

Super-resolution STED and two-photon microscopy of dendritic spine and microglial dynamics

Thomas Pfeiffer

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Thomas Pfeiffer. Super-resolution STED and two-photon microscopy of dendritic spine and microglial dynamics. Neurobiology. Université de Bordeaux, 2017. English. NNT: 2017BORD0743. tel-01967610

HAL Id: tel-01967610 https://theses.hal.science/tel-01967610

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THESIS PRESENTED

FOR THE DEGREE OF

DOCTOR

AWARDED BY

THE UNIVERSITY OF BORDEAUX

DOCTORAL SCHOOL OF LIFE SCIENCES AND HEALTH

SPECIALISATION NEUROSCIENCE

By Thomas PFEIFFER

SUPER-RESOLUTION STED AND TWO-PHOTON MICROSCOPY OF DENDRITIC SPINE AND MICROGLIAL DYNAMICS

Supervised by: Professor U. Valentin NÄGERL

Defended on November 21st, 2017

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THÈSE PRÉSENTÉE POUR OBTENIR LE GRADE DE

DOCTEUR DE

L'UNIVERSITÉ DE BORDEAUX

ÉCOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

SPÉCIALITÉ NEUROSCIENCES

Par Thomas PFEIFFER

IMAGERIE DE LA DYNAMIQUE DES MICROGLIES ET DES ÉPINES DENDRITIQUES PAR MICROSCOPIE SUPER-RÉSOLUTIVE STED ET BI-PHOTONIQUE

Sous la direction de : Professeur U. Valentin NÄGERL

Soutenue le 21 Novembre 2017

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Abstract

Super-resolution STED and two-photon microscopy of dendritic spine and microglial dynamics

Activity-dependent changes in neuronal connectivity are thought to underlie learning and memory. I developed and applied novel high-resolution imaging-based approaches to study *(i)* microglia-spine interactions and *(ii)* the turnover of dendritic spines in the mouse hippocampus, which are both thought to contribute to the remodeling of synaptic circuits underlying memory formation.

(*i*) Microglia have been implicated in a variety of novel tasks beyond their classic immune defensive roles. I examined the effect of synaptic plasticity on microglial morphological dynamics and interactions with spines, using a combination of electrophysiology and two-photon microscopy in acute brain slices. I demonstrated that microglia intensify their physical interactions with spines after the induction of hippocampal synaptic plasticity. To study these interactions and their functional impact in greater detail, I optimized and applied time-lapse STED imaging in acute brain slices.

(*ii*) Spine structural plasticity is thought to underpin memory formation. Yet, we know very little about it in the hippocampus *in vivo*, which is the archetypical memory center of the mammalian brain. I established chronic *in vivo* STED imaging of hippocampal spines in the living mouse using a modified cranial window technique. The super-resolution approach revealed a spine density that was two times higher than reported in the two-photon literature, and a spine turnover of 40% over 5 days, indicating a high level of structural remodeling of hippocampal synaptic circuits.

The developed super-resolution imaging approaches enable the examination of microglia-synapse interactions and dendritic spines with unprecedented resolution in the living brain (tissue).

Keywords: super-resolution STED microscopy, two-photon microscopy, microglia, synaptic plasticity, dendritic spine, structural plasticity, *in vivo* imaging;

Résumé

Imagerie de la dynamique des microglies et des épines dendritiques par microscopie super-résolutive STED et bi-photonique

Les changements des connections neuronales interviendraient dans la formation de la mémoire. J'ai développé de nouvelles approches basées sur l'imagerie photonique pour étudier *(i)* les interactions entre les microglies et les épines dendritiques, et *(ii)* le renouvellement des épines dans l'hippocampe *in vivo*. Ces deux phénomènes contribueraient au remodelage des circuits synaptiques intervenant dans la mémoire.

(*i*) Les microglies sont impliquées dans de nouvelles fonctions en condition saine. J'ai examiné l'effet de la plasticité synaptique sur la dynamique morphologique des microglies, et sur leur interaction avec les épines. En combinant l'électrophysiologie et l'imagerie bi-photonique dans des tranches aigües de souris transgéniques, je démontre que la microglie intensifie son interaction physique avec les épines. Ainsi pour continuer l'étude de ces interactions et leur impact fonctionnel plus précisément, j'ai optimisé l'imagerie STED dans des tranches aigües.

(*ii*) La plasticité structurale des épines est cruciale pour la mémoire, mais les connaissances à ce sujet dans l'hippocampe *in vivo* restent limitées. J'ai donc établi une technique d'imagerie chronique STED *in vivo* pour visualiser les épines dans l'hippocampe. Cette approche a révélé une densité double de celle reportée précédemment à l'aide de la microscopie bi-photonique. De plus j'ai observé un renouvellement des épines de 40% en 5 jours, représentant un taux important de remodelage synaptique dans l'hippocampe.

Les approches d'imagerie super-résolutive permettent l'étude des interactions microglie-épine, et du renouvellement des épines hippocampiques avec une résolution inédite chez la souris vivante.

Mots clés: microscopie super-résolutive STED, microscopie bi-photonique, microglie, plasticité synaptique, épine dendritique, plasticité structurale, imagerie *in vivo*;

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Acknowledgements

I would like to thank the people whose support was crucial in the course of my PhD.

First I wish to thank Professor Valentin Nägerl for hosting me in his laboratory and supervising me over the last 5 years. I am particularly thankful for the freedom he granted me, his support and permanently making me push my boundaries. I will profit from my training in his lab in the future. Thank you, Valentin! I really enjoyed the enthusiastic spirit and international working environment in the lab, and Valentin's incredible repertoire of analogies for all situations in life and when the laser needed to be petted.

I would like to thank the members of my mid-thesis and thesis committee, Prof. Jochen Lang, Dr. Giovanni Marsicano, Dr. Stéphane Oliet, Dr. Valentina Emiliani, Dr. Martin Fuhrmann, Prof. Frank Kirchhoff and Dr. Etienne Audinat for fruitful discussions, guidance and constructive advice throughout my PhD training.

I am particularly grateful to Drs Elena Avignone and Aude Panatier for their mentorship and friendship. Their support has been instrumental. My straight line may still not be straight by Avignone definition, and I may still not pass the Aude factor, but our inspiring discussions and their advice, helped me advance and grow. Thank you for sharing your love for glial cells with me and pushing me to think about "What is your scientific question?". At this place, I acknowledge Dr. Valentin Langlais' help in troubleshooting.

I enjoyed sharing the exciting beginning of my PhD time with Drs Philipp Bethge and Ronan Chéreau. We shared a good handful of pints in their living room – the Sweeney's, and I am grateful for their support, lively discussions, shared sorrows, friendship and literally making me fly.

I feel privileged to have received plenty of support and guidance by Drs Misa Arizono and Mark Sherwood. Arigato for sharing your science enthusiasm. I loved our stimulating scientific discussions and fun time outside the lab.

With the guidance of Dr. Krishna Inavalli I learned how to handle and finally appreciate the complexity of my imaging setup. Thank you, Krishna, for the numerous hours you invested in helping and teaching me the optical ABC. From A like aberration to z like z-axis, or more correctly the famous *Ggggiiii*.

I am grateful to Dr. Ezequiel Saraceno for his support, incredibly positive attitude, friendship and for introducing me to the spirit of Azado. Eze, it has been a pleasure, one could always count on him.

Merci beaucoup à Dr. Julie Angibaud for her constant support and seemingly infinite inner calm. I owe you. She was close, but she never gave up in explaining me over and over again the beauty of the French public and social system.

Many thanks also to old and current lab members, Drs Martin Lenz, Mirelle ter Veer, Lasani Wijetunge and Stéphane Bancelin who made the Nägerl team such a pleasant place to work in and who were always eager to help. At this place also a special thanks to Mirelle with whom I shared a great deal of this exciting journey.

Eventually also merci beaucoup and muchas gracias to the chemists gang, it was always a pleasure to share office with them.

I wish to thank the Boehringer Ingelheim Fonds and the Fondation pour la Recherche Médicale for financing my PhD work.

I was not alone. I engaged in this journey "from puppies to scientific adolescents" (E. Avignone) with the PhD crew, Ben, Corey, Matt, Laetitia, Charline, Anaïs, Mirelle and Tiago. I am grateful for their friendship. Our *only one beer* evenings in Petit Quebec were often legendary. Together with our skiing, hiking trips and other activities they helped to recharge batteries and make my stay in Bordeaux a fun time.

My time in Bordeaux would not have been such a memorable time without my flat mates, in particular Valentin, who received me with open arms and introduced me to the French lifestyle and language.

Thank you, Anaïs, for your love and support, for grounding me and at the same time surpassing ourselves.

My deep gratitude goes to my family for making all this possible. My education has been a family effort. Thank you for supporting my time abroad, always standing behind me, encouraging me, trusting in me and most importantly having a place to always look forward to. Thank you.

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Abbreviations

2P	Two-photon
3D	Three-dimensional
ACSF	Artificial cerebrospinal fluid
ADP	Adenosine di-phosphate
AMPAR	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
AO	Adaptive optics
AP	Action potential
APD	Avalanche photodiode
APV	2-amino-5-phosphonopentanoic acid
ATP	Adenosin tri-phosphate
BDNF	Brain-derived neurotrophic factor
CA	Cornu ammonis
CaMKII	Ca ²⁺ /calmodulin-dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
CREB	cAMP response element-binding protein
CSF1R	Colony stimulating factor 1 receptor
CW	Continuous wave
CX3CL1	Fractalkine
CX3CR1	Fractalkine receptor
DG	Dentate gyrus
EC	Entorhinal cortex
ECM	Extracellular matrix
EM	Electron microcopy
EOM	Electro-optic modulator
EPSC	Excitatory postsynaptic current
fEPSP	Field excitatory postsynaptic potential
GABA	γ-aminobutyric acid
GECI	Genetically encoded calcium indicator
GFP	Green fluorescent protein
HFS	High-frequency stimulus
HyD	Hybrid detector
Hz	Hertz
lba-1	Ionized calcium-binding adapter molecule 1
LTD	Long-term depression
LTP	Long-term potentiation
MD	Monocular deprivation
MHCI	Major histocompatibility complex I
NA	Numerical aperture
NIR	Near-infrared
NMDAR	N-methyl-D-aspartate receptor
OPO	Optical parametric oscillator
PAINT	Point accumulation for imaging in nanoscale topography

PALM	Photoactivated localization microscopy
PKA	Protein kinase A
PMF	Polarization-maintaining fiber
PMT	Photomultiplier tube
PP1	Protein phosphatase 1
PSD	Postsynaptic density
PSF	Point-spread function
ROI	Region of interest
SMLM	Single molecule localization microscopy
SO	Stratum oriens
SP	Stratum pyramidale
SR	Stratum radiatum
STED	Stimulated emission depletion
STORM	Stochastical optical reconstruction microscopy
SUSHI	Super-resolution shadow imaging
TIRF	Total internal reflection fluorescence microscopy
TNF	Tumor necrosis factor alpha
ТТХ	Tetrodotoxin
VIS	Visible light spectrum
YFP	Yellow fluorescent protein

Introduction

Prolog

I received my PhD training in the group of Professor U. Valentin Nägerl at the Interdisciplinary Institute for Neuroscience at the University of Bordeaux, France. The Nägerl group has been a pioneer in developing live STED super-resolution microcopy and its application to synaptic research. My fascination lies in understanding the role of microglia in the healthy brain. Leveraging both, my PhD work thus gave rise to two individual but synergistic lines of research: (i) Investigation of microglia-synapse interactions in the healthy adult brain and (ii) development of chronic *in vivo* STED imaging in the mouse hippocampus to study the turnover of dendritic spines. My work resulted in three manuscripts, of which two are published and one has been submitted. These manuscripts compose the results part of my thesis:

Part 1: Induction of hippocampal long-term potentiation increases the morphological dynamics of microglial processes and prolongs their contacts with dendritic spines. Article published in Scientific Reports.

Part 2: Two-photon STED microscopy for nanoscale imaging of neural morphology *in vivo*. Protocol book chapter published in Methods in Molecular Biology.

Part 3: *In vivo* STED imaging reveals high spine turnover in the hippocampus over days. Manuscript submitted.

In the light of the diverse subjects, I will introduce the background to my research divided in three chapters, as follows:

1. Physiology and function of the excitatory synapse, and structural plasticity of dendritic spines in the living mouse;

2. The dynamic morphology of microglia and microglia-synapse interactions in the healthy brain;

3. The principles of two-photon and super-resolution STED microscopy and their application for imaging of neural morphology in the living brain (tissue).

1. Physiology of the excitatory synapse and structural plasticity of dendritic spines in the living mouse

The brain is made up of 80 to 100 billion neurons, each of them having a particular morphology and function characteristic for its brain region and task involved. These neurons communicate with each other at highly specialized sites called synapses, which are composed of a pre-synaptic and post-synaptic compartment. Their activity-dependent change in size, strength and number are considered to underpin memorizing and forgetting, constituting a ubiquitous process in our daily lives. Despite the omnipresence of this phenomenon, we are still facing fundamental gaps in our understanding of the underlying mechanisms; and that is the animating drive I feel as an early career neuroscientist.

My work focused on the morphology and function of the postsynapse of excitatory pyramidal neurons in the CA1 area of the hippocampus and in cortical layer 5. In the course of my introduction I have therefore placed my emphasis on this neuronal cell type, as follows.

1.1. Function and plasticity of excitatory synapses

1.1.1. Anatomy of the hippocampus

The hippocampus, which is located deep within the brain's medial temporal lobe, is thought to constitute the brain area underlying the formation of episodic and spatial memory. Neurophysiologists appreciate the hippocampus as experimental model because of its simply designed tri-synaptic loop architecture and neatly organized anatomical layers (see *Figure 1*) (Amaral and Witter, 1989).

The hippocampal formation consists of the dentate gyrus (DG), the cornu ammonis (CA), subdivided into CA4, CA3, CA2 and CA1, and the subiculum (SUB). The entorhinal cortex (EC) serves as main in- and output of the hippocampus, and thus represents a hub for the information relay with other cortical structures, e.g. prefrontal cortex and neocortex. Sensory information from the EC layer II conveys input to the DG via the perforant path. The granule cells of the DG project with their axons (mossy fibers) onto the CA3 region, where they synapse with large mossy fiber boutons on the dendrites of the CA3 pyramidal cells (see *Figure 1A, B*). The information is then transferred to the CA1 neurons via the Schaffer collaterals.

Subsequently, the output station CA1 and the downstream subiculum project (via EC layer IV/V) to a variety of brain areas. The fundamental circuitry reflects how information is first integrated (DG), replayed and consolidated as a memory trace (CA3 and CA1), and then relocated to the cortex which functions as the long-term storage device.



Figure 1: The hippocampal formation. **A)** Cajal's drawing illustrates the structural and cellular organization of the hippocampal formation (adapted from (Cajal (translated), 1995)). Inset highlights a CA1 pyramidal neuron. **B)** Scheme of the hippocampal tri-synaptic loop. By stimulation of the CA3 Schaffer collaterals with a high frequency electrical stimulus (HFS) protocol, induction of LTP at the CA3-CA1 synapse can be assessed by recording field excitatory postsynaptic potentials (fEPSPs) in the CA1 *stratum radiatum* (SR). **C)** Hippocampal pyramidal neurons are arranged in laminar layers, which are dedicated to the cellular compartment they contain. *Stratum oriens* (SO), *stratum pyramidale* (SP), *stratum radiatum* (SR), and *stratum lacunosum/molecular* (S-LM).

Finally, the laminar organization of the CA3 and CA1 region is of great convenience for the experimentalist (see *Figure 1B, C*). These layers are easily recognizable at low magnification and the laminar layers are dedicated to the cellular compartments of the pyramidal neurons it contains. The *stratum oriens* contains the basal dendrites and axons of the pyramidal neurons. The *stratum pyramidale* consists of

the neuronal cell bodies. The proximal dendritic arbors of the apical dendrites make up the *stratum radiatum*, where also most of the CA3-CA1 synapses are located. The *stratum lacunosum/moleculare* consists of the distal apical dendrites and is innervated by commissural fibers. Moreover, the laminar organization provide homogeneous ion sinks (dendrites) and sources (cell body layer) and thus allows easy assessment of neuronal network activity and synaptic function through electrophysiological recordings (see *Figure 1B*).

1.1.2. Synaptic physiology and transmission

The information transfer between two neurons takes place at a dedicated functional compartment called synapse, which can be of chemical or electrical nature.

The electrical synapse is composed of gap junctions between two neurons and enables an electro-mechanical coupling between the cells. This synapse type represents only the minority of hippocampal synapses and concerns in general mostly non-pyramidal neurons (Kosaka and Hama, 1985).

In the mature hippocampus, the chemical synapse constitutes the majority of synapses (Voglis and Tavernarakis, 2006). In the vertebrate central nervous system (CNS), most excitatory chemical synapses are built up of a pre-synaptic axonal bouton and a post-synaptic spine, separated by a synaptic cleft (20 - 30 nm in width). Spines are small protrusions on specialized neurites, called dendrites. Conducting EM studies, it has been shown that most spines on pyramidal neurons contain an electron-dense structure that is called postsynaptic density (PSD). The composition of the PSD resembles a mesh-like structure made up of multiple protein complexes, such as scaffolding proteins that anchor neurotransmitter receptors, such as the N-methyl-D-aspartate receptors (NMDAR) and α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPAR), and ion channels in a position facing the pre-synapse. The presence of PSD is an indicator of a mature functional synapse and EM work suggests that visualization of dendritic spines serves as reliable proxy for functional excitatory synapses (~80%) (Harris et al., 1992).

Functionally, synaptic transmission on excitatory synapses works as follows. Upon action potential (AP)-induced depolarization of the pre-synapse and a subsequent Ca²⁺ influx, glutamate-containing vesicles fuse with the membrane and release the neurotransmitter glutamate into the synaptic cleft. Post-synaptically it binds to glutamate receptors, NMDAR and AMPAR, whereas only the latter elevates its

conductance for Na⁺, while the NMDAR remains closed. The Mg²⁺ block of the NMDAR is removed following a sufficient initial depolarization of the resting membrane potential and the presence of a co-agonist (D-serine and/or glycine). Once the Mg²⁺ block is released, NMDAR-mediated Ca²⁺ and Na⁺ influx enable further depolarization of the spine.

Whether the depolarization of the spine elicits a sufficiently large excitatory postsynaptic potential (EPSP) to depolarize firstly the dendrite and ultimately leading to the initiation of an AP at the cell body level (axon initial segment), is multi-factorial. The most prominent factors are (i) localization-dependent integration on the dendrite (proximal versus distal), (ii) co-occurrence and summation with other EPSPs, (iii) coincidence with backpropagating APs, NMDAR and sodium spikes, (iv) hyperpolarization through inhibitory postsynaptic potentials, and likely (v) spine shape (Branco and Häusser, 2010; Chadderton et al., 2014; Magee, 2000; Tønnesen et al., 2014).

1.1.3. Synaptic plasticity

Synapses adapt their strength, i.e. the efficacy of transmission, to their activitydependent use, a phenomenon called synaptic plasticity (see *Figure 2*). It is considered to be the cellular correlate of learning and memory in the mammalian brain (Lynch, 2004). Synaptic plasticity is classically divided in long-term potentiation (LTP) (Malenka and Bear, 2004) and depression (LTD) (Collingridge et al., 2010) of synaptic transmission. While different types of plasticity exist, such as mGluR dependent LTD (Bolshakov and Siegelbaum, 1994; Oliet et al., 1997), pre-synaptic LTP on mossy fibers (Nicoll and Schmitz, 2005), endocannabinoid-dependent LTD (Heifets and Castillo, 2009; Robbe et al., 2002), homeostatic plasticity and metaplasticity (Abraham and Bear, 1996; Rebola et al., 2010; Watt and Desai, 2010; Zhang and Linden, 2003), the NMDAR-dependent LTP and LTD is probably the most abundant form of synaptic plasticity in the CNS, and prevails at the CA3-CA1 synapse (Dudek and Bear, 1992; Mulkey and Malenka, 1992).

LTP has been first described by Bliss and Lomo in the hippocampal dentate gyrus of the anesthetized rabbit (Bliss and Lomo, 1973). It was induced by applying a tetanic stimulation protocol to the perforant path (afferent axonal fibers to the DG granule cells), consisting of a high-frequency electrical stimulus (HFS) protocol, which is still classically used today. The HFS was composed of three 1s trains of stimuli at 100Hz with a 20s inter-train interval. Similar results could be achieved by pairing of

pre-synaptic input and post-synaptic depolarization (Gustafsson et al., 1987). The NMDAR-dependency of this LTP type was established by application of APV, which did not change the basal synaptic transmission, but omitted expression of LTP (Collingridge et al., 1983). Finally, it is the NMDAR-mediated post-synaptic Ca²⁺ rise which permits the potentiation of synaptic transmission, as revealed by application of intracellular Ca²⁺ chelators (Lynch et al., 1983) and Ca²⁺ uncaging (Malenka et al., 1988).

Mechanistically, the large postsynaptic Ca²⁺ influx through the NMDAR following HFS or pairing protocols, results in the activation of the Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) (Lisman et al., 2002). CaMKII in turn, activates a plethora of downstream enzyme complexes, such as protein kinase A (PKA). PKA phosphorylates AMPARs leading to prolonged Na⁺ influx and hence increased EPSPs (Derkach et al., 1999), and activates cAMP response element-binding protein (CREB), a nuclear transcription factor, which leads to the insertion of new AMPARs consolidating a persistently enhanced synaptic strength (Hayashi et al., 2000).

Conversely, LTD leads to a persistent decrease in synaptic strength and is thus often referred to as the opposite of LTP. In terms of functional outcome this holds true, and yet the induction mechanisms resemble closely. Similarly to LTP induction, the commonly studied LTD (at the CA3-CA1 synapse) is also NMDAR-dependent (Mulkey and Malenka, 1992), can be omitted by post-synaptic Ca²⁺ chelators (Mulkey and Malenka, 1992) and induced by post-synaptic Ca²⁺ uncaging (Yang et al., 1999). Importantly, the LTD induction protocol composed of low-frequency electrical stimulation at 1 Hz for 15 minutes, leads to a slower post-synaptic Ca²⁺ rise and thus preferentially activates phosphatases, such as calcineurine and protein phosphatase 1 (PP1) (Lisman, 1989), instead of CaMKII. These phosphatases ensure a persistently reduced synaptic efficacy by de-phospharylating AMPARs (Lee et al., 2000) and triggering AMPAR endocytosis (Lüscher et al., 2000). To conclude, the kinetics of the post-synaptic Ca²⁺ rise are decisive for the expression of either NMDAR-dependent LTP or LTD.

1.1.4. Morpho-functional synaptic plasticity

Using the established LTP protocols paired with electron microscopy (EM), Fifkova and co-workers established in a series of papers that induction of LTP correlates with an increased number of large spines compared to dendrites of un-stimulated control tissue (Fifkova and Anderson, 1981; Fifkova and Van Harreveld, 1977; Van Harreveld and Fifkova, 1975). Yet, restricted to fixed brain tissue, discrimination between LTP induced increase in spine size or de-novo growth was not feasible. Eventually, conducting time-lapse two-photon (2P) microscopy (see Chapter 3), and building up on the first attempts using confocal microscopy (Hosokawa et al., 1995), it was demonstrated that LTP induces the formation of new spines (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Toni et al., 1999). However, while the increased amplitudes in excitatory post-synaptic currents (EPSC), a measure for increased synaptic strength, were recorded within minutes after the LTP induction, the generation of new spines occurred much later (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999). It was thus conceivable that LTP induced changes in morphology and function were not causally linked at the single spine level.



Figure 2: Morpho-functional synaptic plasticity. **A)** Electron micrograph showing a chemical excitatory synapse, composed of a presynaptic bouton and a postsynaptic dendritic spine (adapted from (Korogod et al., 2015)). **B)** Morpho-functional synaptic plasticity describes that persistent changes in synaptic strength (efficacy of transmission), for instance during long-term depression (LTD) and long-term potentiation (LTP), are closely correlated with morphological changes on the postsynaptic level.

To probe whether enlarged (bigger) spines are also the potentiated ones after LTP induction, Matsuzaki et *al.* made use of 2P glutamate uncaging. While patchclamping a pyramidal neuron and specifically activating single spines, a series of papers reported that increased of dendritic spine size is correlated with increased synaptic strength (Matsuzaki et al., 2001, 2004; Okamoto et al., 2004), and thus confirmed the existence of morpho-functional plasticity (see *Figure 2B*).

Conversely, the induction of LTD by either electrical or chemical stimulation induced shrinkage or loss of dendritic spines (Nägerl et al., 2004; Okamoto et al., 2004;

Wang et al., 2007; Zhou et al., 2004). Since spine shrinkage could be rescued by potentiation (Zhou et al., 2004), this indicates that the spines undergoing LTD protocols were still functional.

Thus, changes in structure and function of dendritic spines are correlated, such as that increased and decreased efficacy of transmission go along with increased and decreased spine volume, respectively (see *Figure 2B*). Moreover, a positive correlation exists between spine head volume, PSD area, pre-synaptic active zone area, the number of AMPAR, and the synaptic strength (Bartol et al., 2015; Harris and Stevens, 1989; Matsuzaki et al., 2001; Petralia et al., 1999; Takumi et al., 1999). These correlations indicate that spine structure is overall tightly coupled to synaptic function.

1.2. Structural dynamics and plasticity of dendritic spines *in vivo*

Since the works of Ramon y Cajal in the 19th century, it has been hypothesized and that individual synapses represent the physical correlates of a memory trace (Cajal, 1893; Hübener and Bonhoeffer, 2010; Mongillo et al., 2017). But to what extent can this dogma be underpinned by facts? The existing literature on spine dynamics and their structural plasticity in basal conditions and upon experience-dependent plasticity will be reviewed in the following.

1.2.1. The anatomy of a dendritic spine

Dendritic spines come in different shapes, and are broadly classified in (i) functionally immature filopodia, and mature (ii) stubby and (iii) mushroom spines (see *Figure 3B*). A spine neck connects the spine head to the dendritic shaft. Spine necks of the mushroom-type spine measure ~80 - 250 nm in width (Tønnesen et al., 2014) and likely restrict the free diffusion of ions and molecules between the spine head and the dendritic shaft. Spine necks may thus compartmentalizes post-synaptic signaling both chemically and electrically (Bloodgood and Sabatini, 2005; Noguchi et al., 2005; Tønnesen et al., 2014). Since the reported spine neck measurements are close to the diffraction-limit of light, the application of super-resolution light microscopy (see Chapter 3) is particularly needed for resolving questions relating to the dynamic morpho-functional changes of spines.

1.2.2. Spine density and basal structural dynamics in vivo

Mature hippocampal pyramidal neurons feature a high spine density (~3 μ m⁻¹) in comparison to pyramidal neurons in the sensory cortices (~ 0.25 - 0.4 μ m⁻¹) (Harris et al., 1992; Holtmaat et al., 2006, 2005; Knott et al., 2006). Conducting first longitudinal *in vivo* 2P imaging of Layer 5 pyramidal neurons to track multiple dendritic stretches over months (mostly apical tufts; see *Figure 3A*), groundwork from Gan, Svoboda and Sur laboratories established that structural dynamics of dendritic spines (i.e. turnover; see *Figure 3B, C*) decrease steadily during development into adulthood, and that life-times of spines in visual and somatosensory cortices are distinct (Grutzendler et al., 2002; Holtmaat et al., 2006, 2005; Majewska et al., 2006; Trachtenberg et al., 2002).

In the visual cortex of adult mice the majority of spines (96%) remain stable over 1 month, with an extrapolated half-life time greater than 13 months (Grutzendler et al., 2002). Conversely, in the barrel cortex of young adult mice (6 – 10 weeks old) only 50 - 80% of spines were persistent during a 1 month observational period (Holtmaat et al., 2005; Majewska et al., 2006; Trachtenberg et al., 2002). Thus, spines seem to turn over more slowly in the visual than in the somatosensory cortex (Majewska et al., 2006).

In summary, these reports demonstrated that dendritic spine densities of pyramidal neurons vary dependent on the brain region, due to their persistence (life-time) they may indeed be the potential structural basis of cortical long-term memory storage, and that the kinetics of spine turnover (balance of disappearing/lost and appearing/gained spines; see *Figure 3C*) might be intrinsic to the cortical region. For completeness, although, structural stability is the predominating state in adult visual and somatosensory cortices, there is a smaller population of spines which are exhibiting a lifespan of less than 1 day (Trachtenberg et al., 2002).



Figure 3: Structural plasticity of dendritic spines. **A)** Illustration of a Layer5 cortical pyramidal neuron its dendritic tree and the corresponding nomenclature. **B)** Relationship between dendritic spine structure and morphological dynamics. **C)** Illustration of gained and lost dendritic spines reflecting potential remodeling of the synaptic circuitry. **D)** Cortical brain regions and experimental paradigms to examine the association of synaptic remodeling with learning and memory.

Whether synapses on hippocampal pyramidal neurons underlie similar morphological changes *in vivo* has not yet been entirely elucidated. This is mainly due to the challenging optical access and the lack of sophisticated microscopy techniques that enable to discern single spines on dendrites of hippocampal pyramidal neurons. Employing either a refined hippocampal window preparation (Gu

et al., 2014) or a microendoscopy-based approach (Attardo et al., 2015) it has recently become possible to visualize dendrites of CA1 pyramidal neurons. Formation and elimination of spines in the hippocampus in vivo have been reported on radial obligue (Gu et al., 2014) and on basal dendrites of CA1 pyramidal neurons (Attardo et al., 2015) - yet the described kinetics of spine turnover diverged greatly. While Gu et al. measured 3.4% spine turnover on radial oblique dendrites of CA1 pyramidal neurons over 16 days (Gu et al., 2014), in a comparable time window (~3 weeks), Attardo et al. computed 100% spine turnover on basal dendrites in a simulation-based approach (Attardo et al., 2015). However, using diffraction-limited 2P microscopy both studies could only measure a density of ~1.1 spines per µm (Attardo et al., 2015; Gu et al., 2014). Indeed, 2P imaging with high numerical aperture (NA) objectives (≥ 1.0) offers a resolution at best up to 350 nm, which is not sufficient to detect and properly measure spines in the hippocampal formation, a brain region with a particularly high spine density ($\sim 3 \mu m^{-1}$; (Harris et al., 1992)). Therefore, exact spine densities and structural dynamics in the hippocampus might be underestimated due to methodological restrictions of currently available chronic in vivo imaging approaches.

1.2.3. Structural plasticity of dendritic spines during learning and memory in vivo

To probe whether structural plasticity of spines correlates with experiencedependent learning in superficial cortical areas, longitudinal *in vivo* 2P microscopy has been complemented first by lesion studies and then by sensory stimuli or motor learning (see *Figure 3D*) (for review: (Fu and Zuo, 2011; Hofer and Bonhoeffer, 2010; Mongillo et al., 2017)).

Mice undergoing whisker trimming, a lesion-based model of experienced-dependent plasticity of cortical receptive fields, showed increased synapse turnover (2 - 4 days after whisker trimming), while the average spine density (0.4 μ m⁻¹) remained unaffected (Trachtenberg et al., 2002). Newly appearing spines were stabilized and originally persistent ones removed. Interestingly, the new persistent spines were preferentially added onto layer 5B apical tufts that exhibit a complex dendritic structure rather than on simply structured ones (Holtmaat et al., 2005), potentially indicating that even among dendrites of the same neuron different spine dynamics exist. The Gan Lab expanded on this latter idea, by demonstrating that after motor-learning, when two dendritic branches were examined one typically showed many

more new spines than the other (Yang et al., 2014). Indeed, different skills are likely localized in the form of new spines on different dendritic branches. This has been illustrated elegantly by showing that dendritic branches, which did not show any addition of new spines during a forward-running rotarod-learning paradigm were producing spines subsequently upon backward-running (Yang et al., 2014).

Another loss of function model consists of carrying out a retinal lesion and studying the resulting synaptic circuit remodeling (Keck et al., 2008). Confirming previous work, tracking of dendritic spines of layer 5 pyramidal neurons in mouse visual cortex showed that spine turnover rates are low (5%) in basal conditions. Yet, upon one-sided lesion a complete replacement of spines in the "dis-connected" cortex took place within 2 months. Eventually this was correlated with regained responsiveness of the brain region (also after 2 months) (Keck et al., 2008). As this massive circuit remodeling did not occur when all visual inputs were removed, it likely reflects that the establishment of new synaptic circuits is activity-dependent. However, lesions represent a drastic interference with the brain circuitry and the extent of remodeling might not represent physiological learning processes.

First studies demonstrating a correlation between behavioral learning and spine formation were done by Zuo and Gan labs. Successful motor learning was shown to induce rapid increase in spine density, which is followed by spine pruning. Preferentially learning-induced spines are preserved and finally original spine density is thus restored (Xu et al., 2009; Yang et al., 2009). Further hints towards how experience could be stored in synaptic circuits could be gained by conducting ocular dominance plasticity experiments (Hofer et al., 2009). Previously, it had been shown that monocular deprivation (MD) early in life has a preconditioning effect, and thus renders the adult visual cortex more susceptible for subsequent MD (Hofer et al., 2006). To examine whether this functional plasticity is underpinned by spine structural plasticity, mice were monocularly deprived and apical dendrites of pyramidal neurons were tracked in visual cortex (Hofer et al., 2009). At the synaptic level, during first deprivation the rate of spine formation doubled, thereby increasing spine density. After re-storing vision, spine formation became normal, while the density remained elevated. Many of the deprivation-induced spines persisted during the period of functional recovery. Interestingly, upon a second deprivation no shift in spine dynamics could be observed. Spines produced during the first experience may thus provide the structural basis for the subsequent functional shifts (Hofer et al.,

2009). Hence, while in visual cortex learning results in spine formation and a subsequent increased spine density, motor learning leads to spine turnover, with learning-associated spines and replacing unrelated spines to ultimately re-establish basal spine densities. This may indicate that experience-dependent structural plasticity is not necessarily expressed similarly across brain regions.

On a side note, sleep, accompanied by a particular oscillatory brain network state, stabilizes newly formed spines (Yang et al., 2014). This observation may link the two previously rather unconnected research fields of memory consolidation on a cellular network and synaptic level.

In sum, it is thus conceivable that memory formation is underlined elimination and/or emergence of individual synapses. Indeed, this has been recently shown for hippocampal GABAergic neurons upon contextual fear conditioning, a hippocampus-dependent associative memory test (Schmid et al., 2016). Moreover, the targeted shrinkage of spines and thus elimination of synapses, which have been active during a motor learning task, demonstrated that spine structural plasticity is necessary for successful memory retrieval (Hayashi-Takagi et al., 2015). These two studies support that dynamic formation and elimination of synapses very likely represent a physical correlate of a memory trace.

To investigate whether synapses also constitute the mnemonic unit in hippocampal learning and memory in the living animal it is instrumental to have an exact measurement of individual synapses *in vivo*. The high relevance of obtaining these measurements in the densely packed hippocampus is supported by an *in vivo* study of dendritic spines of pyramidal neurons in the auditory cortex (Loewenstein et al., 2015). In basal conditions, the longevity of spines can be quantitatively predicted using their age and morphological features (Loewenstein et al., 2015). This study thus supports the necessity of precise quantification of basal and learning-induced changes in hippocampal spines.

2. Microglial morphological dynamics and interaction with synapses in the healthy brain

Traditionally, investigating synapse function has been carried out in a neuro-centric manner. This changed ~20 years ago with the recognition of astrocytes for their ability to sense neuronal activity and regulate synaptic function through the secretion of glio-transmitters and uptake of neurotransmitters - a concept called the tripartite synapse (Araque et al., 1999, 2014).

Over the last decade, enabled by the emergence of new tools, including transgenic mice and (*in vivo*) 2P microscopy, the research in microglia, the immune cell of the brain, has yielded a variety of novel microglial functions beyond their classic defensive roles in the healthy brain. With recent findings suggesting a microglial contribution to synaptic plasticity, the cellular substrate of learning and memory, microglia-neuron interaction represents one of the novel hot research topics in neuroscience.

2.1. Introduction to research in microglia

Microglia were recognized and defined as an individual brain cell by Pio del Río-Hortega in 1919. With his seminal works in categorizing the cellular components of the brain Río-Hortega concluded the efforts of his illustrious predecessors Rudolf Virchow, Ramon y Cajal, Alois Alzheimer, and Franz Nissl. To date, Río-Hortega's work constitutes the basis of how CNS glial cells are classified in astrocytes, oligodendrocytes and microglia ((Kettenmann et al., 2011; del Rio-Hortega, 1932) translation of Río-Hortega's papers (Sierra et al., 2016)).

Back then, employing a laborious silver carbon-based labeling strategy, Río-Hortega envisaged the origin, physiology and function of microglia. His vision on microglia can be summarized in 9 postulates (reviewed by (Kettenmann et al., 2011) which were all eventually confirmed and/or still hold true today. Basing myself on Hortega's visionary postulates, I will develop my introduction to microglial biology as follows.

Microglia are derived from primitive macrophages that enter the embryo from the yolk sac early in development. Microglia are thus ontologically distinct from bone-marrow-derived monocytes/macrophages and do not share neuronal and astroglial origin (ectodermal) (Ginhoux et al., 2010, 2013). This is interesting for the

development of tools to selectively manipulate microglia, such as microglial elimination based on the colony stimulating factor 1 receptor (Elmore et al., 2014). Microglia become distributed throughout the embryo as resident macrophages, and invade the CNS at embryonic day 9.5 (Ginhoux et al., 2010, 2013; Mosser et al., 2017). State-of-the-art transcriptomics demonstrate that microglia make up the bulk of resident brain immune cells, complemented by dendritic cells, memory T cells, B, and natural killer cells (Korin et al., 2017). While early in brain development microglia display a rather amoeboid shape complemented by a high phagocytic capacity, in the adult brain microglia are highly ramified cells, consisting of a cell body which gives rise to numerous highly branched protrusions. Microglia are omnipresent throughout the brain with equal distribution patterns in the respective brain regions. Unlike astrocytes, microglia are not electro-tonically coupled. In turn, they also occupy cellular domains in physiological conditions. Similarly to astrocytes, microglia are equipped with a plethora of receptors which enable them to sense signaling factors secreted by the entity of brain cells, and at the same time microglia can influence their surrounding through secretion of a variety of factors, such as chemokines and cytokines (Kettenmann et al., 2011).

Going along with their immune cell duties, microglia are determining the progress of an insult, such as brain infection and disease. In this condition, microglia change progressively their morphology to a more amoeboid-like shape while adapting their cytokine expression, a phenomenon referred to as "activation" (Kettenmann et al., 2011, 2013). In the course of this process, microglia tend to give up their territory, migrate towards the danger site, and exercise an anti- or pro-inflammatory effect. Ultimately, this has an either beneficial or detrimental outcome for the surrounding brain parenchyma.

For long, microglia were considered to exist in two states: passive "resting" and "active" pro-inflammatory mediators. This simplified binary view underpinned the predominant research on their function in pathology, largely neglecting their putative contribution to brain function in health. Fueled by a transgenic mouse labeling microglia specifically with green fluorescent protein (GFP) under the fractalkine promoter CX3CR1 (Jung et al., 2000), in 2005 the groundbreaking discovery that resting microglia are in fact highly mobile (so called 'cops on the beat') has led to a revision of their "passive" physiological purpose (Davalos et al., 2005; Nimmerjahn et al., 2005; Raivich, 2005). Complemented by *in vivo* 2P imaging experiments,

which enable the study of microglia *in situ*, this served as an impetus for research in microglia and their interaction with neurons. In fact, the significance of the cross-talk between neurons and microglia is exemplified by the existence of a designated chemokine, CX3CL1 (fractalkine), whose sole receptor (CX3CR1) is found on microglia (Harrison et al., 1998; Nishiyori et al., 1998).

2.2. Microglia contribute to the development of brain circuitry

Developmental studies were the first demonstrating that microglia contribute to normal brain physiology. Microglia spread throughout the brain at the time of peak synaptogenesis and it turned out that microglia are critically involved in the formation of functionally wired neuronal circuits (Hoshiko et al., 2012; Paolicelli et al., 2011; Schafer et al., 2012a; Stevens et al., 2007; Zhan et al., 2014), as it will be portrayed in the following (see *Figure 4*).

The development of neuronal circuitry is initiated by a period of enhanced plasticity, after which synapses are selectively maintained, formed and remodeled based on synaptic strength (Majewska et al., 2006). Ultimately, mature synapses possess one or two strengthened presynaptic inputs ("synaptic multiplicity") while the remainder (pre- and post-synapses) undergo elimination, and it is in this latter role that microglia emerge to be crucially involved (see *Figure 4A*) (Paolicelli et al., 2011; Schafer et al., 2012a; Zhan et al., 2014). Detection of SNAP25 and PSD95, pre- and postsynaptic markers, contained in microglial processes indicated that microglia indeed phagocytose synapses during development. Yet, despite this convincing correlation of reduced synapse numbers and localisation of synaptic markers inside microglial lumen (Paolicelli et al., 2011), direct proof and real-time observation of microglial removal of synapses is still pending.

Identification of the synapses to be removed ("pruned") is achieved by the unique involvement of the complement system, which is otherwise classically reserved for pathogen identification and destruction (Frost and Schafer, 2016; Schafer et al., 2012a; Stevens et al., 2007). Up-regulation of the initiating factor C1q is stimulated by nearby astrocytes and leads to the deposition of C3 on target synapses. Microglia exclusively express the C3 receptor and are thus attracted by the tagged synapses. During this period, although unchallenged, microglia exhibit strong phagocytic tendencies. Recognition of the C3 "eat me" signal subsequently initiates
phagocytosis of both pre- and postsynaptic elements (Bialas and Stevens, 2013; Schafer et al., 2012a; Stevens et al., 2007; Wu et al., 2015).



Figure 4: Microglia in brain development. A) Microglia contribute to pruning of extranumerary synapses during brain development in a Cx3Cr1- and complement factor-dependent manner. B) In somatosensory cortex, timely arrival of microglia in the barrel centers correlates with a developmental switch of NMDAR subunits. C) Microglial contact of an aspiny dendrite induces filopodia formation in the vicinity.

While the complement system is pivotally involved in brain development, some degree of synapse maturation still occurs in its absence. In this regard, the structurally similar neuronal pentraxin (Biartmar et al., 2006), major histocompatibility complex class I (MHC I) (Shatz, 2009), the chemokine CX3CL1 (Hoshiko et al., 2012; Paolicelli et al., 2011; Zhan et al., 2014) and astrocytes (Chung and Barres, 2012; Chung et al., 2013) have been implicated in synapse elimination and/or neuronal circuit development. In each case knock-out (KO) resulted in a diminished capacity to execute synaptic refinement, leaving an either transient or persistent presence of immature synapses with aberrant excitatory activity (Chu et al., 2010; Hoshiko et al., 2012; Paolicelli et al., 2011; Ragozzino et al., 2006; Shatz, 2009). Moreover, work in CX3CR1 KO mice suggested that the time window for microglial synaptic pruning is narrow and delay or lack of it resulted in persistent abnormal long-range connectivity across brain regions (specifically between hippocampus and prefrontal cortex), which eventually manifested in an autism-like behavioral phenotype (Zhan et al., 2014).

Redundancy within the synaptic pruning / circuit development system may indicate discrete but coordinated functions served by each player, and/or the presence of a safety mechanism to ensure completion of developmental circuit remodeling. However, the fact that microglia can express receptors for each of these leaves little doubt concerning their critical involvement in establishment of mature synaptic circuitry.

Aside phagocytic activity, microglia may contribute to the functional maturation of synapses through the secretion of mediators, as work in the developing barrel cortex might suggest (see *Figure 4B*) (Hoshiko et al., 2012). Deficiency of CX3CR1 led to a delayed microglial inhabitation of the barrel centers, which resulted in delayed functional maturation of the thalamocortical synapses (assessed through AMPAR/NMDAR ratio). This indicates that besides removal of extranumerary synapses, microglial presence is also necessary to release signaling factors which induce maturation of synapses.

Beyond shaping synapse maturation, it has been shown that microglia support the creation of synapses in development (see Figure 4C) (Miyamoto et al., 2016). Miyamota et al. demonstrated elegantly that in mouse somatosensory cortex during postnatal days 8 to 10, a period of intense synaptogenesis, microglial contact of dendrites is followed by filopodia formation in the close vicinity. These filopodia formed approximately 3 minutes after the contact and had an equal probability to persist compared to non-contact induced filopodia. It may not be entirely clear yet whether microglia identify dendritic locations where potentially filopodia will be formed or whether microglia initiate the de-novo growth. But, the latter is supported by the detection of dendritic Ca²⁺ transients exclusively during / after microglial contact. Moreover, elimination of microglia led to reduced spine density and ultimately number of functional synapses, as well as connectivity between layer 2/3 and layer 4 neurons. This conclusion is interesting in the light of the developmental studies conducted in the hippocampus of CX3CR1-deficient mice (Paolicelli et al., 2011; Zhan et al., 2014). Conversely, delayed microglial invasion resulted in persistence of extranumerary synapses. This hints at a sensitive topic, namely the divergence of reported effects manipulation or elimination of microglia has or does not have on the neuronal circuitry. Likewise to studies of neuronal and astrocytic function, besides methods of investigation (visualization or indirect read-out in vitro or in vivo) microglia most likely have different implications across brain regions, in different developmental stages and dependent on the status of the surrounding neuronal network.

In sum, strong evidence has been accumulated that microglia are pivotal for the proper development of functional brain circuits, with recent research implying that this can occur in three flavors: synapse removal, maturation and formation. Although, candidate molecular pathways, such as brain-derived neurotrophic factor (BDNF) (Parkhurst et al., 2013) and tumor necrosis factor alpha (TNF-alpha) (Lewitus et al., 2016) have been identified, future in depth work will aid to elucidate whether and how these or other microglia-derived factors contribute to both maturation and formation of synapses in development and in the adult.

2.3. Microglial morphological dynamics

As highlighted earlier in this chapter, the discovery of the microglial morphological dynamics in physiological ("resting") conditions came as recently as 2005 (see *Figure 5A*) (Davalos et al., 2005; Nimmerjahn et al., 2005). It was catalyzed by the concomitant development of *in vivo* 2P microscopy, cortical window surgery techniques (Grutzendler et al., 2002; Holtmaat et al., 2005) and the CX3CR1-GFP mouse line that specifically labels all microglia with GFP under the fractalkine receptor promotor (CX3CR1) (Jung et al., 2000). Together, this enabled the examination of resting ramified microglia, which are composed of a rather stationary cell body decorated with numerous highly branched extensions (see *Figure 5A*). 1st order processes show only minor morphological dynamics and together with the cell body, they may serve to maintain the territorial distribution (see *Figure 5B*).

In contrast, the fine higher order processes undergo a continuous remodeling which comes down to a balanced process of extension and retraction of the microglial protrusions (Nimmerjahn et al., 2005). During this exquisite navigation through the extracellular space microglial processes establish transient contacts with its surrounding cellular partners, consisting in the cerebral vasculature, astroglia and neuronal cells (Nimmerjahn et al., 2005). During this, what is called *microglial morphological dynamics*, average microglial process velocities of 2.5 μ m/min were measured (see *Figure 5A, C*). Differences in reported velocities are likely dependent on experimental conditions, analysis, brain region, developmental stage and tissue preparation (Avignone et al., 2015; Davalos et al., 2005; Lee et al., 2008; Li et al., 2012; Nimmerjahn et al., 2005; Sipe et al., 2016; Wu et al., 2007).



Figure 5: Microglial morphological dynamics. A) Microglia are dynamically extending and retracting their numerous processes. These morphological dynamics are also described as microglial surveillance and scanning activity. B) Similar to astrocytes microglia maintain a territory / domain. C), D) Changes in microglial process velocity C) and microglial ramification / number of processes D), serve as a measure for changes in microglial morphological dynamics. E) Microglia show increased and decreased morphological dynamics in response to increased and decreased neuronal activity, respectively. F) Microglia preferentially contact highly active neurons, which in subsequently reduce their activity.

Importantly, based on this continuous movement of microglial processes, it has been extrapolated that microglia explore their surrounding and ultimately the entire brain parenchyma in a few hours (Nimmerjahn et al., 2005). These observations gave rise to the commonly used terms of *surveying*, *monitoring*, and *scanning activity*.

The likely reason for this mobility is to be constantly vigilant towards any abnormalities in the vicinity. Such a deviation can range from small local neuronal damage to a large global insult. Insults originating from a mechanical or a laser lesion induce directed microglial process movement. Microglial reaction is composed of a two-step process. Firstly, a rapid migration of microglial processes to the site of injury takes place (Avignone et al., 2015; Davalos et al., 2005; Haynes et al., 2006). These can come from as far as 50 μ m (Davalos et al., 2005) and the reported velocities amount to approximately 4.5 μ m/min (Avignone et al., 2015). The microglial processes isolate the damaged area, and phagocytic clearance is likely reflected by bulbous enlargements in the process terminals. Subsequently, if necessary, in the second stage somatic displacement towards the site of injury may take place.

Likewise, exposure to chemicals elicits microglial chemotaxis and allowed for the dissection of the underlying signaling mechanisms. The rapid extension of microglial processes towards a laser lesion site can be reproduced by local application of purines, i.e. ATP and its derivatives (Avignone et al., 2015; Davalos et al., 2005; Haynes et al., 2006).

A variety of diverse functions are executed by purines, of which regulation of chemotaxis is particularly relevant for microglia. The P2Y12 receptor, which is exclusively and abundantly expressed by microglia in physiological conditions, is highly sensitive to adenosine triphosphate (ATP) and adenosine diphosphate (ADP),

and underlies microglial process motility (Haynes et al., 2006; Honda et al., 2001). ATP induces strong inward Ca²⁺ currents in microglia, which initiate mechanisms promoting ramification and motility (Färber and Kettenmann, 2006a, 2006b; McLarnon, 2005; Wollmer et al., 2001). Application of ATP through a glass pipette is as effective as mechanical injury in stimulating migration of microglial processes *in vivo* (Davalos et al., 2005). However, blockade of astrocytic ATP release abolishes microglial motility, suggesting that the chemoattractant effect is not exclusively induced by neurons (Davalos et al., 2005). In this regard, a complex interplay in which astrocytes act as an intermediary downstream partner in both ATP and glutamate signaling has been proposed (Pascual et al., 2011).

2.4. Perception of neuronal activity and changes in microglial morphological dynamics

Through the expression of purinergic (P2Y and P2X family, and adenosine P1) and glutamatergic (AMPARs and mGluRs, but not NMDARs) receptors (Kettenmann et al., 2011), microglia are able to perceive changes in neuronal activity in their vicinity. Effectively, this may allow microglia to coordinate its surveillance activity with the neuronal activity in its domain.

Indeed, manipulation of neuronal activity through application of neurotransmitters (and their analogues) (Dissing-Olesen et al., 2014; Eyo et al., 2014; Fontainhas et al., 2011; Gyoneva and Traynelis, 2013; Li et al., 2012; Nimmerjahn et al., 2005), pharmacological intervention (Nimmerjahn et al., 2005; Tremblay et al., 2010; Wake et al., 2009), and neuronal depolarization by patch-clamping (Eyo et al., 2014) have proven to influence microglial surveillance activity.

Microglial process extension, ramification, and enhanced basal dynamics were reported during increased neuronal activity elicited by the application of glutamate, NMDA, AMPA, kainate and blockade of GABAergic transmission (Dissing-Olesen et al., 2014; Fontainhas et al., 2011; Nimmerjahn et al., 2005). Analogously, AMPAR block and its related down-regulation of neuronal activity led to a reduction of microglial dynamics (Eyo et al., 2014). These observations were further consolidated in more physiological conditions, when neuronal activity and microglial dynamics were reduced in mice undergoing light deprivation, complete visual deprivation (binocularly and through ocular TTX injection) and reduction of mouse body temperature (see *Figure 5D, E*) (Tremblay et al., 2010; Wake et al., 2009).

Extension of these works in zebrafish larvae showed that microglial processes targeted specifically highly active neurons, which subsequently reduced their firing frequency. Contact of microglial processes can thus have a homeostatic calming effect (see *Figure 5F*) (Li et al., 2012).

In slight contrast with the descriptions above, microglia in dark-adapted mice showed an increased process ramification covering more extracellular space (Tremblay et al., 2010). Moreover, reducing neuronal activity by the application of TTX *in situ* did not lead to changes in microglial surveillance (Nimmerjahn et al., 2005), nor did the application of a HFS to induce hippocampal LTP (Wu and Zhuo, 2008). Yet, these differences might originate from the diverse experimental conditions, such as developmental brain state, brain region and preparation used.

In sum, although, minor inconsistencies exist, increase and reduction of neuronal activity is accompanied by enhanced and reduced microglial surveillance activity, respectively (see *Figure 5E*). It is conceivable that microglial P2Y12Rs underpin the perception of changes in neuronal activity through the postsynaptic ATP release upon postsynaptic activation (Dissing-Olesen et al., 2014; Eyo et al., 2014), an hypothesis which will be revisited in more detail below.

2.5. Microglia-synapse interaction in the adult

Identically to its developmental duties (Hoshiko et al., 2012; Paolicelli et al., 2011; Schafer et al., 2012a), it is conceivable that microglia similarly exercise their regulatory capacities on synapses in the adult healthy brain. This gave rise to the hypothesis that microglia participate to the continuous experience-dependent reorganization of neuronal circuits throughout life.

Indeed, unchallenged mature microglia exhibit a high phagocytic potential and shape adult neurogenesis in dentate gyrus of the hippocampal formation (Sierra et al., 2010). EM studies carried out in the visual cortex of the adult mouse, showed that microglia are in contact with all synaptic compartments (pre-, post- and astrocytic) in the mature brain and 3D reconstruction occasionally showed a finger-like structure wrapped around a dendritic spine (see *Figure 6A*) (Tremblay et al., 2010; Wake et al., 2009). In the cortex 3.5% of synapses are in physical contact with a microglial process tip (Sogn et al., 2013). The majority of these microglial processes in contact with synapses contain Iba-1, a calcium-binding complex that also serves as microglial marker. Importantly various subcellular organelles and

vesicular structures were also detected. Together, this indicates a putative gliotransmitter based communication and clathrin-coated pits may ensure the uptake of secreted material by the synaptic partners (Sogn et al., 2013; Tremblay et al., 2010). Communication between microglia and the pre-, postsynaptic and astrocytic elements is thus possibly bi-directional.

Early on, evidence for a microglial contribution to synaptic function in adulthood was derived from real-time *in vivo* imaging studies. Initial work in mouse visual cortex demonstrated that the contacts between microglial processes and pre- and postsynapses are transient (see *Figure 6A*) (Tremblay et al., 2010; Wake et al., 2009) and the length of the interaction was prolonged upon an ischemic insult. This suggested that microglia-synapse interactions are likely dependent on the state of the neuronal network (Wake et al., 2009) and fueled subsequent efforts in a better understanding of microglia-synapse interactions in the mature brain.

Under basal conditions microglial processes preferentially contact small spines, which initially transiently expand but exhibit a higher probability of disappearance than non-contacted ones 2-3 days after contact (see *Figure 6B*) (Tremblay et al., 2010). In turn, in a model of experience-dependent plasticity, when mice were light-deprived, microglial processes preferentially contacted larger mushroom-like dendritic spines which subsequently persistently shrank, instead of small immature-like spines (Tremblay et al., 2010). Thus, microglial interaction with synapses correlates with subsequent structural changes at the synaptic level. Although, characteristics and functional significance of these interactions are not fully understood yet, they may influence neuronal activity, either acting on the morphology of the synapse through its phagocytic capacity or by releasing modulatory factors, as it has been observed for activated microglia and during development (Hoshiko et al., 2012; Pascual et al., 2011; Trapp et al., 2007).

To achieve this, there is a requirement for a complex regulation of synaptic components and identification of synapses of interest in the first place. In this regard, it is elusive whether microglia can detect single synapses of interest, or whether they are rather attracted to a cluster of synapses. It will be interesting to reveal whether microglia preferentially interact with weak (resembling their role during brain development) or strong synapses during adulthood.





C Postsynaptic ATP release attracts microglia



D Microglia promote learninginduced synapse formation



Figure 6: Microglia-synapse interaction in the healthy adult brain. A) Microglia-synapse interactions (modified from (Wake et al., 2009)). Electron microscopy image showing a microglial process (m) in contact with the pre- and postsynaptic compartment (left panel). *In vivo* two-photon image presents a

microglial process (yellow arrow) contacting a dendritic spines (blue arrow, right panel). **B)** Microgliaspine interaction is dependent on the network state, and correlates with subsequent spine structural changes. **C)** Activation of postsynaptic NMDARs and subsequent ATP release leads to P2Y12Rdependent microglial process extensions. **D)** Microglia-derived BDNF is crucial for motor learningassociated synapse formation.

Moreover, astrocytes may also participate in this signaling. Hence, in sum, precision in the identification of synaptic targets needs answering to the following questions: synapse specific or cluster, and/or network state or astrocytic involvement?

In contrast to what has been shown in brain development, fractalkine signaling seems not to be involved in synaptic remodeling in the adult (Lowery et al., 2017).

Microglial P2Y12 receptor could be the cornerstone regarding the identification of active synapses. Postsynaptic ATP release upon postsynaptic activation and subsequent Ca²⁺ increase in the spine head, steered microglial processes in a P2Y12 receptor-dependent manner towards CA1 pyramidal neurons (see *Figure 6C*) (Dissing-Olesen et al., 2014; Eyo et al., 2014). Moreover, microglial P2Y12 receptor-deficient mice showed aberrant remodeling of synaptic connections during ocular dominance plasticity (Sipe et al., 2016). Interestingly, the first phase of ocular dominance plasticity needs NMDAR-dependent LTD (Bear and Rittenhouse, 1999). Microglia are thus necessary for synaptic plasticity in mouse visual cortex and may need P2Y12 signaling to identify depressed synapses (Sipe et al., 2016).

In turn, a candidate signaling molecule with which microglia may influence synapses is BDNF. Similarly, to contact-induced filopodia growth during brain development (Miyamoto et al., 2016), microglia promote learning-induced formation of glutamatergic synapses and that is mediated by microglial BDNF (see *Figure 6D*) (Parkhurst et al., 2013).

At present observations of modifications by microglia predominate at the level of the post-synapse. The reason for this is probably twofold: Dendritic spines exhibit a higher turnover rate in contrast to pre-synaptic terminals (Majewska et al 2006) and studies to decipher neuronal plasticity were traditionally focusing on the post-synapse (see Chapter 1). These structures can be generally divided into two types depending on their structural and functional roles. Large spines, containing post-synaptic densities, are stable over extended periods of time (Grutzendler et al., 2002; Trachtenberg et al., 2002) and represent at least 80% of the spines in the

adult brain (Majewska et al., 2006). Conversely, small spines and filopodia in mouse visual, somatosensory, motor and frontal cortices are structurally dynamic units which are thought to be rather transient (Alvarez and Sabatini, 2007; Holtmaat and Svoboda, 2009; Trachtenberg et al., 2002; Zuo et al., 2005), but have the potential to develop into more mature spines (Kasai et al., 2003). As discussed above, it is with even greater preference that microglia interact with the latter and exert their modulatory activity (Tremblay et al., 2010). Interactions are shown to induce alterations exclusively to small spines, often resulting in either morphological changes or disappearance immediately after contact. It has been proposed that smaller dynamic spines are those involved in the rapid acquisition of new memory, while larger permanent spines essentially represent the storage devices (Kasai et al., 2003).

Accordingly the effect of microglia on the synaptic morphology may ascribe them a crucial role as housekeeper of learning and memory of the brain. In investigating this hypothesis, there are a number of pressing questions which research will have to address. The studies to date have reported different effects and whether these are dependent on developmental processes, the experimental model of choice or the neuronal network state needs clarification. One suggestion is that supplementary signaling from microglia (such as BDNF) may be important during normal learning paradigms, whereas phagocytosis may predominate in definitive mechanisms of development and disease. However, microglia may employ its phagocytic capacities to remodel extracellular space to accommodate newly growing spines, as it has been adverted prudently (Tremblay et al., 2010).

To solve this question, the initial step will be to establish the nature of contacts by microglia in response to synaptic plasticity, and establish an experimental paradigm which allows for the detailed examination of microglia-synapse interactions.

3. The principles of two-photon and superresolution STED microscopy and their application for imaging in the living brain (tissue)

Seeing is believing - and for neurophysiologists you may want to expand this and provocatively say: seeing is understanding. Fluorescence microscopy, and in particular 2P microscopy, constitutes the cornerstone technology underpinning the progress in the understanding of synaptic plasticity, dendritic spine structural plasticity and microglia function. Yet, some synaptic structures and fine microglial processes are too fine and/or to densely packed to be properly visualized and examined by common confocal and 2P microscopy. To overcome this limitation, I made use of live super-resolution STED microscopy, a technique of which the application to neuroscience questions my PhD lab has pioneered (Bethge et al., 2013; Chéreau et al., 2017; Tønnesen et al., 2011, 2014; Urban et al., 2011). Hence, in the following, I will introduce 2P microscopy, STED microscopy and their application to imaging live brain tissue, with an emphasis on *in vivo* imaging.

3.1. Principle of fluorescence microscopy

"If and to what degree fluorescence microscopy will widen the possibilities of microscopic imaging only the future will show" – inventor Oskar Heimstädt (Heimstädt 1911)

Despite his humble reservation towards the usefulness of his invention, fluorescence microscopy became one of the most widely applied and powerful techniques in the biosciences since it enables the visualization of specifically labeled cellular structures in (living) tissue samples. In brief, fluorescence describes the phenomenon when a fluorophore absorbs a photon and subsequently emits a photon with a higher wavelength (see *Figure 7A*).

The wavelength of light is inversely proportional to its energy level. The shorter the wavelength the more energy it has and this follows a linear relationship. Accordingly, a photon with a wavelength of 400 nm has twice the amount of energy than a photon that has a wavelength of 800 nm. Traditional fluorescence microscopy uses a single photon to excite fluorescent dyes using mainly visible excitation wavelengths (~390 - 700 nm).



Figure 7: Principles of fluorescence microscopy. **A)** Jablonski diagram explaining the transfer of a molecule to its excited state and the subsequent fluorescence emission. The Stokes shift exemplified with GFP. **B)** Limits in optical resolution illustrated by Airy disks.

In more detail, after excitation of the fluorophore from its ground state (S0) to an excited state (S1), that means the fluorophore absorbed the incoming photon and is subsequently in an unstable elevated energy state, the electron will remain there for a few picoseconds. The electron then drops back to S0 and during this process it will release a photon with slightly less energy (and hence a longer wavelength) than the excitation photon (lost due to processes like vibrational relaxation). The gap between the excitation and emission wavelength is called Stoke shift, and is characteristic for each fluorophore (see *Figure 7A*). GFP, utilized in my work, has its peaks of the excitation and emission spectra at 488 nm and 508 nm, respectively.

In principle a fluorophore could undergo this cycle multiple times. But, microscopists often experience progressive fading of the fluorescence intensity obtained from their sample. The phenomenon is called photo-bleaching and this occurs when excited fluorophores converge from the S1 into a triplet state, which is lasting longer than the normal nanosecond time-window of fluorescence emission. Hence, fluorophores risk undergoing chemical reactions with components in their cellular environment, such as oxygen, which ultimately results in the buildup of reactive oxygen species and destabilization of the dye and the cellular milieu.

3.2. Optical resolution and diffraction limit

In traditional fluorescence microscopy the capacity to distinguish two closely spaced emitters (fluorescent point sources) defines the optical resolution of a system, and this is restricted by the diffraction limit of light. When focusing light through an objective a pattern of diffraction is formed, called the point spread function (PSF). In practice, the PSF defines the space in which fluorophores get excited. The theoretically best PSF (and hence the resolution limit *r* in the x-y plane) of a microscope can be calculated by Abbe's law. It is dependent on the excitation wavelength (λ) and the numerical aperture of the objective (NA):

$$\Delta r \cong \frac{\lambda}{2NA}$$

In theory, working with GFP which is excited at 488 nm, and using a NA 1.4 objective, the best achievable lateral resolution is thus approximately 175 nm. Structures lying closer than this diffraction limit will not be distinguishable and hence appear as one. Here, the term airy disk is helpful to describe the minimal distance resolvable between two closely spaced emitters. Described by the Rayleigh criterion two closely spaced objects can only be distinguished if the minimum of the first airy disk coincides with the maximum of the second (see *Figure 7B*).

3.3. Two-photon microscopy: why two photons help

2P microscopy is a fluorescence imaging technique that allows the visualization of living tissue at depths unachievable with conventional (one-photon) fluorescence microscopy. It relies on the principle of two-photon absorption which has been first described by Marie Göppert-Mayer in her doctoral thesis 1930 (Göppert-Mayer, 1930, 1931), and utilized to build the first 2P microscope by Winfried Denk (Denk et al., 1990). Physically, two-photon absorption describes the concept that two photons of identical or different wavelengths can excite a fluorophore from the ground state S0 to a higher energy state S1 in a single quantum event (see *Figure 8*). The energy difference between the two states is equal to the sum of the energies of the two-photons absorbed. For this to happen, it is necessary that the two photons must hit the fluorophore within 1 femtosecond of each other (10⁻¹⁵ seconds). This requires very high laser power and in principle a continuous wave (CW) laser could be used to achieve this. Yet, to circumvent the related damage to the tissue, a pulsed laser

that is able to produce very fast pulses of light (~80 MHz) with a high peak power (~150 000 W) is used in 2P microscopy.



Figure 8: Principles of two-photon microscopy. **A)** Jablonski diagram illustrating the two-photon effect. **B)** In comparison to single photon (1P) excitation, the fluorescent generation in two-photon (2P) excitation is restricted to the focal volume. Therefore, 2P microscopy has intrinsic optical sectioning.

While classical fluorescence microscopy uses a single photon (in the visible light range) to excite fluorescent dyes, in 2P microscopy, two photons with double the wavelength (and hence carrying half of the energy) are used to excite the same fluorescent dye. Only one photon is released when the electron drops down to its more stable ground state. This will have the same wavelength as the equivalent one-photon fluorescence method (i.e. slightly longer than half the 2P excitation wavelength). Since, the most commonly used fluorescent dyes have excitation spectra in the 400 to 500 nm range, the wavelengths used to excite the same dyes with 2P microscopy imply the use of light in the near-infrared spectrum (NIR), thus between 800 to 1000 nm.

Why is 2P microscopy actually beneficial for imaging living (brain) tissue? The pulsed laser necessary to build a 2P microscope renders it rather expensive in comparison to conventional fluorescence microscopes. So what is the advantage of using two-photon instead of one? A key benefit of 2P microscopy is its intrinsic optical sectioning capacity (see *Figure 8*). The objective's focal point is the only space where the density of photons is high enough to ensure simultaneous absorption of two photons by the fluorophore. The excitation is thus restricted to a tiny focal volume and effectively it entails that there is no out of focus emission.

Therefore, any light picked up by the detectors at the identified emission wavelength originates from that focal plane. This makes pinholes in front of the detectors dispensable (as it is done in confocal microscopy) and thus enhances the collection efficiency and reduces the illumination time per pixel (dwell time) and laser intensity. Moreover, in comparison to confocal microscopy where the entire sample is bathed in the excitation light cone, 2P imaging results in less out-of-focus photo-bleaching and photo-toxicity.

Another advantage of 2P imaging is the use of NIR wavelengths. Light from the visible light spectrum (VIS) scatters heavily in biological tissue (the amount of scattering is inversely proportional to the fourth power of the wavelength: λ^{-4}), which restricts its depth-penetration by insufficient delivery of excitation light to spaces located deeper in the tissue. Conversely, NIR laser light scatter much less as defined by Rayleigh's law and brain tissue usually absorbs less light around 800 – 900 nm. Together, it thus allows a superior depth-penetration of up to 1 mm in 2P versus 200 µm in confocal microscopy (Centonze and White, 1998; Helmchen and Denk, 2005).

Finally, similarly to confocal microscopy, by scanning the 2P laser over the specimen or region of interest (ROI), the experimenter obtains a 2D image. Collection of the fluorescence signals over time (time-lapse imaging) and if wished over various focal planes (3D), enables to visualize and track biological processes as they happen. Thereby, scanning speed of the mirrors and the respective minimum need of fluorescent signal to build up a picture (pixel dwell time) are the limiting factors for the temporal resolution achievable.

3.4. Super-resolution STED microscopy

The term super-resolution microscopy qualifies for light microscopy techniques that overcome the so-called diffraction limit of light (~200 nm) and hence allow the visualization of structures smaller and/or closer than 200 nm. Despite, its introduction 20 years ago, super-resolution fluorescence microscopy started to make an impact on the life sciences just very recently. For bringing optical microscopy into the nanodimension and for the future promises super-resolution microscopy entails, Eric Betzig, Stefan W. Hell and William E Moerner were jointly awarded the 2014 Nobel Prize for Chemistry.

Stimulated emission depletion (STED) microscopy, conceptualized (Hell and Wichmann, 1994) and established by Stefan W Hell based on a confocal microscope (Klar and Hell, 1999), utilizes a physical property of fluorophores to bypass the diffraction limit of light and hence achieve super-resolution images, as follows. When a molecule has been moved to the excited state, it can directly be deexcited by a photon that matches the energy difference between the excited and the ground state. For instance, for GFP the corresponding depletion wavelength is 595 nm. After a photon at this wavelength interacts with the molecule in the excited state, the fluorophore returns to the ground state before the regular spontaneous fluorescence emission can occur. This phenomenon is called stimulated emission and STED microscopy elegantly utilizes this photo-physical process to spatially control where fluorescence can be emitted (see *Figure 9A*).

For this purpose, a doughnut-shaped STED PSF is engineered (see *Figure 9A*). This is achieved by passing the Gaussian STED beam through a helical vortex phase mask (of charge one) which forces the beam to destructively interfere in the center and obtain its characteristic doughnut-like shape. For best performance it is crucial that the center (null) of the doughnut intensity needs to be at its absolute minimum, which requires a circular polarization. To ensure this, the STED beam passes through a combination of $\lambda/2$ and $\lambda/4$ wave plates to fine tune the polarization in the focal plane. Hence, by overlaying the diffraction-limited excitation beam (usually at 488 nm) with the STED doughnut (at 595 nm), the residual emitting area is restricted to the center of the doughnut, while the rim of the fluorescence is efficiently quenched. The lateral emission PSF obtained is thus much smaller (40-50 nm) compared to the original diffraction-limited one (200 nm).

The improvement in spatial resolution is dependent on the intensity of the STED beam. Similar to the relationship between excitation and emission intensity, stimulated emission depletion is a non-linear process. This means that the resolution in STED microscopy is in theory unlimited and only restricted by the finite amount of laser intensity and the biological sample (photo-bleaching and toxicity). The achievable resolution can be as good as 5.8 nm (Rittweger et al., 2009) and can be expressed by adding the intensity of the STED beam (*I*) and the STED beam intensity required (I_S) to reduce the fluorescence by a factor of 1/e (Harke et al., 2008) to Abbe's formula:

$$\Delta r \cong \frac{\lambda}{2NA\sqrt{1 + I/I_S}}$$

In practice, especially while experimenting in living (brain) tissue, application of high light intensities endangers the health of the sample and renders time-lapse imaging challenging. The tissue absorbs light (in particular in the VIS), which results in heat development and ultimately photo-toxicity. To circumvent or at least reduce the impact, the use of pulsed laser sources instead of continuous wave (CW) ones is advantageous. This renders gated STED systems dispensable, but necessitates the synchronization of the excitation and the STED beam, and precise manipulation of the temporal delay between both beams (in the pico- to nanosecond range).

Of note, the resolution improvement using standard STED microscopy is restricted to the lateral plane (x-y) (see *Figure 9B*). Consequently, the axial resolution resembles the one of confocal microscopy. However, it is possible to obtain near isotropic resolution by the use of a so called bottle beam, which enables the confinement of the residual PSF in 3D (Wildanger et al., 2009). Thereby, an axial resolution of ~150 nm is achievable.

Finally, how do the other super-resolution techniques differ from STED and why are they not suited for imaging in thick living brain tissue? Technically, in contrast to the other techniques, STED is a point-scanning method (see Figure 9C) which delivers nanoscale resolution in a purely optical way relying on the interplay of optics (phase interference and polarization) and photo physical processes (stimulated emission). The single molecule localization microscopy-based imaging methods (SMLM), such photo-activated localization microscopy (PALM) and stochastic optical as reconstruction microscopy (STORM) (Betzig et al., 2006; Hess et al., 2006; Rust et al., 2006), do not break the diffraction limit optically or by applying illumination patterns, as done by structured illumination microscopy (SIM; (Gustafsson, 2005)). Conversely, PALM and STORM rely on creating super-resolved images by precisely locating individual fluorophores through the definition of their centers of mass. Thousands of consecutive images with sparsely emitting fluorophores are required to convolve a super-resolved map of the molecule of interest through post-hoc image reconstruction. SMLM are based on wide field microscopy and CCD camerabased detection. Thus, to increase signal-to-noise ratio the systems rely on the use of total internal reflection fluorescence (TIRF) objectives/illumination. To date, this

restricts the application of SMLM mostly to cell culture experiments, and hence precludes its application to the imaging of synaptic and microglial structures in living brain tissue (acute brain slices and *in vivo*).



Figure 9: Super-resolution STED microscopy. **A)** Principle of STED microscopy. By overlaying a confocal excitation beam with a doughnut-shaped STED beam, the residual emission is restricted to the center of the doughnut. The size of the resulting super-resolved point-spread function (PSF) is dependent on the laser intensity of the STED beam. The underlying photo-physical principle is depicted in the Jablonski diagram. **B)** The 3D resolution of commonly used advanced fluorescent microscopy techniques. The PSFs are shown in x-z orientation. **C)** STED microscopy is a coordinate-based point-scanning technique. **D)** The principle of 2P-STED microscopy. Notably, through

combination with STED, 2P microscopy gains 5 to 6 times in lateral resolution; as illustrated in **B**). **E**) Setup scheme of the home-built 2P-STED microscope showing the beam path, optical components and light sources.

However, since SMLM provides nanometric resolution in 3D and is compatible with multi-color acquisitions, it is hence well suited for the examination of protein and molecular interactions in the context of molecular biology questions. For more details on SMLM and STED and their possible applications, I recommend the recent review of Sahl *et al.* (Sahl et al., 2017).

3.5. Two-photon STED microscopy

Since most STED microscopes rely on the classical one-photon excitation combined with a pinhole installed in front of the detection, these systems could be provocatively called "ameliorated confocal microscopes". For stability reasons they are built up around an inverted microscope body, which does neither allow the (easy) combination with electrophysiological recordings nor to conduct in vivo brain imaging. To allow the synergetic association of these techniques and complement them with the advantages of 2P excitation (depth-penetration), our group has custom-built an upright 2P excitation STED microscope (see Figure 9D, E) (Bethge et al 2013). Expanding on and learning from the groundwork performed by Moneron et al., Ding et al. and Li et al. (Ding et al., 2009; Li et al., 2009; Moneron and Hell, 2009), our 2P-STED setup relies on tunable pulsed excitation (900 nm) and depletion (598 nm) light sources, which enable the acquisition of super-resolved images in dual-color (GFP and YFP) and over extended periods (~0.5 hrs) of dendritic spines located well below the surface of acutely prepared brain slices (50 -100 µm in depth). The depth penetration could be achieved by static aberration correction using the objective's correction collar (Bethge et al., 2013). Usually, the spatial resolution of a 2P microscope is limited to ~350 nm and hence not sufficient to resolve fine details of spine and microglial morphology. The 2P-STED microscope overcomes these limitations and improves the lateral resolution by 5-6 folds to ~60 nm (Bethge et al., 2013).

3.6. Fluorescence imaging of dendritic spines in the living mouse brain

The structural plasticity of dendritic spines in the superficial cortical layers of somatosensory, visual and motor cortex has been extensively studied *in vivo* using

2P microscopy (see Chapter 1). Different procedures exist to obtain access to the living brain in order to examine cell structure and function and combine it with electrophysiology (Helmchen and Denk, 2005). While an open skull approach allows for ready combination of electrophysiological recordings with imaging, it is rather suited for short-term experiments on a time-scale of hours. For longitudinal monitoring of brain structure and function, it is possible to gain chronic optical access to the brain by employing the thinned skull technique, which consists in thinning the skull bone of the anesthetized mouse. This approach has been established and is to my knowledge solely exercised by the Gan laboratory (Grutzendler et al., 2002). Its advantage lies in leaving the brain intact with limited mechanical interference with the cortical tissue under investigation. However, this comes with the disadvantage of bone re-growth and challenging spherical aberration caused by the inhomogeneous nature and thickness of the bone tissue. Most laboratories make use of a chronically implanted cortical window (Holtmaat et al., 2006, 2009, 2005), which consists in the circular opening of the skull bone and the permanent installation of a #1 coverslip. Even though, bone re-growth can also be an issue in this preparation, the coverslip gives more reproducible optical access (quantifiable refractive index and thickness) than manually performed thinning of the skull. Aided by a low-magnification objective, the region containing the cells or structures of interest can be retrieved through the vascular pattern. ROIs can be thus examined over months in the living mouse. Longitudinal studies can readily be combined with behavioral read-outs and/or the study of the impact of disease progression.

In general, the big challenge for conducting *in vivo* microscopy constitutes the motion artifacts originating from blood pulsation and breathing. A combination of thorough control of the anesthesia levels and, if the experiment allows, a fast image acquisition with optimized scanning patterns complemented with post hoc correction often suffices to minimize the impact of motion artifacts. This is particularly true for the imaging of macroscale cellular structures (e.g. cell bodies and dendrites) and becomes less suitable for smaller structures, such as synaptic compartments.

However, the spatial resolution of 2P microscopy (~350-500 nm laterally) might be insufficient to resolve many important details of spine morphology, including spine necks (Tønnesen et al., 2014). Moreover, insufficient resolution may impede the discrimination of densely arranged spines, and hence result in faulty representation

of spine densities. Yet, to be fair, this is only relevant in brain regions where spine densities are reported to be higher than 1 μ m⁻¹, as it is true for pyramidal dendrites in the hippocampus (Harris et al., 1992) (but not in the cortices).

Due to its deeply embedded location inside the mammalian brain, the hippocampus is classically examined after dissection from the rodent brain in the form of acute brain slices, cultured organotypic brain slices and primary cell cultures (dependent on the scientific question at hand). Following the order of this sequence ease of optical accessibility increases greatly, while conservation and intactness of the neuronal architecture and circuitry is strongly decreasing. To study the hippocampus and its circuitry and synapses in the living animal with the possibility to correlate it with behavioral learning paradigms, hippocampal window (Dombeck et al., 2010; Gu et al., 2014; Schmid et al., 2016) and micro-endoscopy-based (Attardo et al., 2015; Barretto et al., 2009) approaches have recently been established. Spiny dendrites in hippocampal CA1 area could be tracked over extended periods of time (up to 14 weeks (Attardo et al., 2015)), by combining these optical approaches with chronic surgical preparations and Thy1-GFP mice that express GFP in a subset of CA1 pyramidal neurons (Attardo et al., 2015; Gu et al., 2014). Through immunohistochemistry it has been confirmed that after a recovery period following the surgery these approaches are accompanied by minimal / non detectable glial activation. Through behavioral tests it has been verified that mice exhibit normal behavior and perform as their non-operated littermates in hippocampus-dependent memory tests (Attardo et al., 2015; Gu et al., 2014). Yet, spine densities in hippocampal CA1 are high (~3 μ m⁻¹, (Harris et al., 1992)), and employing a 2P microendoscope equipped with a 0.85 NA lens reduces the optical resolution of 2P microscopy considerably (~610 nm, (Barretto et al., 2009)). Consequently, this may result in merging of spines and hence representing artificial spine stability, since two or more of them have to disappear before a merged spine would indeed turnover (Attardo et al., 2015).

The advent of stimulated emission depletion (STED) microscopy (Hell and Wichmann, 1994) and its continuing development enabled the investigation of neuronal structures in living brain slices at unprecedented detail (Chéreau et al., 2017; Nägerl et al., 2008; Tønnesen et al., 2014). Aided by 2P excitation, STED imaging can also be performed in thicker brain preparations (Bethge et al., 2013; Takasaki et al., 2013) and recently first applications of *in vivo* STED imaging in the

cortex of acutely prepared mice have been carried out (Berning et al., 2012; Wegner et al., 2017; Willig et al., 2014). To conduct *in vivo* super-resolution imaging, the substantial challenges are the optical access and the insufficient stability of the brain tissue due to blood vessel pulsation and breathing artifacts. The authors solved the stability problem using an anesthesia complemented by muscle relaxants and a tracheotomy allowed controlling the ventilation of mice by a tracheal tube. Despite the excellent result obtained applying this approach, the tracheotomy precluded repetitive imaging of identified structure over days. Thus, to date, we are lacking a sophisticated imaging approach which allows resolving single spines in the densely packed hippocampal region *in vivo* and which is compatible with the study of spine structural plasticity over days to months.

Aims of the thesis

My thesis work is composed of two synergistic lines of research which aimed at visualizing and better understanding two cellular mechanisms likely contributing to the rewiring of the synaptic circuitry underlying learning and memory in the adult brain: *(i)* Investigating microglia-synapse interactions and *(ii)* the development of chronic *in vivo* STED imaging to measure dendritic spine turnover in the mouse hippocampus.

(*i*) Microglia fulfill a variety of novel functions in the healthy brain, with a very likely implication in remodeling of synaptic circuits during brain development and probably also in the adult brain. It is hypothesized that microglia contribute to the structural plasticity of synapses, which is believed to be an important substrate for learning and memory in the mammalian brain. However, only indirect evidence in support of this hypothesis exists and/or it is obtained from fixed tissue analysis. Moreover, we lack a basic quantification and characterization of microglia-synapse interactions.

My first work thus aimed at determining the impact of synaptic plasticity on microglial dynamics and their physical interaction with neuronal synapses (Part 1).

Subsequently, I set out to examine the impact of the observed altered microglial morphological dynamics on synaptic function. However, research on dynamic microglia-synapse interactions in living brain tissue has been hampered by insufficient spatial resolution of conventional fluorescence microscopy, which cannot visualize important morphological details of the dynamic microglial process tips and dendritic spines. To overcome this obstacle, I took advantage of live STED super-resolution imaging. I set out to learn, master and improve the performance of our home-built 2P-STED microscope, and to optimize the experimental conditions to image the morphological interaction of microglia and dendritic spines at unprecedented resolution over minutes to hours in acute brain slices (Part 2).

(*ii*) Spine structural plasticity contributes to rewiring of synaptic circuits, which is hypothesized to be crucial for memory formation. Yet, we know only little about it in the hippocampus *in vivo*, which is the major memory center of the mammalian brain. Moreover, the two existing studies did not arrive at a consensus on the turnover kinetics of CA1 hippocampal spines in the living mouse. One of the reasons is most likely the lack of optical resolution of conventional *in vivo* 2P microscopy.

To tackle this, we set out to create an imaging approach which enables to properly measure the spine density and structural plasticity in the mouse hippocampus *in vivo* (Part 3). For this purpose, I took the lead in a joint project with the group of Martin Fuhrmann at the DZNE (Bonn, Germany). Despite the challenges of combining super-resolution microscopy and *in vivo* imaging, we aimed at establishing the first chronic *in vivo* STED imaging of hippocampal spines in the living mouse making use of a refined cranial window technique.

Results

Part 1: Induction of hippocampal long-term potentiation increases the morphological dynamics of microglial processes and prolongs their contacts with dendritic spines.

Article published in Scientific Reports.

Background and aim of the study. Microglia, the immune cell of the brain, has recently been shown to be involved in diverse brain functions beyond their classical defensive roles. At the time the project has been designed (2011/2012), first studies indicated that microglia are involved in the establishment of functional synaptic circuits during brain development (Hoshiko et al., 2012; Paolicelli et al., 2011; Schafer et al., 2012a). Likely, they contribute by either using their phagocytic capacity and/or by releasing regulatory factors. Moreover, microglia are dynamically monitoring their environment (Davalos et al., 2005; Nimmerjahn et al., 2005) and thereby establish transient contacts with synapses (Tremblay et al., 2010; Wake et al., 2009). We and others hypothesized that microglia could employ these capacities also in the healthy adult brain and thus contribute to the activity-dependent remodeling of synapses during learning and memory. However, an early study reported that microglia were not responsive towards the induction of synaptic plasticity (Wu and Zhuo, 2008). First, we thus set out (i) to determine whether microglia sense and react towards the induction of synaptic plasticity, (ii) to carry out a quantification of microglia-synapse interactions, and (iii) to test whether the interaction changes in synaptic plasticity.

Methods. For this purpose, we performed simultaneous time-lapse two-photon imaging and extracellular field recordings in acute hippocampal brain slices, obtained from CX3CR1^{+/GFP} x Thy1H^{+/YFP} mice (P30-40). Changes in microglial morphological dynamics and interaction between microglial processes and dendritic spines were assessed before and after induction of hippocampal LTP on the CA3-CA1 synapse.

Results and conclusion. Quantification of microglia-spine interactions indicated that these interactions are a short (~1.4 min) and at first glance a rare phenomenon. Yet, facilitated by their dynamic scanning activity, it can be assumed that microglia

potentially scan all CA1 hippocampal synapses over the time course of a day. Following the induction of LTP, we report an increased microglial surveillance (called scanning density in the manuscript) which is underpinned by an enhanced microglial ramification. Moreover, microglia-spine interactions became less frequent, but established contacts were 50% longer (~2.1 min). The LTP-induced changes could be omitted in the presence of the NMDAR-antagonist APV.

Altogether, our data supports that microglia coordinate their surveillance activity with the surrounding neuronal activity. Microglia react to the induction of hippocampal LTP by changing their surveillance activity and intensifying their interaction with dendritic spines. This study fuels further efforts in (i) dissecting the underlying signaling pathways, (ii) determining whether potentiated dendritic spines were contacted, and (iii) defining the nature of the contact and the functional impact on the synapse.

My contribution. Guided by both supervisors, EA and UVN, I designed and developed the study, conducted the experiments and analysis, and created analysis routines. The Matlab-based spectral unmixing routine and some experiments were contributed by EA. UVN and I wrote and revised the paper, with the input of EA.

SCIENTIFIC REPORTS

Received: 15 February 2016 Accepted: 27 July 2016 Published: 08 September 2016

OPEN Induction of hippocampal long-term potentiation increases the morphological dynamics of microglial processes and prolongs their contacts with dendritic spines

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Recently microglia, the resident immune cells of the brain, have been recognized as multi-tasking talents that are not only essential in the diseased brain, but also actively contribute to synaptic circuit remodeling during normal brain development. It is well established that microglia dynamically scan their environment and thereby establish transient physical contacts with neuronal synapses, which may allow them to sense and influence synaptic function. However, it is unknown whether and how the morphological dynamics of microglia and their physical interactions with synapses are affected by the induction of synaptic plasticity in the adult brain. To this end, we characterized the morphological dynamics of microglia and their interactions with synapses before and after the induction of synaptic plasticity (LTP) in the hippocampus by time-lapse two-photon imaging and electrophysiological recordings in acute brain slices. We demonstrate that during hippocampal LTP microglia alter their morphological dynamics by increasing the number of their processes and by prolonging their physical contacts with dendritic spines. These effects were absent in the presence of an NMDA receptor antagonist. Taken together, this altered behavior could reflect an active microglial involvement in circuit remodeling during activity-dependent synaptic plasticity in the healthy adult brain.

A number of studies over the last few years have indicated that microglia carry out a variety of important functions in the healthy brain, leading to a reappraisal of their role for normal brain physiology¹. Microglia have highly dynamic finger-like processes that are continuously moving through the surrounding brain tissue^{2,3}. During this scanning-like activity, which is modulated by neuronal activity², microglial processes establish transient contacts with synapses. While lowering neuronal activity reduces the number of contacts with synaptic structures, conditions of cerebral ischemia prolong microglia-synapse interactions⁴.

Microglia have been shown to remove synapses ('synaptic stripping') in lesion and inflammation models^{5,6}, and recent evidence indicates that they contribute to synapse pruning during normal brain development, possibly by way of their phagocytic activity^{7,8}. Moreover, microglia appear to be able to influence excitatory synaptic transmission by the release of modulatory factors like ATP9.

Given these observations it stands to reason that microglia might also contribute to activity-dependent synaptic plasticity in the healthy adult brain¹. Evidence in support of such a role has come from $Cx3Cr1^{-/-}$ mice, where the elimination of the fractalkine receptor (Cx3Cr1) from microglia was shown to disrupt hippocampal LTP¹⁰. Moreover, it was recently shown that microglial processes are preferentially steered towards active neurons^{11,12} and that their outgrowth is promoted by the activation of neuronal NMDARs^{13,14}, which are in turn strongly activated during LTP induction. However, an earlier study did not detect any changes in microglial motility in response to glutamate applications or after the induction of LTP, arguing against microglia being involved in synaptic plasticity¹⁵.

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Figure 1. Microglial morphological dynamics are altered during hippocampal LTP. (A) Scheme of the experimental design, showing the location of the stimulation and recording electrodes and the region of interest for the concurrent image acquisitions in the stratum radiatum of hippocampal CA1. Representative region of interest with microglia (green) and pyramidal neurons (red). (B,C) Normalized fEPSP slope with (C, n = 10slices) and without (B, n = 7 slices) the induction of LTP using a HFS (two arrows). The insets represent average fEPSPs in baseline (black), 40–60 min after LTP induction (blue) (C) and without application of a HFS (grey) (B). (D) Cumulative MIPs over 20 min show an increase in GFP-positive pixels, indicating an elevation in the scanning density 40–60 min after HFS (right) compared to baseline conditions (left). (E,F) The scanning density was constant over time in the absence of a HFS (E) (control: repeated measures Anova, factor of time: p = 0.3; n = 9 cells/7 slices), while it was significantly enhanced 20–40 min after LTP induction and remained elevated (40'-60') (F) (LTP: repeated measures Anova and Turkey's multiple comparisons post-test, factor of time: p = 0.0004; 0' - 20': p = 0.48; 20' - 40': p = 0.01; 40' - 60': p = 0.0004, compared to baseline; n = 18 cells/10 slices). (G) Cumulative MIPs showing that the microglial domain is unaffected 40–60 min after LTP induction (right) compared to baseline (left). (H,I) Microglial domain was stable and did not change over 80 min in absence (H) (control: repeated measures Anova, factor of time: p = 0.83; n = 9 cells/7 slices) and presence of the HFS (I) (LTP: repeated measures Anova, factor of time: p = 0.23; n = 18 cells/10 slices).

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Given these incongruent reports, we set out to revisit this issue by directly visualizing the morphological interactions between microglial processes and dendritic spines during synaptic plasticity. To this end, we combined two-photon time-lapse imaging with extracellular field recordings in acute hippocampal brain slices obtained from transgenic mice, where microglia and neurons were labeled by two different fluorophores. We analyzed the morphological dynamics of microglia and their dynamic interactions with dendritic spines of CA1 pyramidal neurons before and after the induction of hippocampal LTP.

We observed that microglia increased the number of their processes and that the duration of microglia-spine contacts increased after LTP induction. By contrast, in the presence of the NMDAR antagonist APV these changes were suppressed.

Our study provides clear evidence for microglia to be able to sense and react to the induction of synaptic plasticity, supporting the notion of a microglial contribution to activity-dependent changes at the synapse in the healthy adult brain.

Results

Microglial morphological dynamics are altered after induction of hippocampal LTP. At first, we verified that time-lapse two-photon imaging together with recordings of evoked field potentials in *Stratum radiatum* of the CA1 area of the hippocampus was compatible with maintaining microglia in their 'resting' state (Fig. 1A,B). We observed microglia with stationary cell bodies and main branches giving rise to highly ramified

and motile processes, which resembled those reported *in vivo*^{2,3}. Prolonged time-lapse imaging (160 z-stacks acquired during 80 minutes) did not induce any signs of microglial activation or neuronal damage.

To investigate whether microglia can sense the induction of synaptic plasticity, we compared microglial morphological dynamics before and after the induction of LTP in hippocampal CA1, which was robustly expressed after electrical high-frequency stimulation (HFS) of the Schaffer collateral afferents (161.6% \pm 1.1% of baseline, n = 10 slices, p < 0.001; Fig. 1C).

To globally quantify these dynamics, we counted the total number of GFP-positive pixels in maximum-intensity projections (MIP) of the two-photon image stacks across space and time (see Methods for details).

After application of the HFS, the number of GFP-positive pixels was significantly increased in the cumulative MIPs (Fig. 1D), which was not observed in control experiments without HFS over time (Fig. 1B,E) (number of GFP-positive pixels; control: Anova with repeated measures and Turkey's multiple comparisons post-tests, factor of time: p = 0.3, 0'-20': $97.1\% \pm 6.1\%$, p = 0.97; 20'-40': $104.1\% \pm 9.2\%$, p = 0.93; 40'-60': $109.6\% \pm 9.3\%$, p = 0.5, all compared to baseline; 0'-20' vs. 20'-40': p = 0.74, 0'-20' vs. 40'-60': p = 0.28, 20'-40' vs. 40'-60': p = 0.85; mean \pm sem, n = 9 cells/7 slices; Fig. 1E). This increase persisted for at least 60 minutes until the end of the recordings (LTP: Anova with repeated measures and Turkey's multiple comparisons post-tests, factor of time: p = 0.0004, 0'-20': $110.2\% \pm 5.9\%$, p = 0.48; 20'-40': $122.7\% \pm 7.7\%$, p = 0.01; 40'-60': $130.5\% \pm 9.4\%$, p = 0.0004, all compared to baseline; 0'-20' vs. 20'-40': p = 0.3, 0'-20' vs. 40'-60': p = 0.03, 20'-40' vs. 40'-60': p = 0.68; mean \pm sem, n = 18 cells/10 slices; Fig. 1F).

By contrast, the size of microglial domains, as defined by the perimeter within which individual microglia project their dynamic processes, was unaffected by the induction of LTP (domain size; control: Anova with repeated measures and Turkey's multiple comparisons post-tests, factor of time: p = 0.83, 0'-20': $103.3\% \pm 4.9\%$, p = 0.97; 20'-40': $101\% \pm 10.1\%$, p = 0.99; 40'-60': $96.6\% \pm 9.6\%$, p = 0.96, all compared to baseline; 0'-20' vs. 20'-40': p = 0.99, 0'-20' vs. 40'-60': p = 0.79, 20'-40' vs. 40'-60': p = 0.93; n = 9 cells/7 slices; LTP: factor of time: p = 0.23, 0'-20': $104.1\% \pm 3.5\%$, p = 0.76; 20'-40': $108\% \pm 4.6\%$, p = 0.24; 40'-60': $107.2\% \pm 5.7\%$, p = 0.32, all compared to baseline; 0'-20' vs. 20'-40': p = 0.79, 0'-20' vs. 40'-60': p = 0.88, 20'-40' vs. 40'-60': p = 0.99; mean \pm sem, n = 18 cells/10 slices; Fig. 1G–I).

Taken together, we observed an increase in microglial morphological dynamics after the induction of LTP, as measured by the integrated number of GFP-positive pixels, while the perimeter within which individual microglia project their processes remained unchanged. In other words, the density at which microglia scanned the surrounding neuropil ('scanning density') was increased as the size of the scanned area ('scanning domain') stayed the same.

LTP induction increases the number, but not the velocity, of microglial processes. The elevated scanning density by microglia could be due to an increase in the velocity or the number of individual microglial processes. To assess the relative contribution of these two factors we measured the velocity of single motile microglial processes (Fig. 2A) and counted the number of processes, during baseline and 40–60 min after the induction of LTP, which coincided with the largest increase in the microglial scanning density.

The average velocity did not change during the control experiments (i.e. in the absence of HFS) (baseline: $3.26 \pm 0.13 \,\mu\text{m/min}; 40'-60': 3.25 \pm 0.06 \,\mu\text{m/min}; \text{mean} \pm \text{sem}$, paired t-test, p = 0.92, n = 7 slices; Fig. 2B). Likewise, we did not detect any change in microglial process velocity after LTP induction (baseline: $3.40 \pm 0.07 \,\mu\text{m/min}; 40'-60': 3.28 \pm 0.12 \,\mu\text{m/min}; \text{mean} \pm \text{sem}$, paired t-test, p = 0.30, n = 10 slices; Fig. 2C).

We then examined whether microglia increased the number of their processes after LTP induction as increased microglial ramification was shown after pharmacological stimulation of neuronal NMDARs¹³. While the number of microglial processes remained constant during control conditions (baseline: median 86.5 [55.25, 159.75]; 40'-60': median 76.5 [48.0, 159.0]; median [25th and 75th percentile], Wilcoxon paired test, p = 0.85, n = 10 cells/7 slices; Fig. 2E), it considerably increased after LTP induction (baseline: median 50.0 [40.25, 84.75]; 40'-60': median 190.5 [90.0, 243.5]; median [25th and 75th percentile], Wilcoxon paired test, p = 0.0004, n = 12 cells/9 slices; Fig. 2D,F).

Taken together, we found that the increase in microglial morphological dynamics, or scanning density, after LTP induction can be accounted for by an increase in the number but not in the velocity of microglial processes.

Microglia-spine interactions are altered during hippocampal LTP. While it was shown before that microglial processes can contact synapses^{4,16}, little quantitative information exists on the frequency and dynamics of these interactions during basal synaptic transmission. To this end, we identified microglia-spine contacts, which we defined as a close physical apposition (i.e. <400 nm), in the time series of 3D image stacks, and analyzed their number and duration (Fig. 3A).

At the beginning of our experiments we observed only very few microglia-spine contacts: 1.61 ± 1.15 per 100 µm of dendrite (mean ± SD). Assuming a spine density of 1.1 spines per µm, which was reported for the *stratum radiatum* of the CA1 area¹⁷, this means that only $1.5\% \pm 1.0\%$ (mean ± SD) of all spines are in contact with a microglial process at any given time (Fig. 3B). However, because of the high turnover of contacts, the cumulative percentage of contacted dendritic spines increased substantially, reaching on average 12.1% ± 7.3% after 80 min of time-lapse imaging, and in some slices even exceeded 20% (Fig. 3B).

Most of the detected contacts were transient during basal synaptic transmission and had short lifetimes (mean contact duration = $1.53 \text{ min} \pm 0.05 \text{ min}$; 390 contacts from 7 slices). Moreover, individual spines were rarely contacted twice within the observation period (4 out of 390 contacts). Although quite variable between different experiments, the average number of contacts was stable throughout the recording period (# contacts per $100 \,\mu\text{m}$ dendrite observed during 20 min of baseline: 5.2 ± 1.2 ; during 40'-60': 5.0 ± 1.4 ; mean \pm sem, paired t-test, p = 0.25, n = 7 slices; Fig. 3C). At the same time, the average duration of these contacts was stable throughout



A Microglial process velocity

Figure 2. LTP induction increases the number, but not the velocity, of microglial processes. (A) Example of a tracked microglial process. The cumulative MIP is a projection over 12 consecutive time points, in which the microglial process tip was tracked over time (left panel, white circles). The magnified insets show the process tip at different time points (right panel). (**B**,**C**) The average velocity of microglial processes did not change over time in the absence of HFS (**B**) (baseline: $3.26 \pm 0.13 \,\mu$ m/min; 40'-60': $3.25 \pm 0.06 \,\mu$ m/min; mean \pm sem, paired t-test, p = 0.92, n = 7 slices) and after LTP induction (**C**) (baseline: $3.40 \pm 0.07 \,\mu$ m/min; 40'-60': $3.28 \pm 0.12 \,\mu$ m/min; mean \pm sem, paired t-test, p = 0.30, n = 10 slices). (**D**) MIP of a microglia in baseline and LTP conditions (left column) and the corresponding skeletons (right column). (**E**,**F**) Quantification of the number of processes per microglia in the absence of HFS (**E**) (baseline: median 86.5 [55.25, 159.75]; 40'-60': median 76.5 [48.0, 159.0]; median [25th and 75th percentile], Wilcoxon paired test, p = 0.85, n = 10 cells/7 slices). Microglia increased the number of their processes upon LTP induction (baseline: median 50.0 [40.25, 84.75]; 40'-60': median 190.5 [90.0, 243.5]; median [25th and 75th percentile], Wilcoxon paired test, p = 0.0004, n = 12 cells/9 slices).

the experiments and similar across different experiments (contact duration during baseline: $1.5 \min \pm 0.1 \min$; during 40'-60': $1.5 \min \pm 0.2 \min$; mean \pm sem, paired t-test, p = 0.96, n = 7 slices; Fig. 3F).

Next, we assessed whether and how the induction of LTP affected the dynamics of microglia-spine contacts. We analyzed contacts in the time window 40–60 min after the induction of LTP, which coincided with the largest increase in microglial dynamics, and compared them to baseline before the HFS. We found that the number of contacts was reduced after HFS (from 3.6 ± 0.4 to 2.2 ± 0.4 contacts; mean \pm sem, paired t-test, p = 0.04, n = 10 slices; Fig. 3D,E). By contrast, the duration of the formed contacts was on average increased by around 50% (from $1.4 \min \pm 0.1 \min t 0.2 \min \pm 0.2 \min;$ mean \pm sem, paired t-test, p = 0.006, n = 10 slices; Fig. 3G). Thus, the induction of LTP led to fewer but more persistent microglia-spine contacts.

HFS-induced effects depend on the activation of NMDA receptors. Subsequently, we tested whether the activity-dependent changes in the morphological dynamics of microglia and their interactions with dendritic spines depended on the activation of NMDA receptors. We repeated the experiments in the presence of the NMDAR antagonist APV, which blocks the expression of LTP at CA3/CA1 synapses, but does not appreciably affect pre- or postsynaptic firing behavior.

As expected, bath application of $50 \,\mu\text{M}$ APV completely blocked the expression of LTP (Fig. 4A). At the same time the HFS protocol did not induce any changes in microglial scanning density and scanning domain (scanning density: Anova with repeated measures and Turkey's multiple comparisons post-tests, factor of time: p = 0.63, 0'-20': $109.6\% \pm 10.1\%$; p = 0.6; 20'-40': $106\% \pm 7.8\%$; p = 0.86; 40'-60': $107.8\% \pm 10.1\%$, p = 0.74; all compared to baseline, 0'-20' vs. 20'-40': p = 0.96, 0'-20' vs. 40'-60': p = 0.99, 20'-40' vs. 40'-60': p = 0.99; n = 9 cells/7



Figure 3. Microglia-spine interactions are modified after LTP induction. (A) A contact between a microglial process tip (green) and a dendritic spine (red) lasting for 90 s captured by time-lapse imaging (white arrow head, 2^{nd} - 4^{th} image). (B) Percentage of spines contacted in the field of view during basal synaptic transmission, integrated over 80 min. (C,D) The number of contacts did not change over time in the absence of the HFS protocol (C) (# contacts per 100 μ m dendrite observed during 20 min of baseline: 5.2 ± 1.2 ; during 40'-60': 5.0 ± 1.4 ; mean \pm sem, paired t-test, p = 0.25, n = 7 slices). The number of microglia-spine contacts was decreased 40–60 min after LTP induction (blue) compared to its baseline conditions (black) (D) (from 3.6 ± 0.4 to 2.2 ± 0.4 contacts; mean \pm sem, paired t-test, p = 0.04, n = 10 slices). (E) MIPs over 20 min showing the number of microglia-spine contacts on a hippocampal dendrite in baseline (5 contacts) (left panel) and LTP conditions (3 contacts, 40'-60') (right). (F,G) The duration of microglia-spine contacts was constant in the absence of a HFS protocol (F) (contact duration during baseline: $1.5 \min \pm 0.1 \min$; during 40'-60': $1.5 \min \pm 0.2 \min$; mean \pm sem, paired t-test, p = 0.96, n = 7 slices). In contrast, contact durations were significantly enhanced $40-60 \min$ after the induction of LTP compared to baseline conditions (G) (from $1.4 \min \pm 0.1 \min to 2.1 \min \pm 0.2 \min$; mean \pm sem, paired t-test, p = 0.006, n = 10 slices).

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slices; scanning domain: factor of time: p = 0.5, 0'-20': $101.8\% \pm 6.1\%$; p = 0.99; 20'-40': $105.7\% \pm 6\%$; p = 0.69; 40'-60': $107.1\% \pm 7.2\%$; p = 0.53, all compared to baseline, 0'-20' vs. 20'-40': p = 0.87, 0'-20' vs. 40'-60': p = 0.74, 20'-40' vs. 40'-60': p = 0.99; mean \pm sem, n = 9 cells/7 slices; Fig. 4B–D).

Likewise, in the presence of APV, the HFS did not induce any changes in microglial process velocity (base-line: $2.93 \pm 0.1 \mu$ m/min; $40'-60': 2.9 \pm 0.12 \mu$ m/min; mean \pm sem, paired t-test, p = 0.83, n = 7 slices; Fig. 4E) and in the number of microglial processes (baseline: median 68.5 [46.25, 102.25]; 40'-60': median 82.0 [56.5, 91.5]; median [25th and 75th percentile], Wilcoxon paired test, p = 0.2, n = 8 cells/7 slices; Fig. 4F). At the same time, APV suppressed the changes in the number and duration of microglia-spine contacts that are triggered by the HFS protocol (contact number during baseline: 3.4 ± 0.6 ; during 40'-60': 3.6 ± 0.6 ; mean \pm sem, paired t-test, p = 0.32, n = 7 slices; Fig. 4G,H; contact duration during baseline: $2 \min \pm 0.2 \min \pm 0.2 \min ; 40'-60'$: $1.7 \min \pm 0.1 \min$; mean \pm sem, paired t-test, p = 0.16, n = 7 slices; Fig. 4I).

In summary, the NMDAR antagonist APV inhibits the HFS-induced changes in microglial morphological dynamics and microglia-spine interactions.

Discussion

We have shown that microglia establish transient contacts with a sizable fraction of dendritic spines within a relatively short period of time under baseline conditions in healthy adult brain tissue. Although the functional significance of these contacts is still elusive, we documented that the induction of synaptic plasticity altered these dynamic morphological interactions: while microglia exhibited enhanced morphological dynamics after LTP



Figure 4. NMDAR antagonist APV prevents HFS-induced changes in microglial morphological dynamics and microglia-spine interactions. (A) Normalized fEPSP slope in the presence of APV during baseline (red) and after the HFS (purple), showing the suppression of LTP (n = 7 slices). The inset presents average fEPSPs traces in the presence of APV during the baseline (red) and 40-60 min after the HFS (purple). (B) Cumulative MIPs over 20 min in the presence of APV showing that the scanning density and domain 40–60 min after application of the HFS (right) were similar to its baseline conditions (left). (C,D) Scanning density (C) (repeated measures Anova, factor of time: p = 0.63; n = 9 cells/7 slices) and domain (D) (repeated measures Anova, factor of time: p = 0.5; n = 9 cells/7 slices) in the presence of APV did not change after the HFS. (E,F) In the presence of APV process velocity (E) (baseline: $2.93 \pm 0.1 \,\mu$ m/min; 40'-60': $2.9 \pm 0.12 \,\mu$ m/min; mean \pm sem, paired t-test, p = 0.83, n = 7 slices) and the number of processes (F) (baseline: median 68.5 [46.25, 102.25]; 40'-60': median 82.0 [56.5, 91.5]; median [25^{th} and 75^{th} percentile], Wilcoxon paired test, p = 0.2, n = 8 cells/7 slices) remained unaffected by HFS. (G) Cumulative MIPs showing the number of microglia-spine contacts in the presence of APV during the baseline (3) and after the HFS (3, 40'-60'). (H,I) Application of the HFS in the presence of APV did not change the number of contacts (H) (baseline: 3.4 ± 0.6 ; during 40'-60': 3.6 ± 0.6 ; mean \pm sem, paired t-test, p = 0.32, n = 7 slices) nor their duration (I) (baseline: $2 \min \pm 0.2 \min$; during 40'-60': $1.7 \min \pm 0.1 \min$; p = 0.16, n = 7 slices).

induction, they formed fewer but more stable contacts with dendritic spines. These changes suggest that microglia scan the surrounding neuropil more intensely by increasing the number of processes and that some of these processes engage more intimately with dendritic spines during synaptic plasticity.

We observed a gradual increase in microglial scanning density after induction of NMDAR-dependent LTP, which is consistent with reports showing that microglial motility is affected by changes in neuronal activity and NMDAR activation^{2,13,14}. Given that microglia under normal conditions do not express functional NMDARs¹⁴, it is likely that activation of NMDARs on neurons due to the HFS and the concomitant increase in neuronal activity caused the changes in microglial morphological dynamics. In addition, the experiments indicate that the NMDAR antagonist APV did not have a direct effect on microglial morphology. The number of processes per microglia under baseline conditions was unaffected by the application of APV (see Supplementary Fig. S1, Figs 2E,F and 4F). The changes in microglial morphological dynamics after LTP induction became observable only 40 minutes after the HFS, and thus were more subtle and gradual than the rapid changes seen after glutamate and NMDA bath application^{13,14}. We speculate that the activation of synaptic NMDARs during the synaptic plasticity paradigm is a more physiological stimulus compared with bath application of exogenous glutamate and NMDA, which may mimick pathological states.

Our findings contrast with an earlier study that failed to detect an effect of LTP induction on the morphology of microglia¹⁵. This discrepancy may be explained by important differences in experimental conditions. Unlike

the previous confocal imaging study, which was conducted at room temperature, the temperature in our experiments was 33 °C, which is important to preserve normal morphological dynamics of microglia¹⁸. Moreover, our two-photon imaging approach in acute brain slices allowed us to image at depths beyond 50 μ m, where microglial morphology is less affected by the slicing procedure¹⁹. Hence, we were able to image microglia continuously without interfering with their health state, as indicated by the control experiments where the ramifications of microglia stayed intact.

Microglial processes have been shown to associate with synaptic structures such as axonal boutons and dendritic spines in a neuronal activity-dependent way in the healthy adult brain^{4,16}. Expanding these original observations, our study provides a quantitative description of the dynamics of these interactions sampled at a higher temporal resolution, which is needed to better assess the potential influence of microglia on synapses.

Microglia-spine contacts were rare and brief during basal synaptic transmission in hippocampal CA1. At any point in time, we estimated that only about 1.5% of CA1 hippocampal dendritic spines were in contact with a microglial process, which is consistent with a recent EM study²⁰. While this percentage seems too low to be of physiological relevance, it must be kept in mind that EM analyses just offer a snap shot in time. As microglia were morphologically highly dynamic, over time they could effectively contact a substantial fraction of spines (>10% within 1.5 hours). In fact, they appeared to accomplish that in a swift and systematic way, as spines were usually contacted briefly (<1.5 min) and only once during the observation period (80 min). By extrapolation, this means that the vast majority of spines on CA1 pyramidal neurons might get visited by a microglial process over the course of a few hours.

Given the effect of LTP induction on microglial morphological dynamics, we wondered whether and how the dynamic interactions between microglial processes and dendritic spines might be affected. To our surprise, the number of microglia-spine contacts decreased after the induction of LTP. However, those contacts that did form became more stable, indicating that microglia engage more selectively and intimately with dendritic spines during LTP. More generally, these observations suggest that microglia can sustain at the same time increases in global dynamics and in contact stability with spines. Both phenomena may be independently regulated and could serve distinct purposes for the surrounding cellular network. Alternatively, the increase in the number of microglial processes might compensate for the more intense interactions with dendritic spines during neuronal plasticity, allowing microglia to maintain the level of surveillance activity.

What could be the role of microglia in synaptic plasticity? The change in contact duration during LTP is suggestive of an involvement of microglia in activity-dependent remodeling of synapses. During LTP, synapses undergo remodeling, which includes morphological and functional changes. Microglia could contribute to both phenomena. Conceivably, microglia utilize their phagocytic capacity to clear the extracellular space to accommodate growing spines and/or release modulatory factors that affect potentiated synapses. Conversely, microglial processes may also target synapses undergoing hetero-synaptic depression, which is a by-product of LTP, and contribute to the weakening or removal of these synapses. Consistent with this idea, microglia have been shown to associate preferably with shrinking spines after changes in sensory experience *in vivo*¹⁶.

In conclusion, we provide clear evidence that microglia increase their scanning activity and modify their interactions with dendritic spines during hippocampal LTP. While this altered behavior may implicate microglia in synaptic remodeling in the healthy adult brain, future studies need to clarify whether microglial processes are targeting a specific subset of synapses and what the functional consequences for these synapses might be.

Materials and Methods

Animals. Cx3Cr1^{+/eGFP}-Thy1^{+/eYFP} mice were used, where GFP is expressed in all microglia under the fractalkine receptor (Cx3Cr1) promoter²¹ and YFP in a subpopulation of principal neurons in the hippocampus under the Thy1 promoter²² (Jackson Labs, Bar Harbor, ME). All experiments were carried out in accordance with the Directive 2010/63/EU of the European Parliament and approved by the Ethical Committee of Bordeaux (#50120201).

Acute slice preparation. Acutely prepared hippocampal slices were obtained from 28–40 days old mice of both sexes. Mice were anesthetized with isoflurane prior to decapitation. Brains were quickly removed and placed in ice-cold, oxygenated (95% O_2 and 5% CO_2) sucrose-based artificial cerebrospinal fluid (ACSF) containing (in mM): 210 sucrose, 10 glucose, 2 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 0.1 CaCl₂ and 6 MgCl₂ (pH 7.4, osmolarity ~315 mOsm/L). Sagittal slices were cut (350 µm thick) and incubated for 1 hour at 33 °C in carbogenated (95% O_2 , 5% CO_2) ACSF containing (in mM): 124 NaCl, 3 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose, 2 CaCl₂ and 1 MgCl₂ (pH 7.4, osmolarity ~305 mOsm/L). Subsequently, slices were stored at room temperature (RT) and used until 4 hours after preparation. Experiments were performed in a submerged recording chamber at 33 °C with continuous perfusion (3–4 ml/min) of carbogenated ACSF.

Slice electrophysiology. Schaffer collateral fibers were electrically stimulated at 0.05 Hz and evoked field excitatory postsynaptic potentials (fEPSP) were recorded in the *Stratum radiatum* of hippocampal CA1. Recording electrodes were carefully positioned in the slice and placed at depths where imaging was performed (Fig. 1A). NMDAR-dependent LTP was induced by applying a high-frequency stimulation (HFS) protocol consisting of 1 s trains of 100 Hz stimulation repeated two times 20 s apart. For APV experiments, 50 µM APV (Tocris Biosciences), an NMDAR antagonist, was continuously bath applied.

Two-photon imaging. Microglia and neuronal dendrites were imaged in the *Stratum radiatum* of CA1 using a commercial two-photon microscope (Prairie Technologies). For simultaneous two-color imaging of GFP (microglia) and YFP (neurons) the two-photon laser (Ti:sapphire, Mai Tai, Spectra Physics) was tuned to 900 nm. Images were acquired using a 40×1.0 NA water immersion objective (Plan-Apochromat, Zeiss). Laser power ranged from 10-25 mW in the focal plane. The fluorescence signal was spectrally divided into two channels by a

dichroic mirror at 514 nm and collected in a non-descanned way by PMT detectors. Z-stacks of 10 μ m with 1 μ m step size were collected consecutively every 30 s for 80 min. Imaging was performed at least 50 μ m below the slice surface, where microglia are less likely to be affected by the slicing procedure. Images were acquired at 512 \times 512 pixels with a pixel size of 200 nm, a dwell-time of 3.6 μ s and two running frame averages.

Image processing and analysis. Images were processed using MATLAB and ImageJ. Spectral unmixing of the fluorophores, GFP and YFP, was carried out using a custom-written MATLAB routine (courtesy of F. Nadrigny²³). Time-lapse image series based on concatenated single maximum intensity z-projections (MIP) were prepared independently for both fluorophores. To correct for x-y drifts during time-lapse image acquisition, the ImageJ plug-in MultiStackReg was applied. For further analysis spectrally unmixed and drift-corrected GFP and YFP channels were merged. For illustration purposes brightness and contrast of the images were adjusted and a median filter was applied.

To assess global changes in microglial morphological dynamics we defined two parameters, the microglial scanning density and the microglial domain (Fig. 1D,G). The time-lapse images were binned into time windows of 20 min, resulting in one baseline (-20-0 min) and three post HFS bins (0-20, 20-40, 40-60 min). Control experiments were carried out similarly without HFS. The resulting cumulative MIPs over 20 min, based on 40 time points, served as a measure of microglial morphological dynamics. Thus, every GFP-positive pixel represents a position in space, which was visited by a microglial process during the respective time window. For evaluation of the scanning density a line was manually drawn around the microglia. To measure the microglial domain the most distant process tips were linked. The changes in each parameter over time were expressed in percent relative to the baseline values.

Microglial process velocity was assessed by tracking single microglial process tips in consecutive MIPs using the ImageJ plugin mTrackJ²⁴. Changes were determined by comparing the average velocity of 10 motile microglial processes before and after HFS (i.e. in 20 min baseline and 40–60 min after HFS). As motile processes underlie a high turnover we were not able to analyze the same process in the two conditions. Processes at the edge of the z-stack were not included in the analysis.

Changes in the number of microglial processes were evaluated by comparing MIPs chosen at one time point during the baseline (0'-20') and after HFS (40'-60'). To minimize variations due to shifts in the z-plane, the two images were chosen in order to have similar cell body shape and major 1st order branches. The spectrally-unmixed image was first binarized applying a custom-written ImageJ plugin, which is based on wavelet-thresholding, and then skeletonized. The number of microglial processes was then extracted using the ImageJ plugin AnalyzeSkeleton²⁵.

A contact between a microglial process and a dendritic spine was defined as a physical apposition closer than 2 pixels (i.e. ≤ 400 nm) in the same z-plane. The number of microglia-spine contacts in each time window was normalized to $100 \,\mu$ m dendrite and the contact durations were evaluated by number of frames and subsequently translated in minutes. Changes were assessed comparing measurements obtained in periods before and after LTP induction.

Statistics. To assess statistical differences in the electrophysiological data Students t-tests were used. One-way Anova with repeated measures and multiple comparisons with Turkey post-hoc test were applied to test changes in microglial morphological dynamics, taking into account the factor of time. Paired t-tests were applied to test changes in microglial process velocity and microglia-spine interactions. Wilcoxon paired test was applied on the non-Gaussian distributed number of microglial processes. The number of slices and microglial cells analyzed is indicated in the text and the respective figures. Data are represented as mean \pm sem. The number of microglial processes was presented by median, 25^{th} and 75^{th} percentile. The percentage of spines contacted by a microglial process was represented by mean \pm SD to indicate the variability. The significance level was chosen to be 0.05. The asterisks in the figures denote *p < 0.05, **p < 0.01 and ***p < 0.001.

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Acknowledgements

This study was supported by a Boehringer Ingelheim PhD fellowship to TP and grants from the Agence Nationale de la Recherche (ANR-09-CEXC-012-01, ANR-2010-BLAN-1419, ANR-12-NEUR-0007-03) to UVN. We are grateful to A. Panatier, V. Langlais and members of the Nägerl lab for fruitful discussions, technical assistance and comments on the manuscript.

Author Contributions

T.P., E.A. and U.V.N. designed experiments; T.P. and E.A. carried out the experiments and analysis; U.V.N. provided supervision and funding; T.P. and U.V.N. wrote the paper.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Pfeiffer, T. *et al.* Induction of hippocampal long-term potentiation increases the morphological dynamics of microglial processes and prolongs their contacts with dendritic spines. *Sci. Rep.* **6**, 32422; doi: 10.1038/srep32422 (2016).

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Supplementary information

Induction of hippocampal long-term potentiation increases the morphological dynamics of microglial processes and prolongs their contacts with dendritic spines

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Supplementary Figure S1. Microglial morphological dynamics are not changed in the presence of the NMDA receptor antagonist APV.

A, *B*, The scanning density of microglia (A) and the size of its domain (B) remained unaffected by the application of 50 μ M APV. For this, 20 min of drug-free baseline were compared to 20 min in the presence of APV (scanning density: 99.2 % ± 6.17 %, p = 0.45, mean ± sem, paired t-test, compared to baseline, n = 16 cells / 11 slices; domain: 95.74 % ± 3.07 %, p = 0.08, mean ± sem, paired t-test, compared to baseline). *C*, *D*, Similarly, microglial process velocity (C) (baseline: $3.2 \pm 0.07 \,\mu$ m/min; APV: $3.07 \pm 0.05 \,\mu$ m/min; p = 0.11, mean ± sem, paired t-test , n = 11 slices) and the number of processes per microglia (D) (baseline: median 100.0 [74, 163]; APV: median 84 [70, 155]; median [25th and 75th percentile], Wilcoxon paired test, p = 0.79, n = 11 slices) were not affected by the application of APV.

Part 2: Two-photon STED microscopy for nanoscale imaging of neural morphology *in vivo*.

Protocol book chapter published in Methods in Molecular Biology.

Background and motivation. Classical diffraction-limited 2P microscopy does not allow the precise examination of the exquisite morphology of dendritic spines, fine microglial processes and their interaction in living brain tissue. This protocol aims at providing hands-on advice of how to conceive and maintain a super-resolution 2P-STED microscope, and run time-lapse super-resolution imaging experiments. The application of the system is exemplified by imaging of dendritic spines and microglial processes in acute cortical brain slices and in the somatosensory cortex *in vivo*.

Specifically, my motivation lied in creating an experimental paradigm which enables the investigation of the nature and functional impact of the dynamic microgliasynapse interactions at its finest detail.

Methods. To this end, MTV and I ameliorated the performance and stability of the setup. I established acquisition parameters which enabled the repetitive imaging of dendritic spines and microglial-spine interactions at a time-scale from minutes to hours.

My contribution. Content and outline of the protocol were planned by all authors. I contributed the data and designed the related graphs of Figure 1, 2, 3 D-G, 5. MTV and I prepared a first draft of the manuscript. UVN and I wrote and revised the protocol.

Chapter 5

Two-Photon STED Microscopy for Nanoscale Imaging of Neural Morphology In Vivo

Mirelle J.T. ter Veer, Thomas Pfeiffer, and U. Valentin Nägerl

Abstract

The advent of super-resolution microscopy offers to bridge the gap between electron and light microscopy. It has opened up the possibility of visualizing cellular structures and dynamic signaling events on the "mesoscale" well below the classic diffraction barrier of light microscopy (10–200 nm), while essentially retaining the advantages of fluorescence microscopy concerning multicolor labeling, detection sensitivity, signal contrast, live-cell imaging, and temporal resolution.

From among the new super-resolution techniques, STED microscopy stands out as a laser-scanning imaging modality, which enables nanoscale volume-metric imaging of cellular morphology. In combination with two-photon (2P) excitation, STED microscopy facilitates the visualization of the highly complex and dynamic morphology of neurons and glia cells deep inside living brain slices and in the intact brain in vivo.

Here, we present an overview of the principles and implementation of 2P-STED microscopy in vivo, providing the neurobiological context and motivation for this technique, and illustrating its capacity by showing images of dendritic spines and microglial processes obtained from living brain tissue.

Key words STED microscopy, Two-photon microscopy, Super-resolution imaging in vivo, Mouse cortex, Cranial window, Acute brain slices, Nanoscale neural morphology, Dendritic spines, Microglial processes, Microglia-synapse interactions

1 Introduction

Ever since Ramon y Cajal's foundational studies on the microanatomical organization of the brain, much of neuroscience research has been devoted to reconstructing brain cells and to understanding how their anatomical complexity and diversity may serve brain physiology and becomes compromised by disease.

Neurons receive input from thousands of neighboring cells via synapses, which are the physical junctions that mediate fast and flexible communication between neurons in the central and peripheral nervous system. Most excitatory synapses terminate on

Mirelle J. T. ter Veer and Thomas Pfeiffer contributed equally to this work.

Holger Erfle (ed.), Super-Resolution Microscopy: Methods and Protocols, Methods in Molecular Biology, vol. 1663, DOI 10.1007/978-1-4939-7265-4_5, © Springer Science+Business Media LLC 2017

dendritic spines that are formed by small protrusions in the postsynaptic membrane of the dendrite and that typically feature a bulbous head and an elongated neck, providing a veritable channel to the parent dendrite for the passage of electrical and biochemical signals.

Conspicuous and unique, spine morphology is thought to play a key role for synaptic physiology, shaping the ability of spines to compartmentalize electro-chemical signaling and enable synapsespecific regulatory mechanisms. Moreover, structural remodeling of spines is thought to be a mechanism for rewiring synaptic circuits and tuning synaptic potentials [1, 2]. Mounting evidence indicates that spine structural changes are an important anatomical substrate of brain plasticity that underlies adaptive processes such as learning and memory. Recent studies indicate that microglia, the immunecompetent cells of the brain, contribute to structural remodeling of synapses, possibly using their hyperfine and motile processes to sculpt dendritic spines [3].

While electron microscopy (EM) revealed the ultrastructural organization of synapses [4], featuring a presynaptic active zone, synaptic cleft, and postsynaptic density, two-photon (2P) microscopy made it possible to study their morphological and functional properties in live brain tissue [5, 6]. Together with transgenic expression of fluorescent proteins for bright and sparse labeling of neurons [7] and "cranial window" surgery techniques for high-resolution optical access to superficial layers of mouse cortex [8], 2P microscopy has become the method of choice for imaging neurons and synapses in the intact brain in vivo.

However, the spatial resolution of fluorescence microscopy, including 2P microscopy, is limited by diffraction to around half the wavelength of the excitation light (~400 nm), which in many instances is insufficient for properly resolving crucial details of synaptic morphology and nearby glial cells such as astrocytes and microglia.

Fortunately, the advent of super-resolution microscopy, in particular STED microscopy [9, 10], which overcame the classic diffraction barrier of light microscopy, has made it possible to visualize synapses with nanoscale spatial resolution in living brain slices [11, 12] and even in vivo [13, 14].

1.1 Basic PrinciplesMore recently, a combination of STED and 2P microscopy (2P-
STED) was developed to reconcile diffraction-unlimited imaging
with high optical sectioning and depth penetration in thick living
brain tissue [15–17], building on proof-of-principle studies that
demonstrated that 2P excitation can indeed be used together with
stimulated emission to improve the spatial resolution of a micro-
scope [18, 19].

As a laser-scanning fluorescence imaging technique, this new approach inherits most of its features from regular 2P microscopy,

except the diffraction handicap. Therefore, the types of fluorescent dyes that are "STED-able" and the useful labeling strategies to visualize interesting biological structures are virtually identical to those of regular 2P microscopy.

2P excitation, which is based on femtosecond lasers, offers superior penetration into biological tissue because the wavelength of the excitation light is about twice as long as compared with one photon excitation, which greatly reduces the amount of light scattering in biological tissue [20]. Moreover, the nonlinearity of the 2P excitation process provides intrinsic optical sectioning and reduces photo toxicity and bleaching because the generation of the fluorescence stays restricted to the focal spot [21].

By simultaneously absorbing two near-infrared photons, the fluorophore is elevated from the ground state (S_0) to the excited state (S_1) , emitting a fluorescence photon during its subsequent relaxation back to the ground state, which occurs spontaneously within a few nanoseconds after excitation in the case of regular 2P microscopy (*see* Fig. 1a). By contrast, in 2P-STED microscopy the excited fluorophore is expressly forced back to the ground state right after excitation, i.e., before spontaneous emission has a chance to occur. This transition is induced by stimulated emission by the STED laser.

Because the wavelength of the STED laser is set to the long wavelength end of the fluorophore's emission spectrum, the STED light produces very little direct excitation. The STED light, including the "copy photons" from stimulated emission that have the exact same wavelength as the STED light, can be discarded by standard optical filters and dichroic mirrors without losing too much of the fluorescence signal.

The phase of the STED beam is spatially modulated in a way that its intensity distribution resembles a doughnut in x-y cross sections. By bringing the STED doughnut into perfect registry with the focus of the Gaussian excitation laser beam (irrespective of whether one or two-photon excitation is used), the fluorescent spot can be cut down in size in a center-symmetric way because of the quenching effect by the STED light on the edge of the excitation spot. It is this spatial constriction that underlies the gain in spatial resolution achieved in a STED microscope.

To achieve a high quenching efficiency, and thus a high gain in resolution, the femtosecond 2P excitation pulses need to be optimally overlaid in time with the picosecond pulses of the STED lasers, which makes it necessary to be able to synchronize laser sources running at high repetition rates (80 MHz).

Whereas the classic "resolution formula" of light microscopy [22] is given by:

$$\Delta r = \frac{\lambda}{2 \times \mathrm{NA}},$$



Fig. 1 2P-STED microscopy: Basic principle and implementation. (a) Simplified Jablonski diagram of the energy transitions during a fluorescence cycle. Simultaneous absorption of two infrared photons excites the fluorophore from the ground state (S_0) to the first excited singlet state (S_1) . After vibrational relaxation to the lowest energy level of S₁, spontaneous emission of a photon usually occurs within a few nanoseconds after excitation, which returns the fluorophore back to S₀. Stimulated emission intercepts this transition by forcing the fluorophore back to S_0 and emitting a photon identical to the incident one. The doughnut-shaped STED beam guenches the fluorescence only in the outer rim of the excitation beam, which effectively enhances the spatial resolution. (b) Scheme of homebuilt 2P-STED microscope, designed for two-color super-resolution imaging of green and yellow fluorophores in living brain tissue. A Titanium:Sapphire (Ti:Sapph) laser tuned to 910 nm at ~80 MHz provides the 2P excitation light. The intensity and beam size are adjusted by an electrical optical modulator (EOM) and a telescope. The polarization of the 2P light is modulated by $\lambda/2$ -wave plate. The 2P and STED beams are combined by a dichroic mirror (DM 1). The STED beam originates from a Ti:Sapph laser tuned to 834 nm at \sim 80 MHz pumping an optical parametric oscillator (OPO) to produce light at 598 nm. The light pulses are stretched by a glass rod and a 20 m long polarization-maintaining single-mode fiber to approximately 70 ps. The characteristic doughnut-shaped STED beam is generated by a helical vortex phase plate and its circular polarization can be adjusted by $\lambda/2$ and $\lambda/4$ -wave plates. The combined beams are scanned across the sample using a beam scanner and a fast nano z-positioner for image acquisition through a water-immersion objective with a numerical aperture (NA) of 1.1 and $60 \times$ magnification. De-scanned detection and spectral separation of the emitted fluorescence is done with a dichroic mirror (DM 2 and 3) and avalanche photodiodes (APDs)

where λ is the wavelength of the excitation light and NA the numerical aperture of the objective, in the STED case, the denominator of the formula contains the term I_{STED} , which is the adjustable intensity of the STED laser:

$$\Delta r = rac{\lambda}{2 imes \mathrm{NA} imes \sqrt{1 + rac{I_{\mathrm{STED}}}{I_{\mathrm{S}}}}},$$

where I_S is the intensity value at which half of the fluorescent molecules are effectively quenched [23].

By implication, as the intensity of the STED laser is increased, the STED "curtain fire" invades successively into the central region of the excitation spot, effectively shrinking it along the radial direction. As the transition from the excited to the ground state reaches saturation, or in other words, as the number of excited molecules gets depleted quantitatively, the relationship between excitation and fluorescence generation becomes highly nonlinear, which effectively decouples the achievable spatial resolution from diffraction.

1.2 Applications of STED Microscopy in Neuroscience The STED concept was proposed in 1994 [10], confirmed experimentally 6 years later [24], and acknowledged by the Nobel Prize in 2014 [25]. Initially, the use of STED microscopy in neuroscience was limited to simple immuno-histochemical analyses of the distribution of proteins in fixed neuronal cell cultures and their synapses [26–28], delivering a "confocal plus" performance, which became available to a broad user community through imaging core facilities. Aided by technical improvements concerning optics and fluorescence labeling, the scope of STED microscopy soon expanded to include more sophisticated live-cell applications [29] and intact tissue preparations [12]. Since then, a series of papers have demonstrated the capacity of STED microscopy to image synapses deep inside living brain slices [30] and in the intact brain in vivo [13, 14]. Notably, the STED approach can be combined with other imaging and physiology techniques, such as 2P uncaging, fluorescent recovery after photobleaching (FRAP), and patch-clamp electrophysiology [31], making it possible to closely correlate super-resolution imaging data with physiological measurements obtained from the exact same cells or structures of interest.

Despite all the advance credit and excitement, STED microscopy has not yet been embraced by a large number of biology labs. However, ongoing technical improvements (concerning userfriendliness and stability of the microscope, signal-to-noise and sensitivity, temporal resolution and depth penetration) and commercialization of the technology are inspiring wider adoption of STED microscopy, turning STED from a fancy curiosity into an effective mainstream research tool.

In this protocol, we present the principle and operation of a homebuilt 2P-STED microscope, describing its main optical architecture and pieces of instrumentation, and providing a how-to guide for reliable performance and maintenance of the microscope. Finally, we demonstrate the capacity of 2P-STED microscopy by showing a couple of specific examples of dual-color imaging in brain tissue, including the intact mouse brain in vivo and thick living acute brain slices, focusing on nanoscale dendritic spines and microglial processes.

2 Materials

In the following, we list the main components and tools needed for constructing a 2P-STED microscope and performing imaging experiments in living brain tissue.

2.1 Microscope Equipment	1. Pulsed laser light sources
	 (a) 2P excitation: 2P laser tuned to a wavelength of 910 nm at a repetition rate of 80 MHz (Millennia eV combined with a Tsunami, SpectraPhysics).
	(b) STED depletion: 2P laser (Chameleon, Coherent) tuned to 834 nm, which is converted by an optical parametric oscillator (OPO, APE) to generate light at 598 nm at 80 MHz.
	2. Microscope base and objective
	(a) Modular research-grade upright microscope (e.g., Olympus BX-51WI).
	(b) Motorized sample stage (e.g., 380FM, Luigs & Neumann).
	(c) High NA water-dipping objective with correction collar (e.g., Olympus LUMFI 60x, NA 1.1, working distance 1.5 mm).
	3. Optical elements
	 (a) Pulse stretching: SF 6 glass rod, 12 cm, to stretch femto- second STED pulses to ~1 ps. 20 m long polarization- maintaining single-mode fiber to further stretch the STED pulses to ~70 ps (Pure silica core, NA 0.12, Schäfter & Kirchhoff).
	(b) Fiber coupler (Schäfter & Kirchhoff).
	(c) Electro-optical modulator for light intensity modulation: Pockels cell (e.g., Conoptics).
	(d) 2π delay vortex phase mask (RPC Photonics).
	(e) $\lambda/2$ -wave plate and $\lambda/4$ -wave plate (e.g., Qioptiq).
	(f) Beam expansion and collimation: STED beam collimation after the fiber with a $f = 40$ mm convex lens; emitted fluorescence is focused with $f = 300$ mm convex lenses; 2P beam expansion = 1:2 ($f = 75$ mm concave and $f = 150$ mm convex), scan and tube lens combination with beam expansion = 1:5 ($f = 40$ mm convex and $f = 200$ mm convex).
	(g) "Telecentric" beam scanner for x,y plane with beam expansion = 2:1, due to the action of a focusing lens and spherical mirror inside the scanner (Yanus IV digital scan head, Till Photonics).
	(h) Piezo-driven objective positioner for z-scans (P-725, Physik Instrumente).
	 (i) Mirrors with the proper coating adapted to the light in the respective beam paths: dielectric mirrors for the STED and 2P excitation light, and silver mirrors for the emitted

fluorescence to optimize performance (spectral reflectivity, polarization insensitivity) and price considerations.

- (j) Pellicle beam splitter (55%/45%, Thorlabs).
- (k) Dichroic mirrors and filters:
 - Band-pass filter (593/40, F37-593, AHF Analysentechnik).
 - Short-pass dichroic (700/10, T700dcspxruv, AHF Analysentechnik).
 - Long-pass dichroic (580/10, 580dcxru, AHF Analysentechnik).
 - Short-pass filter (680, FF01-680/SP-25, Semrock).
 - Single Notch filter (594, NF02-594S-25, Semrock).
 - Emission band-filter (520/70, FF01-520/70-25, Semrock).
 - Long-pass dichroic (514, LP02-514RS-25, Semrock).
- (1) $2 \times \emptyset$ 62.5 µm multi-mode fibers.
- (m) Detectors: Photodiode (3932 BSM, SpectraPhysics) with a spectral responsiveness ranging from 400 to 1100 nm to detect the repetition rate of the STED laser and allow for synchronization with the 2P laser. Photomultiplier tube (PMT, Photosensor modules H10722 series, Hamamatsu Photonics K.K.) with a detection range suited for STED and 2P wavelengths to visualize the reflection of gold beads. Two avalanche photodiodes (APDs, SPCM-AQR-13, PerkinElmer) with a spectral responsiveness ranging from 400 to 1060 nm to detect the emitted fluorescence.
- 4. Opto-mechanical components
 - (a) Optical mounts, post assemblies, optical rails, and other useful standard optical equipment (e.g., from Thorlabs, Newport and Qioptiq).
 - (b) $2 \times$ motorized mirror mounts for high-precision, handsoff steering of the STED and 2P beams (Newport).
- 5. Additional hardware/tools
 - (a) Phase-lock-loop for synchronization of the laser pulses (SpectraPhysics, Model3930 Lok-to-Clock).
 - (b) Nanosphere samples immobilized on regular microscopy glass slides (150 nm gold spheres, 40 nm and 170 nm fluorescent nanospheres).
 - (c) Sea of fluorescence (e.g., 10 mM calcein or fluorescein dissolved in buffered H_2O , pH 7.2).
 - (d) Power meters with sensitivity ranging from microwatts to 3 W.

6. Software

- (a) ImageJ (NIH).
- (b) Imspector (Abberior).
- (c) Agilis (Newport).
- (d) SpectraSuite (Ocean Optics).

2.2 Sample Preparation

- 1. Acute brain slices
 - (a) Mice: Thy1H^{+/eYFP} and Thy1H^{+/eYFP} x Cx3Cr1^{+/eGFP}.
 - (b) Preparation of sagittal brain slices (350 μm thick) using a vibratome (e.g., Leica VT1200S).
 - (c) Artificial cerebrospinal fluid (ACSF): Sucrose-based ACSF (in mM): 210 sucrose, 10 glucose, 2 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 0.1 CaCl₂ and 6 MgCl₂ (pH 7.4, osmolarity ~315 mOsm/L). Experiments are performed in carbogenated ACSF (in mM): 124 NaCl, 3 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 10 glucose, 2 CaCl₂ and 1 MgCl₂ (pH 7.4, osmolarity ~305 mOsm/L). Perfusion (3–4 mL/min) and heated recording chamber (32–33 °C).
 - (d) Dissection tools.
 - (e) Stage for acute slices and electrophysiology (Motorized sample stage (e.g., 380FM, Luigs & Neumann)).
- 2. In vivo preparation
 - (a) Mice: Thy1 $H^{+/eYFP}$.
 - (b) Anesthetics: Lidocaine 2% (Lurocaïne[®])/Lidocaïne 2% (Xylocaïne[®]) and Isoflurane at 4% and 1.5% during induction and surgery/imaging, respectively.
 - (c) Analgesics: Buprecare ten times diluted in PBS, 100 μ L per 30 g bodyweight.
 - (d) Other drugs: Dexamethasone sodium phosphate, 20 μL at 4 mg/mL and Ocry-gel.
 - (e) Cortex buffer (in mM): 123 NaCl, 5 KCl, 10 glucose, 10 Hepes, 2 CaCl₂, and 2 MgSO₄.
 - (f) Surgery tools.
 - (g) Head-holder, in vivo platform and stage (customized from Luigs & Neumann).

3 Methods

3.1 Step-by-Step Procedure for Working with a 2P-STED Microscope The achievable resolution and contrast of the images depends critically on the quality of the optical components, as well as on the precise alignment of the laser beams (in x, y, z, and t) and detectors (*see* **Note 1**). We have described the details of the design and construction of a 2P-STED microscope from scratch previously [15].

3.1.1 Description of the Laser Beam Paths

The 2P beam clears an electrical optical modulator (EOM, e.g., a Pockels cell), which enables fast and convenient power modulation of the transmitted intensity of the 2P light (*see* Fig. 1b). The 2P beam diameter is expanded with a telescope to properly overfill the back aperture of the objective. By slightly de-collimating the beam with the telescope, the z-focus of the 2P beam can be adjusted to coincide with the STED doughnut in the focal plane. In order to increase the depletion efficiency, the polarization of the 2P beam can be adjusted to the polarization of the STED beam by using a $\lambda/2$ -wave plate. Eventually, the 2P beam is combined with the STED beam path via a dichroic mirror.

The STED light pulses are stretched in time by sending them through a 12 cm long SF6 glass rod before passing through an EOM, which allows for rapid power modulation (see Fig. 1b). Subsequently, the beam is launched into a 20 m long polarization maintaining single-mode optical fiber (SMF) to obtain a pulse length of ~70 ps. After the fiber, the re-collimated beam acquires its characteristic doughnut shape by passing through a helical 2π phase mask. The shape of the doughnut is fine-tuned by adjusting a $\lambda/2$ -wave and $\lambda/4$ -wave plate. Specifically, they serve to establish circular polarization of the STED beam (see Note 2). The STED and 2P beams are combined by a short-pass dichroic mirror. The foci of both beams are moved over the sample pixel-bypixel in all three dimensions by the beam scanner and the piezo zpositioner. The emitted fluorescence is de-scanned by passing it through the scanner in reverse and sent toward the detectors by the way of a long-pass dichroic mirror. The emitted fluorescence of green-yellow fluorophores, such as GFP and YFP, can be spectrally separated via a dichroic mirror before getting focused into multimode fibers coupled to avalanche photodiode light detectors (APDs).

- 3.1.2 Preparation of the
 1. Determine the power of the STED and 2P light and calibrate the transmission through the Pockels cells. Correct alignment of the Pockels cell can be checked by the transmission and extinction ratio, which should be >90% and >1000×.
 - 2. Verify regularly that the laser beams are centered on the mirrors and optical elements. The installation of pinholes will facilitate the assessment of beam alignment and its optimization (*see* also **Note 3**). Long-term alignment stability is greatly aided by eliminating thermal gradients and fluctuations on and around the setup.
 - 3. Coupling the STED beam into the single-mode optical fiber (*see* also **Note 4**): At low laser power, ensure that at least 50% of the light is transmitted. If necessary, align the beam ("beam walking") until maximal transmission of the light is reached (>65%, depending on fiber length).

- 4. STED doughnut construction and optimization: A smooth and symmetric shape in all three spatial dimensions featuring a low intensity minimum at the center (the "null") is crucial for obtaining a high gain in resolution and a good signal-to-noise ratio. The doughnut is visualized by scanning the reflection from 150 nm gold nanospheres immobilized on a glass slide. The reflected light is sent via a pellicle beam splitter to a PMT (see Fig. 2b). The quality of the doughnut can be optimized by adjusting the wavefront shaping optical elements. First, rotation of the wave plates can improve the symmetry and diameter of the central minimum. Second, repositioning of the helical phase mask can additionally adjust the position of the null. Preferably, the remaining intensity in the center of the doughnut should be less than 1% of the intensity measured on the rim of the doughnut. Third, the correction collar on the waterimmersion objective should be adjusted to produce a tight z-profile. All these elements should be tweaked iteratively until the optimal doughnut shape is reached in x, y, and z (see Fig. 2a).
- 5. Co-alignment of the 2P and STED beam: Utilizing the reflection of the illuminated gold bead sample, verify that the 2P and the STED beam overlap in x, y, and z. If this is not the case, the 2P beam can be moved using a motorized mirror external to the common beam path (*see* Fig. 5). The telescope in the 2P beam path can serve to overcome misalignment in z by slight de-collimation (*see* Fig. 2a).
- 6. Detector alignment: Emitted fluorescence is de-scanned and spectrally detected by two APDs. For two-channel detector alignment, a highly concentrated sea of fluorescence is used (e.g., 10 mM calcein or fluorescein). Note, when using a waterdipping lens, the objective is in direct contact with the fluorescent solution and needs to be thoroughly rinsed afterward. Large field of view scans (e.g., $100 \times 100 \ \mu m$) can be used to assess whether signals are centered on both the detection channels (see Fig. 2c). If both the channels are not well aligned, the signal can be steered with the last common mirror in the detection path. Ultimately, both the YFP and GFP signals are centered in their dedicated channels by moving the beam with the respective mirrors in front of the multimode fibers connected to the APDs. To verify that both the channels are well superimposed, we check the overlay of the channels in ImageJ (see Fig. 2c).
- 7. Test of the quenching efficacy of the STED doughnut: While performing rapid z-scans across the interface of the sea of fluorescence and the glass slide, we make sure that the fluorescence quenching efficiency by the STED doughnut is higher than 80%. Applied laser powers should be similar to the

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Fig. 2 Setting up the microscope and testing its resolution. (a) Lateral and axial views of point spread functions (PSFs) using gold beads illuminated by 2P and STED light. A symmetric STED doughnut with a zero-intensity center (*left*). Gold bead illuminated by 2P light showing a Gaussian beam profile (*middle*). For 2P-STED imaging, it is crucial to perfectly overlap the two beams in x, y, and z (*right*). (b) Gold bead PSF imaging is performed by using a flip pellicle beam splitter (55%/45% transmission/reflection) to send the reflected light to a photomultiplier tube (PMT). (c) Lateral scans of the sea of fluorescence in a large field of view (FOV) are performed to align the two detectors. (d) The quenching efficiency of the STED beam is assessed by scanning axially the sea of fluorescence across the coverslip interface in 2P and 2P-STED mode. Example z-scan images show approximately 80% quenching in the 2P-STED mode. (e) Two closely spaced fluorescent beads (with 170 nm diameters) can only be resolved in 2P-STED mode. (f) Axial alignment is verified using the fluorescent beads. (g) Consecutive imaging of 40 nm fluorescent beads in 2P and 2P-STED mode aquantitative measure of the spatial resolution, which is around 60 nm. Scale bars: 500 nm (e-g)

situation when the biological sample is actually used. At this stage the co-alignment of the detectors in the z-dimension can be verified by exciting the sea of fluorescence with the 2P beam. An offset of the position of the edge of the interface between fluorophore and glass slide between both the channels is rectified by adjusting the distance of the collection lens in front of the multimode fibers. The synchronization system (Lok-to-Clock, SpectraPhysics) phase-locks the 2P to the STED beam and allows introducing a variable time delay. The use of a rough (12 ns) and fine (2 ns) delay allows for overlapping the 2P and STED pulses in time in order to maximize the quenching effect (i.e., quenching of >80% of the original fluorescent signal; see Fig. 2d). Low quenching efficiency can be improved by adjusting the relative intensities of the excitation and STED beams. While too little STED power will not efficiently quench the 2P fluorescence, excessive STED power can produce direct excitation of the fluorophore, thereby reducing quenching efficiency. If these manipulations do not yield sufficient fluorescence quenching, we recommend going back to improving the doughnut on the gold beads and checking the spatial and temporal registry of the 2P and STED laser PSFs, as low quenching efficacy can originate from a low-quality doughnut and/or from poor co-alignment of the 2P and STED beams.

8. Evaluation of the spatial resolution achieved by 2P-STED imaging using fluorescent nanospheres: First, while imaging 170 nm fluorescent beads in a small field of view (FOV, $10 \times 10 \ \mu$ m) with both the 2P and STED beams, we can test whether the two beams are well aligned. Small corrections can be carried out by using the two motorized mirrors in the 2P and STED beam paths. Z-scans of single beads allow for the validation of the co-alignment of the detectors in the z-dimension (*see* Fig. 2f). As a result, the beads should be resolved at approximately 170 nm (measured by full-width half max (FWHM)) and the peak of the intensity should be centered (*see* Fig. 2e).

To evaluate subdiffraction resolution, we recommend using 40 nm fluorescent nanospheres and image them with a pixel size of 10–20 nm in x-y. They are about ten times smaller than the resolution limit of regular 2P microscopy, yet still fairly bright and produce robust signals with a sufficient signal-to-noise ratio. The same beads are imaged by 2P and 2P-STED light and line profiles with their FWHM measurements are used to quantitatively assess the PSF. The resolution enhancement can be improved by finding the optimal power ratio between the 2P and STED light as mentioned above (*see* step 7). Using our 2P-STED microscope, we achieve a lateral resolution of around 60 nm of beads on a cover slip (*see* Fig. 2g).

3.2 Sample Preparation

The 2P-STED setup is designed for imaging acute brain slices as well as the intact brain through a "cranial window." Below, we shortly describe how to prepare the different samples for imaging.

3.2.1 Acute Brain Slice Acute sagittal brain slices are prepared from P25 - P35 aged Thy1H^{+/YFP} or Thy1H^{+/YFP} x Cx3Cr1^{+/eGFP} mice. In both transgenic mice a subset of pyramidal neurons is labeled with YFP, while in the latter in addition all microglia are labeled with GFP. Microglia are the resident immune cells of the brain and are very sensitive to the cellular damage and debris stemming from the slicing procedure, requiring quick and skillful execution. Slices are prepared as described previously [33]. In brief, after the slicing procedure slices are kept in carbogenated (95% O₂ and 5% CO₂) artificial cerebrospinal fluid (ACSF) until use. Experiments are to be performed in a submerged recording chamber at 33 °C with continuous perfusion of carbogenated ACSF (3–4 mL/min).

3.2.2 Preparation for Standard procedures for in vivo brain imaging include thinned skull preparations, the implementation of a cranial (glass) window, or an In Vivo Brain Imaging open skull preparation. We utilize a cranial window (see Fig. 3a) in P55 - P75 aged Thy1H^{+/YFP} male mice, which allows for keeping the cranial pressure constant, thereby reducing significantly movement artifacts of the brain compared to the open skull preparation. A 170 µm thick glass coverslip serves as a window and permits better optical access (for superior signal/noise contrast and spatial resolution) to structures deeper in the brain than the thinned skull preparation. Mice are chosen in this age-range based on their skull thickness and ease of surgery. At this age the skull is thin enough to easily penetrate it with the drill, but at the same time thick enough to resist breaking and possibly doing damage to the underlying cortical tissue.

In principle, a wide variety of mouse lines expressing different fluorescent proteins can be used for 2P-STED microscopy in vivo, as long as their spectral properties are matched to the microscope and the expression is strong but not too dense. In our case we used the classic Thy1H^{+/YFP} mouse line [7], which transgenically expresses a robust level of YFP in a subset of pyramidal neurons, providing a nearly ideal label for volume-metric STED imaging.

As the YFP molecules can freely diffuse inside the cell's cytosol, the usual bleaching problem encountered when taking z-stacks or time-lapse series is greatly mitigated, because bleached molecules quickly get replaced by unbleached ones from areas outside of the imaging field of view.

Successful in vivo experiments critically depend on the mechanical stability of the sample. A headholder, which is glued to the brain via a helmet-like structure, allows for the fixation of the



Fig. 3 2P-STED imaging in live brain tissue. (**a**) Outlining of the cranial window in Thy1H^{+/YFP} transgenic mice (*top*), and positioning of the mouse below the objective (*bottom left*), and typical view through the cranial window of an operated mouse (*bottom right*). (**b**) 2P and 2P-STED image of YFP-labeled dendritic spines in layer 1 of the barrel cortex in vivo, showing the improvement in spatial resolution. (**c**) Time-lapse STED imaging of the morphology of dendritic spines in layer 1 of the barrel cortex in vivo at 40 μ m depth. (**d**) Comparison between 2P and 2P-STED images (maximum intensity projections) of dendritic spines in an acute cortical brain slice of layer 5 (L5) obtained from a Thy1H^{+/YFP} mouse. (**e**) Time-lapse imaging of spines on apical oblique dendrites in L5. Maximum intensity projections were acquired at a depth of approximately

mouse to the microscopy platform and ultimately for the physical stabilization of the skull. In addition, the sample stability and eventually the quality of the acquired images can be optimized by considering the following points. First, the head of the mouse is turned in such a way that breathing is not obstructed, but the region of interest can still be easily accessed by the objective. Second, the body posture of the mouse is adjusted so that the lungs can easily expand during breathing, while the front paws are still resting on the heating pad for a stable body posture. The anesthesia mask should cover the nostrils of the mouse and anesthesia is set to oxygen supply at 0.6 L/min and ~1.5% isoflurane. At this rate the breathing of the mouse is calm and abdominal, thereby reducing vibrations of the brain compared to diaphragmatic breathing. Having established a stable and optically accessible cranial window, one can image neuroanatomical structures like dendritic spines (see Fig. 3b).

Super-Resolution For STED imaging experiments high-quality samples are of out-3.3 most importance. Cellular debris on the surface of the tissue or an Image Acquisition overall unhealthy state of the tissue will elevate the background fluorescence induced by the excitation and STED lasers. In general, laser intensities, field of view (FOV), pixel size, and dwell time depend on the purpose of the experiment, depth in the tissue, label intensity of the structure of interest and whether single images or time-lapse experiments are envisaged. For imaging dendritic spines we choose FOVs located at least 20 and up to 80-90 µm below the surface of the brain slice and intact brain. Imaging of microglia-spine interactions was performed at least 50 µm below the slice surface, where microglia are less likely to be affected by the slicing procedure. 2P-STED microscopy enables the observation of dynamic microglia-spine interactions at the nanoscale (see Fig. 3g). Slice health and moderate laser powers (2P and STED) are the key for successful super-resolution time-lapse image acquisitions.

During in vivo imaging, breathing (1 Hz) and blood pulsation (5-7.5 Hz) of the mouse can severely degrade the quality of the images. Clearly, motion artifacts are a serious limitation for obtaining high-quality images, especially in the case of super-resolution imaging, which imposes much stricter requirements for mechanical stability than regular 2P microscopy. To minimize the impact of

Fig. 3 (Continued) 30 μ m below the surface of the brain slice. (f) The morphology of dendritic spines can be tracked with a relatively high temporal resolution. Single z-section images were acquired approximately 35 μ m below the surface in an acute brain slice. (g) Two-color 2P-STED time-lapse imaging can be used to record the dynamic morphological interactions between microglial processes and dendritic spines in acute brain slices (Thy1H^{+/YFP} x Cx3Cr1^{+/eGFP} mouse). The image is a maximum intensity projection based on two z-planes acquired 45 μ m below tissue surface. Scale bars: 500 nm (f, g); 1 μ m (b–d); 2 μ m (e)

brain motion, the region of interest should be far from major blood vessels and the image acquisition parameters (pixel size and dwell time) need to be adjusted frequently. By continuously checking and adapting the anesthesia settings, a stable breathing pattern can be ensured. Scanning faster, i.e., the reduction of the FOV and/or the pixel dwell time, can overcome the effects of brain motion. However, scanning faster means higher 2P and STED laser intensities, which can in turn induce photodamage. The needed power depends both on the intensity and the stability of the label, on the imaging depth and whether the sample is an acute brain slice or the intact mouse brain. Typically, they range between $5 \sim 20 \text{ mW} (2P)$ and $15 \sim 30$ mW (STED) (measured at the back aperture of the objective). Image stacks are acquired with voxel sizes between $20 \times 20 \times 500$ nm and $40 \times 40 \times 1000$ nm (x, y, and z) and typical pixel dwell times are $10 \sim 50 \,\mu s$. Time-lapse imaging can be performed at a temporal resolution of $5 \sim 10$ min for a few hours on end. We advise acquiring small stacks of 3 µm in z for in vivo acquisitions in order to guard against drift of the sample out of the focus because of brain motion.

3.4 Analysis of Super-Resolved Images Acquired 2P-STED images of dendritic stretches and spines are exported as 16-bit tiff files from the Imspector software to NIH ImageJ. For illustration purposes, brightness and contrast can be adjusted and a 1 pixel median filter applied to improve image quality. Z-image stacks can be visualized in maximal intensity projections (MIP, see Fig. 3b, d). For the visualization of time-lapse acquisitions (see Fig. 3c, e–g), we use the ImageJ plugin StackReg to correct post-hoc lateral sample drift.

> To quantify the nanoscale morphology of dendritic spines (over time) (*see* Fig. 3c, e–g), it is possible to measure the full width at half maximum (FWHM) of spine neck width (*see* Fig. 4b), which is done on raw data of single z-sections. To automatically measure FWHM a custom-made image analysis program was used. At multiple positions on the spine neck, the program automatically estimates the best orthogonal fit and plots the intensity profile. Measuring across multiple parallel lines guards against noise in line profiles based on single pixels. The FWHM value can be extracted from the nonlinear Gaussian fits of the intensity profiles (*see* Fig. 4a).

4 Notes

1. Backward-forward alignment is not only mandatory when first setting up a STED microscope, but it is also regularly carried out to maintain a good performance of the system. To this end, a collimated beam from a 600 nm light source and an additional optical multimode fiber are used. The beam, which is coupled in the fiber, is sent backward through the beam path by clearing as centrally as possible the objective, the scanner, and



Fig. 4 Quantitative analysis of dendritic spine morphology. (a) A custom-made image analysis program facilitates analysis of morphometric parameters. After defining a region of interest, the program produces an outline of the region and creates a skeleton. Orthogonal lines are drawn on the skeleton and the spine head is fitted with an ellipse. The intensity distribution of the orthogonal lines is plotted and the FWHM is extracted from the Gaussian fit. (b) Example image showing the difference in FWHM of different spine neck widths on the same dendritic stretch. Scale bar: 1 μ m

the detection and excitation beam paths. Ultimately, the backward propagating beam has to be aligned into the 20 m polarization-maintaining single-mode fiber. The two pinholes to be installed before the scanner in the common beam path are positioned accordingly (*see* Fig. 5a). They will serve as a reference for the forward alignment and all minor daily alignment routines.

Subsequently, the STED beam will be aligned forward to the reference pinholes (*see* Fig. 5b). The backward-forward alignment is repeated until both the beams clear the pinholes and couple correctly into the optical fibers. At this stage, the 2P beam is superimposed on the STED beam path. Subsequently, the overlap of the 2P and the STED beam is ensured on a large distance before continuing the regular beam alignment (see the preparation of the microscope).

2. In 2P-STED microscopy, the efficiency of the STED effect depends greatly on the ability to control the polarization of the light, which should be circular when it enters the objective. The STED beam must be circularly polarized so that the focal doughnut can exhibit a deep intensity minimum. This is particularly an issue for high NA objectives, where the marginal rays coming in at shallow angles otherwise do not interfere destructively. A circular polarization is achieved by the insertion of a rotatable $\lambda/4$ -wave plate into the beam path. The circularity can be checked by measuring the intensity of the light after a

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Fig. 5 Microscope alignment procedure. (a) Schematic explanation of the backward alignment procedure. A collimated beam from a laser diode is sent backward through the setup. The beam travels in a centered way through the objective, z-positioner, beam scanner, and the STED beam path. Pinholes are inserted based on the backward beam, and serve as reference for the forward beam. (b) Forward alignment: the STED beam is aligned to the reference pinholes and is guided through all optical elements. Back- and forward-alignment is repeated until the pinholes are perfectly cleared. (c) 2P beam alignment: the 2P beam is subsequently coaligned to the STED beam by utilizing the reference pinholes and a fluorescent target to verify co-alignment is maintained until the sample

rotating polarizer. In case of perfect circularity, a flat line will appear on the oscilloscope, since all polarization directions are equally represented; otherwise, the signal will be modulated by a sine wave, indicating poor circularity.

3. In order to facilitate alignment procedures, we recommend inserting pinholes at strategic positions of the common (*see* Fig. 5a) and external beam paths. This allows verifying whether the laser beams are still aligned to the centers of the optical elements and, more importantly in the common beam path, whether the 2P and STED beam are still co-aligned. By iterative beam walking perfect clearing of the pinholes can be achieved (*see* Fig. 5a, b). Replacing the objective by a fluorescing alignment target makes it possible to check whether the co-alignment of the two beams on the pinholes holds true until reaching the sample (*see* Fig. 5c).

4. Fiber-coupling can be facilitated by using a power meter suitable for the pulsed STED beam in order to measure the input power and the amount of transmitted light. In order to couple the light properly into the fiber, as well as in order to align to pinholes, one should walk the beam. Two different mirrors (each having three degrees of freedom) are used to control translation and rotation of the laser beam separately.

Acknowledgments

This work was supported by a PhD fellowship from the "European Neuroscience Campus" network (ENC) to MJTTV, a Boehringer Ingelheim PhD fellowship and a 4th-year extension grant from the "Fondation pour la Recherche Médicale" (FRM. FDT20160435677) to TP, and grants from the "Agence Nationale de la Recherche" (ANR) (ANR-13-BSV4-0007-01), France-BioImaging (ANR-10-INSB-04), and European Research Area Network NEURON II (ANR-12-NEUR-0007-03) to UVN. We thank Florian Levet (CNRS, University of Bordeaux) for the custom-made image analysis program.

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Part 3: Chronic STED microscopy reveals high turnover of dendritic spines in the hippocampus *in vivo*.

Manuscript submitted.

Background and aim of the study. Dendritic spines constitute the post-synaptic part of most excitatory synapses in the mammalian brain, and play a pivotal role in neuronal information processing. Spine structural plasticity contributes to rewiring of synaptic circuits, which is hypothesized to be a key mechanism of memory formation. Experience-dependent structural plasticity of dendritic spines has been extensively studied in cortical brain areas by 2P microscopy *in vivo*. However, we know surprisingly little about spine structural plasticity in the deeply embedded hippocampus, which is the major memory center.

Studying the morphology and plasticity of hippocampal spines *in vivo* is challenging because of the limited optical access (the mouse hippocampus is ≥ 2 mm below the cortical surface) and the insufficient spatial resolution of 2P microscopy to determine the density of dendritic spines, let alone to reveal their morphological details.

Methods. Combining recent developments in super-resolution microscopy and cranial window techniques, we set out to improve the imaging of hippocampal spines in living mice. We thus modified our home-built 2P-STED microscope and equipped it with a long-working distance objective. Complemented with surgical resection of a small part of the overlaying cortex, we obtained high-quality optical access to fluorescently labeled pyramidal dendrites in the CA1 area of the hippocampus.

Results and conclusion. Leveraging on this new approach we determined the density of dendritic spines and monitored their turnover in anesthetized mice. Spine density was about two times higher than reported in the 2P literature, and ~40% of dendritic spines turned over during a five-day observational period, revealing a surprisingly high level of structural remodeling.

Taken together, we have established chronic super-resolution imaging in the hippocampus *in vivo*, facilitating detailed studies of the anatomical correlates and mechanisms of animal behavior, such as learning and memory, in the living mouse.

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My contribution. MF, UVN and I initiated the collaboration. I performed preliminary tests of candidate objectives and optimized the STED imaging setup. SP and I performed pilot experiments, the imaging experiments, and analyzed the data. SP, SB and I discussed the figure outline. I created the figures and wrote a first draft of the manuscript. All authors contributed to the revision of the manuscript.

Chronic STED microscopy reveals high turnover of dendritic spines in the hippocampus *in vivo*

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Abstract

Rewiring neural circuits by the formation and elimination of synapses is thought to be a key cellular mechanism of learning and memory in the mammalian brain. Dendritic spines are the postsynaptic structural component of excitatory synapses, and their experience-dependent plasticity has been extensively studied in mouse superficial cortex *in vivo* using two-photon microscopy.

By contrast, very little is known about spine plasticity in the hippocampus, which is the archetypical memory center of the brain, mostly because it is very difficult to visualize dendritic spines in this deeply embedded structure with sufficient spatial resolution.

We developed chronic STED microscopy in mouse hippocampus, using a 'hippocampal window' and long working distance objective for optical access. We measured a two-fold higher spine density than previous studies based on two-photon microscopy, and observed that approximately 40% of spines turned over within 5 days, providing direct evidence for a high degree of circuit rewiring in the hippocampus.

Our study demonstrates chronic super-resolution microscopy in the hippocampus, enabling longitudinal and correlative analyses of nanoscale brain structures with genetic, molecular and behavioral experiments.

Introduction

Dendritic spines form the postsynaptic structural component of most excitatory synapses in the mammalian brain. They constitute computational units of information processing that underlie essentially all higher brain functions¹⁻³ and play a crucial role in brain disorders such as autism spectrum disorder and Alzheimer's disease^{4, 5}. Spine structure is closely linked to synapse function, as the size of spine heads scales with synaptic strength^{6, 7} and the shape and number of spines can be modified by the induction of synaptic plasticity^{6, 8-11} and by sensory experience^{12, 13}.

Rewiring of neural circuits by spine plasticity is considered a key neurobiological mechanism of memory formation^{2, 14}. Notably, a recent study showed that optically induced shrinkage of potentiated spines disrupts newly acquired motor skills¹⁵, establishing a causal link between spine plasticity and memory. While experience-dependent plasticity of dendritic spines has been a consistent finding across mouse cortex¹⁴, it has been studied very little in hippocampus *in vivo*. However, this neural structure is considered the archetypical memory center of the brain, and hippocampal brain slices and primary cell cultures are the dominant model systems for the study of synaptic plasticity.

Imaging dendritic spines in the hippocampus *in vivo* is challenging because of its remote location more than 2 mm below the surface in the mouse brain. Only recently did researchers accomplish this with two-photon (2P) microscopy, using a 'hippocampal window'¹⁶ or an endoscopic approach¹⁷. However, 2P microscopy lacks sufficient spatial resolution to visualize important details of spine morphology, such as spine necks¹⁸, and even struggles to distinguish individual dendritic spines on CA1 pyramidal neurons, leaving a high fraction of them undetected¹⁷.

While regular light microscopy typically detects a spine density of around 1 spine/µm^{16, 17, 19}, electron microscopy (EM) reports 3 spines/µm on CA1 hippocampal pyramidal neurons, at least in rats²⁰. By comparison, spine density is about 10 times lower in many cortical areas, e.g. approximately 0.24 spines/µm for pyramidal neurons in layer 5 of mouse barrel cortex¹².

Given the limited spatial resolution of 2P microscopy, we turned to super-resolution light microscopy to improve the study of dendritic spine plasticity in the hippocampus of living mice. We used a home-built stimulated emission depletion microscope based on 2P excitation (2P-STED)^{18, 21} and equipped it with a long working distance objective to reach the deeply located hippocampus in anesthetized mice. We adopted a 'hippocampal window' technique^{16, 22, 23}, where a portion of the overlying somatosensory cortex is surgically

removed and replaced by a metal cylinder sealed with a cover slip, providing stable optical access to the CA1 area of the hippocampus.

We demonstrate that our new approach offers substantially improved spatial resolution and image quality compared to regular 2P microscopy in mouse hippocampus *in vivo*. We measured spine density on basal dendrites of pyramidal neurons in *stratum oriens* of the CA1 area in transgenic mice, where a subset of pyramidal neurons was fluorescently labeled. Repetitive imaging of dendritic stretches over a 5-day period allowed us to determine spine turnover. Spine density was about two times higher than reported by conventional 2P microscopy, and around 40% of all spines turned over, suggestive of a high level of synapse remodeling in the hippocampus.

Results

2P-STED microscopy with a long working distance objective

We set out to establish STED microscopy of dendritic spines in mouse hippocampus to track their morphological dynamics over the course of several days. We used a custom-built 2P-STED microscope (Fig. 1A;^{18, 21}) in combination with a modified 'cranial window' technique to gain optical access to *stratum oriens* in the CA1 area of the hippocampus^{16, 23}. We surgically removed the overlying somatosensory cortex and inserted a metal tube sealed with a cover slip as physical place holder (Fig. 1C;^{16, 23}). To bridge the distance between the surface of the skull and the alveus (~2.3 mm), which is located right above the hippocampus, we used an objective with a long working distance, which at the same time offered a relatively high numerical aperture (Nikon N60X-NIR: WD 2.8 mm, NA 1.0).

To test whether this objective would be suitable for this purpose, we determined the pointspread function (PSF) of the microscope by imaging fluorescent nanospheres (diameter: 40 nm). Their full-width at half maximum (FWHM) was 57 ± 2 nm in the x-y plane in 2P-STED mode, compared with 355 ± 13 nm in regular 2P mode (p<0.0001, paired t-test; n = 17 beads; Fig. 1B), demonstrating that our super-resolution approach delivered a six-fold nominal gain in lateral spatial resolution.

2P-STED microscopy of dendritic spines in the hippocampus in vivo

We then used the 2P-STED microscope to image dendritic spines on basal dendrites of CA1 pyramidal neurons (*stratum oriens*) in anesthetized mice. We used transgenic mice (Thy1- $H^{YFP/+}$ and Thy1- $M^{GFP/+}$, 4 – 12 months old) that expressed yellow and green fluorescent proteins, respectively (YFP and GFP), in a subset of CA1 pyramidal neurons²⁴. Focusing on dendrites close to the cover slip (5 – 20 µm), we obtained a major improvement in image

quality with 2P-STED microscopy (Fig. 1D), where the shape of dendritic spines was much more clearly delineated in 2P-STED than in 2P mode. The difference was especially evident for spine necks that exhibit diameters well below the diffraction limit of regular 2P microscopy^{10, 25}. We more accurately resolved spine necks with 2P-STED than with 2P microscopy, as demonstrated by paired measurements of spine neck widths (2P-STED: 177 \pm 13 nm; 2P: 468 \pm 19 nm; p<0.0001, paired t-test; n = 29 spine necks, 11 dendrites, 6 mice; Fig. 1E).

The *in vivo* acquired images contained only few brain motion artifacts (Supplemental Movie 1). Image distortions caused by blood vessel pulsation and pulmonary respiration normally represent a serious challenge for image analysis, in particular for STED microscopy, which is more susceptible to brain motion due to higher spatial resolution compared to other imaging techniques. The remarkable stability of the preparation spared the need to apply motion correction to the images.

Dendritic spine density of CA1 pyramidal neurons in vivo

To demonstrate that our STED approach achieves a higher accuracy in quantifying dendritic spine density in the hippocampus *in vivo*, we counted spines on basal dendrites of CA1 pyramidal neurons in images acquired in 2P-STED as well as in 2P mode. There was a significant difference between the two modes (2P-STED: $2.13 \pm 0.10 \,\mu\text{m}^{-1}$; 2P: $1.61 \pm 0.07 \,\mu\text{m}^{-1}$; p<0.0001, paired t-test; n = 82 dendrites, 6 mice; Fig. 2B), where 2P-STED detected on average 32% more spines than 2P in a direct comparison (Fig. 2C). The increase was due to the improved detection of spines by 2P-STED, which were otherwise buried in the fluorescence signal of the dendritic shaft in lower resolution 2P mode. Furthermore, 2P-STED improved the discrimination of closely spaced spines which frequently appeared merged in the 2P images (Fig. 2A).

While our 2P-based mean value for spine density (1.61 μ m⁻¹) is around 45% higher than reported in the light microscopic literature (~1.1 μ m⁻¹)^{16, 17}, the mean value based on 2P-STED (2.12 μ m⁻¹) is about twice as large as this. However, the density is still less than what was reported by EM for CA1 pyramidal neurons in rat hippocampus (~3 μ m⁻¹)^{20, 26}.

Dendritic spines undergo high morphological turnover in vivo

We then monitored individual dendritic stretches over 5 days (day 1, 3 and 5). To retrieve a particular dendrite from one imaging session to the next, we used tissue landmarks, such as blood vessels, fluorescent cell bodies and dendrites. Importantly, we did not observe any

overt signs of phototoxicity, such as dendritic "blebbing" during short time-lapse sequences and across the imaging sessions (Supplementary Fig. 3).

First, we quantified average spine density for each time point, which remained stable over the entire observation period (day 1: 2.31 \pm 0.10 μ m⁻¹; day 3: 2.28 \pm 0.08 μ m⁻¹; day 5: 2.30 \pm 0.10 μ m⁻¹; n = 14 dendrites, 3 mice; Fig. 3B).

Next, we examined spine turnover by counting all spines that appeared and disappeared from one imaging session to the next on all dendritic stretches we imaged. Furthermore, we calculated the percentage of spines that were present on day 1 and remained visible, i.e. survived, during the two subsequent imaging sessions. This survival fraction was 78.1 ± 3.6 % for day 3, and 60.8 ± 3.4 % for day 5 (Fig. 3A, C).

We also calculated the fraction of spines, which were lost and gained between the imaging sessions. The fraction of lost spines was 22.2 ± 4.2 % from day 1 to 3, and 18.8 ± 2.1 % from day 3 to 5 (Fig. 3D), while the fraction of gained spines from day 1 to 3 was 20.6 ± 2.6 %, and 20.5 ± 2.5 % from day 3 to 5. This means that the rates of spine loss and gain were practically invariant over time and balanced each other out, which fits with the observation that spine density remained constant.

Taken together, using 2P-STED microscopy in the hippocampus of living mice, we observed a high level of spine turnover over a relatively short period of time while average spine density stayed the same.

Discussion

We established chronic super-resolution imaging of dendritic spines in the hippocampus of living mice. It is based on a home-built upright 2P-STED microscope^{18, 21} equipped with a long working distance water immersion objective and a 'hippocampal window' to reach this deeply embedded brain structure^{16, 23}. STED microscopy has been used before for imaging dendritic spines *in vivo*, but only in superficial cortical areas and for one-off acute imaging sessions^{27, 28}.

Dendritic spines commonly serve as convenient morphological markers for excitatory synapses, allowing inferences on synaptic strength and functional connectivity, for instance when their shape, size or number change over the course of an electrophysiological or behavioral experiment^{3, 14}. However, because spines have nanoscale dimensions and are densely packed in light scattering tissue, an accurate and quantitative anatomical readout is oftentimes hard to come by¹⁸.

Whereas diffraction-limited light microscopy, such as 2P microscopy, fails to visualize important morphological features of spines²⁵ and even struggles to distinguish spines on hippocampal neurons¹⁷, our approach can resolve spine necks and detect spines with an unprecedented level of reliability in the hippocampus *in vivo*.

According to serial section transmission EM, spine density varies between 2 to 4 μ m⁻¹ for proximal apical dendrites in *stratum radiatum* in adult rats^{20, 26} and mice (Erik Bloss, Janelia Research Campus, USA, *personal communication*), and a recent light microscopic study using array tomography reported a spine density of 2 - 3 μ m⁻¹ for basal oblique dendrites in *stratum oriens* in adult mice²⁹. Our mean value (2.12 μ m⁻¹ for *stratum oriens*) is in the range of these measurements, as opposed to the value of around 1.1 μ m⁻¹ recently reported by *in vivo* 2P microscopy^{16, 17}, which corresponds to a detection rate of only about one third.

As our STED approach only improved the spatial resolution in the x-y plane, spines that protruded into the z direction were still difficult to detect, which explains the residual detection gap. However, our method stands to benefit from ongoing technical advances, for instance in adaptive optics, which will improve spatial resolution, in particular in $z^{30, 31}$, and should enable nearly complete spine detection.

We could only image spines in the *stratum oriens* within 20 µm from the cover slip; beyond this distance, image quality degraded rapidly. However, it would be highly desirable to be able to image more deeply and reach dendrites in *stratum radiatum* and other areas in the hippocampus. The depth limitation was likely due to spherical aberrations caused by mismatches in refractive index between the immersion medium of the objective (n = 1.33 for water), the glass cover slip (n = 1.51) and the sample (n ≈ 1.37 for brain tissue³²). Unlike other objectives we have used for STED microscopy in brain tissue^{18, 33}, the new objective did not have a correction collar to reduce spherical aberrations, which otherwise probably would have permitted greater imaging depth.

We were struck by the near total absence of motion artifacts in the images. We suspect that this was primarily due to two effects: firstly, the implanted metal tube probably stabilized the brain mechanically, and secondly, blood pulsations are likely weaker in the hippocampus where the vasculature is mostly made of capillaries and has fewer large arteries than in superficial cortex³⁴.

Faithful and complete detection of spines across space and time is absolutely critical for an accurate assessment of spine turnover. Failure to distinguish closely spaced spines will lead to an underestimate of spine turnover, because any events of individual spines appearing or disappearing within a merged cluster will invariably be missed, giving a false reading of spine

stability¹⁷. Conversely, temporal fluctuations, for instance when spines rotate in or out of the optical axis, will overestimate spine turnover, whereas biased detection of large spines might distort the measurements of spine turnover as large spines are reportedly less structurally plastic than small spines³⁵. Clearly, these pitfalls are compounded by low spatial resolution and poor image quality.

The spine detection problem was highlighted by two recent studies that pioneered chronic imaging in the hippocampus *in vivo*^{16, 17} and reported very different lifetimes for spines on apical and basal dendrites of CA1 pyramidal neurons. Whereas the study by Gu *et al.* reported that 96.4% of spines survived for at least 16 days in *stratum radiatum*¹⁶, the study by Attardo *et al.* concluded that all spines in *stratum oriens* turned over (i.e. survival fraction equaled 0%) within a month¹⁷. Both studies used 2P microscopy with relatively low NA optics and presented images of comparable quality, reporting similar average spine densities (~1.1 µm⁻¹) that were invariant over time. While Gu *et al.* measured spine turnover at 'face value', Attardo *et al.* used mathematical modeling to account for the effect of merged spines on apparent spine turnover. Importantly, without mathematical correction, the survival fraction was around 80% after 21 days, indicating that analyzing 2P data at 'face value' is highly unreliable for determining spine turnover.

Our super-resolution approach registered a spine survival fraction of 60% after 5 days, providing direct and unambiguous evidence that spines turn over extremely rapidly in CA1 *stratum oriens*. The absence of any signs of phototoxicity and the fact that spine density remained constant indicate that the observed turnover was real and not artifactually induced by the metal cylinder or STED imaging. While our results experimentally confirm the modeling-based conclusions of the Attardo et *al.* study, they also make a strong case for the use of super-resolution microscopy, because it allows direct and faithful investigation of spine dynamics.

Our anesthetics included the NMDA-receptor antagonist ketamine, which could be problematic because of the important role of NMDA-receptors in hippocampal synaptic plasticity. However, as the drug was applied only for a couple of hours per imaging session and we did not induce any plasticity, it is unlikely that our results were compromised by it. In the future, it will be preferable to use more innocuous drugs or to dispense with anesthesia altogether.

Even though Gu et *al.* probably underestimated spine turnover because they did not account for merged spines, the fact that the results from the two studies were so divergent suggest that dendritic spines turn over much faster in basal than in apical dendrites of CA1 pyramidal neurons. This may point to, yet unknown, intrinsic structural differences between basal and apical dendrites that control spine structural plasticity. Alternatively, it may reflect anatomical or physiological differences in the quality and level of afferent input to CA1 basal and apical dendrites. *Stratum oriens* not only receives fibers from CA3, but also from entorhinal cortex and amygdala, unlike *stratum radiatum*, where the input primarily comes from CA3^{36, 37}.

Our finding that hippocampal dendrites are subject to ongoing intense anatomical remodeling supports the view of the hippocampus as a highly dynamic structure designed to encode and process new memories, but not as a long-term repository of information³⁸. The situation may be quite different in cortical areas, where typically a large fraction of spines is stable for many weeks in cage-reared adult mice³⁹⁻⁴¹, unless they are subjected to behavioral training^{42, 43} or sensory manipulations^{44, 45}. However, whether and how spine turnover actually reflects memory-relevant functional adaptations in synaptic strength and connectivity is not well known.

In summary, the present work adds up to a substantial advance for the study of hippocampal synapses in living mice. In the future, by correlating spine-level structural changes with genetic, molecular and behavioral interventions and assays, our chronic super-resolution imaging approach opens up manifold opportunities to study the biological mechanisms and functional relevance of spine plasticity in the mammalian brain.

Methods

Animals

We used adult female and male transgenic mice (Thy1- $H^{YFP/+}$ and Thy1- $M^{GFP/+}$, 4 – 12 months old) where a subset of pyramidal neurons is fluorescently labeled²⁴. The mice were group housed by gender at a day/night cycle of 12/12 hours. All procedures were in accordance with the Directive 2010/63/EU of the European Parliament and approved by the Ethics Committee of Bordeaux and by the government of North Rhine Westphalia.

Hippocampal window surgery

Chronic hippocampal windows were implanted as described previously^{16, 22}, providing optical access to the *stratum oriens* of the CA1 region of the hippocampus. In brief, mice were anesthetized with an i.p. injection of ketamin/xylazine (0.13/0.01 mg/g bodyweight) and received subcutaneous injections of analgesics (buprenorphine, 0.05 mg/kg) and antiinflammatory agents (dexamethasone, 0.2 mg/kg) to prevent the brain from swelling during the surgery procedure. Using a dental drill a craniotomy of 3 mm in diameter was performed above the right hemisphere (stereotactic coordinates: anteroposterior, -2.2 mm; mediolateral, +1.8 mm relative to bregma). The dura and somatosensory cortex above the hippocampus were carefully aspirated, while leaving the external capsule of the hippocampus intact. Subsequently, a custom-made metal tube sealed with a coverslip on the bottom side (both 3 mm in diameter, height 1.5 mm and 0.13 mm (#1)) was inserted into the craniotomy and fixed to the skull with dental acrylic. Following the surgery, mice received analgesics for 3 days (buprenorphine, 0.05 mg/kg, s.c.) and were allowed to recover from the surgery for 4 weeks before experiments started.

Two-photon STED microscopy

We used a custom-made upright two-photon STED microscope (2P-STED; Fig. 1A) based on two-photon excitation and stimulated emission depletion (STED) using pulsed lasers^{18, 21}. In brief, a femtosecond mode-locked Ti:Sapphire laser (Chameleon, Coherent, Santa Clara, USA) operating at 80 MHz and emitting light at a wavelength of 834 nm was used in combination with an optical parametric oscillator (OPO BASIC Ring fs, APE, Berlin, Germany) to produce STED light pulses at a wavelength of 598 nm and with a duration of ~150 fs. The pulses were stretched to ~100 ps by passing them through a glass rod and a 20 m long polarization-maintaining fiber. A STED light reflection served to synchronize a second Ti:Sapphire laser (Tsunami, Spectra Physics, Darmstadt, Germany) tuned to 910 nm and running at a repetition rate of 80 MHz, which was used for two-photon excitation (2P laser) of the fluorophores. Synchronization and optimal pulse delay were achieved with phase-locked loop electronics (3930, Lok-to-Clock, Spectra Physics). The STED doughnut was created by passing the STED beam through a vortex phase mask (RPC Photonics, Rochester, USA), which imposed a helical 2π phase delay on the wave front. Wave plates ($\lambda/2$ and $\lambda/4$) were used to make the STED light circularly polarized before it entered into the objective. The 2P and STED beam were combined using a long-pass dichroic mirror. The two laser beams were moved over the sample in all three dimensions using a galvanometric x-y scanner (Janus IV, TILL Photonics) combined with a z-focusing piezo actuator (Pifoc, PI, Karlsruhe, Germany). To bridge the physical distance between the surface of the brain and the deeply embedded hippocampus, we employed a long-working distance water-immersion objective (Nikon CFI Apo 60X W NIR, 1.0 NA, 2.8 mm WD). The epi-fluorescence was de-scanned and imaged onto an avalanche photodiode (SPCM-AQR-13-FC, PerkinElmer, Villebone-sur-Yvette, France). Signal detection and peripheral hardware were controlled by the Imspector scanning software (Abberior Instruments, Göttingen, Germany) via data acquisition cards (PCIe-6259, National Instruments). Optical resolution was assessed by imaging 40 nm fluorescent nanospheres (yellow-green fluospheres, Invitrogen) immobilized on glass slides (Fig. 1B). Regions of interest were consecutively imaged in 2P and 2P-STED mode (10 x 10 μ m²; 10 nm pixel size; 50 μ s pixel dwell time).

In vivo imaging

Mice were anesthetized with an i.p. injection of ketamin/xylazine (0.13/0.01 mg/g bodyweight) and the eyes were protected with ointment (Bepanthen). The mouse was fixed to a custom-made stereotactic frame and kept at body temperature using a heating pad. For the quantification of spine density, 2P-STED and 2P images of basal dendrites of CA1 pyramidal neurons were acquired applying identical acquisition parameters (10 x 10 x 4-8 μ m³; 20-40 nm pixel size; 0.5-1 μ m z-step; 20-70 μ s pixel dwell time). For chronic time-lapse imaging, the position of the field of view (FOV) was registered in the first imaging session with the help of vascular landmarks and cell bodies of CA1 pyramidal neurons. This allowed for subsequent retrieval of the FOV for each mouse. An overview 2P image z-stack (100 x 100 x 20 μ m³; 200 nm pixel size; 1 μ m z-steps) was acquired at each time point starting from the coverslip. Subsequently, identified distal stretches of basal dendrites in CA1 *stratum oriens* (5 – 20 μ m in depth) were imaged in 2P and 2P-STED mode. A single imaging session lasted for ~1 hour and mice woke up in their home cage afterwards. All mice survived the imaging sessions and recovered normally from the anesthesia.

Image analysis

The 2P and 2P-STED images were exported as 16-bit tiff files to ImageJ. Images were spatially filtered (1 pixel median filter) and their brightness and contrast were individually adjusted. Optical resolution of the 2P-STED microscope was assessed by taking images of 40 nm fluorescent nanospheres. We measured 2-pixel line profiles across the nanospheres and perpendicular to the spine neck, and fitted them with a Lorentzian function, whose fullwidth at half maximum (FWHM) served as a measure of the spatial resolution and neck width, respectively. Notably, paired spine neck measurements were restricted to spine necks which were clearly delineated in both, 2P and 2P-STED acquisitions. Spine density was determined as described before ^{16, 46, 47}. All dendritic spines, which extended out laterally from the dendritic shaft by more than 400 nm, were counted by manually scrolling through the z-stacks. Spine density was calculated as the number of spines divided by the dendritic length in micrometers. For the 5-day time-lapse imaging data set, the spine density of each dendrite was determined blindly with respect to the time point of acquisition. Lost and new spines over the 5-day interval were identified by scrolling through the z-stacks in a chronological order. Spines were scored lost, if they were under the 400 nm threshold. Spines were considered new, if their position on the dendrite relative to neighboring spines shifted by \geq 500 nm^{16, 47, 48}. The survival fraction of spines (F_s) was calculated as the number of remaining spines at day t ($N_r(t)$) divided by the number of spines at day 0 (N(0)), expressed in percent:
$$F_s = \frac{N_r(t)}{N(0)} \times 100 \tag{1}$$

The turnover rate of spines (TOR) between two imaging sessions was determined by dividing the number of disappearing spines on day t ($N_d(t)$) by the average of the total number of spines present on day t (N(t)) and the following imaging session (N(t+1)), expressed in percent:

$$TOR = \frac{N_d(t)}{(N(t) + N(t+1))/2} \times 100$$
 (2)

The fraction of new spines (F_n) was assessed for each time point by dividing the number of spines that appeared on day t ($N_n(t)$) by the total number of spines (N(t)):

$$F_{new} = \frac{N_n(t)}{N(t)} \times 100 \tag{3}$$

Quantification and statistics

Quantifications and statistical analysis were performed using Microsoft Excel and GraphPad Prism 7 (GraphPad Software, Inc.). Paired t-tests were performed to establish statistical significance. All values are represented as mean ± sem, unless stated otherwise.

Acknowledgements

This work was supported by the DZNE, grants from the Deutsche Forschungsgemeinschaft (SFB1089; C01 and B06), CoEN (CoEN3018) and the EU (ERA-NET MicroSynDep) to MF, the Fondation pour la Recherche Médicale (DEQ20160334901) and CoEN to UVN. TP was supported by a Boehringer Ingelheim Fonds PhD fellowship and a PhD extension grant from the Fondation pour la Recherche Médicale (634FDT20160435677). We thank Mirelle ter Veer for her contribution to setting up the STED microscope, all lab members for helpful discussions and the DZNE light microscopy and animal research facility for constant support.

Author contributions

TP optimized the 2P-STED microscope, performed the imaging experiments and analysed the data. SP performed the surgery, carried out pilot experiments and analysed the data. SB performed imaging experiments. VGGKI optimized the 2P-STED microscope, KK provided technical assistance. MF and UVN designed and supervised the study. TP, MF and UVN wrote the paper.

Competing financial interests

The authors declare no competing financial interests.

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Figure Legends

Figure 1: 2P- STED microscopy of dendritic spines in the hippocampus in vivo

A) Simplified schematic of the custom-built upright 2P-STED microscope. A Ti:Sapphire laser emits light pulses at 834 nm with 80 MHz repetition rate. The laser pumps an optical parametric oscillator (OPO) to obtain pulsed STED light at 598 nm. A glass rod and a polarization-maintaining fiber (PMF) stretch the STED pulses. The STED doughnut is engineered by a helical 2π phase mask in combination with $\lambda/2$ and $\lambda/4$ wave plates. The second Ti:Sapphire laser emitting light at 900 nm with 80 MHz repetition rate served for twophoton (2P) excitation and is synchronized to the STED light pulses. The 2P and STED beam are combined using a dichroic mirror (DM1). Both beams were scanned over the specimen using a x-y scanner and a z-focusing device. Scan (SL) and tube lens (TL) combination allow for overfilling of the objective's back aperture and for imaging the scanner on the back focal plane of the objective. Fluorescence is de-scanned and guided to the avalanche photodiode (APD) via a dichroic mirror (DM2). Electro-optical modulators (EOM) serve for quick adjustments of the beam intensity. B) Quantification of the resolution on 40 nm fluorescent nanospheres. Upper panel: Representative comparison of 40 nm beads imaged by 2P and 2P-STED microscopy. Lower panel: Paired comparison of mean full-width at half maximum (FWHM) of line profiles obtained from 40 nm nanospheres. C) Schematic visualizing the combined use of the hippocampal window preparation and a long working distance objective (top). A 2P overview image showing the cell body and basal dendrites of a GFP-labeled pyramidal neuron in hippocampal CA1 (bottom). Maximal intensity projection (MIP) of ten z-planes. D) Representative images obtained from a GFP-labeled dendrite acquired by 2P and 2P-STED microscopy. Insets show Lorentzian fitted line profiles and FWHM values obtained from the indicated dotted lines of the dendritic spine neck (MIP of three z-planes). E) Paired measurements of spine neck widths imaged in 2P and 2P-STED mode.



Figure 2: Measurements of dendritic spine density of CA1 pyramidal neurons *in vivo*

A) CA1 basal dendrite imaged with either 2P (top) or 2P-STED (bottom) microscopy. White filled arrowheads highlight spines discerned by 2P-STED, but not 2P microscopy. White open arrowheads indicate spines exclusively resolved by 2P-STED microscopy that were otherwise buried in the blurry dendritic fluorescence in the 2P image. MIP of two z-planes. B) Measured spine densities in subsequent 2P and 2P-STED acquisitions of the same dendrites (n = 82 basal dendrites, 6 mice; p < 0.0001, paired t-test). C) Percentage increase in spine density comparing 2P-STED with 2P imaging. Fine grey lines (A) and circles (B) represent the underlying results for each mouse.



Figure 3: Turnover rate of dendritic spines on basal dendrites in vivo

A) 5-day imaging of basal dendrites in hippocampal CA1 using 2P-STED microscopy. The upper panel shows 2P low-magnification overviews over the 5-day period containing the dendrite of interest highlighted with a white box. The lower panel shows the corresponding 2P-STED images of the dendrite over time. The images represent single z-planes. Dendritic spines with blue arrowheads are stable between imaging sessions. Red arrowheads mark spines which are lost and newly appearing spines are outlined with green arrowheads. The axonal bouton (AB) is marked by a white arrowhead. The numbering of spines is continuously. B) Quantification of the spine density over 5 days (n = 14 dendrites, 3 mice). C) Quantification of the 5-day survival fraction of dendritic spines. D) Turnover rate of spines. E) Fraction of new spines. Fine grey lines represent the underlying measurements of single dendrites.

Supplementary Information

Chronic STED microscopy reveals high turnover of dendritic spines in the hippocampus *in vivo*

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Supplementary Figure 1



Supplementary Figure 1: FWHM measurements of dendritic spine necks

2P-STED image of a dendritic stretch with intensity profiles (orange), Lorentzian fit (white) and FWHM measurements (white horizontal line). 2-pixel line profiles were taken on single planes at the locations indicated by the white dotted lines (for illustrative purposes). FWHM values indicate that 2P-STED microscopy allows for sub-diffraction imaging in the hippocampus *in vivo*. MIP of three z-planes of a GFP-labeled basal dendrite imaged ~10-15 µm below the coverslip.

Supplementary Figure 2







Supplementary Figure 2: 2P-STED imaged dendrites exemplifying the reason for obtaining increased spine densities

A, B, C) Dendrites imaged consecutively with 2P (left) and 2P-STED mode (right panel). The 2P-STED images consistently resolved more details, which led to higher spine density measurements. This was due to the fact, that i) two spines could be distinguished which previously appeared as one in the corresponding 2P image (filled white arrowheads) and ii) that spines could be detected which were previously buried in the fluorescence signal of the dendritic shaft (empty white arrowheads). MIP of three z-planes.

Supplementary Figure 3



Supplementary Figure 3: Short-term time-lapse imaging of hippocampal dendrites *in vivo*

Repetitive 2P-STED imaging of hippocampal dendrites is feasible in the absence of visible photo toxicity and STED light induced spine turnover. MIP of three z-planes of a YFP-labeled dendrite imaged every 10 minutes.

Supplementary Movie 1



Supplementary Movie 1: 2P image z-stack in *stratum oriens* of hippocampal CA1 *in vivo*

The hippocampal window preparation offers excellent tissue stability for imaging in the hippocampus of the anesthetized mouse. The 2P image z-stack (100 x 100 x 60 μ m³; 200 nm pixel size; 2 μ m z-steps) illustrates the virtual absence of major motion artifacts, which would impede the acquisition of super-resolved STED images. A) Surface of the stack. B) MIP of the stack. Note locally restricted vibrations on the neuronal cell body (lower left) originating from blood vessel pulsation.

Discussion and perspectives

In this thesis, I have contributed to the advance in knowledge and tools to study microglia-synapse interactions and dendritic spines in acute brain slices and in the hippocampus of the living mouse at unprecedented resolution.

My microglia work demonstrates that microglia increase their surveillance activity and intensifies its physical interaction with dendritic spines after induction of hippocampal plasticity. My findings are in line with recent work and supportive of a potential microglial contribution to activity-dependent synaptic remodeling in the adult brain (Sipe et al., 2016).

In terms of microscopy-based approaches, I have explored and established first super-resolution 2P-STED imaging of dynamic microglia-spine interaction in acute brain slices and the tracking of hippocampal dendritic spines over days *in vivo*.

The results of my thesis work are organized in three parts, which contain published and submitted manuscripts. The discussion of the results obtained and related technical limitations have been covered in the discussion of each manuscript.

In the following, I will thus take a step back and situate my thesis work relative to the recent developments. In addition, I will illustrate open questions to which the application of the here presented STED-based approaches might act as a bottle opener. Finally, I will conclude with ongoing and planned technological developments which aim at improving the performance of the presented super-resolution-based imaging approaches.

Microglia: Recent developments and ongoing challenges

Research on brain microglia, whose origin and competencies are now becoming clear(er), has diversified and forged ahead in the course of my PhD. Investigating sex differences in microglia is as much *en vogue* as identifying microglia-related gene clusters in disease. Extending the research on the detrimental impact microglia can have on their environment and employing top notch technology (transcriptomics and CSF1R-dependent microglial elimination), aberrant microglial function is considered as one of the likely sources of diseases with a major socio-economic impact on our society, such as Alzheimer's disease, Lupus and bad recovery after

ischaemia following stroke (Bialas et al., 2017; Hong et al., 2016; Szalay et al., 2016).

Despite these new fascinating directions in microglia research, my work integrates in the original and ongoing efforts to determine the nature and functional relevance of microglia-neuron interactions. Its timeliness is reflected by a recent study conducted in a mouse model of Alzheimer's disease, where optogenetically restored brain γ -oscillations re-initiated microglia to remove disease-associated amyloid-beta protein (laccarino et al., 2016). Hence, which factors alert and institute microglia to increase their phagocytic activity constitutes a pressing question. Moreover, the underlying signaling pathway of microglia-neuron crosstalk potentially represents novel avenues for therapeutic treatment.

Microglial heterogeneity - among cells and/or processes?

More fundamental than gender differences, I am intrigued by the idea of microglial heterogeneity. My data on microglial morphological dynamics shows that microglia react to the induction of hippocampal synaptic plasticity by increasing their number of processes and changing their interaction with dendritic spines. However, my experiments also depict that the LTP-induced changes in microglial dynamics are variable (in terms of scanning density and number of processes, as well as the detected onset). Of course, in part the heterogeneous response is most likely due to the experimental paradigm and biological variance. The used HFS protocol does not potentiate all CA1 dendrites and synapses, and in addition we do not know the ones who are. Hence some studied microglia might have been close to potentiated synapses, whereas others not at all.

However, beyond this experimentally-derived heterogeneity, I believe that microglia co-habiting one brain region, such as the hippocampal CA3 and CA1, are potentially composed of a heterogeneous cell population. Indeed, state-of-the-art transcriptomics suggest that across basal ganglia nuclei distinct region and cue specific microglial phenotypes exist (De Biase et al., 2017). However, their function and how flexibly microglia are able to switch between these remains to date elusive. Yet, this may imply, that different microglia carry out different tasks, and indeed based on their juxtaposed cellular neighbors microglia have already been affiliated to the axon initial segment (Baalman et al., 2015), perivascular space (Barón and Gallego, 1972), and satellites to the cell bodies of CA1 pyramidal neurons (Wogram

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et al., 2016). Analogously, "a microglia" may exist which is sensitive to changes in neuronal activity and gets involved in its regulation on the synaptic level. Assuming their existence, their identification and subsequent targeted manipulation would constitute a precious tool to advance our understanding of how microglia contribute to brain function.

Another intriguing possibility, which could co-exist or replace the above scenario, is that microglial processes are actually task-assigned. Similarly to neurons and astrocytes, which are composed of specialized neurites (axons and dendrites) and astroglial processes (juxtaposed to synapses and vascular endfeet), microglia could also possess a diverse set of processes whose responsibilities divide, for instance in surveying blood vessels and scanning synapses. Indeed, EM work indicates that not all microglial processes contain Iba-1 (a microglial calcium binding protein), and that processes highly enriched in Iba-1 preferentially interact with synaptic compartments. Moreover, these processes seem specialized, since they are containing various subcellular organelles and vesicular structures (Sogn et al., 2013), which is suggestive of a gliotransmitter-based communication with the tripartite synapse, a concept called the quad-partite synapse (Schafer et al., 2012b).

An elegant way to investigate whether microglia and/or their processes are affiliated with a specific cellular partner in their environment and to determine the "plasticity" of this assignment constitutes the SUSHI technique. SUSHI, recently developed in my PhD lab, stands for super-resolution shadow imaging. In short, it enables the visualization of literally all brain cells (to date, in an organotypic slice), by labeling the extracellular space through bathing the sample in an artificial cerebrospinal fluid (ACSF) solution containing a fluorescent dye, such as Alexa488 (or similar). Complemented by the use of a custom-built 3D-STED microscope and hence a near isotropic resolution in all three dimensions, it permits an unprecedented view at all cellular structures and their juxtapositions in living brain tissue. Importantly, since cellular structures are not filled with a fluorophore and the dye can freely diffuse throughout the tissue, extensive nanoscale time-lapse imaging is possible without detectable photo-toxicity and bleaching. By translating this technique to the in vivo situation (for proof of principle using 2P imaging see Figure 10) and using a CX3CR1-GFP mouse, it will be feasible to put microglial morphological dynamics and interactions in the context of all brain cells at the same time. While the delivery of the fluorescent dye to the living mouse needs further refinement, optical

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development, such as aberration-correcting optics and 3D-STED, is necessary to achieve this goal (see below for technological development).

SUSHI imaging in hippocampal CA1 in vivo using 2P microscopy



SUSHI imaging enables to visualize putative synapses



Figure 10: *In vivo* SUSHI imaging in hippocampal CA1 using 2P microscopy. Imaging has been performed in CA1 *stratum pyramidale* of anesthesized Thy1-M^{+/eGFP} mice. A subset of pyramidal neurons is labeled with GFP (green). Extracellular space has been labeled with Atto514. The image z-stack was acquired 10 – 17 µm below the hippocampal window. 100 nm pixel size. Fluorophores were spectrally detected and unmixed. The look-up table for the extracellular space was inverted, and thus unlabeled cellular structures appear white. The z-stack shows GFP-positive dendrites and axons, and unlabeled dendrites and likely the cell body of a CA1 pyramidal neuron. The insets 1 and 2 are magnified below. Likely, GFP labeled axons synapse on the unlabeled dendritic spines (white boxes).

Microglia-astrocyte communication in the healthy adult brain

The idea of the quad-partite synapse suggests a reciprocal crosstalk of astroglia and microglia with pre- and postsynaptic elements (Schafer et al., 2012b). While for both, astrocytes and microglia, their separate contribution to synaptic function in the

healthy brain is appreciated (although to different extents), a very fascinating interaction is still understudied: the crosstalk of microglia and astrocytes in the healthy adult brain.

Developmental studies in the retinogeniculate system suggest an intricate cooperation of astrocyte and microglia during synaptic pruning. Up-regulation of the initiating complement factor C1q is stimulated by nearby astrocytes and leads to the deposition of C3 on target synapses, which will then be removed by microglia (Schafer et al., 2012a; Stevens et al., 2007). Besides the legwork, astrocyte can also mediate synapse elimination (Chung and Barres, 2012; Chung et al., 2013, 2016). However, does this redundancy serve as backup strategy, if one of the two cells fails, or do astrocytes and microglia have distinct tasks / targets? If the latter is true, does for this purpose exist a "on and off" signaling between the two glial cells?

Deciphering a potential crosstalk would be relevant for the mature brain. In the hippocampal CA1 approximately 80% of the postsynapses are covered by an astrocytic process. Are non-covered synapses weak or silent synapses, and do microglia preferentially contact these? Hence resembling their function in brain development, aided by astrocytes, it is conceivable that microglia identify and remove weak synapses in the adult brain. Yet, it is still elusive whether the complement factor system is also involved in activity-dependent synaptic circuit remodeling in the healthy adult brain (see *Figure 11*).

Microglia can manipulate synaptic transmission through ATP-induced astrocytic glutamate release and subsequent increased miniature EPSCs (Pascual et al., 2011). In turn, postsynaptic NMDAR activation and subsequent Ca²⁺ influx leads to postsynaptic ATP release (Dissing-Olesen et al., 2014; Eyo et al., 2014), which attracts microglial processes in a P2Y12R-dependent manner. However, given the rapid enzymatic degradation of ATP, astrocytes may act as crucial signal amplifiers. It is thus conceivable that astrocytes point out active synapses to microglia, which in turn exercise their phagocytic capacity and/or release regulatory signaling factors on the synapse (see *Figure 11*). Hence, purinergic signaling likely underpins microglia-astrocyte communication (Eyo et al., 2014; Pascual et al., 2011). However, since purinergic receptors and their agonists (ATP and ADP) are omnipresent in the brain, isolated examination in *in situ* conditions is challenging. Here, ATP uncaging and novel transgenic ATP sensors (Trevisiol et al., 2017) may help to localize sources and targets.



What is the functional impact of microglia-spine interactions?

Figure 11: The role of microglia in spine structural dynamics. Microglia may contribute to structural remodeling of dendritic spines via their phagocytic activity or the release of modulatory factors. Through the complement factor system and purinergic signaling microglia potentially retrieve dendritic spines of interest in the adult brain.

Microglial remodeling of synapses in the healthy adult brain

In the course of my PhD. I established STED imaging of the dynamic microgliaspine interactions in acute brain slices (see Figure 12). Follow-up work (possibly complemented by targeted activation of single dendritic spines through 2P glutamate uncaging) may dissect these interactions more thoroughly, and test the impact on the morphology and function of the dendritic spine. Importantly, through targeted activation of spines, it will allow for probing whether and how microglia contribute to remodeling of the synaptic circuitry (see Figure 11 and 12). Interestingly, resembling the microglia contact-induced filopodia formation in developing somatosensory cortex (Miyamoto et al., 2016), I have observed microglia processes juxtaposed to spinule-carrying dendritic spines of mature Layer 5 pyramidal neurons (see Figure 12A). These spinules (spine head protrusions) are thought to reflect the postsynaptic probing for active presynaptic partners (Richards et al., 2005; Ueda and Hayashi, 2013). Conversely, the contact of a spine cluster can also be correlated with subsequent spine structural changes (see Figure 12B). Yet, whether microglia are causally involved in these kind of synaptic remodeling in the adult remains to be determined.



A Microglia-spine interaction and spinule formation

B Microglia-spine interactions and spine structural changes



Figure 12: Microglia-spine interactions in the healthy adult brain. Time-lapse STED imaging in acute brain slices obtained from $Cx3Cr1^{+/GFP} \times Thy1-H^{+/YFP}$ mice. **A)** Microglia-spine interactions correlate with spinule formation (white arrowheads). MIP of 2 z-planes of a L5 apical oblique dendrite 55 µm below the slice surface. 40 nm pixel size. **B)** Microglial processes (green) engulf a cluster of dendritic spines (yellow). A filopodia-like spine (white arrowhead) shrinks during the observation period. MIP of 2 z-planes of a L5 apical oblique 45 µm below the slice surface. 40 nm pixel size.

Structural plasticity of hippocampal dendritic spines in vivo

Our *in vivo* hippocampus work indicates that unlike cortical dendritic spines which are rather stable in the adult brain, dendritic spines in CA1 *stratum oriens* are structurally very dynamic resulting in 40% turnover in 5 days. A complementary labeling of PSD95 would resolve whether the disappearing spines have likely been functional synapses and thus help to determine whether the reported dendritic spine turnover reflects synaptic circuit remodeling. Moreover, I am particularly excited to examine how astrocytic and microglial coverage correlate in this context with spine turnover and stability.

The hippocampal spine - a physical memory trace?

To date, the most convincing piece of evidence advocating for dendritic spines as physical traces of memory represents work from the Kasai lab. Eliminating few learning-associated spines in the mouse motor cortex proved to be sufficient to omit memory formation (Hayashi-Takagi et al., 2015). For deciphering whether similar to motor cortex interfering with a few learning-associated hippocampal spines would disrupt hippocampal memory, the here presented *in vivo* STED approach needs to be combined with a hippocampus-dependent memory task (such as the Morris water maze). Hence, by making use of AS-PaRac1 (Hayashi-Takagi et al., 2015), an opto-probe which modulates potentiated spines in response to light, the involvement and necessity of single hippocampal spines for memory recall can be probed.

Are hippocampal spine ensembles underpinning CA1 place cell activity?

The orderly activation of hippocampal neuronal ensembles is considered to underpin major cognitive processes, including spatial memory and place cell firing (Colgin and Moser, 2006). CA1 hippocampal ensembles can be readily examined by reading out neuronal Ca²⁺ signals when an awake mice runs on a treadmill (Malvache et al., 2016). However, due to a lack of sophisticated imaging approaches, nothing is known about it on the synaptic level. After extending in vivo STED imaging to the awake mouse and through combination with functional optical readouts (Ca²⁺ imaging using a red-shifted genetically encoded calcium indicator (GECI)), identification of dendritic spines active at specific locations while running on a treadmill would be possible. Such an experimental paradigm, equally difficult and insightful, would for instance allow for testing whether place cell firing is underpinned by an ensemble of co-active "place spines". Secondly, by making use of optogenetic tools, such as the AS-PaRac1 (Hayashi-Takagi et al., 2015), the involvement and necessity of single hippocampal spines for place cell formation could be elucidated. Finally, these spine ensembles may represent the building blocks for encoding or retrieving experience.

AO-based 3D STED imaging of hippocampal dendritic spines in vivo

The presented *in vivo* STED imaging approach builds up on an outstanding tissue stability provided by the chronic hippocampal window preparation (Gu et al., 2014;

Schmid et al., 2016). During image acquisition, artifacts arising from blood vessel pulsation and breathing, were either absent or restricted to the immediate vicinity of blood vessels. However, optically, we can still do better. At the current stage, imaging is performed using a long-working distance, NA 1.0, water-immersion objective without correction collar (Nikon N60X-NIR). We demonstrated that the objective is compatible with the acquisition of super-resolved images of immobilized 40 nm fluorescent beads and hippocampal dendritic spines *in vivo*. Yet, we were restricted to the imaging of dendrites in $5 - 20 \,\mu$ m depth below the coverslip. Due to the degradation of the STED PSF over depth the STED image quality deteriorated rapidly. In addition, gain in resolution using our 2P-STED microscope is still limited to the x-y image plane. Consequently, spines orthogonal to the imaging plane still suffer from a diffraction-limited z-axis resolution.

Hence, aberration-reducing optics (Booth, 2011; Gould et al., 2012) paired with a 3D-STED approach (Wildanger et al., 2009) might help to overcome these limitations. Recent efforts to improve the microscope were thus directed towards implementing adaptive optics (AO). To this end, we purchased a spatial light modulator (SLM)-based system from Abberior (Göttingen, Germany). This device allows interfaced wavefront shaping, and hence, conveniently combines the capacity for compensation of optical aberrations (such as spherical and coma) with the design of a 2D STED doughnut and a 3D bottle beam. We thus expect a considerable boost in lateral and axial resolution (x-y: ~50-70 nm; z: 200-300 nm), and depth-penetration (50 – 100 μ m). Once implemented and aligned, the Abberior SLM system is more intuitive to handle and the STED doughnut quality more reproducible through parameterized PSF design, than manually adjusting a helical phase mask (for 2D STED) and a phase mask imposing a centered π delay (for 3D STED). In sum, through this development, we expect to resolve (almost) all dendritic spines of hippocampal pyramidal neurons in CA1 stratum oriens and tracking them over days to months. Moreover, enabled by an AO-based 3D-STED imaging approach in vivo SUSHI experiments become feasible (see above).

Proximity detection

Besides improving the excitation pathway, advantageous technological refinement of the 2P-STED setup would include the replacement of the avalanche photodiodes (APD) by proximity detection. For historical reasons (demonstrated performance in confocal STED microscopes), APD-based de-scanned detection has been chosen for building the 2P-STED microscope (Bethge et al., 2013). While APDs allow good signal-to-noise ratio and low dark noise, due to the small active area the emitted photons have to be delivered via an optical fiber. Hence, the coupling of the emission beam into the fiber acts as a confocal pinhole, which is not needed in 2P microscopy. Moreover, through loss during the coupling, precious emitted photons get lost. Therefore, to increase significantly the detection efficiency proximity detection should be employed. For this purpose, photo-multiplier tubes (PMTs) have been classically used in 2P microscopy. PMTs have a larger active area and thus when installed close to the objective's back focal plane the collection of scattered photon is considerably improved. However, PMTs are less sensitive than APDs, represented by their lower quantum efficiency for 400 - 500 nm wavelengths (~25% versus ~45%, respectively; quantum efficiency describes the ratio of incident photons and photoelectrons generated). Hybrid detectors (HyD), such as GaAsP-HyD, constitute a compromise between APDs and PMTs and are thus frequently installed on 2P microscopes. HyD have a quantum efficiency of 45%, quicker response time than PMTs, and though they have a smaller active window than PMTs, HyD can be used in proximity detection. The installation of proximity detection on the 2P-STED setup would be rather simple, since it suffices to install a dichroic mirror between the tube lens and objective, and the detectors.

Concluding remarks

Firstly, my microglia work is supportive of a microglial contribution in activitydependent synaptic plasticity in the adult brain, and sets the stage for a more in depth exploration of microglia-synapse interactions. Secondly, the methodological developments provide novel super-resolution-based imaging approaches beyond the classical tool set of neurophysiologists, which enable the detailed examination of dynamic microglia-synapse interactions and hippocampal dendritic spines in the living brain (tissue). Thirdly, longitudinal *in vivo* STED imaging enables the testing of classical hippocampal plasticity mechanisms developed from *in vitro* studies in the living mouse. Finally, through the discussed technological development complemented with awake imaging, Ca²⁺ imaging, SUSHI, and behavioral learning paradigms the quality and the scope of this *in vivo* super-resolution imaging approach will be further augmented. Thus enabling the investigation of cellular mechanisms associated with learning and memory in the behaving mouse.

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Curriculum vitae

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09/2010 – 10/2012	<i>Master of Molecular Biosciences, Major Neuroscience</i> University of Heidelberg, Germany
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Research Experie	ence
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04/2010 – 08/2010	Bachelor Thesis with Dr. Udo Kraushaar and Prof. Gisela Drews, Natural and Medical Sciences Institute NMI, University of Tübingen, Germany
12/2009 – 03/2010	<i>Student Assistant with Prof. Sven Ingebrandt,</i> Department of Informatics and Microsystemstechnology, University of Applied Sciences Kaiserslautern, Germany
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Publications

[1] Two-photon STED microscopy for nanoscale imaging of neural morphology *in vivo*

ter Veer MTV*, <u>*Pfeiffer T**</u> and Nägerl UV; Methods in Molecular Biology **2017** 1663:45-64.

[2] Induction of hippocampal long-term potentiation increases the morphological dynamics of microglial processes and prolongs their contacts with dendritic spines

<u>Pfeiffer T</u>, Avignone E[#] and Nägerl UV[#]; Scientific Reports **2016** 6:32422.

- [3] Optimized temporally deconvolved Ca²⁺ imaging allows identification of spatiotemporal activity patterns of CA1 hippocampal ensembles <u>Pfeiffer T</u>, Draguhn A, Reichinnek S[#], Both M[#]; NeuroImage 2014 94:239-249.
- [4] Rapid functional evaluation of beta-cells by extracellular recording of membrane potential oscillations with microelectrode arrays <u>Pfeiffer T</u>, Kraushaar U, Düfer M, Schönecker S, Haspel D, Günther E, Drews G, Krippeit-Drews P; Pflugers Arch – Eur J Physiol 2011 462:835– 840.

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Awards and Fellowships

- 10/2016 Fondation pour la Recherche Médicale PhD Fellowship (12 months)
- 06/2013 Boehringer Ingelheim Fonds PhD Fellowship (*30 months*)
- 09/2011 Training Fellowship, KOOR BEST Erasmus Mundos, UV Nägerl Group, University of Bordeaux, France (*4 months*)
- 03/2011 Best Poster Award, 90th Annual Meeting of German Physiological Society 2011
- 08//2009 Travel Grant, Auslands-BAföG, JA Rudd Group, The Chinese University of Hong Kong, China (*4 months*)

Invited Seminars and Talks

- 11/2017 Klaus-Armin Nave, MPI Göttingen, Germany
- 06/2017 CNRS Course on Super-resolution Microscopy, Bordeaux, France
- 10/2015 Beth Stevens, Harvard University, USA
- 10/2015 Wenbiao Gan, New York University, USA
- 10/2015 GliSyn, International Bordeaux Neurocampus Conference, France
- 03/2015 13th Synapse Day Meeting, University of Bordeaux, France
- 09/2014 30th Boehringer Ingelheim Fonds Summer Seminar, Hirschegg, Austria
- 11/2013 Stephane Oliet, Neurocentre Magendie, Bordeaux, France
- 01/2013 Frank Kirchhoff, Institute of Molecular Physiology, University of Saarland, Germany

Conference contributions

- 2017 5th Frontiers in Neurophotonics, Bordeaux, France
- 2016 Jacques Monod Conference Optical imaging of brain connectivity, Roscoff, France
- 2015 45th Annual Meeting of the Society for Neuroscience SFN, Chicago, USA
- 2014 9th Forum of Neuroscience FENS, Milan, Italy
- 2013 3rd International Symposium Frontiers in Neurophotonics, Bordeaux, France
- 2013 European Synapse Meeting, Bordeaux, France
- 2013 10th Göttingen Meeting of German Neuroscience Society, Göttingen, Germany
- 2013 92th Annual Meeting of the German Physiological Society, Heidelberg, Germany
- 2011 90th Annual Meeting of the German Physiological Society, Regensburg, Germany
- 2010 7th International Meeting on Substrate Integrated Microelectrode Arrays, Reutlingen, Germany
- 2010 Stem Cells and Neuroregeneration COST-B30 Meeting, Harlem, Netherlands