_3$ 25mM, D-glucose 25mM. ACSF was as follow: NaCl 125mM, KCl 2.5mM, NaH_2PO_4 1.25mM, $NaHCO_3$ 26mM, D-glucose 4.8g/L, $CaCl_2$ 1mM, MgCl 2mM. Pipette internal solution was as follow: KCl 5mM, Potassium gluconate 115mM, HEPES 10mM, Mg-ATP 10mM, Na-GTP 0.3mM, Sodium phosphocreatine 10mM, RNAseOUT 2U/μL. Smart-seq2 lysis buffer in each PCR tube was as follow: RNAseOUT 4U, 0.2% Triton X-100 (2.4μL), dNTP mix 10mM (1μL), ERCC spike-ins 1:500,000 dilution (0.1μL), Smart-seq2 oligo dT 5'-AAGCAGTGGTATCAACGCAGAGTACT30VN-3' 10mM (1μL).

Immunohistochemistry and image processing

Antibodies were used as follow: rabbit anti-cFos (Millipore ABE457) 1:4000, mouse anti-NeuN (Millipore, MAB377) 1:1000; α-Bungarotoxin, Alexa 488 Conjugate (Molecular Probes, Life Technologies, B-13422) 1:500, goat anti-PV (Swant, Bellinzona) 1:5000, rat anti-BrdU (abcam ab6326) 1:500, Mouse anti-Calbindin D28-k (Swant, Bellinzona) 1:100, Mouse anti-Syt2 (Znp-1, ZIRC) 1:100, Chicken anti-GFP (abcam ab13970) 1:2000

Mice were transcardially perfused with ice cold 4% PFA in PBS pH7.4. Brains were collected and kept overnight in PFA 4% at 4°C and subsequently transferred to sucrose 30% until sinking. The next day brains were embedded in OCT and sectioned with a cryostat in 40μm coronal slices. The standard procedure for staining was as follows: sections were blocked at room temperature for one hour with 10% donkey serum in PBS-Triton 0.3% and subsequently incubated overnight with primary antibody-specific dilution in 3% donkey serum and 3%PBS-Triton 0.3%. The following day section were washed 3 times in PBS-Triton 0.3% and then incubated for two hours at room temperature in secondary antibody solution. Following 3 final washing steps slices were mounted on poly-L-Lysin coated slides in Prolong gold antifade reagent (Molecular Probes) and stored at 4°C until imaged.

Generation of Syt2-Cre animals

The annotated genomic sequence of the Synaptotagmin 2 gene was downloaded from ensembl genome browser and the 3'UTR contained in the exon 9 was selected as a target region for the cut by the Cas9 protein. The sgRNA was selected among other candidates based on the low off target score provided by the crispr.mit.edu software. A donor cassette containing an internal ribosome entry site, a Cre recombinase and a SV40 polyadenylation

site and flanked by a 1kb homology region on both ends was synthesized by a commercial vendor and injected intracytoplasmic at 10ng/μL into a donor C57BL6 zygote together with the following reagents:

crRNA ([mC][mG][mG]CCGGGGCCACGCCCCUAGUUUUAGAGCUAUGCUGUU[mU][mU][mG]) 300ng/μL (9μL), tracrRNA 65ng/μL (1μL), mRNA Cas9 (Sigma-1EA) 100ng/μL (6μL) in a total volume of 30μL.

Behavioral testing

All animals tested in behavioral protocols were 80 to 120 days old. Before the start of behavioral testing mice were single housed for at least 24h and had *ad libitum* access to water and food. All animal procedures were approved and performed in accordance with the Veterinary Department of the Kanton Basel-Stadt.

Contextual fear conditioning

During acquisition of contextual fear conditioning mice are allowed to explore the environment for 2.5 minutes and subsequently shocked 5 consecutive times (duration of the shock is 1 second at 0.8mA) with an inter-shock interval of 30 seconds for a total of 5 minutes in the context. Contextual fear memory recall is measured by returning the animal in the same context for 5 minutes during which the percentage of time the animals spent freezing (suppression of all movement) is quantified.

Morris Water Maze

A 140 cm in diameter pool filled with opaque water is used as a maze. The escape platform of 10 cm in diameter is place just above the water level during the first day (visible platform day) and 2 cm below the water level during the following days. Three signs of different shape and color are placed around the pool to provide allocentric distal cue. Mice undergo 4 trials per day, each lasting 60 seconds, spaced by 5 minutes. On visible platform day, if mice fail to reach the escape platform during the 60 seconds they are placed on it. From day 2 onwards, the platform is located in the opposite quadrant respect to day 1 and is kept invisible. The performance is measured by quantifying the time (latency) the animals need to reach the platform. During probe trials the platform is removed from the pool and the performance is quantified as time spent in each quadrant.

Stereotaxic surgeries

All surgeries are conducted using a stereotaxic alignment system. Mice are anaesthetized using isoflurane at 4% for induction of anesthesia and kept at 1.5%-2% during the surgical procedure, during which the body temperature is monitored and maintained stable with an heating system. Localized viral injections are carried out using glass-pulled pipettes containing 200nL of viral solution. Pressure is applied with a picospritzer (Parker Hannifin Corporation) to slowly inject the total volume over a period of 6 minutes, after which the pipet is left in place for the following 10-15 minutes to allow for diffusion of the virus and avoid backflow. The coordinates used are as follows: dCA1 (a.p. -2; m.l. 1.5, d.v. 1.25)

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