UiO : University of Oslo

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Balancing stability and plasticity-perturbations of extracellular matrix and inhibitory activity in the mature grid cell network

Thesis submitted for the degree of Philosophiae Doctor

Institute of Basic Medical Sciences
Faculty of Medicine

Centre for Integrative Neuroplasticity (CINPLA)

2020

Series of dissertations submitted to the
Faculty of Medicine, University of Oslo

ISBN 978-82-8377-699-7

Cover: Hanne Baadsgaard Utigard.
Print production: Reprosentralen, University of Oslo.

To Klara and Iris <3

Preface

This thesis is submitted in partial fulfillment of the requirements for the degree of *Philosophiae Doctor* at the University of Oslo. The research presented here is conducted under the supervision of associate professor Torkel Hafting and professor Marianne Fyhn.

The thesis is a collection of three papers, presented in chronological order. The papers are preceded by an introductory chapter that provides background information and motivation for the work, a chapter listing the overall objectives of the studies, a chapter highlighting challenges and considerations related to some of the methods used in this thesis, and finally a discussion chapter that aims to place findings from the papers in a broader perspective and point out relevant directions for future research.

Acknowledgements

I would like to thank my supervisors associate professor Torkel Hafting and professor Marianne Fyhn. You introduced me to the incredibly fascinating world of "place" and "grid cells" which gave me the opportunity to learn from the very best in the field. You have given me support, inspired me with your enthusiasm, guided me in fruitful directions and at the same time challenged me to become independent. You have been able to balance my pessimistic time realism with your ever lasting time optimism. And, although most of my depicted timetables were completely shattered, I have learned that surprisingly much can be done with very little time, - as long as it's really urgent.

To my colleagues at CINPLA, thank you so much for creating both an inspiring professional environment, but also for being such a sociable and cool group of people. I have looked forward to go to work every single day, much thanks to you guys. The past year has been particularly inspiring since we were able to finally join forces from the computational and experimental world. Thank you so much Dr. Svenn-Arne Dragly for taking the initiative to join my project. Thanks to Mikkel Lepperød for the very productive collaboration on two of the papers in this thesis, and for teaching me the value of "stubbornness". I also want to direct a special thanks to Dr. Kristian Lensjø for bringing me on board the Hafting-Fyhn group to begin with. During the past seven years you have taught me everything from all of the experimental methods I've used, to valuable work hacks like how to avoid booking flight tickets through Egencia. Thanks for your endless patience. I also want to thank Hilde Hyldmo for always saying "yes, I will fix!" to all my animal-related requests, Tove Klungervik for never giving up on impossible genotyping protocols, and to Cathrine Fagernes for being my much needed HSE-advisor during pregnancy.

To friends and family. You've had to endure many late hour lectures on rat behavior, the complexity of the brain and the slow progression in research. Thank you for always listening with enthusiasm and interest. I've found it very inspiring that you take interest in my work. A special thanks to my family for helping out with kids during "impossible quarantine conditions" in the last hours before I handed in my thesis. Last but not least, to David. You and our two beautiful girls are my world and has kept my life balanced, fun and sensible throughout these years. You've accepted every challenge I've thrown at you and never complained about odd working hours or having to be alone with a toddler for more than a week at a time. I'm truly grateful for all your support, and not to forget that you've kept me updated on whatever goes on in the world outside Blindern. You make my life complete. I love you.

Ane Charlotte Christensen
Oslo, April 2020

Summary

A fine tuned balance between inhibitory and excitatory neural activity is essential for proper brain function. Inhibitory interneurons are important for cognitive functions such as memory processing and navigation. Grid cells in the medial entorhinal cortex (MEC) are considered to be the hub in the brains network for spatial representations and are interconnected through inhibitory (PV^+) neurons. However, little is known about how PV^+ cell activity affect grid cell properties.

Every time an animal returns to the same environment, grid cells are active in the same locations tiling the environment with a hexagonal pattern. Furthermore, pairs of grid cells maintain relative spatial and temporal activity patterns across different environments and brain states. All in all this suggests little plasticity in the underlying neural network, and is in stark contrast to the highly plastic network in hippocampus where different populations of neurons are activated in different environments. PV^+ cells are involved in shaping network function during brain development, a role that has been thoroughly demonstrated in sensory cortical circuits. A hallmark of maturing PV^+ cells is the gradual condensation of specialized extracellular matrix molecules called perineuronal nets (PNNs), which embody their cell soma and proximal dendrites. Maturation of PNNs coincides with a marked reduction in plasticity, thus rendering mature neural circuits with a high level of stability. Due to the abundant number of PV^+ cells in MEC, PNNs are densely expressed in this area but it remains unknown if PNNs contribute to development of grid cell activity and their properties in adulthood.

In this thesis, I used immunohistochemistry to label PNNs and describe a timeline for development of PNNs in MEC. Next, I described the expression pattern and cell type specificity of PNNs in MEC, hippocampus and the primary visual cortex. To test if grid cell activity relies on intact PNNs, I enzymatically degraded PNNs in MEC and recorded single unit activity while rats explored familiar and novel environments. The findings I present in this thesis suggest that PNNs are important for maintaining both structural and functional stability in the grid cell network, in particular the rapid stabilization of novel spatial representations. Moreover, I show that removal of PNNs impairs the temporal relation between grid cells, suggesting that PNNs may be important for maintaining the relative stability of firing patterns between grid cells.

It has been postulated that theta oscillations in the local field potential is essential for grid cell coding. Thus, in the last paper I test how manipulations of theta oscillations affect the spatial coding of grid cells. I find that rhytmic optogentic activation of PV^+ cells in the medial septal area cause robust pacing of theta oscillations in MEC, but without changing spatial firing patterns of grid cells. Many grid cells respond with increased phase locking to the new theta

frequency, which completely abolished phase precession. This study therefore challenge both theoretical and experimental work by showing that grid patterns are not altered with increased theta frequency, nor do they rely on phase precession or speed modulation of theta.

In summary, I demonstrate in this thesis that in a mature MEC network, perturbations of plasticity-limiting extracellular matrix or oscillatory activity in the theta frequency range, have modest effects on the overall spatial activity of grid cells. However, these manipulations disturb the temporal spiking dynamics and also stability of novel grid representations. During both these perturbations, the effects are likely caused by the indirect manipulations of PV^+ cell activity, suggesting a critical role of local inhibition for the workings of grid cells.

List of Papers

Paper I

Lensjø, K.K.*, Christensen, A.C.*, Tennøe, S., Hafting, T., Fyhn, M. 'Differential Expression and Cell-Type Specificity of Perineuronal Nets in Hippocampus, Medial Entorhinal Cortex, and Visual Cortex Examined in the Rat and Mouse'. In: *eNeuro* **4(3)** (2017), DOI: 10.1523/ENEURO.0379-16.2017.

* These authors contributed equally

Paper II

Christensen, A.C., Lensjø, K.K., Lepperød, M.E., Dragly, S-A., Sutterud, H., Blackstad, J.S., Fyhn, M., Hafting, T. 'Perineuronal nets stablilize the grid cell network'. *Submitted for publication.*

Paper III

Lepperød, M.E., Christensen, A.C., Lensjø, K.K., Buccino, A.P., Yu, J., Fyhn, M., Hafting, T. 'Optogenetic pacing of medial septum PV cells disrupts temporal but not spatial firing in grid cells'. *Submitted for publication.*

Contents

Chapter 1

Introduction

Regulation of brain activity

The brain contains nearly a hundred billion neurons that each can be connected through thousands of chemical contact points called synapses. The exact number of different neuronal cell types is not yet resolved, but common to all is that they communicate by using one of two fundamentally different methods; either by promoting activity in downstream neurons, termed excitation, or by preventing activity in downstream neurons, termed inhibition. Excitatory neurons are often referred to as principal neurons and generally viewed as the neurons with executive functions. Inhibitory neurons on the other hand, are often called interneurons because they regulate activity between local subsets of principal neurons, creating neural circuits. In order for the immensely complex network of neurons in the brain to perform meaningful computations, a finely tuned balance between excitation and inhibition is essential. A well known example of brain activity out of control is when too much synchronized activity produce epileptic seizures, potentially damaging the brain (Scharfman 2007). Both the timing and the strength of excitatory activity is controlled and balanced by inhibition. This tightly regulated interplay between excitation and inhibition is often referred to as the excitatory/inhibitory balance (E/I).

Excitatory/inhibitory balance

Regulation of the E/I balance is detectable at multiple levels in the brain, even down to balancing the signalling strength of a single synapse (Xue et al. 2014). In the broader sense, the number and activity level of inhibitory synapses are perfectly adjusted to regulate the activity in local populations of principal neurons. However, the E/I balance can be temporarily shifted to allow neural circuits to adjust their connections. This feature, known as plasticity, is essential during development (Takesian and Hensch 2013), but also in adulthood during learning and memory and for updating sensory- and motor function (Hensch 2005). When the E/I balance is shifted, i.e. synaptic drive is changed persistently, it is quickly counterbalanced in a process termed homeostatic plasticity (Turrigiano 2011). Upon increase in signalling strength in one synapse, other nearby synapses can decrease their signalling strength in order to maintain a set activity ratio between excitation and inhibition. Moreover, inhibitory interneurons can adjust their intrinsic excitability through regulation of ion channels in order to match the excitation level of the network (Campanac et al. 2013). Hence homeostatic plasticity helps to maintain the overall E/I balance in the brain, avoiding that change in one microcircuit can cause extended effects on other connected circuits.

Maintaining the delicate balance between excitation and inhibition is vital for the healthy brain. In mental illness or cognitive impairment this balance is often disturbed, and there's multiple strong links between several common mental diseases and malfunctioning inhibition (reviewed in Selten et al. 2018). Therefore, understanding how inhibitory neurons shape the activity of principal neurons is critical for understanding both the healthy and the diseased brain.

Inhibitory networks

Although inhibitory interneurons make up less than 20% of all neurons in the brain (Markram et al. 2004; Meyer et al. 2011), they represent a vastly heterogeneous group and are far more diverse than their excitatory counterparts (Klausberger and Somogyi 2008; Tremblay et al. 2016). Inhibitory interneurons differ greatly in morphology, electrical properties, protein expression and connection schemes. GABA is the most common neurotransmitter used by inhibitory neurons in synaptic signalling, while excitatory neurons generally use glutamate. In addition, many neurons release neuromodulators that operate on longer timescales and can have multiple effects in downstream neurons (reviewed in Avery and Krichmar 2017). Interestingly, it is becoming apparent that different inhibitory cell types not only contact highly specific subcellular domains of postsynaptic neurons (Bloss et al. 2016; Royer et al. 2012), but they also control separate functional subgroups of principal neurons (Fuchs et al. 2016; Miao et al. 2017), meaning that they work in concert to regulate the activity of principal cell populations. The variety of connection schemes provided by interneurons can modulate gain, bursting, tuning properties and filtering of excitatory activity, thus providing the flexibility needed for complex cognitive operations (Figure 1.1). Recent technological developments over the last decades has provided new tools to perform targeted perturbations of genetically defined interneuron subtypes, opening up the possibility to investigate their assumed roles in intact brain circuits.

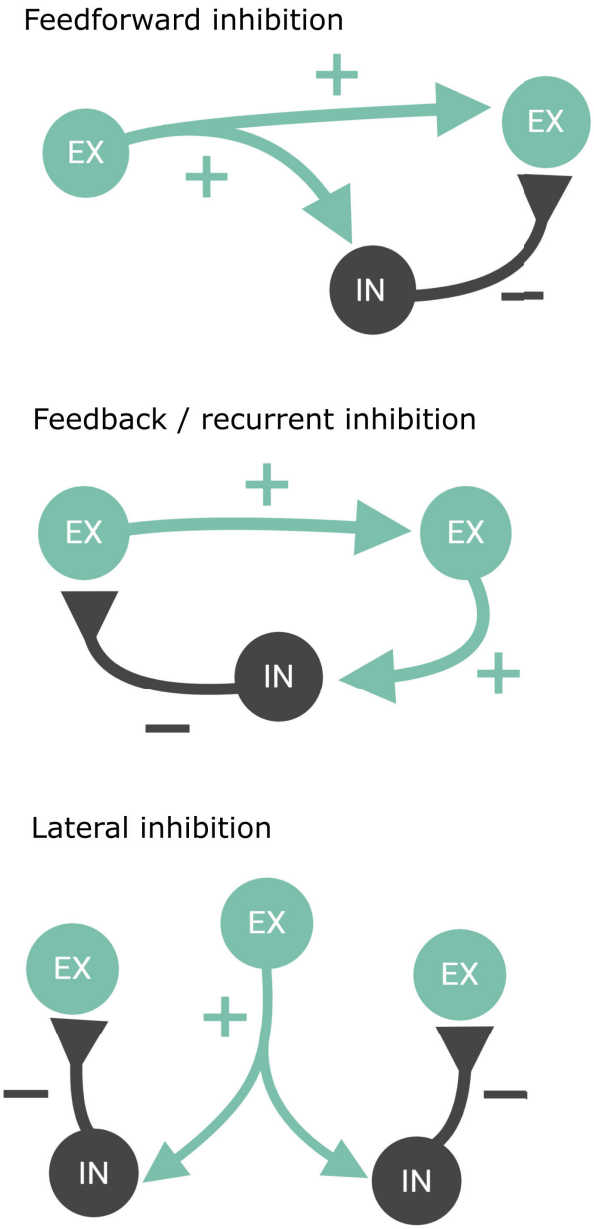

Figure 1.1: **Inhibitory circuits create distinct coding properties**. Feedforward inhibition limits the time window for when a cell can be active, increasing spike-timing dependent tuning. Feedback inhibition limits excitation in a circuit. Lateral inhibition implements a winner-takes-it all dynamics where activation of one principal neuron leads to suppression of surrounding principal neurons.

Parvalbumin positive inhibitory interneurons

The population of inhibitory neurons expressing the calcium binding protein parvalbumin (PV), is the most numerous group of inhibitory interneurons. PV^+ cells can be either basket cells contacting cell soma and the proximal part of dendrites, or chandelier cells synapsing onto the initial segment of axons. This connection scheme gives basket and chandelier cells a powerful role in controlling the spiking output of connected cells (reviewed by Hu et al. 2014). Most PV^+ cells project locally, contacting a large majority of excitatory neurons in their vicinity. In addition, some PV^+ cells have long distance projections such as the projection from the medial septal area (MSA) in the basal forebrain, to the hippocampus and parahippocampal areas (Freund 1989; Fuchs et al. 2016; Kiss et al. 1990).

PV^+ cells coordinate local neuronal networks

Due to their perisomatic inhibition, PV^+ basket cells are well suited to regulate spike timing of principal neurons. Since they also express fast spiking properties, PV^+ cells are recognized as controllers of brain oscillations, particularly oscillations in the gamma frequency band (30-90 hz) (Cardin et al. 2009; Sohal et al. 2009). In the hippocampus and parahippocampal areas, inhibitory neurons receive input from pace-making PV^+ cells in MSA that drive local theta oscillations (6-10 hz); a frequency band that is prominent among rodents and associated with spatial navigation and episodic memory formation (Buzsáki 2005; Jeffery et al. 1995).

The activity of neurons in the hippocampus and parahippocampal areas, is shown to be strongly modulated by local theta oscillations. This adds an additional layer of information by organizing single spikes into specific temporal windows (reviewed in Colgin 2013). In addition, many spatially modulated neurons express phase precession, where spikes falls progressively earlier in the theta phase as the animal runs through a firing field (Hafting, Fyhn, Bonnevie, et al. 2008; O'Keefe and Recce 1993). Phase precession is not yet properly understood but could provide the animal with accurate velocity information (Zutshi, J. K. Leutgeb, et al. 2017), enable sequence encoding (Jaramillo and Kempter 2017) or improve position estimate (Reifenstein et al. 2012).

Although the role and significance of different oscillation frequencies is still debated, both gamma and theta oscillations are associated with specific behaviors. In particular, theta oscillations are more prominent during active sampling behavior than during passive behavior (Colgin 2013). Furthermore, gamma oscillations co-occur with theta oscillations and are believed to enhance the efficacy of information transfer between the medial entorhinal cortex (MEC) and hippocampus (Colgin et al. 2009; Pastoll, Solanka, et al. 2013), which is likely to be important for spacial memory (Yamamoto et al. 2014). Synchronized oscillatory activity is also seen across interconnected brain areas during different stages of memory processing. For example, memory consolidation is believed to rely on information transfer between hippocampus and neocortex, and certain

oscillatory frequencies becomes synchronized in the hours following a learning task. If PV^+ cells are inactivated by pharmacogenetics during this time period, synchronization is disrupted and memory consolidation impaired (Ognjanovski et al. 2017; Xia et al. 2017). Therefore, PV^+ cells seem to play a pivotal role in coordinating activity both within and across brain areas.

PV^+ cells are essential during development

During development, PV^+ cells are central in shaping the activity of principal neurons (Fagiolini 2004; Takesian and Hensch 2013). This has been demonstrated for primary sensory areas where enhanced inhibitory activity marks the onset of periods with increased activity-dependent plasticity. During these so-called critical periods, sensory input drives synaptic pruning and refines neural circuits so that the mature network is optimized for processing incoming information (Hensch 2005). The timeline for critical period plasticity has so far been well defined for sensory cortices. However, similar windows of activity-dependent plasticity are likely to be crucial for development in other brain areas as well. For instance, various memory systems seem to have a critical period which marks a division between whether memory traces are stored as separate memories or are subjected to generalization (that is animals below a certain age show fear responses in all contexts, not just the one they have learned to associate with an aversive stimuli), or overwriting (Alberini and Travaglia 2017; Gogolla et al. 2009; Ramsaran et al. 2019; Slaker et al. 2015; Travaglia et al. 2018). In sensory cortices, the closure of critical periods depends on the maturation of extracellular matrix structures called perineuronal nets (PNNs) (Rowlands et al. 2018). PNNs appear at the end of critical periods which indicate that they are vital for regulating activity-dependent plasticity. In contrast to sensory cortices, plasticity regulation in brain areas associated with higher level cognitive function is not well understood. Thus, the role for PNNs in shaping the function of memory systems remains unknown.

Perineuronal nets

Towards the end of critical periods, the PNNs, which are specialized extracellular matrix components consisting largely of chondroitin sulfated proteoglycans, condense around the cell soma and proximal dendrites of primarily PV^+ cells (Härtig, Brauer, et al. 1992; Kosaka and Heizmann 1989) (Figure 1.2). This is accompanied by a dramatic reduction in plasticity. Moreover, critical period plasticity can be reactivated experimentally by removing PNNs in adult animals (Lensjø et al. 2016; Pizzorusso 2002), thus, PNNs are believed to act as a brake on plasticity in adult animals (Carulli et al. 2010, reviewed in Wang and Fawcett 2012). Several properties of PNNs may contribute to their plasticity- limiting role. First, since they enwrap the cell soma and the proximal part of dendrites, leaving holes only at synaptic contact points, they may stabilize existing synapses and limit synaptic turnover (reviewed in Dityatev and Schachner 2003). Therefore, PNNs may contribute to maintaining stable inputs to a large part of the neuron.

Second, they facilitate the fast spiking activity of PV^+ basket cells, possibly by buffering ions and reducing the capacitance of the cell membrane (Härtig, Derouiche, et al. 1999; Tewari et al. 2018). Indeed, removing PNNs reduces spiking probability in PV^+ cells and putative inhibitory neurons (Balmer 2016; Favuzzi et al. 2017; Lensjø et al. 2016). Lastly, PNNs restrict the lateral diffusion of AMPA receptors and thereby reduce the exchange of AMPA receptors across synaptic membranes (Frischknecht et al. 2009).

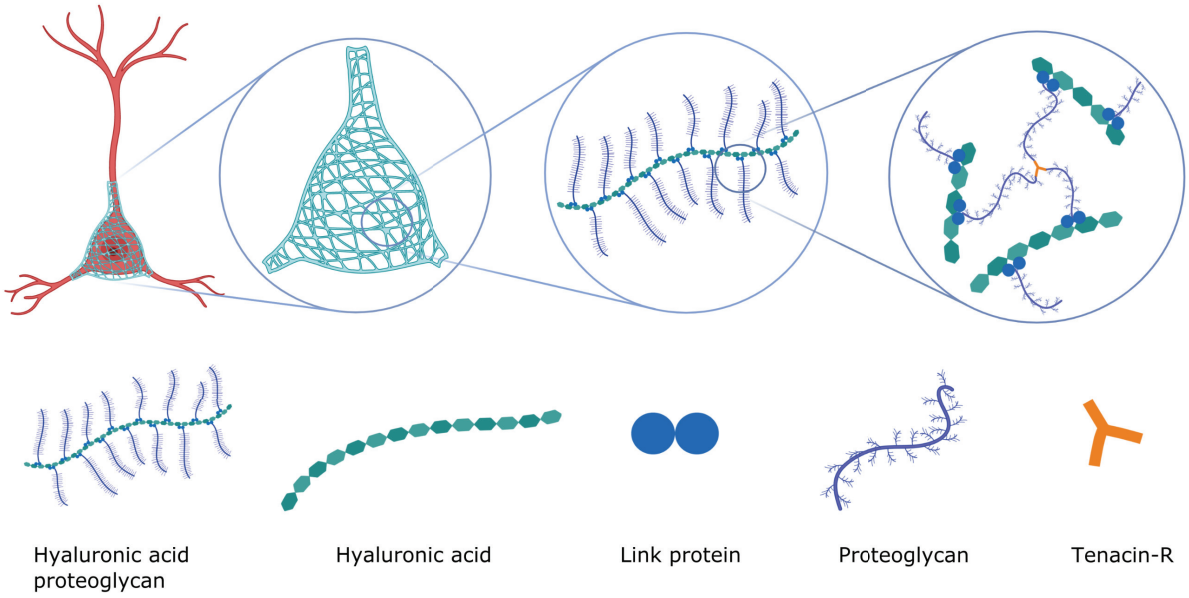

Figure 1.2: **Structure and molecular components of perineuronal nets (PNNs).** The condensed ultrastructure of PNNs forms a dense mesh enwrapping the cell body and proximal dendrites of mainly PV^+cells. PNNs consist of a backbone of hyaluronic acid attached with various proteoglycans (aggrecan, neurocan, brevican and versican) by link proteins. The proteoglycans are again cross-linked by tenacin-R resulting in a highly stable structure.

PNNs show variation in expression pattern and cell-type specificity

Reduced plasticity in adulthood may be beneficial for maintaining consistency in how we experience and respond to the environment. On the other hand, plasticity is necessary in order to learn and make new memories. Accordingly there seems to be a hierarchy in the brain where higher order cognitive brain areas retain high levels of plasticity throughout life, whereas primary sensory areas become more hard-wired during late adolescence and early adulthood. This may be reflected in the local levels of PNNs. Since PNNs are reported in many brain areas and implicated not just in sensory and motor processing (Lensjø et al. 2016; McRae et al. 2007; Pizzorusso 2002), but also learning and memory (Gogolla et al. 2009; Happel et al. 2014; Morellini et al. 2010; Romberg et al.

2013; Slaker et al. 2015; Thompson et al. 2017), their expression pattern could be indicative of functional properties of individual brain areas.

Notably, PNNs do not only surround PV^+ cells, but are shown to occasionally be associated with pyramidal cells in neocortex (Alpár et al. 2006) and amygdala (Morikawa et al. 2017), in addition to ensheathing all pyramidal cells in the hippocampal region CA2 (Carstens et al. 2016; Celio 1993). Such expression patterns may form highly specific circuits with distinct coding properties (Morikawa et al. 2017). The heterogeneity, cell type specificity and functional role of PNNs in different brain areas are still largely unexplored.

This is addressed in paper I where we aimed to map out differences in PNN expression and morphology in three different brain areas, the primary visual cortex (V1), the hippocampus and the medial entorhinal cortex (MEC). We found that PNNs were particularly dense in MEC, an area which contains a large population of PV^+ cells, in addition to many well known functional cell types that contribute to spatial navigation. Interestingly, MEC is postulated to play an essential role in spatial memory and receives integrated information from many areas of the cortex (Witter et al. 2017). Still, the MEC network and its spatially modulated output appears to be particularly hard-wired, suggesting that low plasticity in this network could be determinant for its computational role. With this starting point, we went on to perform functional studies of PNNs and the role of plasticity and inhibition for the spatial cells in MEC.

Medial entorhinal cortex

The MEC is an essential part of the brain's navigation system and postulated to contribute to encoding the spatial aspect of episodic memories (Eichenbaum and Lipton 2008). MEC contains a large variety of spatially modulated cells, and single cell activity correlates specifically with different animal behaviors. Along with grid cells, that are place modulated neurons with highly regular periodic firing patterns, the activity of spatially modulated cells in MEC correlate with head-direction (Sargolini 2006), speed (Kropff, Carmichael, et al. 2015), proximity to borders in the environment (Solstad et al. 2006), and distance and direction from objects (Hoydal et al. 2018). In addition to space, a recent study demonstrate that a subset of neurons in MEC code for elapsed time when the animal is immobile (Heys and Dombeck 2018). Another study has shown that grid cells can represent particular sound frequencies, which strongly suggests that MEC could be involved also in non-spatial tasks (Aronov et al. 2017). The combined activity of neurons in MEC map self-location and self-movement and provides necessary information to support orientation and spatial navigation. Neurons in MEC send their information about self-movement to the hippocampus, a brain region that is essential for acquiring and consolidating episodic memories embedded in a spatial context (Smith and Mizumori 2006) (Figure 1.3). Together with the lateral entorhinal cortex (LEC), the MEC provides the largest cortical input to the hippocampus (reviewed in Witter et al. 2017), and both principal cells and inhibitory PV^+ cells are found to project to specific subregions of the

hippocampus (Ye et al. 2018; S.-J. Zhang et al. 2013).

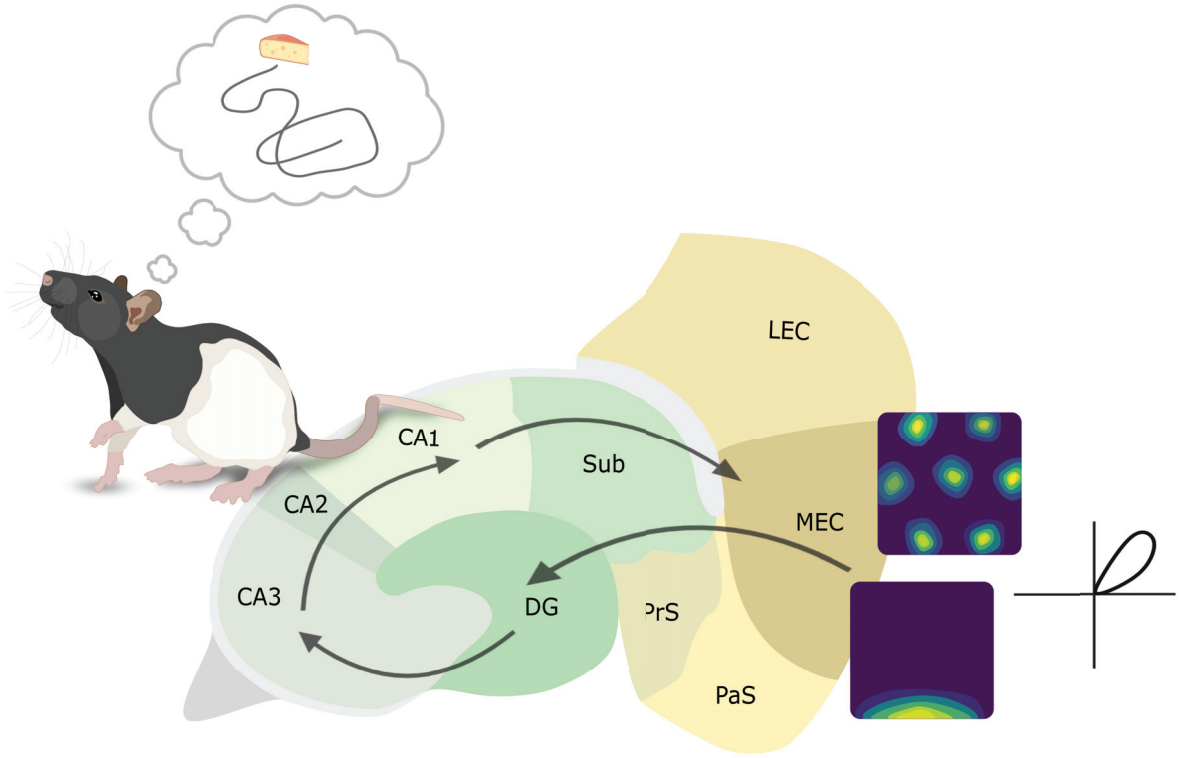

Figure 1.3: **The hippocampus receives input from spatially modulated cells in medial entorhinal cortex (MEC) that contributes to spatial navigation and memory.** Spatial information (here represented by a grid cell, border cell and head-direction cell) from superficial layers of MEC is received through the dentate gyrus (DG), where information is passed to CA3 and CA1 in the so-called three-synaptic loop. Some spatially modulated information from MEC also reach the CA1 directly (arrow not shown). The hippocampus ultimately sends information back to deeper layers of MEC. Together, this flow of information is believed to support episodic memory formation embedded in a spatial context.

In addition to principal cells, MEC contains a prominent population of inhibitory cells, where PV^+ cells constitute the largest group (approximately 50% of all inhibitory neurons in this area) (Witter et al. 2017; Wouterlood et al. 1995). Feedback inhibition is particularly widespread in the MEC network. In recurrent networks such as this, activation of an excitatory neuron provides disynaptic inhibition to neighbouring principal neurons (Figure 1.1). In layer II of MEC, the number of monosynaptic connections between principal neurons are almost negligible for certain cell types, and excitatory connections between principal cells seem to be provided solely by pyramidal cells (Couey et al. 2013; Fuchs et al. 2016; Zutshi, M. P. Brandon, et al. 2018). This highly organized, inhibitory dominated network is believed to enable the distinct spatial activity

of principal neurons in MEC. Nevertheless, we are still lacking insights into how functional connectivity supports the activity of the different functional cell types in MEC.

Grid cells

Grid cells are the most abundant of the spatially modulated cells in MEC. Grid cells possess multiple repeated activity nodes in the environment making up a striking hexagonal activity pattern covering the area visited by the animal (Hafting, Fyhn, Molden, et al. 2005) (Figure 1.4, a). Grid cells are active in all environments and maintain the hexagonal activity pattern when animals move between different environments. Grid cells have therefore been suggested to provide a universal, self-motion based system for navigation (Hafting, Fyhn, Molden, et al. 2005; McNaughton et al. 2006). The hexagonal pattern of grid cells can vary in their relative position of firing fields, called *phase*, in their *orientation* relative to borders in the environment and in the size of -and *spacing* between individual fields (Figure 1.4, b). When moving ventrally from the dorsal border of MEC, grid fields increase in size (Brun et al. 2008)(see also Figure 1.7). This increase is not gradual, but happens in discrete steps where the inner circle of the central field doubles (Stensola et al. 2012). Accordingly, grid cells with similar spacing and orientation are organized in non-overlapping modules, both with regards to properties and anatomy (Gu et al. 2018; Stensola et al. 2012). Interestingly, PV^+ cell terminals seem to follow a similar anatomical organization, where the number of PV^+ inputs to MEC layer II is reduced along the dorsoventral axis (Beed et al. 2013). This suggests that the density of inhibitory inputs may confine the spatial firing pattern of grid cell modules. When animals move from one environment to another, grid cells show a coherent shift in phase and orientation which is called remapping or realignment (Fyhn et al. 2007). At the same time, different place cell ensambles are recruited in the hippocampus leading to a concurrent remapping of the place code (Fyhn et al. 2007; S. Leutgeb 2005). If the animal explores a new environment for the first time, the grid fields may also expand temporarily and slowly return to its original scale as the animal continues to explore (Barry et al. 2012). The stability in spatial coherence between grid cells is striking and the pairwise ratio between the different spatial parameters are maintained from familiar to novel environments (Yoon et al. 2013). Recently, grid cells were also found to maintain pairwise temporal spiking relationships, regardless of change in input to the cells, brain state and even when grid cell firing patterns are lost (Almog et al. 2019; Gardner et al. 2019; Trettel et al. 2019). This indicates that grid cells are likely to be embedded in a highly stable, low plasticity network which maintain the spiking relationship between groups of cells across all environments and regardless of signal input.

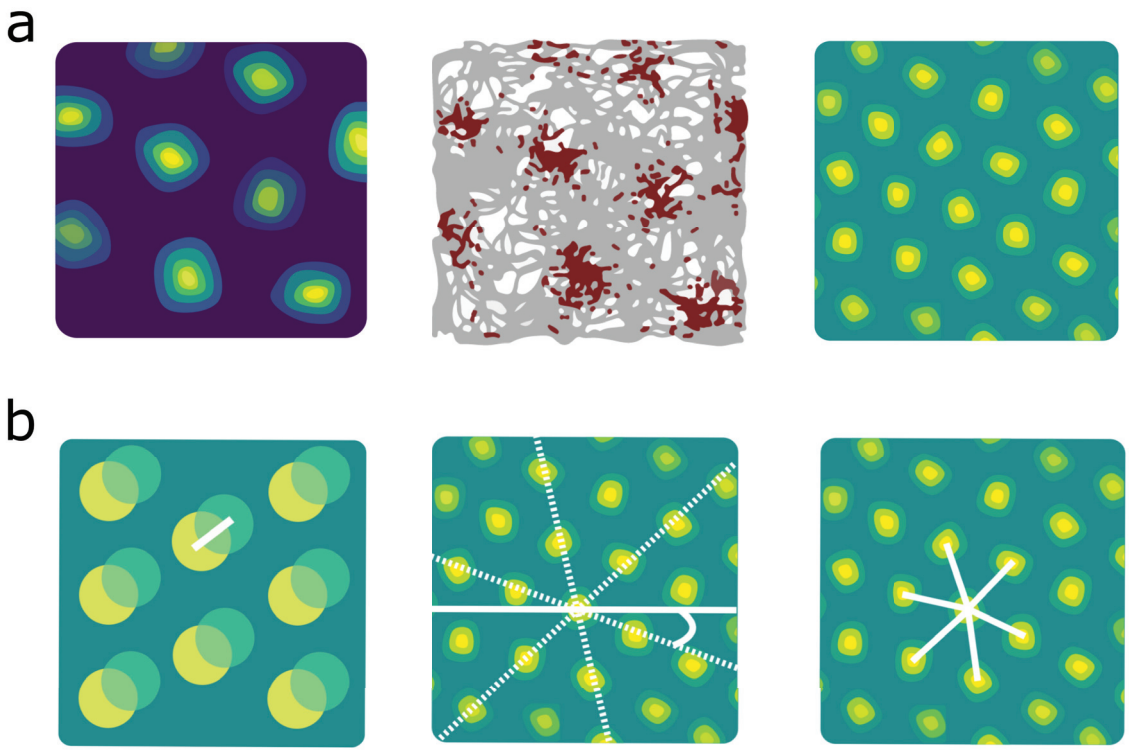

Figure 1.4: **Spatial properties of grid cells. a)** Grid cells' firing fields are commonly represented using rate maps (top left) where the lightest colors represent the highest firing frequency and the darkest color is where the cell is silent. Top middle map shows the trajectory of the animal with spikes (red) superimposed. Top right map shows the spatial autocorrelation of the rate map that is often used to calculate various spatial properties of individual cells. **b)** Bottom row illustrates the difference in *phase* of two grid cells, the *orientation* of a grid cell, given by the smallest angle between a horizontal axis and the three main axes in the autocorrelation map, and lastly the *spacing* of a grid cell that is calculated as the average distance from the center field to the six closest fields in the autocorrelation map.

Role of inhibition in spatial coding of grid cells

Grid cell activity is not restricted to a single anatomical cell type but is found among the two principal cell types in superficial layers of MEC; stellate cells and pyramidal cells (Domnisoru et al. 2013; Gu et al. 2018; Rowland et al. 2018a; Sun et al. 2015). Stellate cells express reelin (Fuchs et al. 2016; Rowland et al. 2018a; Winterer et al. 2017) and are shown to be interconnected through inhibitory cells, mainly fast spiking PV^+ cells (Couey et al. 2013; Fuchs et al. 2016) (Figure 1.5). Calbindin expressing, pyramidal cells in layer II receive inhibition primarily from 5H3a/CCK positive interneurons (Fuchs et al. 2016; Rowland et al. 2018a; Varga, Lee, et al. 2010), but have been reported to receive inhibition also from PV^+ cells (Armstrong et al. 2016; Varga, Lee, et al. 2010). Inhibition from PV^+ cells is suggested to underlie the precise spatial firing pattern of grid cells (Burak and I. R. Fiete 2009; Pastoll, Ramsden, et al. 2012). Pharmacogenetic inactivation of PV^+ cell activity reduce the spatial selectivity of grid cells by increasing the spiking rate outside the grid fields, but does not have a similar affect on non-periodic spatially selective cells (Miao et al. 2017). Furthermore, inactivation of somatostatin expressing interneurons, another major inhibitory cell type in MEC, does not cause similar grid field impairments and suggest that inhibition of grid cells is mediated primarily by PV^+cells. Another study using optogenetics to activate local PV^+ cells in MEC provide further support for the relationship between grid cells and PV^+ cells by showing that PV^+cells receive most of their input from grid cells (Buetfering et al. 2014). However, this study concludes that PV^+cells provide local inhibition to all functionally defined cell types in MEC, thus contradicting the finding reported by Miao and colleagues.

Additionally, PV^+ interneurons are pivotal for mediating local network oscillations (Varga, Oijala, et al. 2014). Input from pacemaking cells in MSA drive theta oscillations in hippocampus and MEC, possibly through long range projecting PV^+ cells terminating on local GABAergic cells (Fuchs et al. 2016; Gonzalez-Sulser et al. 2014). However, how the local networks of hippocampus and MEC generate and sustain theta oscillations, particularly fast theta rhythms observed during active behavior, is not well understood. Interestingly, inactivation of MSA selectively disrupts grid cell spiking activity in addition to strongly reducing theta oscillations (M. P. Brandon et al. 2011; Koenig et al. 2011). It has therefore been suggested that grid cell activity depends on theta oscillations. However, the specificity and temporal resolution of these studies is not sufficient to properly understand how theta oscillations modulates grid cell activity at behaviorally relevant time scales.

From grid cells to place cells

Place cells in the hippocampus (O'Keefe and Dostrovsky 1971), one synapse downstream from MEC, are suggested to derive from grid cell activity (Fuhs 2006; Rolls et al. 2006; Savelli and Knierim 2010; Solstad et al. 2006) (Figure 1.6). Place cells differ from grid cells in that they generally have one or few activity fields (in commonly sized experimental arenas), that correlate with the position

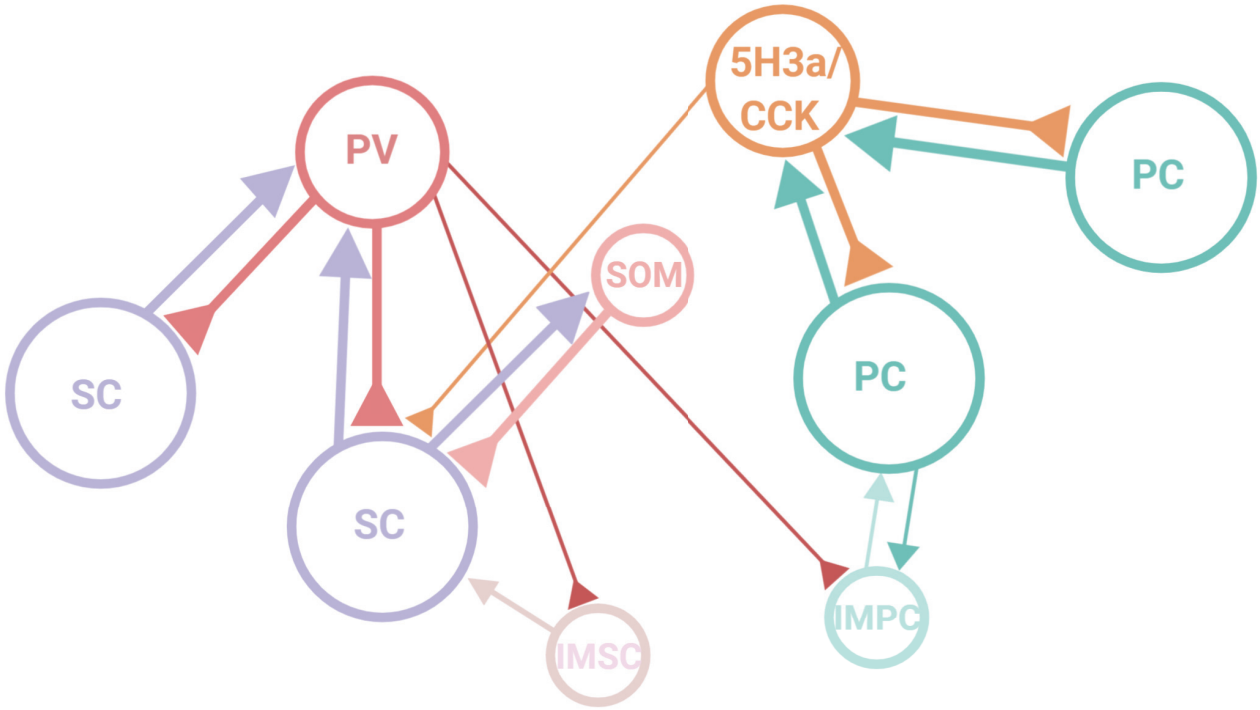

Figure 1.5: **Inhibitory circuits in medial entorhinal cortex layer II**. Stellate cells (SC) are inhibited primarily by parvalbumin (PV) and somatostatin (SOM) expressing cells, while pyramidal cells (PC) are inhibited primarily by 5H3a/CCK expressing cells. Stellate cells and pyramidal cells may therefore belong to separate networks. Intermediate stellate (IMSC) and pyramidal cell (IMPC) types have also been reported and is possibly connecting the two networks (illustrated by smaller arrows). Adapted from Witter et al. 2017

of the animal in the environment. Indeed, the hippocampus receives direct input from principal cells in layer II and III of MEC (many of which are grid cells)(S.-J. Zhang et al. 2013) but experimental lesioning or inactivation of MEC suggests that place cell activity can also be sustained by alternative sources of input, although with slightly less spatial precision (Hales et al. 2014; Rueckemann et al. 2015; Schlesiger et al. 2018). Furthermore, directed manipulations that exclusively disrupts grid cell firing results in only subtle changes in place cell firing and does not interrupt the appearance of new and stable place codes (M. Brandon et al. 2014; Koenig et al. 2011). This has made it challenging to sort out the particular contribution of grid cells to place cell activity, but also their role in navigation and spatial memory. Nonetheless, grid cells probably provide essential input to place cell activity without being the sole driver. A recent study shows that enhancing the activity of stellate cells results in altered firing rate amplitudes of individual grid fields and in turn leads to remapping of place cells in hippocampus CA1 (Kanter et al. 2017). Also, enlarging the scale of grid cell firing fields leads to an enlargement and subsequent instability in place fields

and reduced spatial learning of a Morris water-maze task (Mallory et al. 2018). This suggests that the spatial output of grid cells is an important determinant of place cell activity, and that grid cells provides one of several lines of input to the hippocampus that is needed in spatial memory.

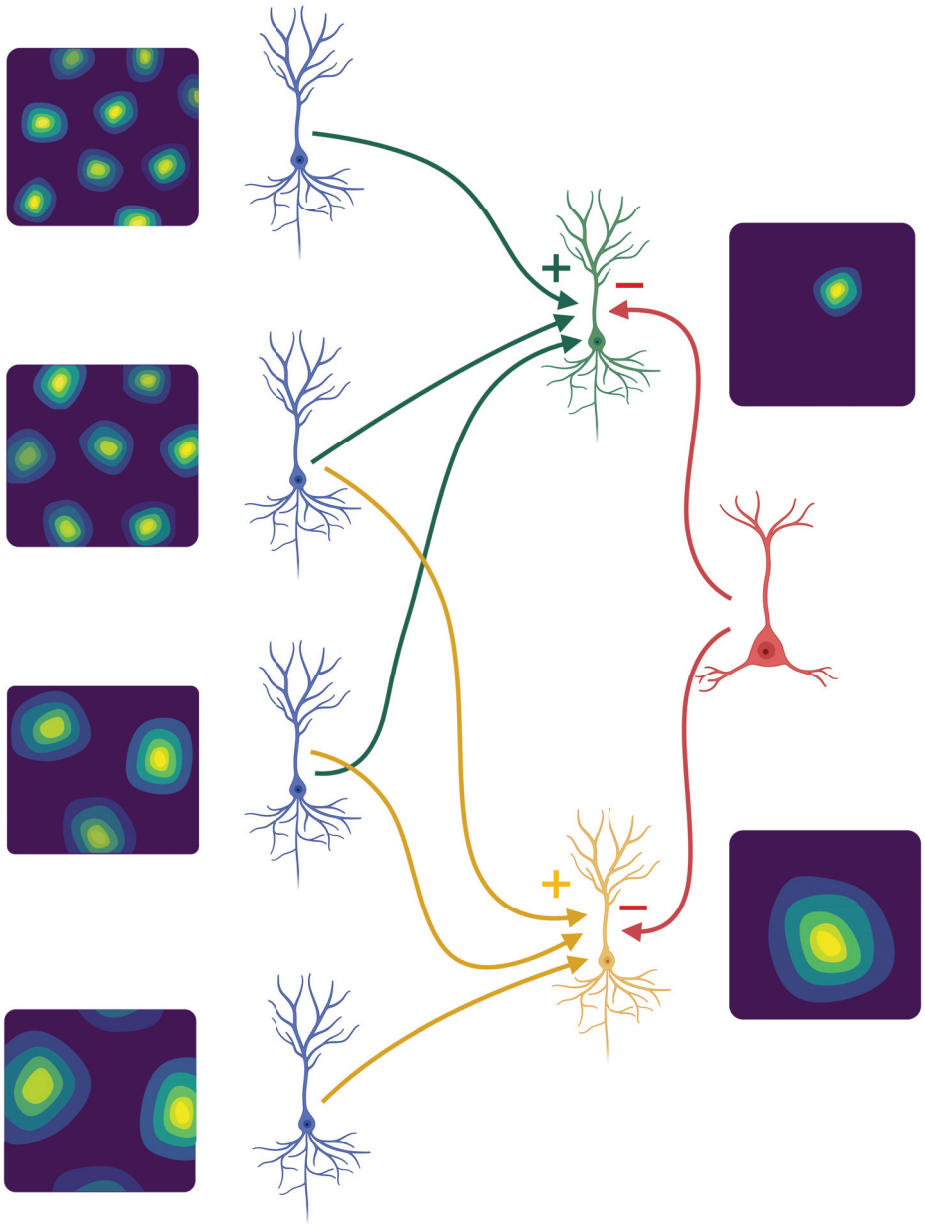

Figure 1.6: Proposed mechanism for grid cell to place cell activity.

Place fields could be formed by linear summation of input from grid cells (blue cells) of different spacing and orientation, but with overlapping phases. Input from grid cells with small spacing leads to small place fields (green place cell), while input from grid cells with larger spacing leads to larger place fields (yellow place cell). Adapted from Solstad et al. 2006.

Interestingly, during development, place cell activity appear many days before grid cells can be detected (Bjerknes et al. 2014; Langston et al. 2010; Muessig et al. 2015; Wills et al. 2010). While place cells and head-direction cells display adult-like activity as soon as rat pups leave their nest, grid cell activity does not fully mature until weeks later. The late and rather abrupt appearance during development (Bjerknes et al. 2014; Langston et al. 2010) poses an interesting question of what drives the formation of grid cell activity. It could be that grid cells are part of a hard-wired network from birth and several sources of input must reach a certain level of maturation before grid cell activity appears. A different possibility is that the activity pattern of grid cells is learned by the MEC network through experience, similar to what we observe for sensory information (Kruge et al. 2018).

Once grid cells display adult-like activity they remain remarkably stable. The same activity pattern is present every time an animal visits the same environment (Diehl et al. 2018; Fyhn et al. 2007), and the relative firing rate amplitude of individual fields is maintained (Ismakov et al. 2017). There could therefore be a critical period for grid cell maturation, where adult-like activity of grid cells depends on low plasticity and stable synaptic contacts.

This is addressed in paper II where we investigated how the stabilizing element of PNNs around PV^+ cells contribute to spatial coding in grid cells of adult animals. We postulated that PNNs could be essential for maintaining low plasticity and balance inhibition in the MEC network and thus support stable grid cell representations. To investigate grid cell properties during encoding of new information, we introduced animals with and without PNNs in MEC to a novel environment and assessed the spatio-temporal stability of grid cells during increased network plasticity.

Modelling grid cells

In the search for mechanisms explaining grid cells' activity patterns, several computational models have been developed (reviewed in L. Giocomo et al. 2011; Moser et al. 2014). In particular, two major classes of models have received much attention. The first is based on local connectivity that determines grid fields, and is called continuous attractor network (CAN) models (Burak and I. R. Fiete 2009; Fuhs 2006; McNaughton et al. 2006). The second creates the hexagonal grid pattern through interference between velocity controlled oscillatory frequencies oriented in different directions, and is called oscillatory interference (OI) models (Burgess et al. 2007; Jeewajee, Barry, et al. 2008; Zilli and Hasselmo 2010). Both these types of models are able to explain separate parts of experimental observations but lack specific evidence for some assumptions. For example, the CAN model is well suited to explain how grid cell pairs can maintain their spatio-temporal relationship despite prominent changes in the response of individual cells. Furthermore, the model can be organized into separate modules where cells behave coherently within the attractor and independent of other attractors, which strongly resembles the modular organization of grid cells found in experiments (Stensola et al. 2012). Nevertheless, anatomical evidence of the

connectivity pattern supporting a CAN network, is still scarce (Gu et al. 2018). In addition, experimental studies find no evidence for grid like firing patterns among inhibitiory interneurons (Buetfering et al. 2014), which they are predicted to have in the CAN model. The OI model on the other hand is successful at explaining temporal firing properties of grid cells such as theta phase precession and firing frequency modulation by running speed (Jeewajee, Barry, et al. 2008). However, the OI model has been criticized for relying on velocity controlled oscillators separated by a 60°orientation, in addition to being challenged by findings of grid cells in animals that lack continuous theta oscillations (Yartsev et al. 2011). Most importantly, OI models can not account for the relative stability of grid cell firing patterns within a module (Fyhn et al. 2007; Yoon et al. 2013). As a result of these issues, a hybrid model combining the two frameworks seems to be able to encompass much of the findings from existing experimental data (Figure 1.7). *In vivo* patch clamp recordings from stellate cells of head-fixed mice show that a membrane potential ramp, anticipated by the CAN model, is a good predictor of grid fields positions (Domnisoru et al. 2013; Schmidt-Hieber and Häusser 2013). Theta oscillating membrane potentials on the other hand correlates well with spike timing within each field, suggesting that spatial and temporal coding of grid cell can be explained separately.

In paper III we set out to test this prediction in freely behaving rats by pacing theta outside of its endogenous range. We stimulated PV^+ cells locally in MSA using optogenetics, while we recorded grid cells from superficial layers of MEC.

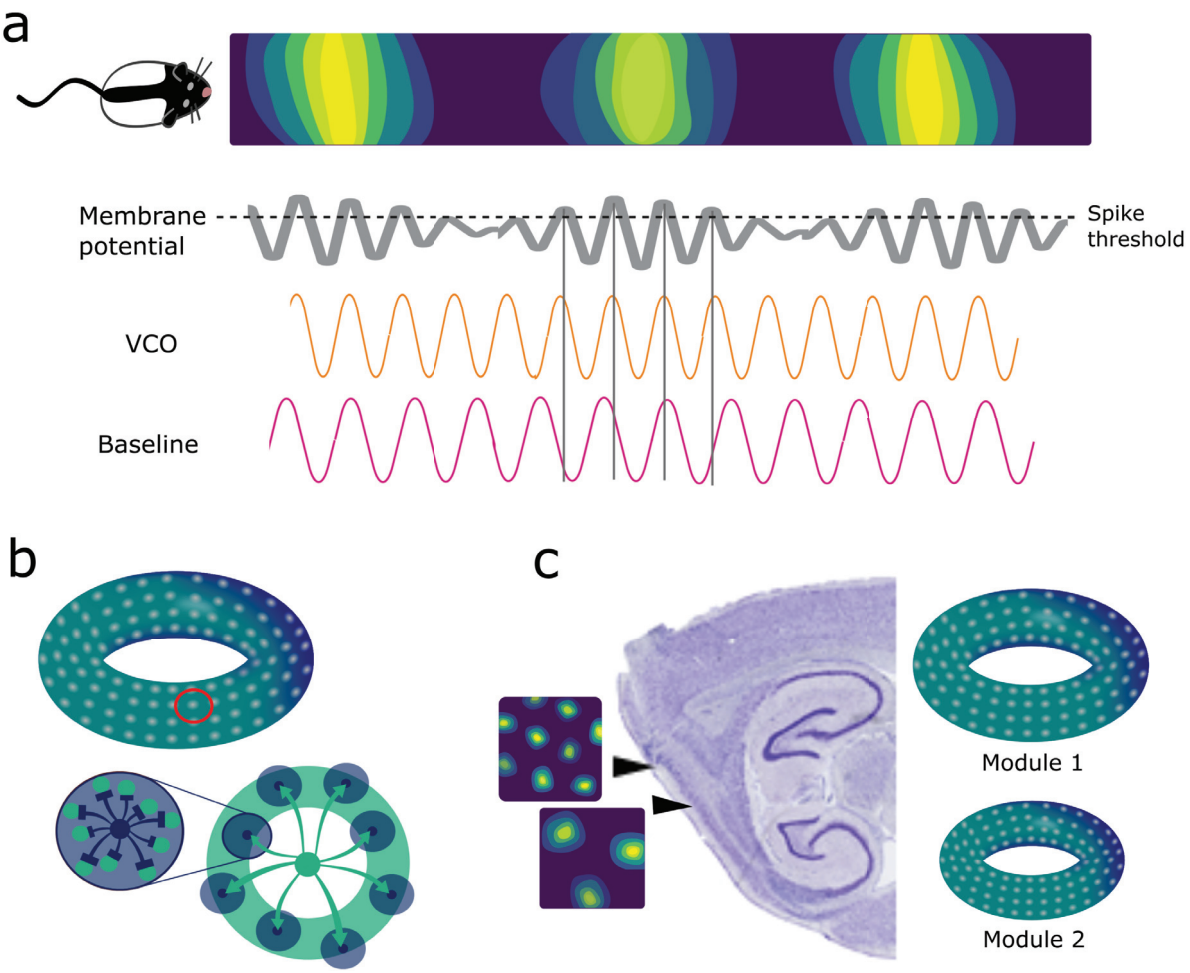

Figure 1.7: Principles of oscillatory interference and continuous attractor network models

a) In the hybrid model, oscillatory interference is used to shift the activity bump and create phase precession. In a 1D environment, the mean of the theta oscillation (purple) and a velocity controlled oscillation (VCO) (orange) at slightly higher frequency, creates a membrane potential oscillation. The threshold for spiking is indicated by a dotted line and the resulting grid fields are shown in the ratemap. Vertical lines show phase precession relative to the theta oscillation when the rat traverses a firing field. Adapted from Bush and Schmidt-Hieber 2018. **b**) In a continuous attractor network (CAN), recurrent connectivity between neurons is topographically arranged to create attractor dynamics. Typically, an excitatory neuron (green) activates distant inhibitory neurons (blue) that again inhibit all excitatory neurons within a fixed radius. **c**) Grid cells are functionally arranged in modules where grid cells share similar spacing and orientation. A sagittal section from a rat brain stained for cell bodies shows likely recording locations of grid cells belonging to two different modules. In the hybrid model, continuous attractor dynamics ensure relative stability between grid fields within a module.

Chapter 2

Objectives

The overall objective of this thesis is to investigate how plasticity-regulating extracellular matrix molecules and inhibitory activity impact the activity in the grid cell network. To achieve this I first (Paper I) mapped the distribution of perineuronal nets (PNNs) in three brain areas, including the MEC. I then went on to indirectly manipulate the activity of (PV^+) interneurons in MEC using two different approaches; in Paper II by degrading plasticity regulating matrix molecules (PNNs) associated with PV^+ cells and then in Paper III by stimulating pacemaking PV^+ cells in the MSA using optogenetics.

Paper I: Aimed to classify the expression pattern and cell-type specificity of PNNs in three different brain areas of rats and mice; the primary visual cortex, hippocampus and medial entorhinal cortex. To establish a timeline for maturation of PNNs in MEC that could be compared with development of spatially selective neurons, I used immunohistochemistry to quantify the gradual development of PNNs in MEC of postnatal rats and their co-localization with PV^+ cells.

Paper II: To test if PNNs are needed to maintain stable grid cell representations in adult animals, I measured single unit activity in MEC of rats where PNNs were enzymatically degraded. Because PNNs are shown to regulate plasticity, I examined if encoding of novel information was altered in a state of enhanced network plasticity. To achieve this, rats with and without PNNs in MEC were introduced to a novel environment arena while I assessed the development of novel grid cell representations. Lastly, I collected single unit data from hippocampus CA1 in rats with PNNs degraded in MEC to explore if altered stability in grid cell representations was reflected in place cell coding one synapse downstream.

Paper III: The objective of this paper was to test if theta oscillations in MEC are needed to create the spatial activity pattern of grid cells. To achieve this I specifically targeted PV^+ cells in MSA and used optogenetics to pace their activity outside the endogenous range of the theta frequency (>10 Hz). Optogenetic pacing of theta oscillations in combination with single unit recordings in MEC was used to assess grid cell responses to increased 'theta'.

Chapter 3

Summary of papers

Paper I Late in postnatal development a specialized form of extracellular matrix called perineuronal nets (PNNs) assemble on primarily, but not exclusively, parvalbumin positive (PV^+) interneurons. This correlates with decreased plasticity and reduced spiking probability in PV^+ cells, thus PNNs are likely to play an important role for the activity of different brain areas. Despite having a widespread yet region specific expression in the adult brain, studies comparing anatomical properties of PNNs in different brain areas are lacking.

Paper I focuses on the anatomical properties of PNNs in three different areas of the brain; the primary visual cortex, the medial entorhinal cortex (MEC) and the hippocampus. These areas represent distinct computational roles and receive a variable degree of processed information, with V1 acquiring direct sensory information, while the hippocampus sits on top of the hierarchy in processing of episodic memories. We found that PNNs were highly expressed in V1 and MEC where they mainly enwrapped PV^+ cells, while they were almost devoid in hippocampus area CA1 of rats. Because mice are widely used as a model organism, we repeated the same experiments in mice and found species specific variations in PNN expression in hippocampus. In mice, large PNNs could be observed in all subregions of the hippocampus which contrasted findings from rats. However, for both species, area CA2 had the highest expression of PNNs. Interestingly, in CA2 the majority of neurons enwrapped in PNNs were principal cells. We also investigated other cell types associated with PNNs in V1 and MEC. Rats and mice showed a similar expression pattern where many PNNs in MEC were enrapping non-PV^+ cells. These were found to be immunopositive for reelin and calbindin suggesting that they could be principal cells. Retrograde labeling of projections from hippocampus suggested that principal cells with PNNs in MEC were not projecting to the hippocampus.

To investigate the development of PNNs in MEC, we stained brain sections from rats at different stages of development. The earliest observations of PNNs were at P12 and the largest increase in expression was observed between P17 and P25. This closely follows maturation of spatially selective activity in MEC.

To test if PNNs maintain structural organization of synapses, we injected a separate group of animals with the enzyme chondroitinase ABC (chABC) in V1 and MEC to remove PNNs. We found changes in both inhibitory and excitatory postsynaptic markers after PNN removal, suggesting that

removing PNNs increased structural rewiring in both V1 and MEC. To our knowledge, this study is the first to describe properties of PNNs in MEC.

Paper II Grid cells are shown to be interconnected via PV^+ cells which is likely to play an important role for their highly specific firing patterns. We show in paper I that the maturation of PNNs on PV^+ cells in MEC closely follows the timeline for development of grid cell activity. Expression of PNNs could therefore be important for development and maintenance of adult grid cell activity. Paper II investigates the role of PNNs for spatial and temporal firing patterns of grid cells. We combined enzymatic removal of PNNs using chABC, with large-scale extracellular recordings of single units in MEC. We first showed that removing PNNs decreased the activity in narrow spiking, putative inhibitory cells, similar to what is reported in V1 and several *in vitro* studies. Grid cells from animals with disrupted PNNs showed reduced spatial specificity, in addition to a large decrease in bursting and spiking variability in familiar environments. We then introduced the animals to a novel environment to test if encoding of new information depended on intact PNNs. Introducing animals to a novel environment led to decreased spatial stability of novel grid cell maps in both control and chABC treated animals. However, in contrast to control animals, the spatial stability of grid fields remained low for the chABC treated group for the entire duration of the novel environment experiment. Furthermore, we also found decreased stability of the familiar representations after animals had been exploring the novel environment, indicating that PNNs may be important for maintaining distinct representations for different environments. In addition, we found reduced temporal cross-correlations in pairs of grid cells from the familiar to the novel environment. All in all this suggests that PNNs could be necessary for quickly balancing inhibition and/or synaptic weights during encoding of new information, and that removal of PNNs renders the network more liable to modifications during abrupt change in input. Interestingly, when recording place cells in hippocampus CA1 during the same experimental paradigm, we observed reduced spatial stability in place cells. This indicates that destabilisation of grid cell activity is reflected in place cell coding one synapse downstream.

Paper III examines the relationship between theta oscillations in the MEC network and spatial and temporal properties of grid cell activity. Theta oscillations in hippocampus and entorhinal cortex depend on rhythmic input from medial septal area (MSA). Previously it has been shown that lesions or inactivation of MSA, significantly reduce theta oscillations and completely abolish the hexagonal firing pattern of grid cells. However it is still unknown if spatial firing patterns of grid cells can be separated from local theta oscillations. We used a novel $Pvalb^{Cre}$ knock-in rat to optogenetically stimulate PV^+ cells in the MSA. This allowed us to pace 'theta' in MEC in frequencies higher than the endogenous theta and we combined this with large-scale extracellular recordings of grid cell activity. We found that grid cells were disynaptically activated through

disinhibition, supporting data from tracing and *in vivo* studies finding that GABAergic input from MSA primarily targets inhibitory neurons in MEC. During stimulation sessions, grid cells strongly phase locked to the stimulation frequency and phase precession was disrupted. In addition, the speed modulation of theta frequency and power was impaired. Interestingly, despite being activated by the stimulation, grid cells showed slightly reduced peak rates within fields and little activation outside fields and therefore maintained stable field positions. This indicates that theta oscillations controls spike timing but not spiking position of grid cells and that the spatial and temporal properties of grid cells can be dissociated.

Chapter 4

Methodological considerations

Electrophysiology and spike sorting: manual vs automatic

In vivo extracellular recordings enable the study of both population- and single cell responses in freely moving animals, which is a prerequisite when studying spatially modulated cells. It also enables sampling of large data sets from individual animals. This greatly increase the gain from each experiment compared to for example in-vivo patch clamp experiments. However, large-scale extracellular recordings are blind to what cell types you record from and since grid cells can belong to different anatomical cell types, it is difficult to know if you record from stellate cells or pyramidal cells. Grid cells possess several specific activity profiles that can be used to separate them into different subpopulations (Dickson et al. 2000; Newman and Hasselmo 2014; Ray, Naumann, et al. 2014), but means of separating stellate from pyramidal grid cells in extracellular recordings has so far produced mixed results (Rowland et al. 2018b; Tang et al. 2014). Due to the lack of accepted methods to do so, we did not attempt to separate stellate cells from pyramidal cells in either of the papers where we performed extracellular recordings.

Although producing large amounts of data, large-scale extracellular recordings require labor intensive spike sorting that is normally done offline after the end of experiments. In paper II we used offline manual spike sorting (Tint, Axona, UK), while in paper III we performed an offline automatic spike sorting (Kilosort2[1], run with SpikeInterface framework (Buccino et al. 2019)). The manual spike sorting is time-consuming and is subjected to experimenter bias. On the other hand, when performed by experienced researchers, the data can be curated to contain little noise and the process of manually going through each recording provides a valuable overview and in-depth quality control. Automatic spike sorting efficiently avoids the problem with experimenter bias, although most methods still require manual curation to merge falsely separated clusters (we experienced that automatic spike sorting often produced more clusters than manual spike sorting for the same datasets), remove noisy spikes and get rid of clusters containing non-biological noise. With the increasing amount of data that can be sampled simultaneously (using probes with increased number of electrodes, larger implants etc.), there is also an increasing need for high quality automatic spike sorters.

Identification of single units

In both paper II and paper III we were interested in following the same units over several recording sessions. When doing manual spike sorting this was achieved

[1] https://github.com/MouseLand/Kilosort2

by visually comparing cluster position, waveform amplitudes and when possible, the spatial output of the unit. Units can then be manually assigned with unique ID's. For automatic spike sorting this was done quantitatively by creating a waveform dissimilarity score between two sessions and calculate a threshold for assigning units to being either the same or different units. In addition we limited the number of waveforms being compared by saying that units recorded more than 100 microns apart in the dorsoventral direction, could not be the same. From experience, we've seen that tetrodes can sometimes move between sessions but still detect spikes from the same unit as a previous session. This can create drift in cluster space and the relative waveform amplitudes between electrodes can shift. For data with a clear behavioral output such as spatially modulated cells, the problem with drift can be circumvented when comparing units manually by looking at the spatial output. Although, it can sometimes be very hard to determine whether a unit is the same or not. The fully automated unit waveform comparisons gives unbiased data and allows for quantification of uncertainty. However, we ran the risk of unknowingly separating data from the same unit. Automatic spike sorting and unit identification might therefore be a better suited tool for data with less clear behavioral output or where the analyses do not depend on comparing responses from individual units across many sessions.

Specificity of PNN degradation

The bacterial enzyme chABC is widely used to disrupt PNNs, but the reported effects can be challenged due to the unspecific degradation of matrix molecules performed by chABC. More specifically, chABC brakes down all chondroitin sulfates in the extracellular space, not only the approximately 2% contained in PNNs (Deepa et al. 2006). This makes it difficult to separate off-target effects from the specific effects of PNN degradation. On the other hand, experiments using genetic knock-down of PNN components (Carulli et al. 2010; Rowlands et al. 2018; Vivo et al. 2013) have confirmed findings from studies that use chABC which indicates that the effects of chABC treatment can be ascribed to disruption of PNNs. While we can not rule out off-target effects of removing PNNs using chABC, a previous paper from our lab specifically knocked out aggrecan, a major component of PNNs, and verified that chABC treatment gave similar results as genetic knock-out in visual cortex (Lensjø et al. 2016; Rowlands et al. 2018). Unfortunately, this mouse line was not available when we performed most of the experiments for paper II.

Despite being a more direct approach, transgenic and conditional knock-outs face several challenges. First, removing PNNs through genetic knock-outs requires many weeks of waiting until matrix turnover has ensured that PNNs are removed. Thus, the only current way to study acute effects of PNN removal is by using enzymes. Second, it is difficult to identify and control for possible compensatory mechanisms that can be activated after silencing expression of a protein. This is even more pronounced when using transgenic models that never develops proper PNNs and the compensatory mechanisms can therefore

be substantial. A good example is found in Favuzzi et al. 2017, where siRNA silencing of the extracellular matrix molecule brevican, has a strong effect on neuronal physiology, while the transgenic brevican knock-out only gives subtle effects. In addition, the transgenic knock-out has a strong upregulation of another matrix molecule, neurocan, that could possibly be rescuing the physiological phenotype. All in all, chABC has been used in several papers (Gogolla et al. 2009; Pizzorusso 2002; Thompson et al. 2017, and continues to be a powerful tool when studying the role of PNNs. Likely, genetic engineering tools will make it easier to perform both rapid and specific manipulations of PNNs and their regulators, in the near future.

Optogentic stimulation

Optogentics has revolutionized biological and medical research in the past decade (Boyden 2011). The ability to control selected subsets of neurons with millisecond precision using light, has greatly improved our understanding of circuit structure and functions. In paper III we activated PV^+ cells in MSA and measured responses far away in MEC. Based on response delay we assumed that PV^+ cells in MSA contacted inhibitory neurons in MEC, which again led to grid cell activation. Given complementary results from other studies (Fuchs et al. 2016; Gonzalez-Sulser et al. 2014), this is likely to be the connectivity of the MSA-MEC circuit. Still, inferring connectivity based on response time after optogenetic stimulation is complicated by several factors that should be taken into account when interpreting these results. First of all, non-directed light delivery induce temporally variable action potentials depending on the intensity of the light, the type of neurons affected and the expression of opsins in each neuron. Also, response efficacy for individual neurons will vary depending on the membrane potential at the time of the light pulse. In effect, cross-correlations of neuronal response delay can look the same for both connected and unconnected cell pairs when many neurons are stimulated at the same time (Lepperød et al. 2018). Second, stimulating axons can cause antidromic activation which could activate neurons and collaterals outside the targeted circuit (Sato et al. 2013). In paper III we addressed this issue by comparing neuronal responses after stimulation of PV^+ cell bodies in MSA, with the stimulation of PV^+ cell axons locally in MEC. Since stimulating PV^+ cell axons in MEC largely caused similar responses in grid cells as stimulating in MSA and did not drive theta oscillations, we concluded that antidromic activation of other cell types was unlikely. We therefore assume that the specificity of our manipulation var largely restricted to activation of PV^+ cells in MSA.

An obvious limitation to our experimental design was that we lacked light controls. A recent study have shown that laser light itself can cause remapping in hippocampus (Zutshi, M. P. Brandon, et al. 2018), thus light stimulation is likely being perceived by the animal despite being delivered deep into the brain tissue. However, this study also tested behavioral effects of pacing PV^+ cells in MSA. Although light caused hippocampal remapping, it did not have an effect on memory performance.

Lastly, we used optogenetics to pace MEC oscillations in specific frequencies, thus causing non-physiological activity in the network. A strong manipulation such as this could induce plasticity which is challenging to control for. We did observe some indications of long-term effects of MSA stimulation since we saw large discrepancies between our two baseline recording sessions (Baseline I and Baseline II). To control for this, we primarily used Baseline I recordings in comparisons with the two stimulation frequencies (11 Hz and 30 Hz).

Chapter 5

General discussion

In this thesis I characterize PNNs in entorhinal cortex and show how grid cells are affected by indirect perturbations of PV^+ cell activity. From my findings, I suggest that PNNs contribute to maintain a stable network in MEC, possibly through its role in supporting PV^+ cell spiking properties. I observed that removing PNNs, likely setting the stage for increased activity-dependent plasticity, decreased the stability of grid cell activity when animals were introduced to a novel environment. This indicates that synaptic plasticity in MEC may perhaps be enhanced by novelty stimuli when PNNs are not present. Furthermore, the work in this thesis shows that the grid cell network maintains its spatial spiking pattern despite perturbing theta frequency in the local field potential oscillations. Grid cells' spiking was enhanced through disinhibition when we performed optogenetic stimulation of PV^+ cells in MSA. This suggests that MSA input controls theta oscillations in MEC through local inhibitory neurons that also control grid cell activity. Interestingly, during stimulation, grid fields were largely confined to their original positions with a short-term increase in out-of-field spiking. This indicates that even though optogenetic stimulation caused robust grid cell responses, their spatial activity patterns were still subjected to the rules posed by local network dynamics. The activity and stability of grid cells therefore seem to depend on intrinsic network connections and strong inhibitory control.

In the following, I will discuss findings from the papers in a broader context and focus on future perspectives. Detailed discussions of results are found in the discussion section of each paper.

The balance between plasticity and stability

The combination of fast learning and stable memory poses a stability-plasticity-dilemma. Decreased plasticity following PNN maturation reduces the brains' capacity for change, but at the same time it might conserve acquired skills and useful experiences. Both memories and acquired skills may be maintained for a lifetime, somehow requiring connections in the brain to be stable. Given that synapses show an extensive capability for change, both short-term (reviewed in Fisher 1997; Zucker and Regehr 2002) and long-term (Bliss and Lømo 1973; Engert and Bonhoeffer 1999), it seems unlikely that long-term information can persist without facing an extensive risk of decay over time. As a possible solution to this problem, PNNs have been suggested to provide the necessary stability for synapses to be maintained over very long periods of time (Thompson et al. 2017; Tsien 2013). When PNNs mature during development, they close critical periods and decrease learning rates, but at the same time they ensure that necessary skills are maintained. For example, fear memories are prone to

complete deletion before PNNs develop in amygdala, but are retained and can quickly be reactivated throughout life once PNNs are present (Gogolla et al. 2009). This ensures that potentially harmful experiences are remembered and can be avoided in the future, and may represent a general concept for PNN function also outside the amygdala (Thompson et al. 2017). The exact way that PNNs contribute to memory stabilization is not understood. Memories could be maintained in specific subsets of synapses surrounded by PNNs as proposed by Roger Tsien (Tsien 2013). Or, PNNs could assist in regulation of synaptic input and excitation level of PV^+ cells (Shi et al. 2019), to ensure that they respond consistently and efficiently to reactivation of a memory trace. How PNNs contribute to network processing and how long-term memories are stored without compromising new learning are research questions of great interest.

Grid cells show strong resilience to indirect manipulation of PV^+ cell function

In paper II and III we find evidence for a highly stable network underlying grid cell spiking. When we manipulate the activity of PV^+ cells in MEC either by stimulating their inputs from MEC or degrade PNNs around PV^+ cells locally in MEC, we find only slight changes to grid cell firing properties. With both manipulations within familiar environments, we see that temporal spiking properties of individual grid cells are more affected than their spatial correlates, suggesting that the position of grid fields is determined by intrinsic properties within the MEC network. The frequency of spiking within each field on the other hand, seems to be sensitive to the strength and timing of inhibition. Interestingly, we find that PNNs might perform an important role in maintaining network stability. When PNNs were removed, the temporal correlation between pairs of grid cells was reduced both between familiar and novel environment sessions, and also when the animal was returned to the familiar environment after exploring the novel arena. This suggests that PNNs assist in preserving cell-to cell-spiking relationships even in situations that induces increased plasticity. The importance of this stability for spatial navigation remains to be determined in tasks testing path integration or spatial memory performance.

What type of network can create grid cell activity?

To understand how grid fields emerge and how the spatial activity patterns are maintained, we must obtain insights into the underlying network mechanisms. Unfortunately, existing experimental data is too limited for conclusive results regarding the network structure that produce grid cell patterns. Even so, experimental data are essential to test and falsify computational models predicting how a network can be wired to produce grid cell activity. Likewise, revising computational models to come up with testable predictions moves the field forward and will bring deeper understanding of underlying mechanisms. Recent experimental findings suggest that the connectivity within MEC is sufficient to elicit periodic firing (Couey et al. 2013; Fuchs et al. 2016; Gu et al.

2018). In addition, units recorded from nearby tetrodes maintain highly stable cell-to-cell relationships across environments (Yoon et al. 2013). Hence, an attractor-like network with a combination of inhibitory-excitatory connections and driven by a non-spatial external excitation is a theory with substantial experimental support (Bonnevie et al. 2013; Couey et al. 2013; Pastoll, Solanka, et al. 2013). Many attractor network models are based on experimental findings of connectivity between layer II stellate cells and layer II inhibitory (mainly PV^+) cells. However, the model can be extended to incorporate excitatory to excitatory connections (Widloski and I. Fiete 2014) such as between pyramidal cells and stellate cells in layer II (Fuchs et al. 2016; Winterer et al. 2017), and between pyramidal cells in layer III (Dhillon and Jones 2000). This is important since grid cells belonging to different cell types might be wired differently. Accordingly, there might be several parallel ways of creating periodic hexagonal firing which coexist within the MEC network.

There are, in contrast to the continuous attractor network (CAN) model, several models suggesting that information required to produce grid cell activity is provided from outside MEC. For example, spatially tuned input from hippocampus (e.g. place cells)(Kropff and Treves 2008) or sensory cortices, in combination with spike-timing dependent or experience-dependent plasticity, can generate grid patterns (Dordek et al. 2016; Monsalve-Mercado and Leibold 2017; Weber and Sprekeler 2018). Another model suggests that stripe or band cells from neighbouring areas such as the parasubiculum (PaS) provides necessary spatial input allowing MEC to self-organize into producing grid cell spiking patterns (Grossberg and Pilly 2014; Mhatre et al. 2010). Stripe-like grid cells have been found in PaS (Krupic et al. 2012), thus providing some experimental support for this model.

The oscillatory interference (OI) models predict that interference between oscillations of different frequencies and directional inputs, result in grid cell spiking patterns. The source of different oscillations typically arise from MSA input and from membrane potential oscillations of local principal cells. Stellate cells are known to have subthreshold membrane oscillations in the theta frequency range (L. M. Giocomo et al. 2007). However, *in vivo* patch clamp experiments have shown that the resulting theta envelope fail to predict the position of grid fields, but theta oscillations rather predicts spike timing within individual grid fields (Domnisoru et al. 2013; Schmidt-Hieber and Häusser 2013). In paper III, we pace the theta frequency of the local field potential (LFP) without affecting the spatial position of grid fields, which corresponds well with the above-mentioned intracellular recordings. Furthermore, we find that phase precession is disrupted during increased pacing of LFP theta frequency, thus oscillatory interference could be directing temporal spiking such as phase precession within individual fields. This possibility has been explored in a hybrid model combining elements from the CAN and OI models (Bush and Burgess 2014). The hybrid models predicts that the attractor network generate fields within the MEC network and maintain cell-to-cell relationships, while oscillatory interference is used for path integration so that grid fields can be moved with the movement of the animal. Interestingly, because oscillatory interference is used to move the grid in these

models, phase precession and grid cell firing patterns must be co-dependent because phase precession is generated by the difference in LFP theta and velocity controlled oscillatory inputs (Bush and Schmidt-Hieber 2018). Thus, when phase precession is impaired by pacing LFP theta in paper III, grid fields should also be eliminated according to the OI and hybrid models. Since we find no experimental evidence for this, our data suggest that a CAN/OI hybrid model is not completely predicting mechanisms for grid cell activity. This means that alternative sources are necessary to update path integration based on findings from our study.

Currently, no grid cell model captures all of the biological features found in experimental data. However, they still provide an important theoretical framework which can be used to develop testable predictions or test out different coding schemes, all which will build knowledge for insight about the grid cell network.

Development of grid cell activity

In computational models of grid cells, noise in synaptic weights can easily cause grid patterns to disperse, although moderate noise levels are tolerated (Solanka et al. 2015). A critical question is how these synaptic connections are tuned during development and later maintained in the mature grid cell network?

In paper I, we establish a timeline for development of PNNs in MEC of postnatal rats and we observe the appearance of PNNs which is overlapping with the onset of grid-like activity (Bjerknes et al. 2014; Langston et al. 2010). In addition, this corresponds with the time of sensory critical periods (Hensch 2005). Grid cell activity can be observed in rats as early as 16 days after birth (P16), but despite the abrupt appearance, adult-like grid cell activity is not established until around P25 (Langston et al. 2010; Wills et al. 2010). Experimental data exploring developmental plasticity in MEC is limited. However, if we assume that plasticity in this area follows a similar progression as seen in sensory cortices, where plasticity is enhanced until it is restricted by PNNs, there might be a critical period for synaptic plasticity also in MEC. During this critical period, activity-dependent plasticity may contribute to refine and ultimately stabilize grid cell activity. The idea that development of grid cell activity could be experience-dependent has been tested experimentally (Kruge et al. 2018). Kruge and colleagues raised rat pups in opaque spherical environments that were meant to limit their experience with distinct environmental boundaries. This delayed the onset of grid cell activity until the animals were introduced to environments with defined boundaries. Letting regularly housed, adult rats live in the spherical environment for several months had no effect on the established grid cell activity patterns (Kruge et al. 2018), suggesting experience-dependent learning is involved in creating grid cell activity within a confined window during development. Still, the type of plasticity necessary for development of grid cells in MEC is unknown. In theoretical work, spike-timing dependent plasticity in combination with spatial exploration produce an attractor-network with grid

cell activity (Widloski and I. Fiete 2014). However, this remains to be explored in *in vivo*.

Another experimental study suggests that synaptic plasticity through NMDA receptor signalling could be essential for development of grid cell activity and possibly for the modular organization of the grid cell network (Gil et al. 2017). Genetic ablation of NMDA receptors in parahippocampal areas of postnatal mice, specifically reduce grid cell periodicity in addition to reduce peak rate and stability (Gil et al. 2017). However, NMDA receptors may also be important for improving spatial and temporal precision of grid cells in mature networks by mediating dendritic integration and nonlinear responses of grid cells. Adding NMDA receptors to an attractor-network model is shown to greatly improve gridness and reduce network drift (Schmidt-Hieber, Toleikyte, et al. 2017). Thus, whether ablation of NMDA receptors disrupt grid cell activity mainly due to developmental impairments or because they improve grid cell precision is an open question.

The structural organization of layer II principal cells is present from birth (Ray and Brecht 2016), suggesting that the gross anatomical structuring of MEC is not experience-dependent. However, functional development of different cell types follows different timelines, with head-direction cells being present before pups leave the nest, and grid cells being the last functional cell type to develop (Langston et al. 2010). Interestingly, stellate cells (of which many are grid cells ((Domnisoru et al. 2013; Rowland et al. 2018a; Schmidt-Hieber and Häusser 2013; Sun et al. 2015)) are found to initiate maturation of the hippocampal and parahippocampal network (Donato, Jacobsen, et al. 2017). Stellate cells express mature electrophysiological properties by P14, but without expressing grid like activity (Bjerknes et al. 2014). At the same time (P10-P15), the cell soma and perisomatic terminals of PV^+ basket cells increase in number (Berggaard et al. 2018). At a later stage (P15-P30) the size of PV^+ basket cell terminals increase, including the number of terminals containing mitochondria (Berggaard et al. 2018). This indicates that development during this period could be structured by experience-dependent inputs that ultimately directs development of grid cell activity through maturation of inhibitory circuits. Since there are no established electrophysiological markers of the developmental stages of grid cells, it is difficult to determine if removal of PNNs (paper II) resets the network to an immature state. Since grid cell firing patterns remained stable in familiar environments after removal of PNNs, the presence of PNNs is likely not necessary for grid like activity, but they may still be essential for maintaining long-term stability and synaptic connections. The increased synaptic turnover (paper I and II) after injection of chABC provides supports for this prediction. It is possible that a more demanding intervention than the novel open field exploration would induce larger effects on the MEC network and perhaps reveal if grid cell activity is returned to an immature state without PNNs. In primary visual cortex, removing PNNs in adult animals does not cause immediate effects on normal visual processing, but has a striking effect on ocular dominance after closure of one eye (Lensjø et al. 2016; Pizzorusso 2002). This suggests that PNN removal opens up for increased plasticity, but that adequate stimuli is

still required to cause changes in the network. The findings from visual cortex provides some parallel to our findings from MEC in that that removing PNNs does not alter spatial activity patterns in familiar environments, but impacts stability of spatial and temporal activity patterns after animals are introduced to a novel environment.

Grid cells in a novel environment

The mechanisms causing grid cells to realign in a novel environment is currently unknown. When introduced to a novel environment, grid cells show e.g. temporary expansion of the grid and reduced spatial stability (Barry et al. 2012), accompanied by theta frequency reduction (Jeewajee, Lever, et al. 2008). This indicates that plasticity may underlie the development of new grid maps. On the other hand, despite being somewhat unstable at first, new grid maps appear as soon as the animal start to explore which is too fast for long-term plasticity to direct learning of the new grid fields. The fast appearance of new grid maps also contradicts most plasticity-based theoretical grid cell models (Dordek et al. 2016; Kropff and Treves 2008). However, fast learning could in theory be obtained with only small changes in synaptic weights as long as inhibitory and excitatory inputs are tightly balanced (Yger et al. 2015). In a balanced network, small shifts in E/I balance to a given neuron has a strong effect on spiking probability even with minimal change in the input. In our novel environment experiments (paper II), we find that rapid stabilization of new grid maps is reduced when PNNs are removed. After removal of PNNs, it is likely that the E/I balance in MEC is more easily shifted as indicated by the observed structural remodelling of synaptic inputs. This again may allow faster learning rates. Interestingly, in a plasticity-based model of grid cells in a novel environment, learning rates can only be enhanced up to a certain level without limiting the stabilization rate of new grid fields (Weber and Sprekeler 2018). Although only theoretical evidence, the model of Weber and co-workers might supply some explanation to our findings in the novel environment. In addition, the rapid appearance of new grid cell firing patterns does not necessarily mean that they will remain stable. For place cells, a certain level of exploration time is required in order for stable place maps to be reactivated during later survey of the same environment (Frank 2004). Also, long-term stabilization of place fields is shown to depend on intact signalling through NMDA receptors (Kentros 1998). It is likely that a similar mechanism is needed to stabilize grid fields in a novel environment where spatial inputs are less precise (Barry et al. 2012; Schmidt-Hieber, Toleikyte, et al. 2017), but since grid cell activity probably depends on intact NMDA receptors (Gil et al. 2017; Schmidt-Hieber, Toleikyte, et al. 2017) this prediction is challenging to test experimentally.

Fine tuned properties of grid cell activity could be important for learning and memory

Although grid cells in MEC are generally thought to provide a metric for space, there is emerging evidence that grid cells also perform an essential function in learning and memory. In two recent studies, the grid cell map is shown to be modulated by learning (Boccara et al. 2019; Butler et al. 2019). In an open field, grid cells display higher activity close to a learned reward location (Butler et al. 2019). In addition, the increase in firing rate near the reward location correlates with how well the animal performs in the learning task (Butler et al. 2019). Grid maps are also shown to be attracted to goal locations in a cheeseboard maze, suggesting a role in goal-directed navigation (Boccara et al. 2019). Interestingly, in the study by Boccara and colleagues, learning a goal location caused long lasting reorganization of grid cell activity lasting beyond changes seen in place cell activity in CA1. Furthermore, the grid map was flickering between different versions of the task, suggesting that grid cells can store multiple representations of the same environment. In regular open field recordings, individual grid fields are found to have different relative firing rates that are stable across recordings in the same environment (Ismakov et al. 2017). Furthermore, theta phase precession is found to vary across grid fields of the same cell to such an extent that each field can be used as an isolated encoder for space (Reifenstein et al. 2012). This indicates that the grid map can provide both an invariant spatial metric supporting self-motion based orientation, but also has a large capacity for coding and storing local information within the environment. It is likely that properties of the grid map holds more information than previously discovered. Given that reorganization of grid field firing rates can cause remapping of hippocampal place fields (Kanter et al. 2017), stability of grid cell representations could be pivotal for consistent learning and memory performance (Weiss et al. 2017) but this remains to be explored.

The role of grid cells and the challenges of studying them

Despite intensive investigations during the past 15 years, the role of grid cells is still not fully understood. There is however, quite substantial consensus regarding their role as a neural code for path integration, i.e. the ability of an animal to memorize overlapping vectors in its travel path and use these to return home in a straight path (Redish and Touretzky 1997). Even with the compelling theoretical framework describing how grid cells can be used for path integration, very few attempts have been made to prove this experimentally (Gil et al. 2017; Tennant et al. 2018). There are several reasons for this. First, grid cells are intermingled with other functional cell types and does not belong to one particular neuronal cell type. This makes it challenging to perform targeted manipulations of grid cell activity. Second, there is a lack of suitable tasks for testing strategies of path integration in animals. In general, there is no established behavioral task known to depend solely on intact MEC function, or that can robustly dissect the contribution from different areas of the brains

navigation system. Third, since MEC both provides input to and receives output from hippocampus, it is hard to dissect the autonomous role of entorhinal grid cells in this network. The first problem is slowly being addressed by the discovery of various manipulations that mainly disrupts grid cell firing, e.g. disconnecting input from hippocampus (Bonnevie et al. 2013), silencing the MSA (M. P. Brandon et al. 2011; Koenig et al. 2011), inactivation of the head-direction input (Winter et al. 2015) and knocking out local NMDA receptors (Gil et al. 2017). Emerging technologies for genetic targeting make it possible to identify and construct targets for manipulating unique genetic sequences and is already starting to overcome the current challenge of studying only one particular cell type in MEC (Heidenreich and F. Zhang 2015; Kanter et al. 2017).

The role of PV$^+$ cells in learning and memory

PV$^+$ cells are found to be central players in regulation of plasticity and consolidation through their connections with other inhibitory neurons. During behavioral learning tasks or environmental enrichment, which increases plasticity, a subset of PV$^+$ cells in hippocampus area CA3 seems to shift to a 'high plasticity-state' (Donato, Rompani, et al. 2013). This is recognized as a decrease in the amount of GAD 67, an enzyme involved in GABA synthesis, and PV that the cell expresses. It is further accompanied by increased recruitment of inhibitory synapses from cells expressing vasointestinal peptide (VIP) (Donato, Rompani, et al. 2013). Unfortunately, it has not been tested how the reduced PV level and increased input from VIP cells affect PV$^+$ cell spiking properties, but the correlation between PV expression and behavior seems evident. After a task is learned, the same population of PV$^+$ cells returns to a 'low plasticity-state' suggested to ensure efficient utilization of the acquired skills. This dynamic interplay of inhibitory neurons fits well with observations that downregulation of PV$^+$ cell function can initiate plasticity and long lasting changes in cortical neural circuits (Kuhlman et al. 2013), and that disinhibition is necessary for some forms of learning (Wolff et al. 2014). Exploring if disinhibition of PV$^+$ cells is similarly regulated in MEC, may provide insight in grid cell function and potentially MEC-dependent learning.

Can the effect of increased plasticity seen after PNN removal be separated from reduced PV$^+$ cell function?

In paper II we see various effects on grid cell activity after removing PNNs. However, it is challenging to determine if these effects belong to change in PV$^+$ cell physiology or increased structural plasticity. It is well known that the level of inhibition is important for plasticity (Hensch 2005), thus reduced PV$^+$ cell activity and enhanced plasticity may be two sides of the same story. In general, it is unknown if the dramatic increase in plasticity observed after chABC treatment can be dissociated from the effect of reduced activity of PV$^+$ neurons. There are however indications of increased network plasticity in our data from paper II. For example, there seems to be long-term effects on grid cell activity patterns

after introduction to a novel environment. Indeed, fast stabilization of novel grid maps could depend on inhibitory activity, and reduced inhibition could therefore reduce the rate of stabilization. However, we also observe reduced stability in the already established familiar maps after the animals have spent some time in the novel environment. If this is also explained by reduced inhibition, it would be reasonable to assume that familiar maps would be unstable also before introduction to the novel environment. We also see a reduced number of inhibitory synapses onto PV^+ cells after injection of chABC, indicating that structural remodelling is enhanced by removal of PNNs. We also saw similar effects in paper I when we investigated the number of synapses in an entire volume of tissue and not strictly on PV^+ cell somas. In addition to reduced number of inhibitory gephyrin punctas after PNN removal, we also found reduced number of excitatory PSD-95 punctas indicating that there might be activation of homeostatic mechanisms to balance excitation and inhibition.

Reduced inhibition onto PV^+ cells does not immediately fit with the reduced firing in narrow spiking cells (putative interneurons). However, in paper II we see a tendency for reduced number if excitatory VGLUT1 puncta onto PV^+ cells, although not significant. PNN integrity is implicated to be important for maintaining glutamatergic input to PV^+ cells (Bozzelli et al. 2018). Thus it is likely that PV^+ cells receive less excitation after removal of PNNs. Further studies should assess the changes in E/I balance, such as alterations in release probability or quantal size of either inhibitory or excitatory synapses. There are most likely multiple mechanistic changes in the network i.e. reduced capacitance (Tewari et al. 2018), synaptic remodelling (Vivo et al. 2013), and reduced PV^+ cell excitability (Balmer 2016). Therefore, it is not straight forward to estimate the network effects, especially in a recurrent network where changes in E/I balance affects all connected neurons in the recurrent population.

Inhibitory neurons as key players in plasticity and regulation of brain activity

It is becoming evident that inhibitory neurons are not merely passive attenuators of brain activity, but play essential roles in performing advanced computations. At a network level, inhibitory neurons can group the activity of principal neurons through maintenance of oscillatory frequencies. This allows for formation of functional cell assemblies and efficient transmission of information across brain areas (reviewed in Roux and Buzsáki 2015). In addition, change in inhibitory activity is often seen as the first response to altered sensory input, eventually leading to long lasting changes in principal cell signalling (Kuhlman et al. 2013). In particular, PV^+ cells seems to play a central role in inhibitory plasticity since they ensure widespread and precise regulation of excitatory activity and thereby maintain the E/I balance (Dehorter et al. 2017). It is likely that the PNN is an essential co-player for maintaining this balance in mature neural networks. Interestingly, although PNNs are robust suppressors of adult plasticity, they are far from permanent structures and are constantly subjected to fine scale regulation through various regulatory proteins. Matrix metalloproteinases are

synthesized and released in an activity-dependent manner and cut the bonds between components of PNNs at specific sites (reviewed in Bozzelli et al. 2018; Meighan et al. 2006), allowing synapses to grow or rewire. Intriguingly, PV^+cells are shown to produce several of the proteinases themselves, meaning that they are part of the fine tuned regulation of their own synaptic inputs (Rossier et al. 2014). The dynamic interplay between PV^+ cells and PNNs is still an emerging field of research and abnormalities in this interplay is implicated in the pathophysiology of several psychiatric disorders, particularly in schizophrenia (Berretta 2012; Pantazopoulos et al. 2010) and addiction disorders (Slaker et al. 2015). Future studies aiming to perform directed manipulations of PNN and PV^+ cell function is likely to reveal new and important functions of PV^+ cells in development and for normal brain functions.

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Papers

Paper I

First Author, Second Author

Submitted for publication. arXiv: 1708.04101.

Neuronal Excitability

Differential Expression and Cell-Type Specificity of Perineuronal Nets in Hippocampus, Medial Entorhinal Cortex, and Visual Cortex Examined in the Rat and Mouse

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DOI:http://dx.doi.org/10.1523/ENEURO.0379-16.2017

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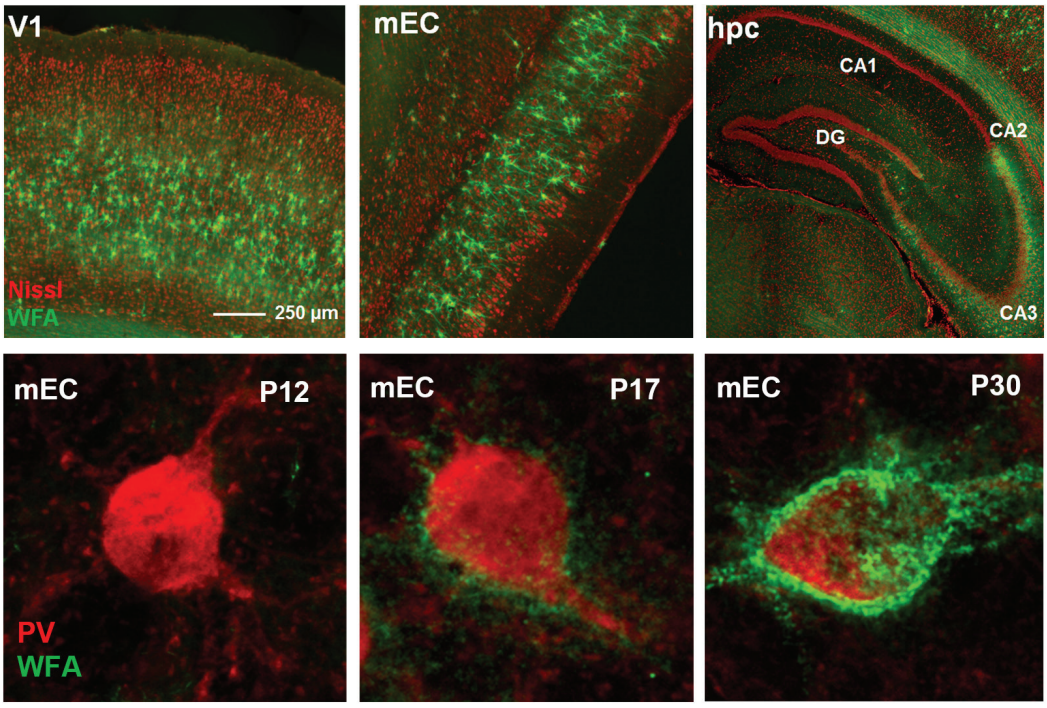
Visual Abstract



Significance Statement

Perineuronal nets (PNNs) are proposed as a key regulator of plasticity in the adult brain. However, PNNs are not uniformly expressed and their prevalence and cell-type specificity is lacking for several well-studied brain regions. Here, we examine the visual cortex, hippocampus and the medial entorhinal cortex (mEC) and show that the density and structure of PNNs as well their cell-type specificity are different between brain areas and between rats and mice. We show for the first time a dense expression of PNNs in mEC and that their assembly during postnatal development coincides with reports on development of the grid cell's activity pattern. The variable expression patterns of PNNs may reflect different levels of plasticity and wiring of the neural networks.

Perineuronal nets (PNNs) are specialized extracellular matrix (ECM) structures that condense around the soma and proximal dendrites of subpopulations of neurons. Emerging evidence suggests that they are involved in regulating brain plasticity. However, the expression of PNNs varies between and within brain areas. A lack of quantitative studies describing the distribution and cell-specificity of PNNs makes it difficult to reveal the functional roles of PNNs. In the current study, we examine the distribution of PNNs and the identity of PNN-enwrapped neurons in three brain areas with different cognitive functions: the dorsal hippocampus, medial entorhinal cortex (mEC) and primary visual cortex (V1). We compared rats and mice as knowledge from these species are often intermingled. The most abundant expression of PNNs was found in the mEC and V1, while dorsal hippocampus showed strikingly low levels of PNNs, apart from dense expression in the CA2 region. In hippocampus we also found apparent species differences in expression of PNNs. While we confirm that the PNNs enwrap parvalbumin-expressing (PV+) neurons in V1, we found that they mainly colocalize with excitatory CamKII-expressing neurons in CA2. In mEC, we demonstrate that in addition to PV+ cells, the PNNs colocalize with reelin-expressing stellate cells. We also show that the maturation of PNNs in mEC coincides with the formation of grid cell pattern, while PV+ cells, unlike in other cortical areas, are present from early postnatal development. Finally, we demonstrate considerable effects on the number of PSD-95-gephyrin puncta after enzymatic removal of PNNs.

Key words: CA2; entorhinal; hippocampus; parvalbumin; WFA

Introduction

Perineuronal nets (PNNs) are specialized structures of extracellular matrix (ECM) that condense around the cell soma and proximal dendrites of subpopulations of neurons (Hockfield and McKay, 1983; Celio and Blumcke, 1994). Recent work indicates that PNNs play a role in regulating plasticity during development, learning, and memory processing (Bartus et al., 2012). Moreover, dysfunctional regulation of PNNs may be linked to impaired synaptic function in some psychiatric disorders such as schizophrenia (Pantazopoulos and Berretta, 2016).

The PNNs are found throughout the brain with profound variations in expression pattern and morphology between brain regions (Seeger et al., 1994). Their main components are hyaluronic acid, link proteins, tenascins, and chondroitin sulfated proteoglycans (Deepa et al., 2006; Giamanco and Matthews, 2012), that are heterogeneously expressed causing differences in the PNN structures (Seeger et al., 1994; Lander et al., 1997; Wegner et al., 2003; Deepa et al., 2006; Dauth et al., 2016). The PNNs mainly enwrap a subpopulation of inhibitory neurons, the fast-spiking parvalbumin-expressing (PV+) inhibitory neurons, but the overlap is not exclusive (Kosaka and Heizmann, 1989; Härtig et al., 1992; Wegner et al., 2003; Alpar et al., 2006). In the neocortex, the PV+ cells mature in parallel with the assembly of the PNNs late in postnatal development as the critical period comes to an end (Hockfield et al., 1990; Pizzorusso et al., 2002; Ye and Miao, 2013). The PV+ cells have been proposed as key regulators of plasticity, both during development and in adulthood (Hensch, 2005; Donato et al., 2013). The PNNs may support the high activity of PV+ cells through ion buffering, protection against reactive oxygen radicals and their role in plasticity through capture of the transcription factor Otx2 (Beurdeley et al., 2012; Cabungcal et al., 2013; Liu et al., 2013; Morawski et al., 2015). Thus, PNNs may restrict plasticity either indirectly through their effect on PV+ cells or directly by acting as a structural barrier inhibiting synapse remodeling and axonal sprouting. Recent work has demonstrated that PNN removal reduces excitability and spiking activity of putative PV+ neurons (Balmer, 2016; Lensjø et al., 2017). Removing the PNNs enzymatically in adult animals increases plasticity in several brain areas, including the visual (Pizzorusso et al., 2002) and auditory cortices (Happel et al., 2014), perirhinal cortex (Romberg et al., 2013), hippocampus (Hylin et al., 2013), and amygdala (Gogolla et al., 2009).Taken together, this suggests that the function of PNNs is tightly linked to PV+ cell function. However, PNNs enwrapping other cell types have been found in several brain areas (Alpar et al., 2006; Carstens et al., 2016; Morikawa et al., 2017). Furthermore, it remains unclear if the level of PNN expression in a brain area is correlated with the level of plasticity in that area.

Despite increased attention to PNNs recently, quantification of PNN expression and their cell-type specificity is limited. We have therefore examined PNNs in three areas of the rodent brain with known different functions: the dorsal hippocampus, medial entorhinal cortex (mEC) and primary visual cortex (V1). The hippocampus is critical for encoding new memories and is reciprocally connected with mEC. The mEC is believed to be the hub in a distributed neural network coding for spatial representation that is fed into the hippocampus and used as a critical component of episodic memories (Buzsaki and Moser, 2013). The V1 has been a canonical system to study sensory

Received December 20, 2016; accepted May 18, 2017; First published May 25, 2017.

The authors declare no competing financial interests.

Author contributions: K.K.L., A.C.C., M.F., and T.H. designed research; K.K.L. and A.C.C. performed research; S.T. contributed unpublished reagents/analytic tools; K.K.L., A.C.C., and S.T. analyzed data; K.K.L., A.C.C., S.T., M.F., and T.H. wrote the paper.

This work was supported by Research Council of Norway Grants 204939, 217920, and 250259 (to M.F.) and 231248 (to T.H.) and by the University of Oslo.

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Acknowledgements: We thank Anne Marte Kvello and Dr. Gunnar Dick for discussions and Frode M. Skjeldal at NorMIC UiO for technical assistance regarding image analysis.

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DOI:http://dx.doi.org/10.1523/ENEURO.0379-16.2017

Table 1: Overview of antibodies used

		Primary antibodies used				
Primary antibody	Reagent	Dilution	Provided by	RRID	Postfix	Other
WFA	N-acetylgalactosamine	1:200	Sigma	AB_2620171	ON	
Rabbit anti-PV	Parvalbumin	1:2000	Swant	AB_10000344	ON	
Rabbit anti-CamKII	CamKII α-subunit	1:500	Abcam	AB_447192	ON	
Rabbit antigephyrin	Gephyrin	1:1000	Abcam	AB_2112628	2h	0.05% Tween 20
Mouse anti-PSD-95	PSD-95	1:500	Abcam	AB_303248	2h	
Mouse anti-CS-6 after chABC digestion	Chondroitin 6-sulfate stubs	1:1000	Millipore	AB_11214309	ON	
Mouse antireelin	Reelin	1:500	Abcam	AB_1603148	ON	
Rabbit anti-GFAP	Glial fibrillary acidic protein	1:500	Dako	AB_10013382	ON	
Rabbit anticalbindin	Calbindin	1:5000	Swant	AB_10000340	ON	
Neurotrace Nissl	Nissl bodies	1:100	Dako	AB_10013382	ON	
Rabbit anti-PCP4	Purkinje cell protein 4	1:200	Sigma	AB_1669533	ON	

processing and critical period plasticity for decades (Hensch, 2005; Wandell and Smirnakis, 2009). While PNNs have been investigated in detail in the V1, both in adults and during postnatal development, it remains elusive how the findings from this primary sensory cortex correspond to the development and expression pattern in mEC.

We show that the expression of PNNs is highly variable within and between the hippocampus, V1, and mEC. We have identified several distinct cell types associated with PNNs, large differences in PNN expression between mice and rats, and considerable but variable effects on the density of PSD-95 and gephyrin-positive puncta after enzymatic removal of PNNs in V1 and mEC in rats. Our results demonstrate that PNNs are heterogeneous in expression, cell-type specificity and morphology. This variability may point to different roles of the PNNs perhaps reflecting different levels of plasticity or wiring of the neural networks.

Materials and Methods

Animals

Fourteen adult (three to five months old) and seven juvenile (postnatal day 10 (P10) to P30) male Long Evans rats and five male c57/bl6 mice (three to five months old) were used for this study. The animals were locally bred and maintained at the animal facility at the Department of Biosciences, University of Oslo. The animals were housed two to three (rats) or five to eight (mice) together, with a 12/12 h light/dark cycle and food and water *ad libitum*. All animal procedures were approved by the Norwegian Animal Research Committee before initiation.

Brain sectioning

The animals were anesthetized in an induction chamber with isoflurane mixed with air. They were then deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and intracardially perfused with 0.9% NaCl followed by 4% paraformaldehyde (PFA) in 1× PBS. The brains were dissected out and postfixed in 4% PFA overnight (except for staining synaptic markers PSD-95 and gephyrin, as well as for tissue injected with retrograde tracer; Table 1). The tissue was then cryoprotected in a solution of 30% sucrose in 1× PBS for 3 d at 4°C, flash frozen, and sectioned with a cryostat in 40-μm-thick sections. The sections were collected from the cryostat with a fine brush and placed in 1× PBS.

Immunohistochemistry

Staining was performed on free-floating sections with constant agitation. Sections were initially blocked with a solution containing 1-2% bovine serum albumin and 0.03% Triton X-100 for 1 h and incubated with the primary antibody overnight (primary antibodies are listed in Table 1). Sections were rinsed 3 × 5 min in 1× PBS and incubated with secondary antibody for 1-4 h. After being washed in 1× PBS, sections were mounted with Fluor-Save Reagent (Merck Millipore).

Microscopy

Overview images were acquired through an Axioplan 2 microscope (Carl Zeiss) with a 10× objective, and high-resolution images were stitched together using the MosaiX extension in the AxioVision software (Carl Zeiss).

Detailed images of PNNs, synaptic markers and colocalization analysis were acquired using a 20× or 60× PlanApo objective on a FluoView FV 1000 confocal microscope (Olympus) using the FV1000 software (version 1.7a). Images were acquired in a stepwise manner through the z-plane, each step separated by 1.4 μm (20× objective) or 0.45 μm (60× objective).

Image analysis

Image analysis was performed using Adobe Photoshop CS4 (Adobe Systems) and ImageJ (NIH). Analysis of puncta and 3D visualization was performed in Imaris (Bitplane).

As a general note for quantification of the number of PNNs or number of cells, and their colocalization, we

attempted an automated approach using ImageJ and by manual counting. Both yielded similar results, but the automatic approach required the removal of background before analysis, which resulted in many false negatives when compared with the raw data. Without background subtraction, the automated approach gave rise to a substantial number of false positives. We therefore chose to perform all colocalization analysis by manual counting, by overlaying the sections with a grid and using the counter tool. All images were counted by two investigators independently. To investigate the fraction of PNN-enwrapped cells we used sections stained with *Wisteria floribunda* agglutinin (WFA) and fluorescent Nissl. In the hippocampus, the cells were too densely packed to be able to separate them and get an accurate cell number; this region therefore was left out of the analysis.

For analysis of both number and distribution of PSD-95 and gephyrin puncta, we always compared the treated and untreated hemispheres from the same rat and normalized values to the mean of the same area in the control hemisphere. This was important to account for variability between animals and staining quality. The control measurements from chondroitinase ABC (chABC)-treated rats were not different from measurements in control or treated sham animals. The sections from each rat were treated in an identical fashion, and stained and imaged in parallel. We used the mean from two to three sites in each of two sections per hemisphere to determine the number of puncta. Analysis of the number of PSD-95 and gephyrin puncta was conducted using the built-in spot detection algorithm in Imaris (γ 1.79, quality 80). The coordinates of the puncta were then exported for further analysis of position and clustering using custom written Python code.

Clustering analysis

The data from the imaging of gephyrin and PSD-95 puncta were clustered with the friends-of-friends (FOF) algorithm described by Davis et al. (1985). The FOF algorithm has one free parameter, the linking length between two points. Any two puncta that lies closer than this length are linked together. A cluster is then all puncta that are connected to each other through a network of linked puncta. Measurements of the distance between a selected set of puncta showed that 2 μm was a good choice of linking length, as a shorter or longer distance would yield clusters of very few puncta or only a few clusters with almost all puncta clustered, respectively. As the objective was to perform a comparison between the two datasets (chABC treated vs control), the exact choice of linking length is not important as long as it is kept constant.

Injections of chABC, artificial cerebrospinal fluid (aCSF), and retrograde tracer

chABC from *Proteus vulgaris* (Amsbio) was diluted in filtered 1× PBS to an initial concentration of 61 U/mL, and stored at -20°C in smaller aliquots before surgery. Anesthesia was induced by placing the animals in an induction chamber with 5% isoflurane concentration. Animals were then placed in a stereotaxic frame and provided with isoflurane mixed with air at a constant flow of 2 l/min, through an anesthesia mask. They were given subcutaneous injections of buprenorphine (Temgesic, 0.04 mg/kg) and local subcutaneous injections of bupivacaine/adrenaline (Marcain adrenaline, 13.2 mg/kg) in the skin of the scalp before surgery began. The scalp was shaved and cleaned with 70% ethanol and chlorhexidine, and a small incision was made in the skin. Small craniotomies were made with a hand held dental drill. The microinjector (NanoJect II, Drummond Scientific) was mounted onto the stereotaxic frame and a glass pipette was filled with chABC mixed with fast green FCF (Sigma-Aldrich Chemie) to a final concentration of 48 U/ml, or aCSF (Harvard Apparatus) as a sham injection. A total of four unilateral injection sites were used for MEC. Stereotaxic coordinates were 0.5 mm anterior of the transverse sinus, 4.5 and 4.7 mm lateral of the midline, and 3.0 and 2.5 mm below dura mater with the pipette positioned at 15° angle in the sagittal plane and the tip pointing in the anterior direction. For V1 injections, we used three coordinates, all relative to lambda: 0.25 mm posterior and 4.5 mm lateral, 0.25 mm posterior and 4.9 mm lateral, and 0.75 mm posterior and 4.7 mm lateral. All the V1 injections were made at a depth of 0.6 mm depth, relative to the dura mater.

Injections at each site were performed in steps of 23 nl each, with a total volume of 368 nl for each position. The pipette was kept in place for 1-2 min to increase diffusion of chABC before the wound was cleaned and sutured shut. Animals were given subcutaneous injections of carprofen (Rimadyl, 5 mg/kg) and local anesthetic ointment (Lidocain) was applied. This was repeated for 3 d. Animals were sacrificed 7 d after surgery.

To perform retrograde labeling of neurons projecting from the mEC to the hippocampus, we used cholera toxin subunit B, conjugated to Alexa Fluor 594 (C22842, Life Technologies), diluted in 1× PBS (10% wt/vol). The procedure was conducted as described above. To target the projections from Layer II of mEC to dentate gyrus (DG) we used the following coordinates relative to bregma: 4.1 mm posterior, 2.6 mm lateral, and 3.5 mm below dura. While for injections in the CA1 aiming for projections from Layer III of mEC were 4.1 mm posterior, 2.6 mm lateral, and 2.1 mm below dura. We injected a total of 0.2 μl at each site over a period of 10 min. Medication procedures were identical to those described above. The animals were sacrificed after 5 d.

Statistical analysis

All statistical analysis was performed using GraphPad Prism 6 (GraphPad) or SigmaPlot 12.0 (Systat Software). All data were tested for normality by Wilks-Shapiro test, and further analysis was performed accordingly (Student's *t* test or Mann–Whitney *U* test).

Results

To investigate the expression of PNNs, we stained brain sections with WFA in the V1, the mEC and the cornu ammonis regions (CA1, CA2, and CA3) of the dorsal hippocampus. We found that both the level of expression and laminar distribution of PNNs differed greatly between these areas (Fig. 1*A*,*B*). In the rat, PNNs were distributed across cell Layers in V1, apart from Layer I which is largely

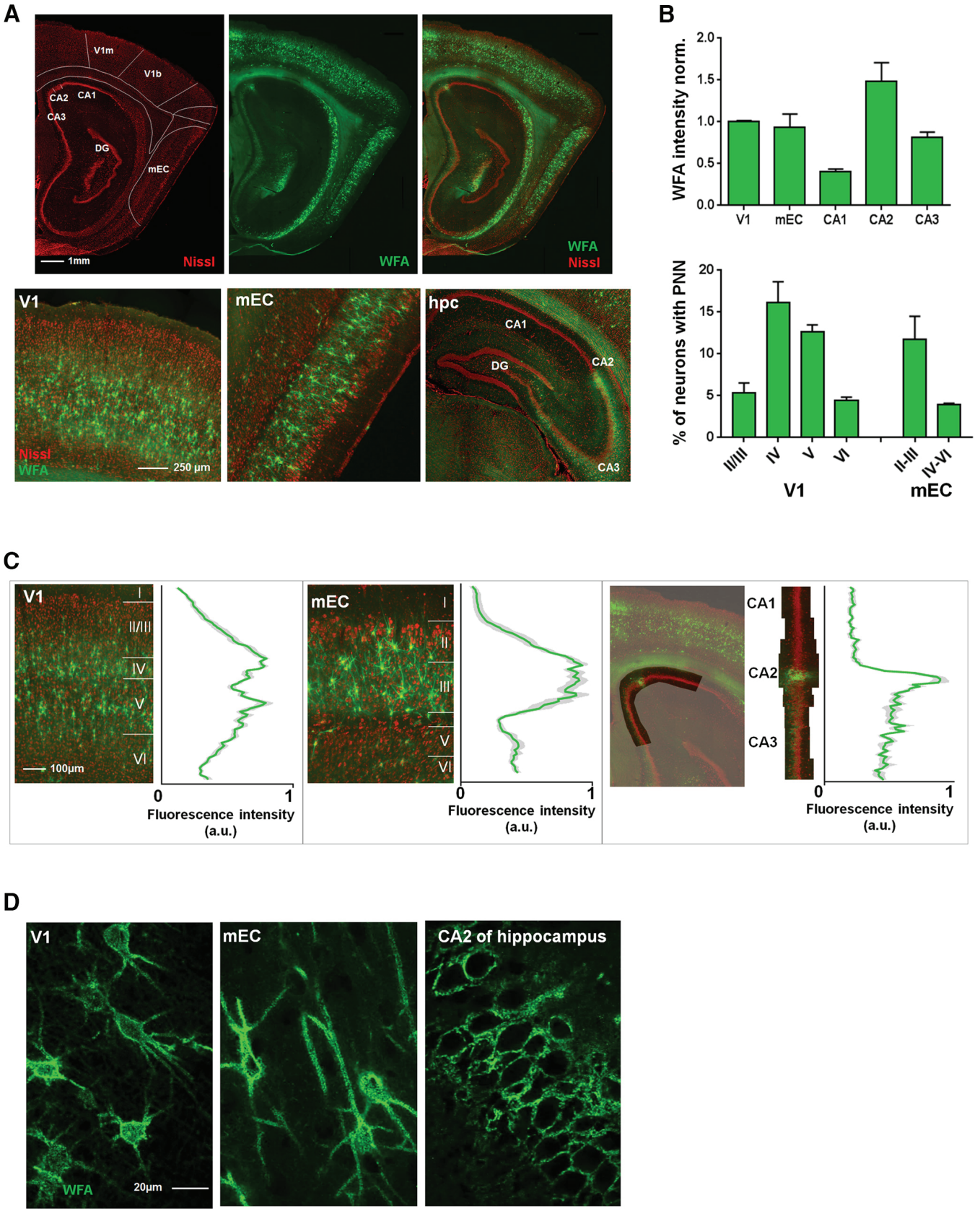

Figure 1. Laminar distribution and structural differences of WFA-positive PNNs in V1, mEC, and hippocampus. ***A***, Sagittal section of a rat brain stained with Neurotrace (Nissl bodies) and WFA. Brain areas are indicated based on the Paxinos atlas of the rat brain, with the areas of interest denoted. Lower panel shows the areas of interest highlighted, mEC in a sagittal plane, while V1 and hippocampus are shown in coronal plane. ***B***, Expression of PNNs was quantified by intensity measurement and counting the fraction of

continued
PNN-enwrapped cells. The intensity measurements showed that expression was highest in V1 and CA2. WFA intensity from all areas was normalized to the values from V1. The fraction of neurons enwrapped by PNNs were quantified by manual counting and showed a layer-specific expression of PNNs. While the area averages (8.4% for V1, 7.9% for mEC) of PNN-positive neurons were similar between the areas, the layer-specific expression was different. Measurements were performed in three sections from three rats, bar graphs shows mean ± SEM. ***C***, In V1, PNNs are uniformly expressed across cell layers, with the highest expression in Layer IV and the lower part of Layer V (left). In mEC, PNNs are densely expressed in Layer II/III with only sparse labeling in Layer V and VI (middle). The dorsal hippocampus (right) is largely void of PNNs, with the exception of area CA2 and partially CA3, which shows dense labeling of diffuse PNN-like structures. Intensity measurements across cell layers in V1 and mEC was performed in three sections from three rats and is shown as mean ± SEM. ***D***, The structure of WFA-positive PNNs is highly variable between brain areas. High-resolution images from the V1 (left) show PNNs enwrapping the soma and the most proximal dendritic segments; from the mEC (middle) where the PNNs enwrap the cell soma extending far out in the proximal dendrite, and from the CA2 (right) where the PNNs are diffuse in structure and only embody the cell soma and neuropil.

void of cell bodies, and the upper part of Layer V. The highest expression was found in Layers IV and V (Fig. 1*B*,*C*, left). In contrast, the superficial layers of mEC showed dense WFA labeling, while few PNNs were located in the deeper layers. The total fraction of PNN enwrapped neurons was, however, similar between V1 and mEC (8.4% in V1 and 7.9% in mEC, 5997 neurons counted in total).

The PNN expression of the dorsal hippocampus stands out with a very sparse and area-specific expression pattern (Fig. 1*A*,*C*, right). Only a few weak PNNs were found in CA1 and they were located in the stratum pyramidale, and some in stratum oriens. In CA2, however, the WFA staining was intense, enwrapping all cell bodies in the stratum pyramidale. The CA2 showed the highest density of WFA-enwrapped cells of all areas investigated, but the microstructure of the PNNs were more diffuse compared with the cortex (Fig. 1*B*,*D*). While a similar diffuse staining was observed in CA3 and in the granular cell layer of the DG, the intensity was much lower than in the CA2. In V1, the PNNs enwrapped the cell soma and a short section of the proximal dendrites. In contrast, PNNs in the mEC were similarly present around the cell soma but continued along long stretches of the dendritic segments (Fig. 1*D*, left and middle panel). In the CA2 of the hippocampus, on the other hand, PNNs located in the neuropil of the pyramidal cell layer enwrapped all cell somas but only a minor part of the processes (Fig. 1*D*, right).

To investigate whether the dense labeling in the dorsal hippocampus was limited to the CA2 region, we stained sections with the neuronal marker PCP4, which selectively label excitatory neurons in CA2 of the hippocampus (Botcher et al., 2014; Kohara et al., 2014). By counterstaining sections with WFA, we found that these markers overlapped, supporting that the PNN-positive neurons in CA2 differ from those in other sub-regions of the hippocampus (Fig. 2*A*). As the PNNs in CA2 were structurally different from the cortical areas, we went on to investigate if they also differed in cell-type specificity. Sections were stained for PV and CamKII, the latter labeling putative excitatory neurons. In CA2, we found that the large majority (~80%) of neurons enwrapped in PNNs expressed CamKII (Fig. 2*B*). The PNNs in CA2 also enwrapped PV-expressing synaptic boutons onto the cell somata (Fig. 2*C*, lower image).

Overall, the special morphology and low density of PNNs in the hippocampus diverged profoundly from the other brain areas; hence we wanted to investigate if this was specific to the rat. While PNNs have been described in a range of species from birds to humans (Adams et al., 2001; Balmer et al., 2009), it remains unknown how the distribution and cell-type specificity varies between the widely used laboratory models mice and rats. The two species have nonetheless been used interchangeably in functional studies of PNNs. Indeed, comparing the distribution of PNNs in the rat and mouse hippocampi revealed large differences. In the mouse, PNNs were present in all sub regions of the hippocampus, with a structure similar to those in the cortical areas (Fig. 3*A*,*B*, right panels). In the rat hippocampus, however, we found very few discernable PNNs apart from that of the CA2 and to some extent in the CA3, which both showed a dense but diffuse matrix (Fig. 3*A*,*B*, left panels). Furthermore, while a large fraction of PNNs in mouse hippocampus colocalized with PV+ neurons, this was not the case in rats, with the exception of the few weak PNNs in the CA1 (Fig. 3*B*; Table 2). <20% of PNNs in CA2 in rats colocalized with PV+ neurons. The CA2 of mice was similar in terms of PNN structure, but not as densely packed as in the rat, and as much as 75% of PNNs enwrapped PV+ cells (Fig. 3*B*; Table 2).

The species comparison of the identity of PNN-positive cells was also conducted in the V1 and the mEC. In the V1, there was an almost exclusive colocalization between PNNs and PV+ in both species (Fig. 4*A*, left panel). In mEC, however, we found significant species differences, both in the fraction of PNNs colocalizing with PV+ cells and also the fraction of PV+ cells without PNNs (Fig. 4*A*, right panel). More PNNs colocalized with PV+ cells in mice (87%) compared with rats (74%). In rats, ~25% of the PNNs in mEC colocalized with other cell types (Fig. 4*A*; Table 3). The mEC contains neurons with common morphologic characteristics but different phenotypes such as the spatially tuned grid-, border-, and head direction cells, and nonspatial cells (Sargolini et al., 2006; Kroppf et al., 2015; Ebbesen et al., 2016). The grid cells are found both among reelin-positive stellate cells that project to the DG, as well as the calbindin-positive pyramidal neurons (Kitamura et al., 2014; Tang et al., 2014; Sun et al., 2015). In an attempt to reveal the identity of the PNN-positive (non-PV) neurons and examine if some of

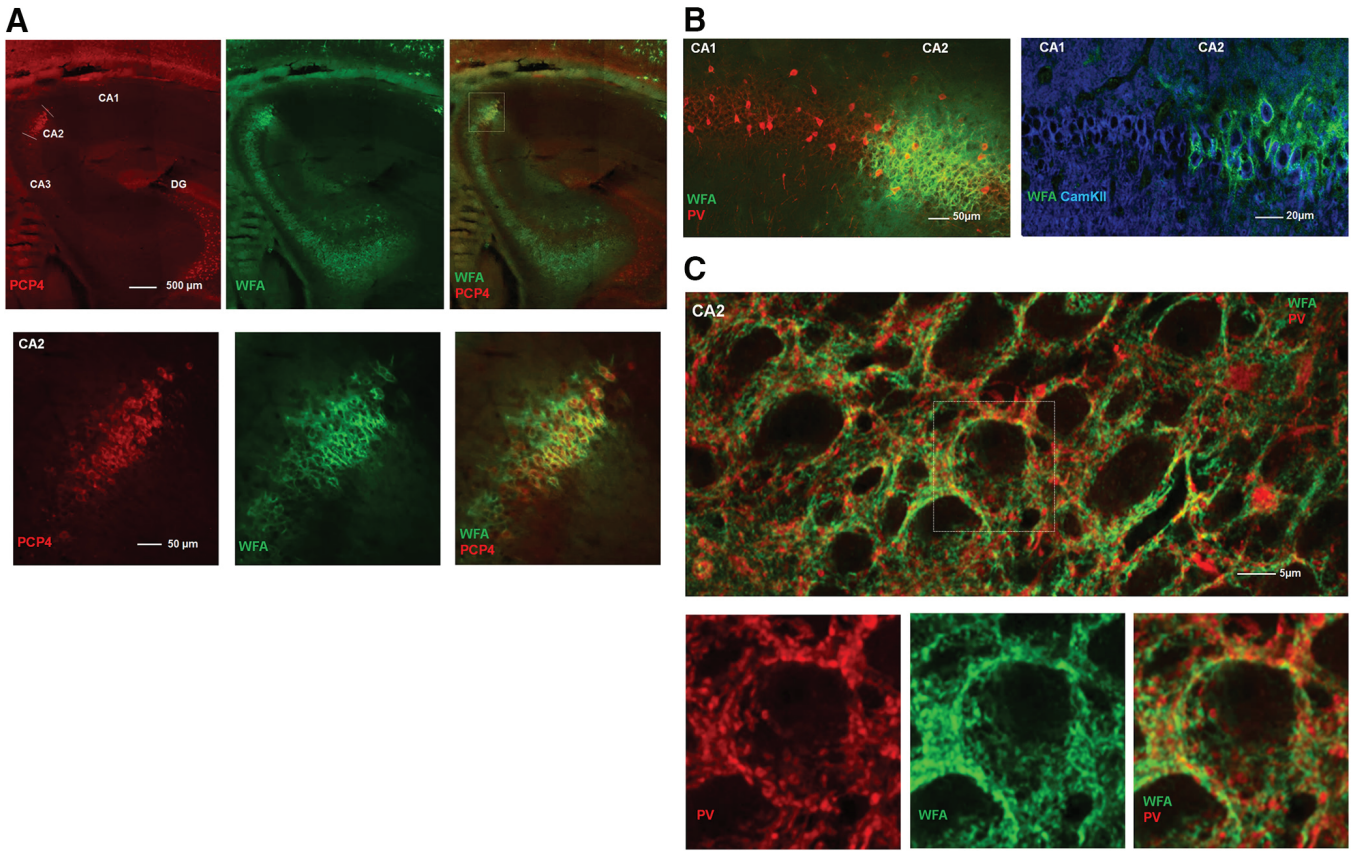

Figure 2. The hippocampal area CA2 shows dense expression of PNNs. ***A***, Sagittal section of a rat brain stained for PNNs (green) and the CA2-specific marker PCP4 (red) show complete overlap, indicating that the dense PNN-structures are selectively expressed in CA2. ***B***, The PNN-like ECM structures in CA2 of rat hippocampus primarily colocalize with CamKII-expressing, excitatory neurons and not PV+ neurons. Note the sharp border in WFA labeling between CA1 and CA2. ***C***, Maximum intensity projection from a z-stack in CA2. While the PNNs in CA2 are mostly associated with CamKII-expressing neurons, they also embed PV+ synaptic boutons surrounding CamKII-expressing principal neurons in the pyramidal cell layer.

them are potential grid cells, we stained brain sections from rats with several markers, including calbindin and reelin. In the most dorsomedial part of mEC we found considerable overlap between PNNs and reelin-positive cells (28% of PNNs colocalized with reelin; Fig. 4*B*,*C*) while only a small fraction (~5%) of calbindin-expressing cells colocalized with PNNs (Fig. 4*B*). To test whether the reelin-positive neurons with PNNs were projecting to the hippocampus, we injected a retrograde tracer in CA1 and DG of dorsal hippocampus respectively, and stained sections with WFA. As expected (Witter, 2007) when injecting the tracer in DG, we found projecting neurons strongly labeled with the tracer in Layer II of mEC, and some labeling throughout Layer III (Fig. 5*A*). Counter-staining with reelin revealed an almost complete overlap between the tracer and reelin in mEC (Fig. 5*B*). The sections from the tracer experiments were also stained with WFA, revealing overlapping cells with the tracer and WFA, in particular in Layer II (Fig. 5*C*). However, only few such colocalized cells were found, indicating that most of the reelin-expressing WFA-positive cells are intrinsic to the entorhinal network, and only a subpopulation project to the dorsal hippocampus.

In the cortex, the assembly of PNNs coincides with the closure of the critical period and may be linked to stabilization of functional phenotypes whose development is experience-dependent, such as ocular dominance and binocular matching in V1 (Pizzorusso et al., 2002; Wang et al., 2010). In the mEC, some head-direction cells show spatial tuning from early age, whereas the characteristic grid cell activity pattern appear much later (Langston et al., 2010; Wills et al., 2010; Bjerknes et al., 2014), suggesting experience-dependent development of grid cells similar to that of the sensory cortices. If the PNN assembly in the mEC follow a similar postnatal development as reported for V1 (Ye and Miao, 2013) remains unknown. To explore this we followed the PNN formation in mEC in postnatal development and found that the development of PNNs was strikingly similar to what has been observed in V1 with diffuse WFA-positive PNNs starting to appear around P12 but not fully developed until P30 (Fig. 6*A*). The most notable difference occurred between P17 and P20 when the PNNs became more clearly defined, after which we observed a gradual increase in WFA intensity and PNNs forming along longer stretches of the dendrites (Fig. 6*B*). We also costained sections with PV and quantified the overlap at different stages of development. In contrast to what has been reported from other cortical areas (Alcántara et al., 1993; Sugiyama

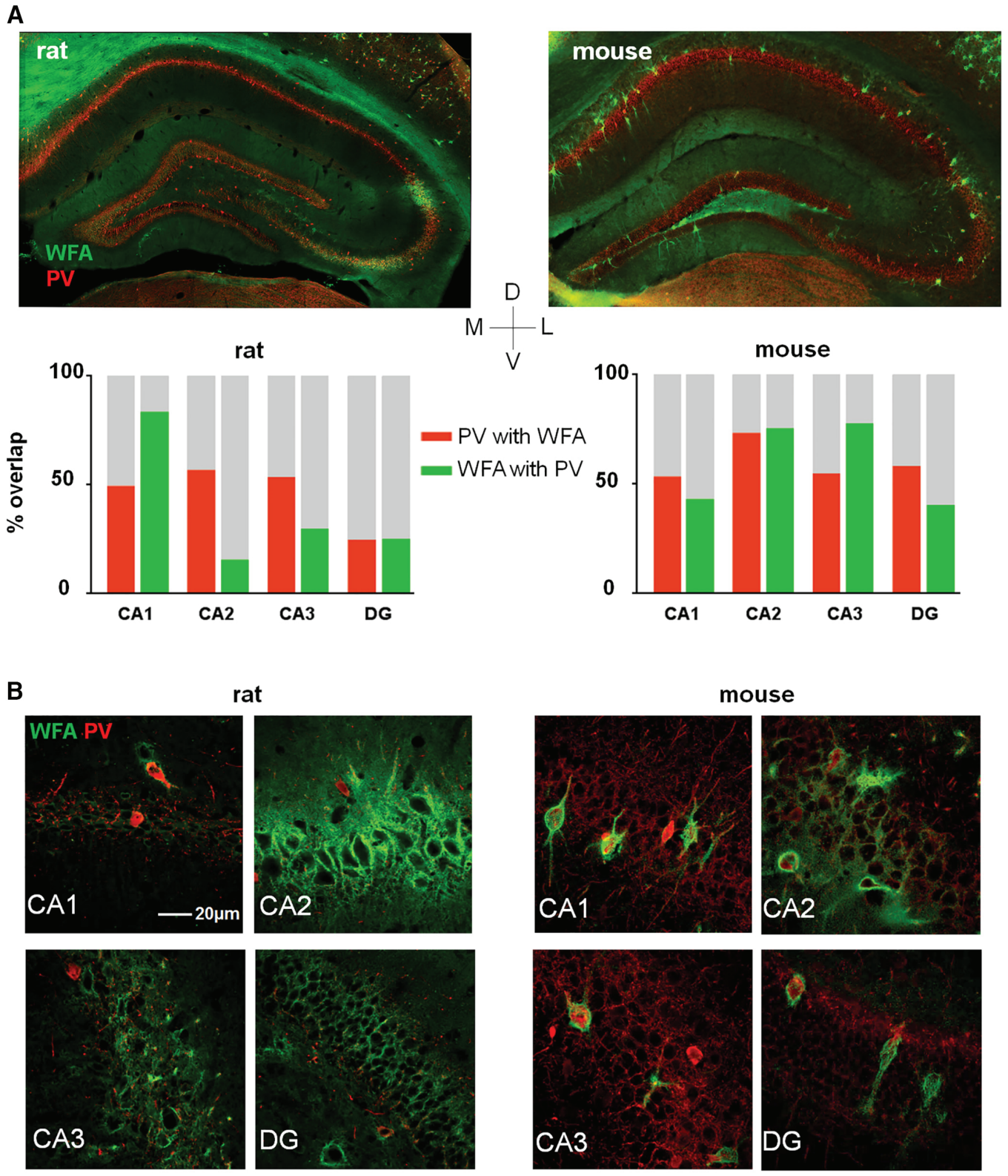

Figure 3. Expression of PNNs in the dorsal hippocampus varies between rats and mice. ***A***, ***B***, Coronal sections from a rat (left) and a mouse(right) brain stained with WFA to label PNNs (green) and parvalbumin (PV+; red). In the rat, almost no PNNs are expressed in the dorsal hippocampus with the exception of CA2, and part of the CA3, which is densely stained with diffuse PNN structures. In mouse, all subfields of the dorsal hippocampus show expression of large, structured PNNs. The overlap between PNN and WFA was calculated from counting identified PNNs and cell bodies in three sections from three animals (bar chart).

Table 2: Colocalization between parvalbumin and WFA-positive PNNs in hippocampal areas of mice and rats

% PV overlapping with WFA			
Area	Rat	Mouse	*p* value
CA 1	49.6 ± 7.7	53.5 ± 4.7	0.34
CA 2	56.8 ± 3.5	73.4 ± 4.5	0.01
CA 3	51.3 ± 2.9	54.9 ± 4.1	0.53
DG	24.8 ± 6.8	58.2 ± 7.1	0.01
% WFA overlapping with PV			
Area	Rat	Mouse	*p* value
CA 1	83.6 ± 3.2	43.3 ± 4.8	<0.001
CA 2	15.7 ± 2.1	75.4 ± 5.9	<0.001
CA 3	29.3 ± 1.5	77.9 ± 6.6	0.002
DG	25.3 ± 5.8	40.5 ± 6.6	0.14

Table 3: Colocalization between parvalbumin and WFA-positive PNNs in mEC and V1 of mice and rats

% PV overlapping with WFA			
	Rat	Mouse	*p* value
V1	71.5 ± 2.4	71.6 ± 1.4	0.9
mEC	83.7 ± 1.4	91.5 ± 0.9	<0.001
% WFA overlapping with PV			
	Rat	Mouse	*p* value
V1	86.7 ± 2.4	85.6 ± 1.9	0.7
mEC	74.2 ± 2.8	87.1 ± 0.9	<0.001

et al., 2008), PV cells in mEC were present from the first time point (P10) we investigated (Fig. 7*A*, upper left panel). Due to the diffuse and weak appearance of the immature PNNs at the early stages an objective quantification of overlap between PNNs and PV+ neurons were not expedient (Fig. 7*B*). From P17 the overlap increased in a similar timeframe to PNN development.

Several lines of evidence point to a role of PNNs in stabilizing synaptic connections between neurons (Dityatev et al., 2010; de Vivo et al., 2013). Despite the profound increase in plasticity after enzymatic degradation of PNNs in the adult brain, it remains unclear how removal of PNNs affects the stability and organization of synapses. To investigate this, we used the enzyme

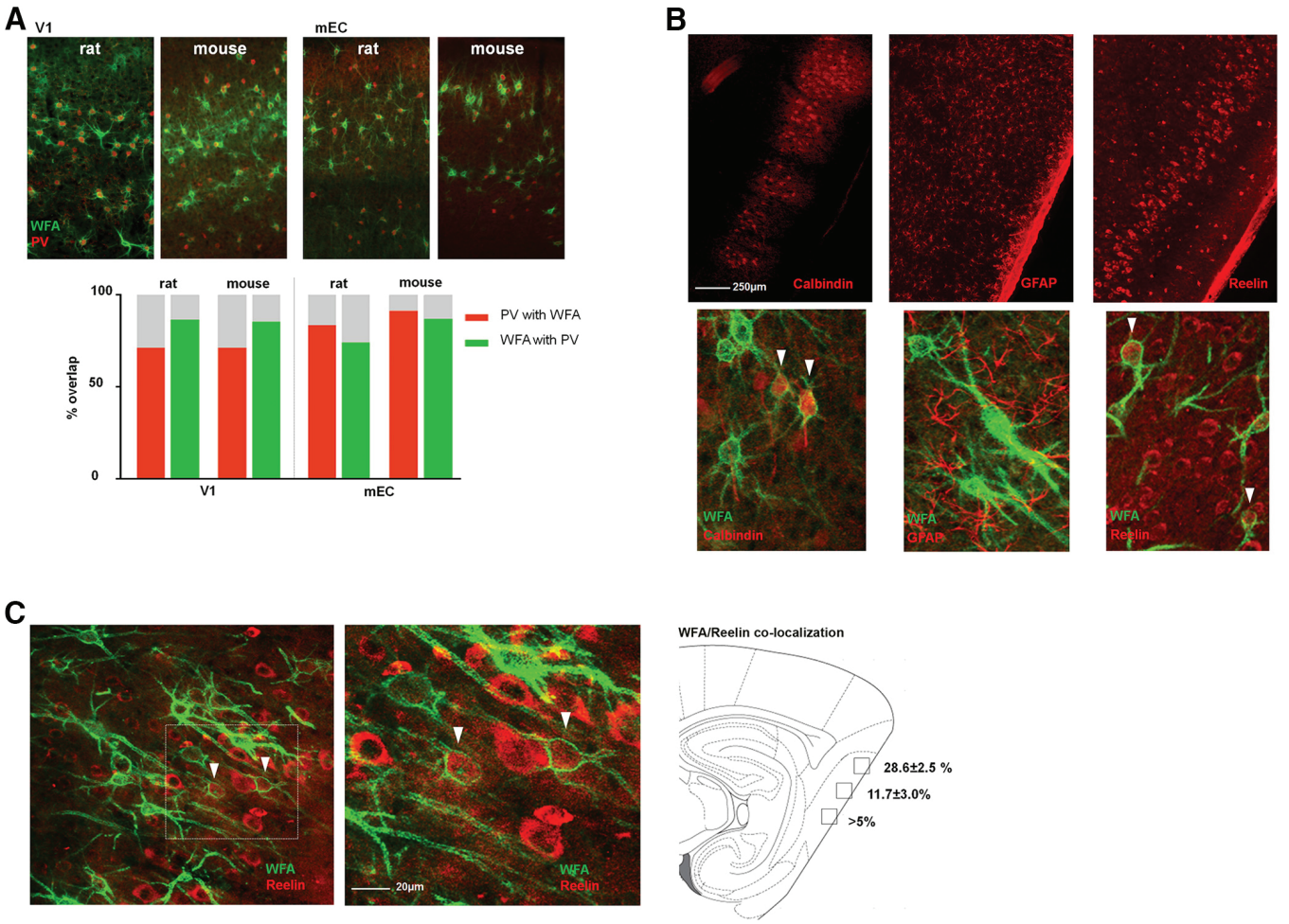

Figure 4. Colocalization of PNNs and cell type-specific markers show species and area-specific differences. ***A***, A majority of PNNs (WFA, green) colocalize with PV+ neurons (parvalbumin, red) in both V1 and mEC (left and right panels, respectively), but the overlap in mEC is different between mice and rats. The overlap (%) was calculated from counting identified PNNs and cell bodies in three sections from three animals (bar chart). ***B***, Sagittal sections from rat brain costained for PNNs and either calbindin (left), the astrocyte marker GFAP (middle), and reelin (right). Calbindin and reelin showed overlap with PNNs while no overlap was seen with GFAP. ***C***, A substantial part (28.6 ± 2.5%) of PNNs in the most dorsal part of mEC colocalizes with reelin-expressing cells.

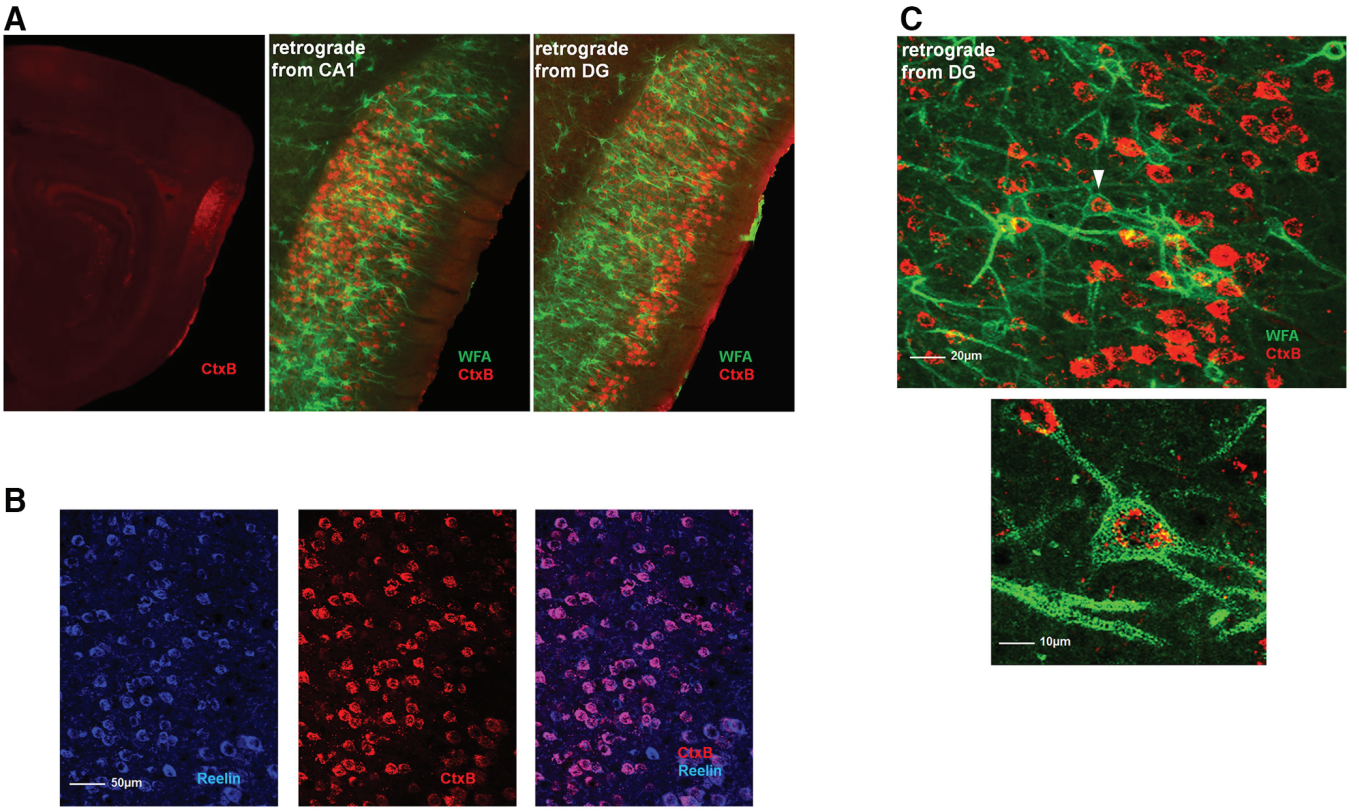

Figure 5. Retrograde tracing reveal projecting neurons from mEC to hippocampus. ***A***, We injected Cholera toxin subunit B (CtxB) into different areas of the dorsal hippocampus. Sagittal sections from a rat brain show PNNs (green) and cell soma of neurons in the mEC (red) filled with the retrograde tracer indicating that they project to dorsal hippocampus. ***B***, All neurons labeled by the tracer in mEC were reelin-expressing cells (blue), but not all reelin-expressing cells were labeled by the tracer. ***C***, Some neurons labeled by the tracer were enwrapped by PNNs, but a lower fraction than reelin cells.

chABC to degrade PNNs. Adult rats were injected unilaterally with chABC or aCSF (sham treatment) in V1 and mEC, and euthanized after 7 d. Brain sections were stained with WFA in combination with the postsynaptic markers PSD-95 or gephyrin, labeling putative excitatory and inhibitory synapses, respectively. The effects of PNN removal showed large differences between brain areas. While removal of the PNNs from the mEC caused a reduction in the number of PSD-95-expressing puncta ($p < 0.002$, Mann–Whitney *U* test; Fig. 8*B*, left panels), the effect of chABC treatment in V1 was an increase in the number of gephyrin puncta ($p < 0.03$, Mann–Whitney *U* test; Fig. 8*A*,*B*, right panels). Sham-treated animals were not different from controls for any marker in any of the areas examined.

Given that chABC treatment caused dramatic changes to the number and inhibitory-excitatory distribution of puncta, we wanted to investigate whether the organization was affected. We used the positional data of the different synaptic markers and an adapted version of the FOF algorithm to cluster the data (Fig. 9*A*). The fraction of puncta located in clusters after treatment with chABC were calculated for each synaptic marker in both areas and compared with controls. Although PSD-95 puncta in mEC showed a tendency to be less clustered after chABC treatment, we found no significant changes in synaptic organization for either of the two brain areas (Fig. 9*B*,*C*).

Discussion

We have shown that the expression, microstructure, and cell specificity of PNNs is highly variable between three brain areas that differ in connectivity, architecture and cognitive functions. Our data show that the general assumption that PNNs primarily colocalize with PV+ neurons is not evident for all brain areas or across different species. In particular, while PNNs mainly colocalize with PV+ neurons in visual cortex, they predominantly colocalize with CaMKII and PCP4-expressing neurons in CA2 of the hippocampus. In the grid cell network of the mEC, PNNs embody several cell types, predominantly PV- and reelin-expressing cells. Moreover, the differences between PNN expression in rats and mice emphasize the importance of taking into account species differences when dissecting the functional roles of PNNs.

The PNNs are believed to serve several functions in the brain, from ion buffering to providing a rigid structure to maintain synaptic integrity and organization (Frischknecht et al., 2009; Beurdeley et al., 2012; Cabungcal et al., 2013; Dick et al., 2013; Tsien, 2013; Morawski et al., 2015). In more general terms, PNNs seem to have an important function in restricting adult brain plasticity. In line with this, removal of PNNs, both by enzymatic degradation and by partial genetic knock-out, has been shown to increase plasticity comparable to juvenile levels in several

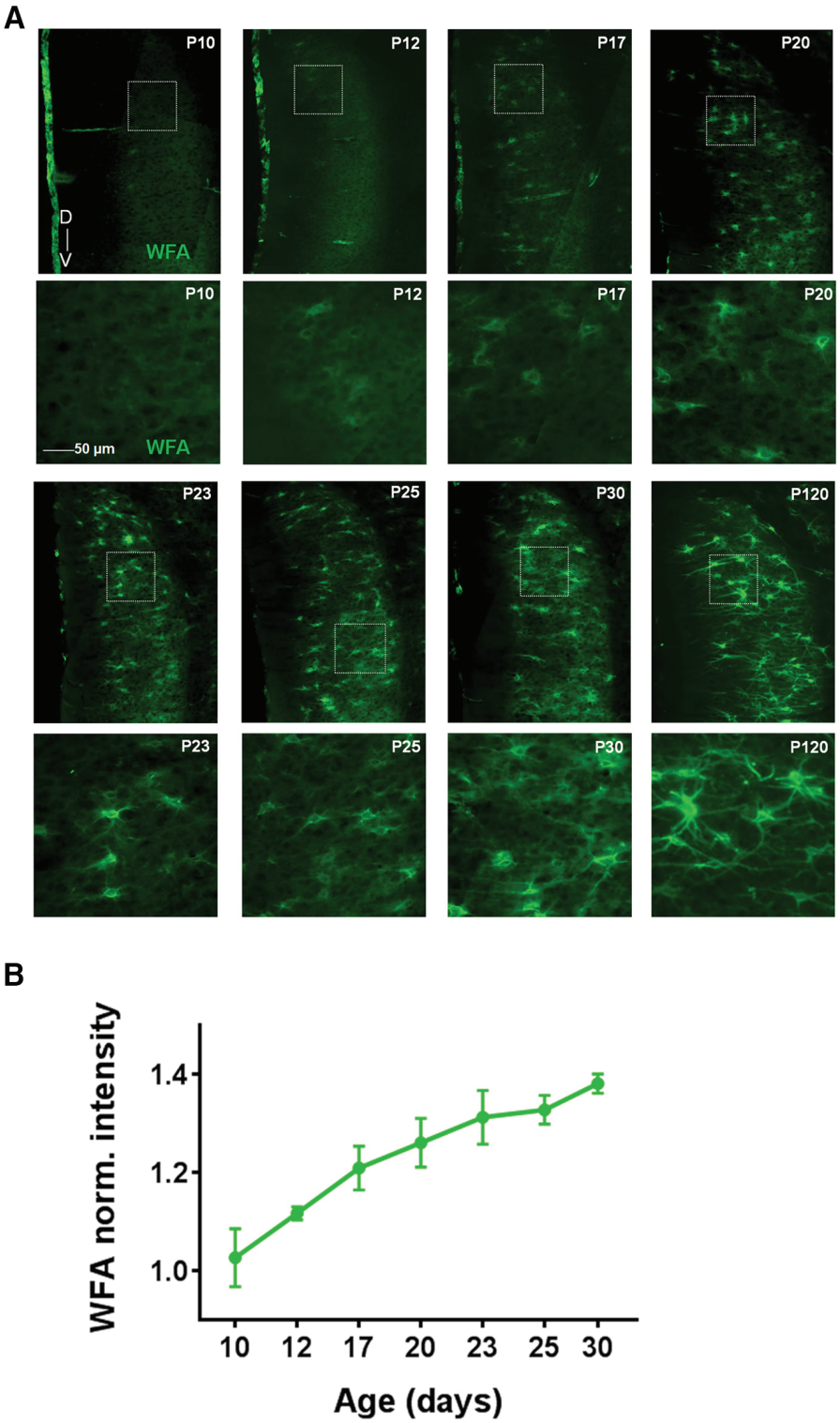

Figure 6. Development of WFA-positive PNNs in mEC of rats. ***A***, Sagittal sections from a rat brain stained for PNNs (WFA, green) at eight different time points during development. Insets are outlined under each time point. The first PNN-like structures appear around P12, and continue to condense throughout late postnatal development; first around the cell soma and later along the proximal dendrites. At P30, PNNs have developed an adult-like structure. ***B***, WFA intensity gradually increases during development. Fluorescence intensity across Layer II/III was measured for each time point at three different dorsoventral positions and normalized to background intensity in Layer I.

areas of the brain (Pizzorusso et al., 2002; Gogolla et al., 2009; Carulli et al., 2010; Happel et al., 2014; Slaker et al., 2015; Lensjø et al., 2017).

PNN expression and plasticity

In line with the functional differences between the three areas investigated, we found large differences in the ex-

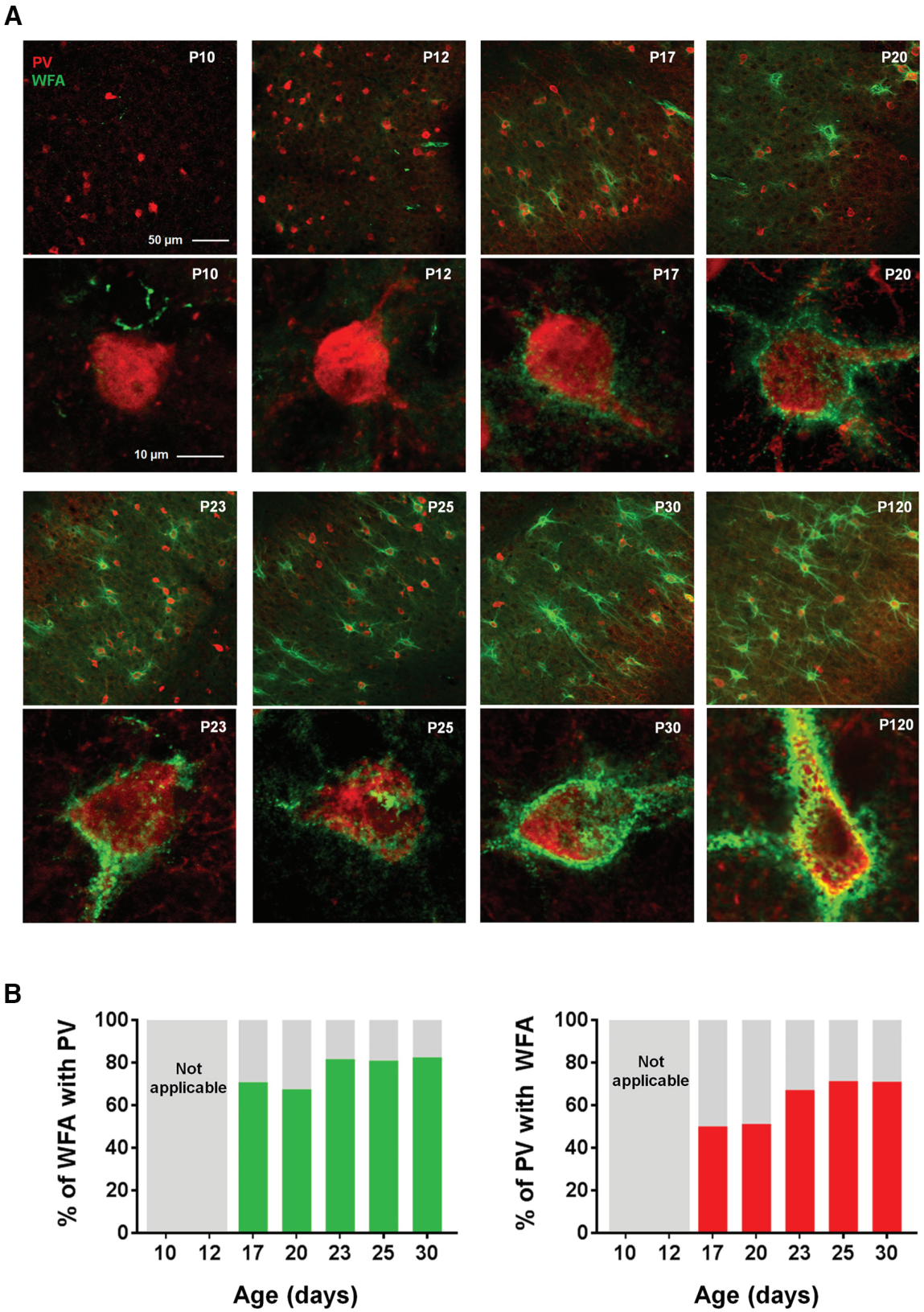

Figure 7. Development of PV+ cells and overlap with WFA-positive PNNs in mEC. ***A***, PV cells in mEC are present at P10, while the first clearly discernable PNNs appear at P17. ***B***, The colocalization between PV and PNNs increases gradually from the first point of measurement at P10 and stabilizes from P23, consistent with the development of PNNs.

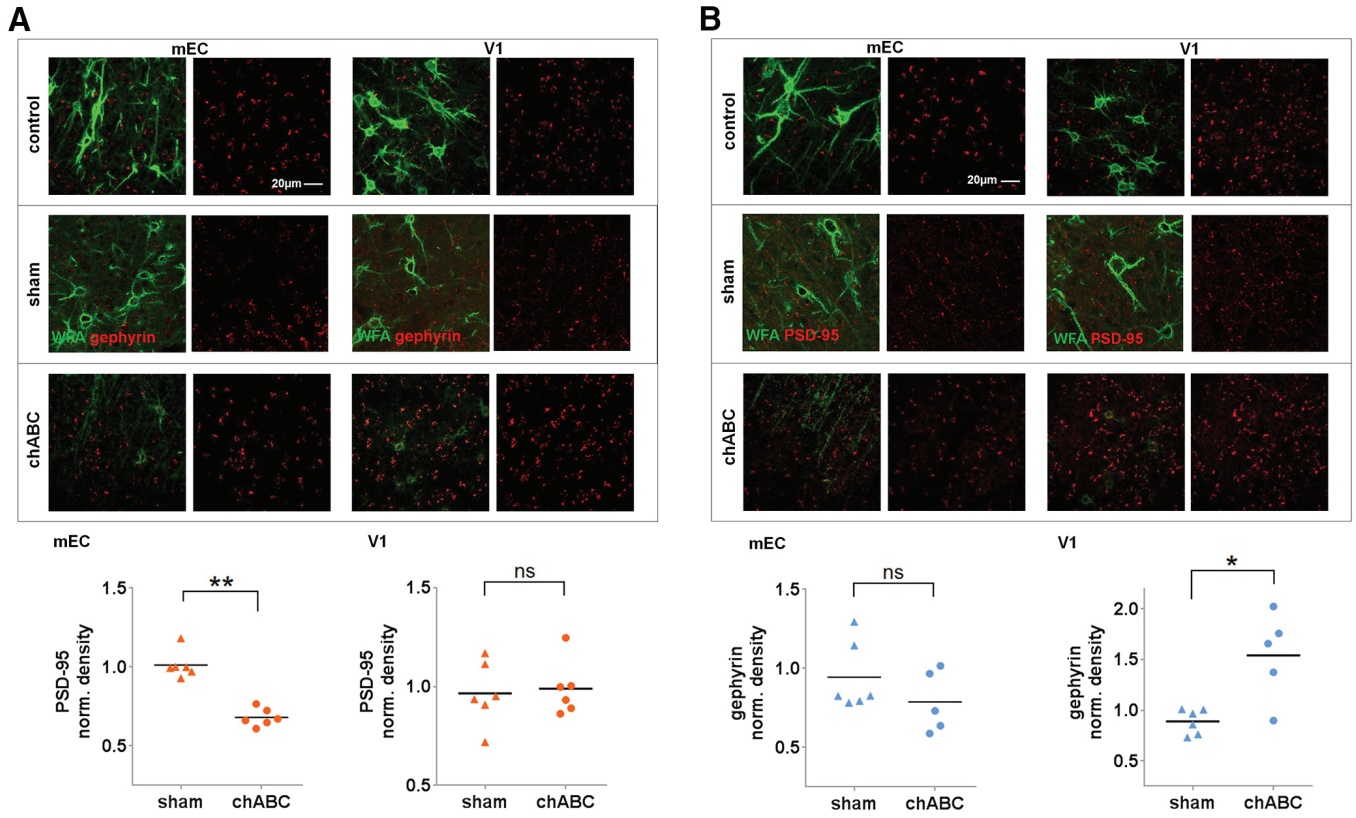

Figure 8. Local injections of the enzyme chABC into the rat brain disintegrate the PNNs and affect the number of PSD-95 and gephyrin puncta. ***A***, Sagittal sections of the brain from a rat treated unilaterally with chABC (bottom row) or aCSF (sham, middle row) and control hemisphere (top row), and stained for PNNs (green) and gephyrin puncta (red) in V1 and mEC. Quantification of puncta was conducted by using the spot detection algorithm in Imaris, and performed on two to three sections from three rats injected with chABC and three rats injected with aCSF (***A***, ***B***, lower panels). In mEC, there were no significant changes in the number of gephyrin puncta ($p = 0.25$, Mann–Whitney *U* test), but an increase in V1 ($p = 0.03$, Mann–Whitney *U* test). ***B***, The chABC treatment caused a significant reduction of PSD-95 puncta (red) in mEC ($p = 0.002$, Mann–Whitney *U* test), and no significant effect in V1 ($p = 0.7$, Student's *t* test). Data were normalized to the contralateral control hemisphere for each animal used, line indicates mean values.

pression of PNNs (Fig. 1). The PNNs in V1 had a uniform distribution with a structure usually described packing around the soma and proximal dendrite (Wegner et al., 2003). In the mEC, however, PNN expression was limited to Layers II and III. The nets in mEC also packed around the soma but extended along long stretches of the dendrites. This difference in distribution pattern may reflect layer-specific functional roles of neurons in the mEC with, e.g., grid cells being most abundant in Layers II and III. Approximately 10% of all the neurons in V1 and mEC of rats were enwrapped in a PNN. This is comparable with previous work in macaque, albeit somewhat higher (5% in V1; Mueller et al., 2016). In contrast, the dorsal hippocampal areas CA1 and CA3 have very low expression of PNNs, which may be indicative of the high level of plasticity in these areas. Local ensembles of hippocampal place cells can take part in different cell assemblies in an unpredictable manner (Leutgeb et al., 2004). This stands in contrast to local ensembles of entorhinal grid cells which show a rigid spatial and relationship with coherent realignment during remapping (Fyhn et al., 2007; Stensola et al., 2012). Further work should clarify if the lack of PNNs in CA1 and CA3 support a flexible population code. Moreover, a potential role of PNNs for stabilizing the grid cell network needs further investigation.

The hippocampus maintains a high degree of plasticity throughout life. This is documented through easy induction and strong long-term potentiation of synapses, NMDA receptor-dependent plasticity of many hippocampus-dependent cognitive processes and the ability of cell ensembles to independently reorganize and change in response to small changes in external inputs (remapping). Within the hippocampus we found large variation in the expression of PNNs. The low expression of PNNs in the CA1 region of the dorsal hippocampus contrasted with the dense expression of PNN-like structures in the CA2 region. Interestingly, the CA2 area is characterized by low levels of plasticity compared with the surrounding areas (Dudek et al., 2016).

Taken together, it is tempting to speculate that there is a relationship between PNN expression and the level of plasticity, where neural networks with lower levels of plasticity correlate with abundant expression of PNNs while the opposite seem to be true for regions with high degree of plasticity such as the CA1 region of the dorsal hippocampus. However, it should be noted that PNNs only surround a small fraction of the neurons, suggesting that the increased plasticity observed after enzymatic degra-

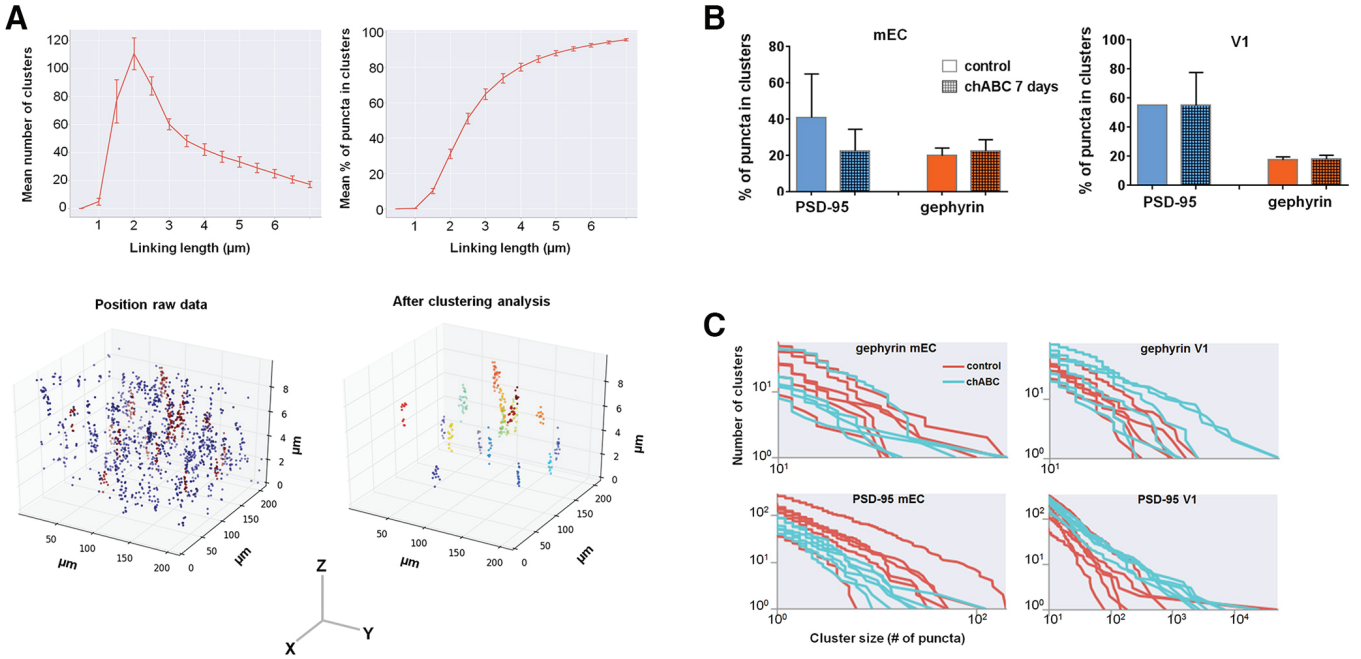

Figure 9. Distribution and clustering of PSD-95 and gephyrin puncta in mEC and V1 after chABC treatment. ***A***, Overview of the clustering method and differences in clustering as a result of different linking length. Lower left panel shows the raw data of identified gephyrin puncta in mEC from a z-stack, visualized in 3D space. The nonclustered puncta are shown in blue, while clusters are shown in red. Lower right side shows the identified clusters, when using 2 μm as linking length. Note the difference in axis range between *z* and *x y*, causing the elongated shape of the clusters. ***B***, The percentage of puncta within clusters in controls and chABC-treated rats in mEC and V1. We did not detect significant differences between the groups. There was a tendency toward reduced clustering after chABC treatment in mEC, but overall, high variation between the data sets, in particular for excitatory puncta (PSD-95). ***C***, Examples of distribution of cluster sizes, from six measurements (z-stacks) in mEC and V1.

dation of PNNs is likely to be network effects including more than the neurons surrounded by PNNs.

PNNs in hippocampus of rats and mice

In accordance with previous work in mice (Yamada and Jinno, 2013, 2016), we found sparse but clearly defined PNNs across most areas of mouse dorsal hippocampus, in particular in CA1. This was in contrast to the rat CA1, where only few and weakly labeled PNNs were discernable (Fig. 3). The species difference was also prominent for the colocalization between PNNs and PV-expressing neurons. Among the few PNNs found in the rat hippocampus, only a relatively small fraction were colocalized with PV+ neurons, while a high fraction colocalized in most areas of mouse hippocampus. It remains an open question if these differences have any functional consequences for network computations in the two species.

The dense expression of PNNs with a diffuse microstructure in the CA2 region of the hippocampus was remarkably different from PNNs in CA1 and neocortex. The PNN-like structures in CA2 of rats did not primarily colocalize with PV+ cells but rather with pyramidal neurons expressing CamKII, indicative of excitatory neurons. The complete overlap in colabeling of WFA and the CA2 neuron-specific marker PCP4 confirmed that the expression of this dense matrix was selective to CA2, which is in accordance with recent work (Carstens et al., 2016). These observations add to a growing body of evidence indicating that the CA2 is a functionally and structurally distinct region of the hippocampus with unique features (Jones and McHugh, 2011; Dudek et al., 2016). The principal neurons found in this area differ from the areas around in terms of morphology, gene expression, physiologic properties, and connections to other areas (Caruana et al., 2012; Hitti and Siegelbaum, 2014; Mankin et al., 2015), and CA2 is more resistant to ischemia-induced cell death (Sadowski et al., 1999; Zao et al., 2007). The CA2 has proven highly resistant to experimentally induced long-term potentiation (at Schaffer collateral synapses) compared with CA1 (Zao et al., 2007). Recent work shows that enzymatic removal of PNNs in CA2 of juvenile mice (P14-18) increases plasticity in this area, to a level comparable to CA1 (Carstens et al., 2016). Similar to chABC treatment, caffeine has also been shown to induce potentiation of synaptic responses in CA2 through its effect of adenosine receptors which are highly expressed in this area (Simons et al., 2011). Thus, it appears that while the CA2 is an area resistant to synaptic change under normal conditions, it may be that a state switch such as that induced by PNN removal or caffeine opens for synaptic plasticity in the circuitry. Taken together, this suggest a highly important functional role for PNNs in the CA2, and that while the PNNs of the CA2 are structurally different to other areas, they likely serve many of the same functions.

Several studies have been using the hippocampus as a model area for investigations of PNN function (e.g. Hylin et al., 2013). However, the remarkable low expression of PNNs in the hippocampus of both mice and rats argues against using the hippocampus as a model to understand the role of PNNs. despite this recent work claims that the

effects of chABC treatment in dorsal hippocampus on behavioral performance and gene expression were due to removal of the PNNs (Donato et al., 2013; Hylin et al., 2013; Yamada et al., 2015). While the effects observed were statistically significant, they may be attributed to chondroitin sulfate proteoglycans in general, rather than the few PNNs present in the area. However, for cortical areas such as visual cortex and the perirhinal cortex, the plasticity effects of chABC treatment have been verified by genetic deletion of the link protein, Crtl-1 (Carulli et al., 2010; Romberg et al., 2013), which is a key component and specific for the PNNs. It may be that it is the lack of PNNs that allows for the hippocampus to retain the unique plasticity necessary for learning and memory processing throughout life.

PNNs in V1

The V1 is the brain area where the function of PNNs has been most investigated. Here, PNNs have been shown to assemble in parallel with the maturation of the inhibitory network of PV+ neurons as the critical period for ocular dominance plasticity ends (Pizzorusso et al., 2002; 2006; Liu et al., 2013; Ye and Miao, 2013). Perturbations of PNNs in V1 by chABC or deletion of the link protein Crtl-1 reopens for ocular dominance plasticity in V1 (Pizzorusso et al., 2002; Carulli et al., 2010). We found strong expression of PNNs in V1 across all cell layers, except for Layer I (Fig. 1), and high degree of colocalization with PV in both mice and rats, with values very similar to what has previously been reported (Fig. 4*A*; Beurdeley et al., 2012; Liu et al., 2013; Ye and Miao, 2013). While the adult V1 network shows limited degree of experience-dependent plasticity in adulthood, prolonged sensory deprivation does cause a shift in ocular dominance (Sawtell et al., 2003; Tagawa et al., 2005; Sato and Stryker, 2008). This process differs from critical period plasticity in that it is largely dependent on other cellular mechanisms, and on the action of matrix metalloproteinases (MMPs) (Ranson et al., 2012; Pielecka-Fortuna et al., 2015). These enzymes are activated on changes in activity and degrade ECM molecules locally. Blocking the action of MMPs abolishes the effect of sensory deprivation in adult animals (Pielecka-Fortuna et al., 2015), suggesting that PNN modification is an important part of the process.

PNNs in mEC

PNNs have been reported in the mEC of humans (Pantazopoulos et al., 2010; Lendvai et al., 2013). To our knowledge, the current study is the first to quantify PNNs in mEC of rodents. The dense expression of PNNs in Layers II and III and high colocalization with PV+ cells that are known to be a key player in the grid cell network, indicates a role for the PNNs in the circuitry for spatial navigation (Fig. 4). The distribution of PNNs corresponds to the PV expression pattern (Wouterlood et al., 1995; Fujimaru and Kosaka, 1996) and to the distribution of PNNs in human mEC where the expression is also mainly located to Layers II and III (Lendvai et al., 2013). The mEC has been proposed to be a hub in a distributed network for navigation in the brain. A subpopulation of neurons, the grid cells, shows a remarkable activity pattern of repetitive firing fields spanning the course of the environment visited by the animal (Hafting et al., 2005). The grid map is stable over time and ensembles of grid cells operate in synchrony and retain its temporal relationship indicating a hard-wired neural network. The grid cells are likely interconnected through a network of monosynaptically coupled PV+ cells, which is believed to contribute to the rigid activity pattern of the grid cell network (Couey et al., 2013, Buetfering et al., 2014). The large overlap between PV+ cells and PNNs described here and the plasticity limiting role of PNNs in other cortical areas, suggests that the PNNs could play a role in grid cell stability. Emerging evidence suggests that the grid cells are likely to be both stellate (reelin-positive) and pyramidal (calbindin-positive) cells (Sun et al., 2015), both of which partly colocalized with PNNs. While the overlap with calbindin was small, we found a substantial part of the PNNs colocalizing with reelin-expressing cells (Fig. 4*B*,*C*). Based on our tracer experiments it appears as if there are different populations of stellate cells, with a subset of hippocampus projecting stellate cells that colocalized with WFA (Fig. 4*C*). While this could also be an effect of the efficiency of the tracer injection, it seems likely that most of the WFA-expressing stellate cells do not project to CA1. It remains to be investigated if the PNN expression of these subgroups is associated with functional properties.

While the underlying mechanisms remain mostly unknown, the occurrence in time of stable spatial representations of the grid cells in mEC (Langston et al., 2010; Wills et al., 2010; Bjerknes et al., 2014) coincide with the closure of critical period plasticity in other cortical areas. We show that the maturation of PNNs in this region closely follows the timing reported for development of grid cells, and is very similar to the PNN maturation seen in primary sensory cortices (Ye and Miao, 2013; Fig. 6). However, unlike in other cortical areas (Alcántara et al., 1993), we found that PV+ neurons in mEC were present already at P10 (Fig. 7*A*). This might appear contradictive to the notion that PV+ neurons and PNNs mature together, but recent work investigating the development of the neuronal network in mEC has demonstrated that PV+ cells show adult phenotypes from P14-P20 (Donato et al., 2017), i.e., during the same period that the colocalization between PV+ cells and PNNs increases dramatically and stable spatial representations appear (Fig. 7*B*). Hence, our data suggests that PNNs may contribute to secure a rigid neural network which may be a prerequisite for the stable spatial representations of grid cells in adulthood. This also suggests that PV itself is not a reliable marker for activity and cellular maturation. Whereas functional investigations of mEC after PNN manipulation are needed to reveal their role in spatial navigation, their dense expression in mEC and the cell types being enwrapped in PNNs point toward a role in stabilizing the network.

Synaptic stability and distribution

To study whether PNNs affect synaptic stability we used the bacterial enzyme chABC to degrade chondroitin sulfate glycosaminoglycans unilaterally in V1 and mEC.

This treatment effectively collapses the PNNs and abolishes WFA staining (Brückner et al., 1998). Previous work has shown that chABC treatment causes changes to the number of synapses and spine motility in hippocampus and V1, respectively (de Vivo et al., 2013; Donato et al., 2013). We found large effects on the number of putative inhibitory and excitatory puncta, and the ratio between these after chABC treatment. However there was large variability between brain areas (Fig. 8). In mEC, there was a reduction in the number of identified PSD-95 puncta, while V1 showed a shift toward more gephyrin puncta. These changes may be indicative of a network in a high plasticity state, as similar effects have been observed after a period of environmental enrichment, a paradigm known to cause increased plasticity (Donato et al., 2013). The different effects of PNN removal on puncta between V1 and mEC may point to different functional effects of the treatment in the two regions. This may be explained by differences in the neural architecture of the networks and composition of sub-populations of neurons. It may be that removal of the PNNs set the network in a plastic state where the plasticity changes that may occur are network specific. Whereas the effect of chABC on V1 is known, the functional effects of chABC treatment in mEC remain elusive and require further investigations. We interpret these results with some caution as the chondroitin sulfate proteoglycans embedded in PNNs only make up 2-5% of the total chondroitin sulfate proteoglycans in the rat brain. It is therefore likely that some of the effects we observe are due to degradation of ECM in general (Deepa et al., 2006). However, as the PNNs enwrap individual synapses onto the soma of neurons (Fig. 2; Mueller et al., 2016), their removal by itself could produce effects on synapse organization and integrity.

To analyze the distribution of puncta, we used an adapted version of the FOF algorithm to define clusters of puncta. The distribution of puncta may indicate if the treatment of chABC caused any changes to the distribution of synapses onto the neurons. To the best of our knowledge, this is a novel tool in the study of synaptic organization. Compared with other clustering algorithms, the FOF algorithm has the advantage of directly returning which puncta belong to a given cluster. Furthermore, it is purely geometric and does not impose any assumptions on the data in the calculations, and is reasonably fast with a time complexity of $O = (n^2)$, where n = total number of puncta. This can be further improved by using an already existing algorithm that compares puncta on a grid, giving a time complexity of $O = N\log(n)$. This may be a highly useful tool for the study of structural network organization and stability.

We did not detect significant differences between the control and chABC-treated areas, but a tendency for PSD-95 puncta in mEC to be less clustered after chABC treatment (Fig. 9*B*). As a general note, however, there was large variability in the data between different animals, in particular for the PSD-95 puncta, and our analysis does not take into account the reduction in total number of puncta. additional experiments using repeated spine imaging are needed to understand the role of PNNs in organization and stability of the synaptic circuitry.

The different levels of expression, cell-type specificity and appearance of the PNNs between brain regions and species may indicate that the PNNs may have different functions depending of the network architecture and function. To reveal the function of the PNNs a systems neuroscience approach is needed combining structural mapping of their distribution and microstructure with functional investigations using targeted manipulations of the PNNs and direct assessment of effects on network function and structure.

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