Analysis of the synaptic localization of the FUS RNA-binding protein with high- and super-resolution microscopy

Dissertation to achieve the medical doctoral degree of the medical faculty of Ulm University

Michael Schön
Wasserburg am Inn
2017
Current dean of the faculty: Prof. Dr. rer. nat. Thomas Wirth

1. Supervisor: Prof. Dr. med. Tobias M. Böckers
2. Supervisor: Prof. Dr. med. Christine von Arnim

Day doctorate awarded: 08.02.2018
Declaration with reference to the Creative Commons Attribution License

Parts of this dissertation have already been published (Schoen et al. 2016; https://doi.org/10.3389/fncel.2015.00496) and are protected under the Creative Commons Attribution License (CC BY 4.0). The affected figures or parts of figures (figures 3, 6, 11, 12, 13, 19, 20, 22, and 24) are indicated with the following term: CC BY 4.0, https://creativecommons.org/licenses/by/4.0/

Gene and protein nomenclature

Genes, gene transcripts, and proteins are denoted according to the HUGO Gene Nomenclature Committee.
Dedication

This is to my beloved eldest brother Johannes (*26.02.1978 – † 05.08.2003) and to Marie-Christine Pfaffendorf (*22.09.1981 – †23.01.2000). Tini was an angel. My brother Hansi awakened the enthusiasm for science in me and showed me how to get along with difficulties in life. He was the most sincere, disciplined, and creative person, I had the privilege to encounter in my life. Our conversations about astronomy and philosophy belong to the greatest moments in my life. A big part of me is strongly influenced by him.

This is also dedicated to my parents, to my twin sister Anna, my brother Tobias with his three lovely children, Lukas, Felix, Aurelia, and to my godson Johann. I consider it as a privilege to have a family I can always rely on. Anna helped me through tough times, and Tobias gave us his wonderful children. They offer our family confidence and happiness. My siblings supported me overwhelmingly in my life. My parents blessed me with love and have always stood by me in a way I cannot express in words. Their infinite and unconditional love offered me a childhood, which gives me strength for the rest of my life. My parents’ sincerity and moral principles serve as a model. They encouraged me to work on the realization of my dreams to become a physician and researcher.
Contents

Contents

Abbreviations

1 INTRODUCTION

1.1 The conformational diseases amyotrophic lateral sclerosis and frontotemporal dementia

1.2 The RNA-binding protein FUS

1.3 FUS pathologies in neurodegenerative diseases

1.4 Synaptic FUS in the central nervous system

1.5 Super-resolution microscopy

1.6 Aims of the thesis

2 MATERIALS and METHODS

2.1 Materials

2.1.1 Chemicals

2.1.2 Solutions and mixtures

2.1.3 Tools, devices, softwares, and webpages

2.1.4 Organisms and biological materials

2.1.5 STED setup

2.1.6 dSTORM setup

2.2 Methods

2.2.1 Cell culture and immunocytochemistry

2.2.2 Conventional fluorescence microscopy and image analysis

2.2.3 Super-resolution fluorescence microscopy and image analysis

3 RESULTS

3.1 Analysis of synaptic FUS in mouse brain and spinal cord tissue

3.1.1 Description of the analyzed mouse CNS structures

3.1.2 FUS with synaptic markers in mouse hippocampus

3.1.3 FUS with synaptic markers in mouse neocortex and cerebellar cortex

3.1.4 FUS with synaptic markers in mouse lumbar spinal cord
3.2 FUS in primary rat hippocampal neurons
   3.2.1 FUS expression in primary neuronal and glial cells
   3.2.2 FUS localization at synapse subtypes of primary hippocampal neurons
3.3 Sub-synaptic localization of FUS with super-resolution microscopy
   3.3.1 FUS and synaptic markers in STED microscopy
   3.3.2 Optimization of the dSTORM staining protocol
   3.3.3 Super-resolved imaging of reference synaptic markers with dSTORM
   3.3.4 Super-resolved FUS with pre- and postsynaptic markers in dSTORM
   3.3.5 Axial synaptic distributions of FUS and reference synaptic markers in dSTORM
   3.3.6 Distribution analysis of FUS at synaptic vesicles with dSTORM
   3.3.7 FUS distribution in glutamatergic hippocampal synapses

4 DISCUSSION
   4.1 Main findings
   4.2 FUS localizes to excitatory synapses in various regions of the mouse CNS
   4.3 Glutamatergic synapses of primary rat hippocampal neurons contain FUS
   4.4 SMLM reveals presynaptic FUS localization at glutamatergic synaptic vesicles
   4.5 Outlook: Putative roles of presynaptic FUS in physiological and in diseased states
      4.5.1 Possible functions of FUS at the presynapse
      4.5.2 Putative roles of presynaptic FUS in pathological states
   4.6 Conclusion

5 SUMMARY

6 REFERENCES

Acknowledgments

Curriculum vitae
**Abbreviations** (in alphabetical order)

Ø  on average; average distance (dSTORM data)
2D-COM  center(s) of mass in a two-dimensional area
aFTLD-U  atypical frontotemporal lobar degeneration with ubiquitin inclusions
ALS  amyotrophic lateral sclerosis
ALS-FTD  amyotrophic lateral sclerosis comorbid with frontotemporal dementia
ALS-FUS  FUS-associated amyotrophic lateral sclerosis
ALS-TDP  amyotrophic lateral sclerosis with TDP-43 inclusion pathology
AOTF  acousto-optical tunable filter
approx.  approximately
bassoon(C)  C-terminal epitope of bassoon
bassoon(N)  N-terminal epitope of bassoon
BDNF  brain derived neurotrophic factor
BIBD  basophilic inclusion body disease
BSA  bovine serum albumin
*C. elegans*  *Caenorhabditis elegans*
C9orf72  chromosome 9 open reading frame 72
CA1 – 3  cornu ammonis, sectors 1 to 3
CHAT  choline O-acetyltransferase
CNS  central nervous system
COM  center(s) of mass
DAB  3,3’-diaminobenzidine
DAPI  4’,6-diamidino-2-phenylindole
DG  dentate gyrus
DIV  day(s) *in vitro*
DMEM  Dulbecco’s modified eagle medium
DNA  deoxyribonucleic acid
*Drosophila*  *Drosophila melanogaster*
dSTORM  direct stochastic optical reconstruction microscopy
e.g.  for example
EDTA  ethylenediaminetetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>EM</td>
<td>electron microscopy</td>
</tr>
<tr>
<td>EMCCD</td>
<td>electron multiplying charge coupled device</td>
</tr>
<tr>
<td>epi</td>
<td>epifluorescence microscopy</td>
</tr>
<tr>
<td>EWS</td>
<td>Ewing sarcoma; also EWSR1 (HUGO Gene Nomenclature Committee)</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FET</td>
<td>FUS, EWS, TAF15</td>
</tr>
<tr>
<td>FTD-FUS</td>
<td>FUS-associated frontotemporal dementia</td>
</tr>
<tr>
<td>FTLD</td>
<td>frontotemporal lobar degeneration</td>
</tr>
<tr>
<td>FTLD-FUS</td>
<td>frontotemporal lobar degeneration with FUS inclusions</td>
</tr>
<tr>
<td>FUS</td>
<td>fused in sarcoma; also FUS RNA-binding protein (HUGO Gene Nomenclature Committee)</td>
</tr>
<tr>
<td>FUSopathy</td>
<td>neurodegenerative disease with FUS contribution</td>
</tr>
<tr>
<td>FWHM</td>
<td>full width(s) at half maximum</td>
</tr>
<tr>
<td>GABAergic</td>
<td>gamma-aminobutyric acid transmitting</td>
</tr>
<tr>
<td>GAD65</td>
<td>glutamate decarboxylase 2 (65 kilodalton); also GAD2 (HUGO Gene Nomenclature Committee)</td>
</tr>
<tr>
<td>GCL</td>
<td>granule cell layer</td>
</tr>
<tr>
<td>geph.</td>
<td>gephyrin</td>
</tr>
<tr>
<td>GFAP</td>
<td>glial fibrillary acidic protein</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GluA1</td>
<td>glutamate ionotropic receptor AMPA type subunit 1; also GRIA1 (HUGO Gene Nomenclature Committee)</td>
</tr>
<tr>
<td>GRR</td>
<td>glycine rich region</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank’s balanced salt solution</td>
</tr>
<tr>
<td>i.a.</td>
<td>inter alia</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
</tr>
<tr>
<td>iPSCs</td>
<td>induced pluripotent stem cells</td>
</tr>
<tr>
<td>KO</td>
<td>knockout</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule associated protein 2</td>
</tr>
<tr>
<td>MAPT</td>
<td>microtubule associated protein tau</td>
</tr>
<tr>
<td>ML</td>
<td>molecular layer</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
</tbody>
</table>
MND  motor neuron disease
mRNA  messenger ribonucleic acid
mRNP  messenger ribonucleoprotein particle
mU  milliunit
NES  nuclear export signal
NIFID  neuronal intermediate filament inclusion disease
NLS  nuclear localization signal
NMDAR  N-methyl-D-aspartate receptor; also GRIN (HUGO Gene Nomenclature Committee)
PALM  photoactivated localization microscopy
PAR  poly(ADP-ribose)
PAZ  presynaptic active zone
PBS  Dulbecco’s phosphate buffered saline
PC  Purkinje cell layer
PFA  paraformaldehyde
pH  potentia Hydrogenii
PLL  poly-L-lysine hydrobromide
PO  polymorph layer
pre-mRNA  precursor messenger ribonucleic acid
PSD  postsynaptic density
PSD-95  postsynaptic density protein 95; also DLG4 (HUGO Gene Nomenclature Committee)
PSF  point spread function
RBP  RNA-binding protein
RGG  arginine glycine rich region
RNA  ribonucleic acid
RRIDs  Research Resource Identifiers
RRM  RNA recognition motif
RT  room temperature
S100B  S100 calcium binding protein, beta (neural)
sALS / fALS  sporadic / familial amyotrophic lateral sclerosis
SD  standard deviation
SEM  standard error(s) of the mean
sFTD / fFTD sporadic / familial frontotemporal dementia
SG stratum granulare
SM stratum moleculare
SMA spinal muscular atrophy
SMLM single molecule localization microscopy
SO stratum oriens
SOD1 superoxide dismutase 1
SP stratum pyramidale
SR stratum radiatum
STED stimulated emission depletion
STORM stochastic optical reconstruction microscopy
SYP synaptophysin
SYQG serine, tyrosine, glutamine, glycine rich region
TAF15 TATA-box binding protein associated factor 15
TARDBP see TDP-43
TDE 2,2′-thiodiethanol
TDP-43 TAR DNA binding protein (43 kilodalton)
TIFF tagged image file format
TIRF total internal reflection fluorescence
U/µl units per microliter
U/ml units per milliliter
UV ultraviolet
VGLUT1 vesicular glutamate transporter 1; also SLC17A7 (HUGO Gene Nomenclature Committee)
VGLUT2 vesicular glutamate transporter 2; also SLC17A6 (HUGO Gene Nomenclature Committee)
vol% volume percent
wt/vol weight per volume
ZF zinc finger domain
1 INTRODUCTION

Light microscopy represents one of the most important tools in life sciences. Ever since the initial inventions of microscopic setups dating back over 300 years, researchers are enabled to observe cells and even the structures within, such as organelles and protein complexes. Certain dyes were developed to enhance the contrast of cellular and extracellular structures, whereas specific antibody-based labeling of proteins with fluorescence dyes – also referred to as fluorescence immunolabeling – facilitated specific and quantifiable analyses of proteins. For more than a century, it was believed that light microscopy is restricted by inherent limitations of resolution as a result of the wave nature of light rays (Abbe 1873). As a consequence of this, a maximum resolution – the minimum distance between two discernible objects – of 200 nm in conventional fluorescence light microscopy impedes the observation of structures and single molecules with subdiffraction-scaled distances. The so called diffraction barrier of limited resolution in light microscopy has recently been broken with different technical approaches, which implement either temporally or spatially depleted fluorophores to reduce the overlap of molecules with unresolvable distance. These inventions are summarized under the umbrella term super-resolution microscopy. In this study, two techniques, namely, stimulated emission depletion (STED) microscopy and direct stochastic optical reconstruction microscopy (dSTORM) were employed to analyze the localization of an RNA-binding protein named fused in sarcoma (FUS) at hippocampal synapses. This protein is associated with the devastating neurodegenerative diseases amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Moreover, the study offers a comprehensive overview of synaptic FUS localization with markers for different subtypes of synapses in the central nervous system of mice.

1.1 The conformational diseases amyotrophic lateral sclerosis and frontotemporal dementia

Conformational diseases are characterized by protein accumulations in neuronal cells and underlie a variety of neurodegenerative diseases, including ALS and FTD.

1.1.1 Conformational diseases

As an accepted concept, cells use partly self-organizing, membrane-surrounded organelles for compartmentalization. These organelles fulfill the needs for a restricted
molecular assembly within their membranous boundaries as a prerequisite to orchestrate diverse biochemical reactions. Moreover, the exchange of macromolecules across membranes is strictly regulated and, thereby, time- and energy-consuming. The formation of such organelles is oftentimes complex and tightly organized by nuclear signals.

Recently, a second, membrane-independent concept of compartmentalization in cells has emerged. It involves a variety of prion-like proteins with intrinsically disordered domains, which are also referred to as prion-like domains (Malinovska et al. 2013, Altmeyer et al. 2015, Kai 2016). Under certain physiological conditions, these proteins undergo conformational changes in order to self-aggregate, which results in a phase transition between the accumulating proteins and the surrounding medium. This process is called liquid demixing. These structures are not as well-defined as membranous compartments but offer temporary and versatile protein interactions (Malinovska et al. 2013). Moreover, it is believed that these protein aggregates are highly dynamic in assembly and decomposition, and that they confine the molecular setup within these aggregates in stark contrast to the surrounding cytoplasm. This process can be locally triggered, as has been shown for the prion-like protein FUS to immediately respond to deoxyribonucleic acid (DNA) damage. More precisely, poly(ADP-ribose) (PAR) acts as a sensor for the DNA damage and also a seed for the rapid aggregation of FUS. As a consequence of this, possible interactions of molecules at the damaged site are restricted (Altmeyer et al. 2015, Kai 2016).

Dysregulated self-assembly of proteins due to prion-similar mechanisms underlies a whole class of neurodegenerative diseases, which are not infectious in contrast to prion disease (Jucker and Walker 2013). Neurodegenerative diseases are defined by a progressive loss of neurons and an often sequence-specific affection of certain parts of the central nervous system (CNS). The underlying pathomechanisms include misfolding and aggregation of proteins (Carrell and Lomas 1997, Kopito and Ron 2000, Costanzo and Zurzolo 2013). Therefore, they are also called conformational diseases (Carrell and Lomas 1997) or protein misfolding disorders (Costanzo and Zurzolo 2013). Sharing factors between prion disease and aggregations in neurodegenerative diseases are “nucleation, templating, growth, multiplication and spread” (page 49) (Jucker and Walker 2013).
In the following, a list of conformational, neurodegenerative diseases is presented with the contributing prion-like proteins and their familiar names in brackets: Alzheimer’s disease (microtubule associated protein tau (MAPT = tau) and amyloid beta deposits), Parkinson’s disease (SNCA = synuclein alpha), prion disease (PRNP = prion protein), trinucleotide repeat expansion diseases, including Huntington’s disease (HTT = huntingtin), spinocerebellar ataxia (ATXN = ataxin 1), and ALS together with FTD (TAR DNA binding protein 43 (TARDBP or TDP-43) and FUS) (Kovacs 2016). FUS ranks thirteenth among more than twenty thousand proteins with regard to prion-like properties (Couthouis et al. 2011). This might, in part, explain the contributions of FUS to the pathogenesis of both FUS-associated ALS and FTD.

1.1.2 Amyotrophic lateral sclerosis and frontotemporal dementia

The association of FTD and ALS was neglected for half a century although a 'paralytisches Irresein' (author’s comment: German for paralytic insanity) was already known as a combined disease entity (Ludolph et al. 2012). Later, histopathological and genetical commonalities further confirmed this connection. Nowadays, both diseases are considered as being cross-linked on several levels and can occur as a disease continuum. Mixed phenotypes are, therefore, referred to as ALS-FTD (amyotrophic lateral sclerosis comorbid with frontotemporal dementia). About 15 to 20 % of all ALS patients show signs of FTD and about the same proportion vice versa (Burrell et al. 2016). FTD usually occurs later than the voluntary motor system degeneration. In comparison to the single diseases, survival time is reduced in ALS-FTD. An important pathogenic link between the two interconnected disorders are identical genes as, for instance, FUS, TDP-43, and chromosome 9 open reading frame 72 (C9orf72) (Ludolph et al. 2012). In the following, ALS and FTD will be separately approached with respect to their different clinical picture and partly individual pathomechanisms.

1.1.2.1 Amyotrophic lateral sclerosis

Motor neuron diseases (MNDs) represent a subgroup of neurodegenerative diseases with a selective demise of motor neurons and MNDs consist of spinal muscular atrophy (SMA), progressive bulbar palsy, primary lateral sclerosis, and ALS (Nishimura et al. 2004). Charcot initially described ALS in 1869. The name is derived from his observation of a diminution of axons, which is observable in the lateral masses of the spinal cord’s white matter in patients with progressive paralysis (Boillée et al. 2006).
INTRODUCTION

On average, first symptoms become obvious at the age of 45 to 60 years with only a short time period from initial symptoms to the full-blown picture, which results in an average life expectancy of 1 to 5 years. The lifetime risk is as high as 1 per 1,000. To date, ALS has a fatal prognosis with insignificant therapeutic options to prolong lifetime. Death mostly results from respiratory failure (Boillée et al. 2006).

While 90% of the cases present as sporadic ALS (sALS), approximately 10% are considered to be inherited – mostly in a dominant manner – and are, therefore, termed familial ALS (fALS) (Boillée et al. 2006). More than 30% of all fALS cases can be assigned to a known mutation (Liscic and Breljak 2011). fALS variants with identified genetic causes or known risk loci have been classified as ALS1 to ALS10 as well as a combination of ALS and frontotemporal dementia (ALS-FTD). In this respect, cases with superoxide dismutase 1 (SOD1) mutations are classified as ALS1, FUS and TDP-43 mutations as ALS6 and ALS10, respectively (Ince et al. 2011).

The first causative ALS-associated mutations were found in the gene SOD1 (Rosen et al. 1993), which contributes to about 20% of fALS (Boillée et al. 2006). In the last decade, a variety of studies have identified several novel ALS genes, including TARDBP, FUS, ATXN, UBQLN2, and C9orf72 (Ludolph et al. 2012). DeJesus-Hernandez et al. initially reported about the most common genetic cause in fALS (ca. 25%), which is associated with hexanucleotide expansions in the C9orf72 gene (DeJesus-Hernandez et al. 2011).

Although different genes are involved in ALS, several shared pathogenic mechanisms are hypothesized. A variety of predisposing factors in motoneurons have been discussed, including mitochondrialopathy, hindered axonal transport, glutamate excess, ribonucleic acid (RNA) processing deficits, protein misfolding, and neuroinflammation. Taken together, it is assumed that none of these hypotheses are superior, but that rather a combination of mutually dependent mechanisms most likely represents the processes leading to ALS. This paradigm, hence, increases potential therapeutic targets (Boillée et al. 2006).

1.1.2.2 Frontotemporal dementia

Main symptoms of FTD include cognitive deficits, language impairment, behavioral changes, and social deficits (Dormann and Haass 2011, Aoki et al. 2012). In general, FTD cases are very heterogeneous in terms of the clinical picture, histopathology, and underlying genetic causes (Pan and Chen 2013).
In individuals younger than 65 years, FTD is the second most common variant of presenile dementia (Seelaar et al. 2011, Aoki et al. 2012). FTD is considered to be the most common neurodegenerative diseases after the Alzheimer’s disease (Braak et al. 2013). FTD is largely intractable and has an inevitable fatal prognosis.

Almost 40 to 50 % of all cases exhibit a familial background and are referred to as familial FTD (fFTD) with two outstanding genes, which each account for ca. 50 % of fFTD: mutations in \textit{GRN} and \textit{MAPT} genes (Seelaar et al. 2011, Halliday et al. 2012). Furthermore, in quest of novel causative genes, mutations have been identified in the genes \textit{FUS, VCP, C9orf72,} and \textit{CHMP2B} (Pan and Chen 2013).

\textbf{1.1.3 Histopathology in ALS and FTD}

The histopathology in ALS has to be reviewed with regard to similarities in histological alterations observed in FTD. While FTD denotes the clinical syndrome, frontotemporal lobar degeneration (FTLD) describes the histopathological findings of a predominant deterioration of the frontal and temporal lobe (Neumann et al. 2009a). A ubiquitous phenomenon in ALS and FTLD histopathology is the occurrence of inclusions in neuronal and glial cells. The composition of these protein accumulations depends on the heritability status (sporadic versus familial forms).

A pioneer work from Neumann et al. adverts to a putative link between ALS and FTLD inclusion pathology. Most ubiquitin-positive, tau-negative inclusions in both diseases are TDP-43-positive. Primarily affected regions in both disease entities are the cortex, the hippocampus, and the spinal cord (Neumann et al. 2006). Thus, a new class of neurodegenerative diseases, called TDP-43 proteinopathies, was postulated. A central role of TDP-43 is hypothesized due to its presence regardless of the individual genetic causes (Cairns et al. 2007). The contribution of ALS with TDP-43-positive inclusions (ALS-TDP) is as high as 97 % of all ALS cases (Mackenzie et al. 2007). In clear contrast, SOD1- and FUS-immunoreactive inclusions in fALS cases are only positive for the respective mutated gene products (Dormann and Haass 2011).

While MAPT- and TDP-43-positive inclusions contribute almost to the same extent (each ca. 50 %) to the pathology in FTLD, those cases with FUS-immunoreactive inclusions (FTLD-FUS) are rather rare (Rohrer et al. 2011). Therefore, FTLD can be divided into three major entities according to the underlying immunoreactivity of inclusions: 1.)
FTLD-TDP-43, 2.) FTLD-tau (inter alia (i.a.) Pick disease), and 3.) FTLD-FUS (Lagier-Tourenne et al. 2010, Seelaar et al. 2011, Pan and Chen 2013).

1.1.4 Common pathomechanisms in ALS and FTD
Not only do ALS and FTD share considerable similarities of histopathological phenomena, but the underlying mechanisms also overlap strongly according to Monahan et al. (2016). Hypothesized pathomechanisms linked to ALS and FTD share two major commonalities, while at least one of the two mechanisms is always present: 1.) a defect in RNA processing, which oftentimes results from mutations of prion-like RNA-binding proteins (RBPs), and 2.) pathological accumulations of prion-like proteins with a (concomitant) affection of the protein degradation system of cells. A common link could be the exacerbated formation of stress granules in cases with mutations in an RBP (FUS, TDP-43, MATR3, and HNRNPA1 / A2) or the perturbed clearance of these granules (OPTN, TBK1, and UBQLN2), which could also be caused by mutations of genes involved in the protein degradation machinery (C9orf72, VCP) (Monahan et al. 2016).

1.2 The RNA-binding protein FUS
In 1993, two groups reported contemporaneously on the involvement of the gene locus of FUS in a translocation-caused myxoid liposarcoma, which was eponymous for fused in sarcoma (FUS) (Rabbitts et al. 1993, Crozat et al. 1993).

1.2.1 The FET protein family
The FET family of RNA-binding proteins comprises FUS, Ewing sarcoma (EWS), and TATA-box binding protein associated factor 15 (TAF15) (FET). All three members of the protein family were initially investigated in their roles as fusion oncogenes. The common functions of these proteins are the processing of RNA and the integrity of genomic stability. FUS and TAF15 are present in most human body cells, especially in the nucleus but also in the cytoplasm. Upon stress, FET proteins are recruited to stress granules (Andersson et al. 2008), which represent a certain type of messenger ribonucleoprotein particles (mRNPs) (Bowden and Dormann 2016).

1.2.2 The protein structure of FUS
The FUS gene is located on chromosome 16 and encompasses 15 exons, which are expressed with a final protein size of 526 amino acids (DeJesus-Hernandez et al. 2011).
The FUS protein harbors the following domains, beginning from the N-terminus as depicted in figure 1: The SYQG rich region with an abundance of serine (S), tyrosine (Y), glutamine (Q), and glycine (G). This so called transcription activation domain binds nuclear hormone receptors, which, thereby, regulates transcription and is also involved in precursor messenger ribonucleic acid (pre-mRNA) processing. Both the SYQG and the following glycine rich region (GRR) are regarded as the prion-like domain, which enables FUS to self-aggregate (Fushimi et al. 2011). This sequence is followed by several motifs, which are capable of binding RNA sequences, including an RNA recognition motif (RRM), two arginine glycine rich regions (RGGs) with an enrichment of arginine-glycine-glycine triplets, and a zinc finger domain (ZF). The whole C-terminus with the DNA- and RNA-binding domains regulates DNA damage repair, RNA processing, and RNA transport (Kwiatkowski et al. 2009, Yang et al. 2010, DeJesus-Hernandez et al. 2011). Furthermore, the very C-terminus harbors a nuclear localization signal (NLS). In combination with the nuclear export signal (NES), FUS is able to shuttle between the cytoplasm and the nucleus (Dormann et al. 2010, 2012).

Figure 1: Domains of the human FUS protein
The sequence of the human FUS protein spans 526 amino acids and comprises several domains from the N- to the C-terminus: SYQG = serine, tyrosine, glutamine, glycine rich region; GRR = glycine rich region; NES = nuclear export signal; RRM = RNA recognition motif; RGG = arginine glycine rich region; ZF = zinc finger domain; NLS = nuclear localization signal. The black bar indicates the antigenic epitope of the FUS antibody, which was used in this study (Resource Research Identifiers: Sigma-Aldrich Cat# HPA008784 RRID:AB_1849181). FUS = fused in sarcoma. Display of domains is mainly based on Lattante et al., Lim et al., and Schoen et al. (Lattante et al. 2013, Lim et al. 2016, Schoen et al. 2016).

1.2.3 Functions of FUS in DNA damage control and RNA processing
Among the various functions of FUS, two are outstanding: a direct regulatory function in DNA damage repair and a multifaceted role in DNA and RNA processing (DeJesus-Hernandez et al. 2011).

Nuclear activities of FUS in RNA metabolism
FUS is involved in transcription events in a rather general manner by being in a complex with the RNA polymerase II (Lagier-Tourenne et al. 2010). FUS-regulated genes harbor
recognition sites for FUS in their promotor regions and can, thereby, be positively or negatively regulated (Tan et al. 2012).

FUS directly participates in various steps of RNA processing, for instance, in pre-mRNA splicing, in polyadenylation, in processes at the splicesome, and in modifying the 3’-end (Masuda et al. 2015). More precisely, FUS binds to a plethora of pre-mRNAs and regulates alternative splicing as part of the splicesome (Rogelj et al. 2012, Lagier-Tourenne et al. 2012, Nakaya et al. 2013). Target RNAs of FUS-dependent RNA splicing are mainly those relevant for proper neuronal development (axonal development, axonal guidance, and cytoskeletal arrangements in dendritic spines) and neuronal function (cell adhesion and vesicle transport). Via the involvements in RNA processing steps, FUS has a crucial function to increase the transcriptomic versatility, which is assumed to be reduced in the pathogenesis of FUS-associated neurodegenerative diseases (Orozco and Edbauer 2013, Masuda et al. 2015). Of note, alternative splicing extensively increases the diversity of proteins in an organism. Neuronal cells hold an exceptional position by splicing almost half of all genes (Orozco and Edbauer 2013).

Extranuclear activities of FUS in RNA metabolism
FUS shuttles between the cytoplasm and the nucleus. It can be found in RNA granules, and carries cargo transcripts to remote cytoplasmic sites (Lagier-Tourenne et al. 2010). RNA granules or mRNPs can be regarded as non-membranous compartments, which serve several crucial steps in extranuclear RNA processing, including local translation (Bowden and Dormann 2016). Typical components include a variety of different RBPs, such as FUS, TDP-43, RBM45, and HNRNPs (Li et al. 2016). FUS-containing RNA granules are transported in both antero- and retrograde directions along microtubules (Kanai et al. 2004).

1.3 FUS pathologies in neurodegenerative diseases
Both localization and functions of the FUS protein are altered in cases of FUS-mediated neurodegeneration, which will be covered in this section. Neurodegenerative diseases with FUS contribution (FUSopathies; singular FUSopathy) demonstrate several remarkable features with regard to the aforementioned functions.
1.3.1 FUS-associated amyotrophic lateral sclerosis (ALS-FUS)

Before $FUS$ was identified as a causal gene in fALS, a distinct ALS entity was referred to as ALS6, which was defined by an involvement of a risk locus on chromosome 16 (Abalkhail et al. 2003, Ruddy et al. 2003). The discovery of ALS-associated mutations in the $TDP-43$ gene in 2008 (Sreedharan et al. 2008, Kabashi et al. 2008) gave rise to studies, which aimed to analyze the aforementioned risk locus including the $FUS$ gene, because the FUS protein shares structural and functional homologies with TDP-43. These efforts resulted in the initial identification of $FUS$ mutations in fALS, thereby, ALS6 was renamed as ALS-FUS (Vance et al. 2009; Kwiatkowski et al. 2009). Later, $FUS$ mutations were also detected in sALS (Corrado et al. 2010, DeJesus-Hernandez et al. 2010).

The prevalence of FUS mutations was estimated between 3 to 5 % within fALS (Damme et al. 2010, Blair et al. 2010, Rademakers et al. 2010, Ince et al. 2011) and approximately (approx.) 1 to 2 % in sALS cases (Corrado et al. 2010, DeJesus-Hernandez et al. 2010, Rademakers et al. 2010, Sproviero et al. 2012). Most ALS-associated FUS mutations reside at the C-terminus, which disrupt the NLS, thus, leading to a mis-localization of FUS into the cytoplasm and to subsequent FUS aggregation. Usually, the basophilic inclusions in the central nervous systems of patients are immunoreactive for ubiquitin and FUS but not for TDP-43 or SOD1 (Kwiatkowski et al. 2009, Vance et al. 2009, 2013, DeJesus-Hernandez et al. 2010, Bäumer et al. 2010, Ince et al. 2011). The disease severity in ALS-FUS seems to correlate with the amount of FUS-positive inclusions in the affected motoneurons and glial cells (Mackenzie et al. 2011a, Suzuki et al. 2012).

Missense mutations of the $FUS$ gene in ALS-FUS are regularly accompanied by an early-onset of the disease and an aggressive clinical course (Suzuki et al. 2010, Sproviero et al. 2012, Kent et al. 2014, Leblond et al. 2016, Shang and Huang 2016). Inheritance is usually dominant with a penetrance of almost 100 % (Suzuki et al. 2010). Of note, ALS-FUS represents the most common cause of early-onset, juvenile fALS (Hübers et al. 2015).

1.3.2 FUS-associated frontotemporal dementia (FTD-FUS)

FTD-FUS is markedly different from ALS-FUS due to the prevailing independence of $FUS$ mutations (Lashley et al. 2011). In 2009, Neumann et al. further scrutinized an FTLD
subgroup previously denominated as atypical frontotemporal lobar degeneration with ubiquitin inclusions (aFTLD-U), which they renamed in FTLD-FUS (Neumann et al. 2009a). This disease entity is neither TDP-43- nor tau-positive. A typical disease course includes an early-onset together with severe behavioral abnormalities. Therefore, the clinical picture is referred to as behavioral variant FTD (Neumann et al. 2009a, Urwin et al. 2010, Lashley et al. 2011, Rohrer et al. 2011, Baborie et al. 2012). FTD-FUS accounts for approx. 5% of all FTLD specimens (Rohrer et al. 2011, Baborie et al. 2012).

FTLD-FUS comprises four histopathologically distinguishable variants: neuronal intermediate filament inclusion disease (NIFID), basophilic inclusion body disease (BIBD), aFTLD-U, and FTLD with FUS mutations (Neumann et al. 2009b, Halliday et al. 2012).

1.3.3 Distinct and overlapping hallmarks in FUSopathies

ALS-FUS and FTD-FUS differ in several features. While ALS-FUS inclusions are only FUS-positive, protein aggregates in FTLD-FUS are positive for all members of the FET protein family, including FUS, EWS, and TAF15 (Neumann et al. 2011). Hence, different pathomechanisms are conceivable with varying degrees of nuclear loss-of-function of these predominantly nuclear proteins (Neumann et al. 2011, Dormann and Haass 2013, Davidson et al. 2013). It remains elusive whether these constellations of an exclusive FUS immunoreactivity (ALS-FUS) or a co-immunoreactivity of all FET proteins (FTD-FUS) results from the presence or absence of FUS mutations, respectively.

Nonetheless, ALS and FTD with underlying FUS pathologies share some remarkable commonalities. More precisely, subgroups of FTD-FUS concomitantly exhibit MND symptoms, in particular, the subtypes NIFID (Neumann et al. 2009b) and BIBD (Munoz et al. 2009). Moreover, a regular feature of FTLD-FUS is the presence of FUS inclusions in spinal cord motoneurons, also in cases that do not exhibit motor symptoms (Mori et al. 2012). At the same time, there have been several reports about few ALS-FUS cases with clinical signs of FTD (Blair et al. 2010, Akiyama et al. 2016). As of late, a hallmark of FTD-FUS seems to be challenged. By analyzing the FUS gene in a large number of FTD, FTD-ALS, and ALS patients, a likely pathogenetic mutation was found in one sporadic FTD (sFTD) case (Van Langenhove et al. 2010).
1.3.4 Models of FUS pathology

FUSopathies have been extensively modelled in several vertebrate and invertebrate animal models. Moreover, patient-derived induced pluripotent stem cells (iPSCs) serve as a new model to scrutinize FUS-associated pathologies.

1.3.4.1 iPSCs-derived FUS pathology models

The vulnerability of neurons toward FUS pathologies is demonstrated by Lim et al. who report of cytoplasmic FUS accumulations in iPSCs-derived neurons but not in the original fibroblasts (Lim et al. 2016). FUS-positive inclusions in stem cell-derived neurons from patients seem to reflect an age- and mutation-dependent severity (Japtok et al. 2015). Applying stress to differentiated motoneurons from ALS-FUS patients is followed by a mis-localization of FUS into stress granules, which is a typical hallmark in FUSopathies and in respective animal models (Ichiyangi et al. 2016).

1.3.4.2 FUS pathologic effects in cell lines and primary cells

FUS pathologic effects, mimicked in vitro, can be divided in dysfunctional nuclear activities and in those related to the pathologic accumulations of FUS.

Disturbance of the DNA and RNA processing in FUS pathologies

FUSopathies have been associated with disturbances in the RNA metabolism (Liscic and Breljak 2011). A knockdown of FUS in mouse neurons is accompanied by an altered splicing pattern (Lagier-Tourenne et al. 2012, Nakaya et al. 2013). Mis-spliced messenger ribonucleic acid (mRNA) is usually recognized at ribosomes and degraded in a process called nonsense-mediated decay (Nakaya et al. 2013). Under knockdown conditions, in particular those mRNAs are affected, which are implicated in action potential transmission and proper motor neuron function (Reber et al. 2016).

FUS mis-localization and aggregation pathology

The amount of FUS in cytosolic stress granules from cell lines, which overexpress different mutant FUS proteins, correlates with the disease onset in the respective ALS-FUS patients (Dormann et al. 2010). The formation of FUS granules is dependent on the presence of the prion-like N-terminal domain (Fushimi et al. 2011, Sun et al. 2011, Shelkovnikova et al. 2014) and is enforced after induction of cellular stress (Vance et al. 2013). In cell lines and rat hippocampal neurons, similar requirements of FUS and TDP-
43 to bind to stress granules are a cytoplasmic mis-localization and their prion-like and RNA-binding domains (Bentmann et al. 2012).

1.3.4.3 Animal models of FUSopathies

Most of the described phenotypes from investigations in vitro and post mortem have also been mirrored in animal experiments, which will be presented in the following.

Vertebrate FUSopathy models

Overexpression of both mutant (Verbeeck et al. 2012, Sephton et al. 2014) and wildtype human FUS in mice leads to MND-like symptoms and early death (Sephton et al. 2014). Transgenic FUS mice lacking the C-terminus demonstrate a severe motor phenotype with premature lethality (Shelkovnikova et al. 2013). Another study alleges a predominant loss of motoneurons when overexpressing human wildtype FUS in mice, which replicate the inclusion pathology and glial reactions in humans. These mice suffer a premature death, most likely due to an aggressive motor phenotype (Mitchell et al. 2013). Intriguingly, when comparing knockout (KO) and mutant FUS mice, Scekic-Zahirovic et al. find overlapping phenotypes in their models with increased lethality, respiratory deficits, lower body weights, and lengths. Only heterozygous animals survive after birth. Additionally, both homozygous FUS KO and mutant models exhibit similar mRNA patterns, which are dramatically altered in comparison to control animals. Taken together, these results highlight both gain- and loss-of-function mechanisms in states of FUS depletion and also FUS mis-localization (Scekic-Zahirovic et al. 2016).

In a study from Huang et al. from 2011, different transgenic rats expressing human FUS were compared. One strain harbored an ALS-associated FUS mutation in the NLS. These animals develop a progressive loss of voluntary motor functions as a consequence of motor neuron degeneration. Moreover, these rats also exhibit a loss of neurons in the cortex and hippocampus with ubiquitin-positive inclusions and glial cell reaction. In turn, those rats expressing human wildtype FUS protein demonstrate a normal development, though a reduced spatial learning and memory, which is accompanied by a substantial loss of neurons in the cortex and hippocampus. The inference is that the mutation is more toxic than wildtype FUS. In summary, both ALS- and FTD-like phenotypes could be reproduced with mutated and wildtype FUS, respectively (Huang et al. 2011).
Invertebrate FUSopathy models

Further extensively analyzed species for FUS pathologies are the invertebrate species *Drosophila melanogaster* (*Drosophila*) and *Caenorhabditis elegans* (*C. elegans*), which will be dealt with in this paragraph.

Overexpressed mutant human FUS in *Drosophila* leads to ubiquitinated protein accumulations, FUS mis-localization to the cytoplasm, neurodegeneration, decreased motility, and early death. On the contrary, wildtype human FUS is not found in the cytoplasm and effectuates no strong phenotype. As a consequence of these observations, mutant FUS may have a toxic gain-of-function effect, which might be contrived in the cytoplasm (Lanson et al. 2011).

Mutant human FUS in *C. elegans* elicits a non-rescuable motor phenotype with a mutation-dependent severity. Co-expressed wildtype human FUS localizes correctly but is not able to overcome the phenotype. Hence, the authors postulate a dominant gain-of-function mechanism of mutant FUS in the cytoplasm (Murakami et al. 2012). Overexpression of wildtype human FUS or TDP-43 in *C. elegans* motoneurons, likewise, results in a severe degeneration of these neurons (Vaccaro et al. 2012). In another *C. elegans* model, the knockdown of the FET proteins orthologue leads to a motor neuron degeneration phenotype, reduced lifespan, and diminished cellular stress resistance (Therrien et al. 2016).

1.3.4.4 General pathomechanisms in models of FUSopathies

FUS models demonstrate remarkable overlaps with findings in patients with FUSopathies. Some of the commonalities are presented in the following passage, which are based on a discussion on gain- and loss-of-function mechanisms in FUSopathies from Halliday et al. published in 2012. Several observations hint toward a gain-of-function mechanism: 1.) In human necropsy material, inclusion-bearing cells harbor remaining nuclear FUS. 2.) In several animal models, an increase of cytoplasmic FUS leads to neurodegeneration, although the nucleus is not completely devoid of FUS. 3.) FUS in yeasts is toxic as a result of cytoplasmic localization. 4.) Furthermore, the amount of cytoplasmic FUS correlates with the severity of symptoms. At the same time, a loss-of-function is supported by the following observations: 1.) There is a correlation between the loss of nuclear FUS and the strength of symptoms. 2.) Also FTD-FUS cases without mutated FUS exhibit reduced nuclear FUS. 3.) Several brain areas with neuronal cells...
bearing cytoplasmic inclusions seem to be phenotypically unaffected. 4.) In FTD-FUS, other FET proteins co-segregate with FUS, which hints toward a multimolecular failure. 5.) Some of the neurodegenerative phenotypes are rescuable in animal models by restoring FUS (Halliday et al. 2012).

In order to unify major hypotheses on FUS pathomechanisms, Dormann and Haass postulated a two-hit model (displayed in figure 2). It includes a first hit, which leads to a homogeneous distribution of FUS. The underlying event can be a mutation in FUS with an effect on the nuclear import of the protein. Also age-dependent defects of the nuclear shuttling machinery or posttranslational modifications of FUS might be causative. A second hit, eventually, results in a trapping of FUS proteins into stress granules. Contributing factors for this process can be cellular stress or the absence of protective factors. This process is reversible, but long-lasting stress might lead to irreversibly large inclusions (Dormann and Haass 2011).

![Figure 2: A model of FUS distribution in healthy and pathological states](image)

FUS (red) localizes to the nucleus of almost all body cells, including neurons (left). However, in disease states (right) – triggered by ALS-associated mutations or other stressors – FUS mis-localizes into the cytoplasm (first hit, bright red) and later forms aggregates (second hit, red aggregates). ALS = amyotrophic lateral sclerosis, FUS = fused in sarcoma.

**1.4 Synaptic FUS in the central nervous system**

Recent studies have averted the attention away from the functions of FUS in the nucleus by discovering a remarkable difference between FUS in neuronal and non-neuronal cells. While being present in the nuclear compartment in most body cells, FUS additionally localizes to synapses in neuronal cells. To date, the function there is merely investigated.
As can be seen in figure 3, the expression of FUS in the CNS is detectable at different steps of rat development with high concentrations in the cortex, cerebellum, and hippocampus (Putz 2013).

**Figure 3: FUS expression in the rat central nervous system**

FUS expression in the central nervous system of rats is displayed both in terms of mRNA and protein expression. (A) *In situ* hybridization targets *Fus* mRNA at embryonic day 19 (left, sagittal section), postnatal day 9 (middle), and adult (right; both horizontal sections). (B) Brain sections are depicted after immunohistochemistry with DAB labeling of FUS at postnatal day 12. The three magnifications (right) refer to respective regions of the sagittal section (left), from left to right: cerebellum, hippocampus, and cortex. *Fus* mRNAs (A) as well as expressed proteins (B) can be abundantly detected in the central nervous system of rats at different developmental stages. The immuno-detection is particularly intensive in the cerebellum, hippocampus, and cortex. DAB = 3,3′-diaminobenzidine, *Fus/FUS* = fused in sarcoma, mRNA = messenger ribonucleic acid. Modified from Schoen et al. (Schoen et al. 2016), open access article: CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

It has been reported that FUS localizes to dendrites in mouse brains (Aoki et al. 2012) and in cultured hippocampal cells (Fujii et al. 2005, Fujii and Takumi 2005). Fujii et al. could detect overexpressed FUS in spines (Fujii et al. 2005). FUS-containing RNA granules move along microtubules in dendrites. Microtubular transport is mediated via binding to KIF5, whereas movement within spines is accomplished by MYO5A (the familiar name is myosin VA), an actin-based motor protein. Synaptic stimulation leads to a recruitment of FUS to dendrites and spines (Fujii et al. 2005, Bowden and Dormann 2016). Intriguingly, spine numbers and maturity of hippocampal neurons in FUS KO mice is reduced (Fujii et al. 2005). In line with this, mRNAs bound to FUS in mouse brain are identified, among them *Nd1-L* as the transcript for an actin-stabilizing protein. The mRNA for *Nd1-L* as well as for β-actin is translocated MYO5A-dependently into dendrites after synaptic stimulation but not in FUS KO neurons, which purportedly explains the spine phenotype (Fujii and Takumi 2005). Moreover, by inducing long-term potentiation (LTP) with brain derived neurotrophic factor (BDNF) in primary rat cortical neurons, proteomes of subsequently isolated postsynaptic densities (PSDs) display...
upregulated RBPs, including FUS (Bowden and Dormann 2016). Therefore, a function of FUS in local translation at spines is hypothesized (Belly et al. 2005).

In addition to the nuclear localization, Aoki et al. describe the distribution of FUS with a granular pattern in the grey matter of the brainstem and spinal cord of the human and mouse CNS. In synaptosomal fractions of mouse brains, FUS is predominantly detected in the PSD compartment. Moreover, immunolabeling unveils a strong overlap of FUS with the presynaptic marker synaptophysin (SYP) in a distribution adjacent to microtubule associated protein 2 (MAP2) (Aoki et al. 2012).

The hitherto published data on synaptic FUS localization have several inherent limitations. The affiliation of FUS to the postsynaptic compartment was investigated with biochemical methods of tissue subfractionation and with diffraction-limited light microscopy. In particular, the latter is incapable of distinguishing the pre- and postsynaptic compartments. Both approaches can be imprecise in exactly localizing proteins. In contrast to previous studies, the employed super-resolution microscopy techniques in the present work offer a localization precision on a scale of single molecules.

1.5 Super-resolution microscopy

A biological structure can be regarded as the sum of its constituents. If precise localization of each molecule could be determined with imaging techniques, one would be able to reconstruct it (Huang et al. 2010a). Positron emission tomography, magnetic resonance imaging, and optical coherence microscopy are able to resolve structures in dimensions of 1 mm, 100 µm, and 10 µm, respectively. Electron microscopy (EM) almost comes down to a single molecule resolution, however, rigid fixation is a prerequisite, which most widely restricts combinations with specific immunolabeling of proteins. The achievable resolution of conventional fluorescence microscopy resides between the initially mentioned methods and EM (Fernández-Suárez and Ting 2008).

The point spread function (PSF) restricts the resolution of a fluorescence microscopy setup. It is a mathematical description of the acquired blurry signals of spot-like light sources – for instance a fluorophore or excitation laser light – and has the characteristics of a Gaussian function (Fernández-Suárez and Ting 2008). In figure 4 (upper right), the signals of isolated fluorophores are displayed with maximum intensity at the center but blurry appearance in the periphery according to their PSFs. As a
consequence of the wave nature of light, excitation with a laser elicits simultaneous fluorescence of many fluorophores with an overlap of their PSFs (figure 4, upper left). Per definition, the full width at half maximum (FWHM) in the underlying Gaussian function of a PSF defines the achievable resolution; beyond this distance, two neighbored fluorophores can be distinguished (Fernández-Suárez and Ting 2008). A focal spot emitting fluorescence light in conventional light microscopy usually appears no smaller than approx. 250 µm in the $xy$-plane and 550 nm in the $z$-axis as a result of the PSF. This is referred to as diffraction-limited resolution. Fluorophores exceeding these distances appear as separable objects, whereas fluorescent dyes with a distance smaller than their respective FWHM considerably overlap with their PSFs, thereby, become unresolvable and appear as single entities (figure 4, upper left).

The Nyquist frequency criterion implies that specific antibody labeling of molecules can be an even more limiting factor of resolution than the microscopy setup itself. Along these lines, the labeling density must be at least twice as high as the desired resolution (Betzig 2015), and for optimal conditions during acquisitions, a scanning step size is recommended half as high as the aimed resolution (Huang et al. 2010a).

1.5.1 The history of super-resolution microscopy

After Abbe implicated the diffraction-caused limitations of resolution in lens-based light microscopy in 1873 (Abbe 1873, Hell 2007), which allows for a resolution power not below the half of the applied wavelength, Synge was the first to theoretically speculate about subdiffraction microscopy by exploiting an extreme nearfield technique. This idea was realized in the 1980s by near-field scanning optical microscopy, which makes use of sub-wavelength apertures (Betzig et al. 1986). This technique exploits the fact that diffraction only occurs when the excitation source and the detection unit are farther away than the wavelength of light (Betzig et al. 1986, Huang et al. 2010a, Betzig 2015). Thereby, a resolution in the range of scanning EM can be achieved (Betzig et al. 1986). However, these devices require inappropriate efforts to scan in a sub-wavelength distance to the imaged object. In addition, near-field scanning optical microscopy is incompatible with many modern immunocytochemical methods as well as with time-lapse microscopy (Huang et al. 2010a). In the last decades of the twentieth century, several technical solutions increased the resolution in fluorescence light microscopy to approx. 100 nm in the $xy$-plane with so called 4Pi microscopy by Hell and Stelzer and
ISM microscopy. These similar techniques apply two opposing objectives and, thus, add their resolving powers, but these approaches only bend the Abbe’s law (Hell and Stelzer 1992, Hess et al. 2009, Huang et al. 2010a).

Recently, fluorescence-based super-resolution light microscopy techniques have been introduced to break the diffraction limit with basically unlimited resolution by exploiting peculiarities of the fluorescence properties of fluorophores (Huang et al. 2010a). The principle of these techniques is the precise localization of single fluorophores (Thompson et al. 2002). Super-resolution microscopy could, in theory, determine the positions of molecules with a precision of a few nanometers, but inherent drift in biological specimens limits the resolution to a few dozens of nanometers (Nägerl and Bonhoeffer 2010). In most farfield super-resolution microscopy techniques, the goal is to isolate the signals of single fluorophores with their underlying PSFs. This is accomplished by either narrowing down the spot where excitation takes place (STED) or by temporally separating the emission of adjacent fluorophores in a series of stochastic activations and inactivations. The latter technique is also known as single molecule localization microscopy (SMLM), in which positions of fluorophores are reconstructed in silico by extracting the centers of the signals of single fluorophores with maximum intensity (Huang et al. 2010a, Sanders 2015). The concepts of STED and SMLM are displayed in figure 4. In addition, both applications require largely photostable fluorophores. Moreover, since SMLM necessitates non-overlapping spectra of dyes, it is usually limited to two different colors (Huang et al. 2010a).

Figure 4: The principles of STED and SMLM

The technical backgrounds of super-resolution techniques in fluorescence microscopy are displayed in a simplified manner. A synapse is composed of a presynaptic and a postsynaptic compartment. The following symbols correspond to fluorescence-labeled markers for synaptic compartments: presynaptic markers (red), postsynaptic markers (green), overlap of both markers (yellow). In conventional fluorescence imaging (upper left), signals of fluorophores in close proximity largely overlap due to their
diffraction-blurred signals. Thereby, a single fluorophore can usually not be distinguished while many neighbored fluorophores are excited simultaneously, which results in an overlap of their PSFs (yellow). STED microscopy (lower left) circumvents this drawback by an overlay of two lasers: one usual excitation laser and a second laser with a doughnut shape (red ring), which allows for fluorescence emission only in the center with zero intensity of the so called depletion laser. In turn, in SMLM a conventional farfield microscopy setting is employed (right half). By bringing fluorophores in a stochastic on / off state and by recording these events in a movie (upper right), it is possible to distinguish single fluorophores. The centroid position of a single fluorophore is much smaller than the underlying PSF. In a subsequent approach, all the in silico calculated centroid positions of the acquired fluorophores can finally be superimposed onto one image (lower right). PSF = point spread function, SMLM = single molecule localization microscopy, STED = stimulated emission depletion.

1.5.2 Stimulated emission depletion (STED) microscopy

Hell and Wichmann were the first to theoretically propose a novel subdiffraction light microscopy technique named STED, in which stimulated emission depletion is used to inhibit fluorescence in the periphery of the PSF of a fluorophore by reducing the excitable volume to a subdiffraction-sized spot (Hell and Wichmann 1994). The first implementation of STED microscopy followed more than ten years later (Westphal and Hell 2005). STED employs a doughnut-shaped depletion laser, which is superimposed onto the excitation laser beam (Huang et al. 2010a). The depletion laser is also diffraction-limited, however, by increasing the laser power, the center with zero intensity exhibits a sub-wavelength size (see also figure 4, lower left) (Hess et al. 2009, Huang et al. 2010a). Exclusively within the few nanometer wide center of this laser, fluorophores can be activated. In theory, STED resolution would be unlimited if the depletion light source had an unlimited intensity. In practice, it is restricted to approx. 20 nm in biological samples; in diamonds a 6 nm resolution can be attained (Huang et al. 2010a).

1.5.3 Single molecule localization microscopy (SMLM)

SMLM is the super-resolution microscopy technique with the highest spatial resolution (approx. 20 to 30 nm in the xy-plane, 60 nm in the z-axis). Further advantages are the rather simple and affordable microscopy setup (Nägerl and Bonhoeffer 2010). Molecules can only be distinguished when two simultaneously blinking fluorophores are farther apart than the maximum resolution of the microscopy setup. Acquired photons from single fluorophores can be fit into a Gaussian function to determine the centroid
position, which is identical with the center of mass (COM) of the PSF. The localization of the COM of a fluorophore represents the most likely position of a single molecule (figure 4, right half) (Heilemann et al. 2008). The spatially distinguishable fluorescence emission of single fluorophores is accomplished by decelerating the on/off states of fluorophores in an iterating process. Requirements for fluorescent probes in SMLM are a clear ability to switch between on and off states with high photon emission during the on states (Huang et al. 2010a).

In historical terms, the discovery of certain properties of the green fluorescent protein (GFP), which allow to control the states of fluorescence emission with reference to the on and off states of blinking, inspired Betzig and Hess to create the first SMLM technique (Betzig 2015). The initial implementation was called photoactivated localization microscopy (PALM) with a 2 to 25 nm resolution (Betzig et al. 2006). Together with Stefan Hell, Eric Betzig was awarded the Nobel Prize in chemistry in 2014 for the invention of super-resolution fluorescence microscopy techniques. SMLM is an umbrella term, which includes PALM, stochastic optical reconstruction microscopy (STORM), and direct STORM (Huang et al. 2010a). They are based on the same principle and employ blinking fluorophores, but they differ in the type of the fluorophores. While PALM uses genetically expressed photoswitchable fluorophores, STORM employs an excitation and a reporter dye coupled to the same antibody. In turn, dSTORM uses standard dyes. These methods include the possibility to measure the localization and amount of proteins in high precision (Ehmann et al. 2015).

Heilemann et al. initially introduced direct STORM (dSTORM), which was mainly employed in this study. 'Direct' in the term dSTORM refers to the independence to use double-labeled dyes as described in conventional STORM. They could demonstrate that even conventional fluorescent dyes can be used as photoswitchers (Heilemann et al. 2008). A photoswitcher is a fluorophore, which changes between the off and on states upon illumination, which is also referred to as blinking. This behavior is usually triggered by using two separate light beams with different wavelengths. Molecules turn into an active state by the so-called activation laser with a short wavelength. Succeedingly, only a subset of fluorophores becomes excited to the on state by the excitation laser, which has a longer wavelength and a low intensity. The density of simultaneously blinking fluorescing dyes has to be lower than the resolution power of the optical system in order to temporally separate spatially unresolvable structures. In other words, the
spatial distances between on state fluorophores are ideally greater than the FWHM of their acquired PSFs (Heilemann et al. 2008, Van De Linde et al. 2011, Sanders 2015).

1.5.4 Super-resolution microscopy of synapses

To date, the only completely unraveled wiring diagram of an organism’s nervous system is from *C. elegans*. The reasons why it is so challenging to map connectivity in more complex organisms are the obstacles to make densely packed neurites in tissue recognizable. Moreover, there is a lack of knowledge about the properties of the complex protein assemblies at synapses. These limitations are closely connected to conventional light microscopy, which is incapable to resolve structures or molecules within the diffraction zone, in which the diffraction blobs of fluorophores overlap (see figure 4, upper left). Along these lines, super-resolution imaging was first applied in neuroscience (Huang et al. 2010a).

1.5.4.1 STED imaging of synapses

Nägerl et al. were the first to report of *in vivo* acquisitions of spines with super-resolution-based time-lapse farfield microscopy (Nägerl et al. 2008). This approach is especially interesting for the 3D imaging of spine necks with a diameter ranging from 50 to 150 nm and a head size of approx. 1 µm, which represent values at or below the diffraction limit (Loew and Hell 2013). For instance, via time-lapse super-resolution microscopy, it is shown that in the course of LTP, spines undergo unpredicted morphological changes with shorter and wider spine necks, paralleled by alterations of the molecular composition and electrophysiological properties of spines (Tønnesen et al. 2014). STED is also suitable to determine localizations of synaptic proteins in relationship to each other. In that respect, it has been demonstrated that bassoon and piccolo, two presynaptic proteins, are molecularly separated but exhibit an aligned orientation in paralleled stripes at neuromuscular junctions (Nishimune et al. 2016).

1.5.4.2 SMLM of synapses

The most comprehensive and seminal super-resolution study analyzing protein distributions at synapses was conducted by Dani et al., which will be further addressed in this section (Dani et al. 2010). The applied methods and results represent the most relevant reference for the investigations with super-resolution imaging of this thesis. Dani et al. employed STORM to analyze the precise localization of ten pre- and
postsynaptic proteins. Of note, it was one of the first studies demonstrating STORM imaging of neuronal tissue. Membrane-adherent protein masses in the pre- and postsynaptic compartments, the presynaptic active zone (PAZ) and the postsynaptic density (PSD), respectively, shape the inner sides of the opposing membrane areas of a synaptic contact. As a consequence of this, these protein densities exhibit a discoid shape. Thus, two extremes of viewing angles on a synapse can be achieved: 1.) In a so-called side view, the trans-synaptic axis is parallel to the imaging plane, thereby, the protein densities appear as bar-shaped structures. The trans-synaptic axis is a direct line between the pre- and the postsynaptic compartments and is perpendicular to the synaptic cleft (see rectangles in figure 6 in 2.2.3.3 and the dashed rectangle in figure 24 in 3.3.7). 2.) In a 90° rotation to that perspective, a face view is obtained with complete co-localization of the protein populations in the projection of both compartments. The trans-synaptic axis is then in a perpendicular orientation to the imaging plane. Referring to the discoid shape, the protein densities then appear in a circular form. These considerations in mind, Dani et al. measured distances between accumulations of certain synaptic proteins. In order to determine the positions of these molecules in the side view, which is referred to as the axial distribution of synaptic proteins, peak-to-peak distances of the acquired signal intensities from labeled synaptic proteins were analyzed across the trans-synaptic axis. Along these lines, two antibodies for classical markers of the pre- and postsynaptic protein densities were applied, namely, bassoon and HOMER-1B/C, respectively. For instance, the average distance between bassoon and HOMER-1B/C along the trans-synaptic axis is 153 nm in the main the orbital cortex. The axial distribution between these two proteins is surprisingly homogeneous in the different analyzed brain areas. In marked contrast to these results, the localizations of proteins in cross-section areas of the disks (face view) are very variable, intra- and interregionally. Such an evaluation represents the lateral distribution of synaptic proteins within the PAZ and PSD (Dani et al. 2010). The reported distance values between synaptic proteins served as a reference for the detailed analysis of FUS localization at synapses in the present study.

**1.6 Aims of the thesis**

The RNA-binding protein FUS has various and crucial functions in all steps of RNA processing in the nucleus but lately also attracted attention as a putative regulator of
local translation at chemical synapses in neuronal cells. The clinical importance of FUS is based on its involvement in various neurodegenerative diseases. In the course of these pathologies, the physiologically nuclear localization is widely abandoned for inclusions in the cytoplasm, which have the propensity to further propagate. Taking the high importance of FUS in physiological and pathological states into account, I aim at elucidating the localization of FUS at murine synapses with immunolabeling methods, which is a rather neglected aspect of FUS research so far.

Conventional fluorescence light microscopy, including confocal microscopy, was employed in order to scrutinize the following aspects of FUS at murine synapses:

1.) I intend to investigate synaptic FUS in combinations with multiple synaptic markers in several mouse CNS regions. The selection of CNS areas included different cortical areas and the spinal cord and was a priori reasoned by a focus set on those areas, which display high FUS expression levels and are affected in FUS-associated FTD and ALS.

2.) Furthermore, I aim to thoroughly analyze FUS localization at different synapse classes in a more uniform neuronal model than neuronal tissue: primary hippocampal neurons derived from rat embryos. The applied antibodies are capable to detect both excitatory and inhibitory scaffolding proteins of the pre- and postsynaptic compartments in combinations with FUS.

Super-resolution microscopy, namely, STED microscopy and dSTORM, were employed to investigate the following facets of FUS localization:

3.) I want to describe the necessary establishment of a working protocol for optimal immunostainings and image acquisitions with super-resolution microscopy. Furthermore, I aim to introduce a novel method to determine protein positions at synapses.

4.) Finally, I intend to perform a detailed localization analysis to determine the affiliation of FUS to either the post- or presynaptic compartment, which might have important cues on the functions of FUS at the synapse. The achievable resolution of the dSTORM is at the level of single molecules.

To sum up, state-of-the-art microscopy techniques were used to achieve a deeper understanding of the localization and role of the FUS RNA-binding protein at synapses.
2 MATERIALS and METHODS

2.1 Materials
In the following, comprehensive information is presented on all applied chemicals, derived solutions, technical devices, softwares, and biological materials.

2.1.1 Chemicals
This section contains all used chemical compounds, buffers, and media, which were provided by the indicated companies.

<table>
<thead>
<tr>
<th>Chemical compounds</th>
<th>Supplier / provided by</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,2′-thiodiethanol (TDE)</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
</tr>
<tr>
<td>4′,6-diamidino-2-phenylindole (DAPI)</td>
<td>Thermo Fisher Scientific, Darmstadt, Germany</td>
</tr>
<tr>
<td>B-27</td>
<td>Life Technologies, Grand Island, USA</td>
</tr>
<tr>
<td>bovine serum albumin (BSA)</td>
<td>AppliChem, Darmstadt, Germany</td>
</tr>
<tr>
<td>deoxyribonuclease I</td>
<td>Life Technologies, Carlsbad, USA</td>
</tr>
<tr>
<td>200 units per microliter (U/µl)</td>
<td></td>
</tr>
<tr>
<td>carbon dioxide, frozen (dry ice)</td>
<td>Linde, Pullach, Germany</td>
</tr>
<tr>
<td>carbon dioxide, gaseous</td>
<td>Linde, Pullach, Germany</td>
</tr>
<tr>
<td>catalase</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
</tr>
<tr>
<td>cysteamine</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
</tr>
<tr>
<td>deionized water</td>
<td>home-made (with a water purification device, see 2.1.3)</td>
</tr>
<tr>
<td>Dulbecco’s modified eagle medium (DMEM)</td>
<td>Life Technologies, Paisley, United Kingdom</td>
</tr>
<tr>
<td>Dulbecco’s phosphate buffered saline (PBS)</td>
<td>Life Technologies, Paisley, United Kingdom</td>
</tr>
<tr>
<td>without calcium and magnesium chloride</td>
<td></td>
</tr>
<tr>
<td>fetal bovine serum (FBS)</td>
<td>Life Technologies, Paisley, United Kingdom</td>
</tr>
</tbody>
</table>

24
**MATERIALS and METHODS**

<table>
<thead>
<tr>
<th>Material</th>
<th>Supplier</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>glucose</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
<td></td>
</tr>
<tr>
<td>glucose oxidase</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
<td></td>
</tr>
<tr>
<td>glycine</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
<td></td>
</tr>
<tr>
<td>Hank's balanced salt solution (HBSS)</td>
<td>Life Technologies, Paisley, United Kingdom</td>
<td></td>
</tr>
<tr>
<td>with / without phenol red</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ketamine</td>
<td>Wirtschaftsgenossenschaft deutscher Tierärzte, Garbsen, Germany</td>
<td></td>
</tr>
<tr>
<td>L-glutamine</td>
<td>Thermo Fisher Scientific, Darmstadt, Germany</td>
<td></td>
</tr>
<tr>
<td>200 millimolar (mM) (= 100x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>methanol</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
<td></td>
</tr>
<tr>
<td>Neurobasal medium</td>
<td>Life Technologies, Paisley, United Kingdom</td>
<td></td>
</tr>
<tr>
<td>nitrogen, gaseous</td>
<td>Linde, Pullach, Germany</td>
<td></td>
</tr>
<tr>
<td>paraformaldehyde (PFA), EM grade</td>
<td>Science Services, München, Germany</td>
<td></td>
</tr>
<tr>
<td>32 % solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>penicillin / streptomycin (Pen Strep)</td>
<td>Life Technologies, Paisley, United Kingdom</td>
<td></td>
</tr>
<tr>
<td>10,000 units per milliliter (U/ml) penicillin / 10,000 µg/ml streptomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PFA</td>
<td>Merck, Darmstadt, Germany</td>
<td></td>
</tr>
<tr>
<td>poly-L-lysine hydrobromide (PLL)</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
<td></td>
</tr>
<tr>
<td>sodium chloride</td>
<td>Sigma-Aldrich Chemie, Steinheim, Germany</td>
<td></td>
</tr>
<tr>
<td>sucrose</td>
<td>Carl Roth, Karlsruhe, Germany</td>
<td></td>
</tr>
<tr>
<td>TetraSpeck Microspheres</td>
<td>Thermo Fisher Scientific, Darmstadt, Germany</td>
<td></td>
</tr>
<tr>
<td>0,1 µm size</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
MATERIALS and METHODS

Tissue-Tek O.C.T. Compound  Sakura Finetek Europe, Alphen aan den Rijn, Netherlands
Triton X-100  Roche Diagnostics, Mannheim, Germany
trypsin-ethylenediaminetetraacetic acid (EDTA)  Life Technologies, Paisley, United Kingdom

2.5 %
VectaMount AQ aqueous mounting medium  Vector Laboratories, Burlingame, USA
xylazine (Rampun)  Bayer Vital, Leverkusen, Germany

2 % solution

2.1.2 Solutions and mixtures

This section contains all solutions, which are based on the aforementioned chemicals.

**Names** (in alphabetical order)  **Recipes**
blocking / permeabilization solution  3 % weight per volume (wt/vol) BSA
0,1 – 0,3 % (wt/vol) Triton X-100 in PBS, finally filtered
deoxyribonuclease I solution  0,05 volume percent (vol%)
in HBSS (with phenol red)
DMEM +  10 vol% filtered FBS
in DMEM
DMEM +++  10 vol% filtered FBS
1 vol% L-glutamine
1 vol% penicillin / streptomycin
in DMEM
ketamine / xylazine  25 vol% ketamine (ca. 300 mg/kg)
5 vol% xylazine (ca. 12 mg/kg)
in saline solution
Neurobasal +++  2 vol% B-27
1 vol% L-glutamine
1 vol% penicillin / streptomycin
in Neurobasal medium
oxygen scavenger buffer  400 U/ml catalase
**MATERIALS and METHODS**

100 U/ml glucose oxidase

4 % (wt/vol) glucose

100 mM cysteamine

in PBS ad pH (potentia Hydrogenii) 7,5; finally degassed (air was substituted by nitrogen)

prepared by the collaborators from the Institute of Biophysics, Ulm University

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration/Weight Percentage</th>
<th>Solution/Condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS + glycine</td>
<td>50 mM glycine</td>
<td>in PBS</td>
</tr>
<tr>
<td>PFA fixation solution (3 %, EM grade)</td>
<td>3 vol% PFA, EM grade</td>
<td>in PBS</td>
</tr>
<tr>
<td>PFA fixation solution (4 %)</td>
<td>4 % (wt/vol) PFA</td>
<td>in heated (ca. 70 °C) PBS ad pH 7,4; finally filtered with a pre-folded filter paper</td>
</tr>
<tr>
<td>PLL solution</td>
<td>70,000 – 150,000 molecular weight</td>
<td>in 50 – 100 ml HBSS (with phenol red), finally filtered</td>
</tr>
<tr>
<td>saline solution</td>
<td>0.9 % (wt/vol) sodium chloride</td>
<td>in deionized water</td>
</tr>
<tr>
<td>sucrose solution</td>
<td>30 % (wt/vol) sucrose</td>
<td>in PBS</td>
</tr>
<tr>
<td>VectaMount + DAPI</td>
<td>0.002 vol% DAPI</td>
<td>in VectaMount AQ aqueous mounting medium</td>
</tr>
</tbody>
</table>

### 2.1.3 Tools, devices, softwares, and webpages

This section lists the employed technical devices, softwares for writing the manuscript, for image processing and analysis, and also webpages.

**Tools and devices** (in alphabetical order)

<table>
<thead>
<tr>
<th>Tool</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>autoclave</td>
<td>Systec, Münster, Germany</td>
</tr>
</tbody>
</table>

for sterilization of the coverslips and instruments for the preparation of animals
MATERIALS and METHODS

brush
VWR International, Fontenay-sous-Bois, France

cell culture plates
12 wells
Greiner Bio-One, Frickenhausen, Germany
24 wells
Corning, Durham, USA

containers, beakers, glass bottles
B. Braun Melsungen, Melsungen, Germany

most equipment; for the production and storage of solutions

conventional microscopes
Nikon Eclipse TS.100
Nikon, Düsseldorf, Germany
Zeiss Axioskop 2
Zeiss, Oberkochen, Germany
Zeiss Imager.Z1
Zeiss, Oberkochen, Germany
Zeiss LSM 710*
Zeiss, Oberkochen, Germany
Zeiss Stemi 2000-CS
Zeiss, Oberkochen, Germany

* provided by the Institute of Molecular Virology, Ulm University

coverslips
12 mm
Carl Roth, Karlsruhe, Germany
13 mm
Glaswarenfabrik Karl Hecht, Sondheim, Germany
18 mm
Carl Roth, Karlsruhe, Germany
cryotome CM3050 S
Leica, Wetzlar, Germany
dry heat sterilizer
Binder, Tuttlingen, Germany

for sterilization of glass coverslips and instruments for tissue preparation

embedding molds
Polysciences Europe, Hirschberg an der Bergstraße, Germany

filters (bottle top filters)
Sarstedt, Nümbrecht, Germany

for the FBS in the blocking/permeabilization solution and in DMEM + / +++; for the PLL solution

fixation plate with clamps
home-made (Wissenschaftliche Werkstatt Feinwerktechnik, Ulm University)

freezer (-20 °C)
Liebherr, Biberach an der Riß, Germany
MATERIALS and METHODS

freezer (-80 °C)  Thermo Fisher Scientific, Darmstadt, Germany

glass pipettes  VWR International, Fontenay-sous-Bois, France

for the media and for washing steps in cell culture and tissue processing

hollow needles  B. Braun Melsungen, Melsungen, Germany

for the injection of the animal

for preparing the narcosis solution  Henke-Sass Wolf, Tuttlingen, Germany

ice cube maker  ZIEGRA Eismaschinen, Isernhagen, Germany

incubator  Thermo Fisher Scientific, Darmstadt, Germany

long plastic pipettes  Greiner Bio-One, Frickenhausen, Germany

for the media and for washing steps in cell culture and tissue processing

magnetic stirrer  IKA, Staufen, Germany

for the preparation of solutions

microscope cover glasses (22 x 22 mm)  Carl Roth, Karlsruhe, Germany

microscope slides  VWR International, Fontenay-sous-Bois, France

for conventional imaging

for super-resolution imaging*  Carl Roth, Karlsruhe, Germany

* provided by the Institute of Biophysics, Ulm University

microtome blades  pfm medical ag, Köln, Germany

for the cryotome

nail polish  cosnova, Frankfurt, Germany

Neubauer counting chamber  Brand, Wertheim, Germany

perfusor (and connected tubes)  Ismatec, Berrington, USA

petri dishes  Greiner Bio-One, Frickenhausen, Germany

pipetboy  Hirschmann Laborgeräte, Eberstadt, Germany
MATERIALS and METHODS

for the use with glass and long plastic pipettes

pipettes (2.5 / 10 / 100 / 1000 µl) Eppendorf, Hamburg, Germany
pipette tips (10 / 200 / 1000 µl) Eppendorf, Hamburg, Germany
pre-folded filter papers GE Healthcare Europe, Freiburg, Germany

razor blades Aesculap AG, Tuttlingen, Germany

for embryonic brain dissection

reaction tubes (0.5 / 1.5 / 2 ml) Eppendorf, Hamburg, Germany
reaction tubes (15 / 50 ml) Sarstedt, Nümbrecht, Germany
refrigerator (+ 4 °C) Liebherr, Biberach an der Riß, Germany

set of instruments for tissue preparation Fine Science Tools, Heidelberg, Germany

most equipment

shaker Sigma-Aldrich Chemie, Steinheim, Germany
sterile bench Thermo Fisher Scientific, Darmstadt, Germany
sterile cell sieves Greiner Bio-One, Frickenhausen, Germany

100 µm pore size

super-resolution microscopes

dSTORM setup (see 2.1.6) home-made (Institute of Biophysics, Ulm University)
STED setup (see 2.1.5) home-made (Institute of Biophysics, Ulm University)

syringes (1 / 2 / 5 ml) B. Braun Melsungen, Melsungen, Germany

for the preparation and injection of the narcosis solution; for the filtering of the FBS (5 ml)
two-component adhesive picodent, Wipperfürth, Germany
water bath Grant Instruments, Cambridgeshire, United Kingdom

for the heating of solutions

water purification device Merck Millipore, Darmstadt, Germany
MATERIALS and METHODS

weighing scales (fine and coarse) Sartorius, Göttingen, Germany

**Softwares** (in alphabetical order)

Adobe Photoshop CS5.1 Adobe Systems, San Jose, USA
AutoQuant X3 X3.0.4 Media Cybernetics, Rockville, USA
AxioVision 4.8 Carl Zeiss, Oberkochen, Germany
Excel 2010 Microsoft, Redmond, USA
GraphPad Prism 5 GraphPad Software, La Jolla, USA
ImageJ 1.46r NIH, Bethesda, USA
Imaris 8.1.2, 8.2, and 8.3 Bitplane, Zürich, Switzerland
LSM Image Browser 4.2.0.121 Zeiss, Oberkochen, Germany
Matlab software MathWorks, Natick, USA
Mendeley Desktop 1.16.1 Mendeley, New York, USA
Origin 9.0G OriginLab, Northampton, USA
PowerPoint 2010 Microsoft, Redmond, USA
PPT Drawing Toolkit – Neuroscience* Motifolio, Ellicott City, USA
Word 2010 Microsoft, Redmond, USA
ZEN Zeiss, Oberkochen, Germany

* The collection was used for the sketches of neurons and synapses in the figures 2, 24, and 25.

**Webpages**

https://scicrunch.org/resources for determination of Research Resource Identifiers (RRIDs) of primary antibodies (see table 1)
https://www.google.de for literature research
https://www.ncbi.nlm.nih.gov/pubmed/ for literature research

**Applications**

for gene and protein sequences
for determination of Research Resource Identifiers (RRIDs) of primary antibodies (see table 1)
for literature research
for literature research

**2.1.4 Organisms and biological materials**

Analyzed animals included several pregnant rats and an adolescent male mouse. Furthermore, a list of used antibodies and specifics of the applications will be presented.

**2.1.4.1 Organisms**

Pregnant rats (species *Rattus norvegicus* from strain Sprague Dawley) for primary hippocampal cell culture were purchased from Janvier Labs (Le Genest-Saint-Isle,
France) 13 days after mating and kept in the animal facility of the university (Tierforschungszentrum, Ulm University). The mouse (species *Mus musculus* from strain C57BL/6) was bred in the animal facility of the university (Tierforschungszentrum, Ulm University).

Investigations including animals were conducted in agreement with the guidelines for the humane welfare of experimental animals issued by the Bundesrepublik Deutschland, the national institutes of health, and the Max Planck Society. I have attended a course in advanced training in animal handling in 2014, certified by the GV-SOLAS (level FELASA, category B). Animal experiments were permitted by the review board (animal ethics committee) of the Land Baden-Württemberg (Regierungspräsidium Tübingen, Referat 35), permit number Nr. O.103.

### 2.1.4.2 Antibodies

A set of primary (listed in table 1) and secondary antibodies were thoroughly tested and applied in this study.

**Primary antibodies**

Primary antibodies were mainly purchased from Synaptic Systems (see table 1).

**Table 1**: Primary antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>RRIDs citation</th>
<th>Dilutions</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>bassoon(C)</td>
<td>Synaptic Systems Cat# 141 003 RRID:AB_887697</td>
<td>1:500</td>
<td>dSTORM</td>
</tr>
<tr>
<td>bassoon(N)</td>
<td>Enzo Life Sciences Cat# ADI-VAM-PS003 RRID:AB_10618753</td>
<td>1:500</td>
<td>confocal</td>
</tr>
<tr>
<td>FUS</td>
<td>Sigma-Aldrich Cat# HPA008784 RRID:AB_1849181</td>
<td>1:500</td>
<td>confocal</td>
</tr>
</tbody>
</table>

**Secondary antibodies**

Secondary antibodies were mainly purchased from Enzo Life Sciences (see table 2).

**Table 2**: Secondary antibodies

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>RRIDs citation</th>
<th>Dilutions</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>FUS</td>
<td>Sigma-Aldrich Cat# HPA008784 RRID:AB_1849181</td>
<td>1:500</td>
<td>confocal</td>
</tr>
</tbody>
</table>
### MATERIALS and METHODS

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Catalog Number</th>
<th>RRID</th>
<th>Dilution</th>
<th>Imaging Technique</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAD65</td>
<td>Abcam Cat# ab85866 RRID:AB_1860505</td>
<td>1:500</td>
<td>STED</td>
<td></td>
</tr>
<tr>
<td>gephrin</td>
<td>Synaptic Systems Cat# 147 011 RRID:AB_887717</td>
<td>1:500</td>
<td>STED</td>
<td></td>
</tr>
<tr>
<td>GFAP</td>
<td>Sigma-Aldrich Cat# G3893 RRID:AB_477010</td>
<td>1:500</td>
<td>epi</td>
<td></td>
</tr>
<tr>
<td>GluA1</td>
<td>Synaptic Systems Cat# 182 011 RRID:AB_1630258</td>
<td>1:500</td>
<td>epi</td>
<td></td>
</tr>
<tr>
<td>HOMER-1B/C</td>
<td>Synaptic Systems Cat# 160 022 RRID:AB_2619857</td>
<td>1:1000</td>
<td>STED</td>
<td></td>
</tr>
<tr>
<td>MAP2</td>
<td>Synaptic Systems Cat# 188 004 RRID:AB_2138181</td>
<td>1:500</td>
<td>STED</td>
<td></td>
</tr>
<tr>
<td>PSD-95</td>
<td>Abcam Cat# ab2723 RRID:AB_303248</td>
<td>1:500</td>
<td>confocal</td>
<td></td>
</tr>
<tr>
<td>S100B</td>
<td>Sigma-Aldrich Cat# S2532 RRID:AB_477499</td>
<td>1:500</td>
<td>epi</td>
<td></td>
</tr>
<tr>
<td>SYP</td>
<td>Synaptic Systems Cat# 101 004 RRID:AB_1210382</td>
<td>1:500</td>
<td>confocal</td>
<td></td>
</tr>
<tr>
<td>VGLUT1</td>
<td>Synaptic Systems Cat# 135 304 RRID:AB_887878</td>
<td>1:500</td>
<td>dSTORM</td>
<td></td>
</tr>
<tr>
<td>VGLUT2</td>
<td>Synaptic Systems Cat# 135404 Lot# RRID:AB_887884</td>
<td>1:500</td>
<td>dSTORM</td>
<td></td>
</tr>
</tbody>
</table>

**Secondary antibodies**

Secondary antibodies conjugated with Alexa Fluor fluorophores (excitation wavelengths: 488 nm, 568 nm, and 647 nm for conventional imaging; 532 nm and 647 nm for dSTORM imaging) were purchased from Thermo Fisher Scientific, Eugene, USA. The applied dilution was 1:750 in blocking / permeabilization solution if not otherwise specified. For STED microscopy, ATTO fluorescent dyes (excitation wavelengths: 590 nm and 647 nm) were purchased from Sigma-Aldrich Chemie, Steinheim, Germany; and ATTO-TEC, Siegen, Germany (provided by the Institute of Biophysics, Ulm University). These antibodies were used in dilutions of 1:750, 1:1000, and 1:2000 in PBS.

**2.1.5 STED setup**

The STED microscopy setup was home-made by Dr. Christian Osseforth from the Institute of Biophysics, Ulm University (director Prof. Dr. Jens Michaelis). The FWHM of
the setup was determined as 35 nm. The core features are a dual-color 3D table-top acquisition system. All lasers are provided by a super-continuum laser source (Osseforth et al. 2014). The presented images were all taken in one plane.

2.1.6 dSTORM setup

The home-made table-top 2D dSTORM setup (constructed by Jochen M. Reichel, Institute of Biophysics, Ulm University) includes two activation and excitation lasers (excitation wavelengths: 532 and 640 nm) and an additional UV (ultraviolet) laser (402 nm), which represents an activation laser. Dichroic mirrors and emission filters are applied to distinguish excitation and emission light of both channels (see figure 5). Acquisition was conducted by feeding each channel onto one half of a high-resolution widefield EMCCD (electron multiplying charge coupled device) camera (pixel size 131.9 nm). The obtained FWHM was determined as approx. 20 to 30 nm (Schoen et al. 2016).

Figure 5: The dSTORM laser setup

The laser system comprises a UV, a green, and a red laser, which are directed by a consecutive set of dichroic mirrors. The laser lights are conveyed through circular apertures to the objective (left side, not displayed on the image). dSTORM = direct stochastic optical reconstruction microscopy, UV = ultraviolet.
The setup was built by Jochen M. Reichel, Institute of Biophysics, Ulm University. The image was acquired with the permission of the Institute of Biophysics, Ulm University.

2.2 Methods
The applied methods comprise primary rat hippocampal culture and processing of mouse CNS tissue for immunohistochemistry, the subsequent various applied microscopy techniques, and the in silico analysis of acquired images.

Contributions: Image acquisitions were performed in cooperation with Dr. Christian Osseforth (STED microscope), Jochen M. Reichel, and Dhruva Deshpande (dSTORM; all Institute of Biophysics, Ulm University). dSTORM-specific processing procedures to in silico create super-resolved images, including the calculations of centroid positions of fluorophores, background signal subtraction, were performed by Jochen M. Reichel and Dhruva Deshpande. Programing of the macros for ImageJ to calculate the statistical values of dSTORM data and the implementation in Origin data files, which underlies the graphs in the figures 19, 20, and 22 was performed by Jochen M. Reichel. PD Dr. Francesco Roselli, the technical assistants Renate Zienecker (tissue processing) and Ursula Pika-Hartlaub (processing of primary neurons) provided sporadic help in some of the methods.

2.2.1 Cell culture and immunocytochemistry
Preparations of primary rat hippocampal cells and of mouse brain tissue for fluorescence immunolabeling are addressed in the following sections.

2.2.1.1 Primary hippocampal cell culture from embryonic rats
The contents of the following passage were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). Hippocampus tissue was harvested from rat embryos 18 to 19 days after mating. Pregnant rats were anesthetized and hypnotized by narcosis with carbon dioxide, followed by decapitation. The degree of the narcosis was evaluated by testing the interdigital reflex. After the extraction of the embryos and subsequent decapitation, the brains were removed and kept in a petri dish filled with HBSS (without phenol red) on ice. Under a light microscope (Zeiss Stemi 2000-CS), the hippocampal formation was dissected out on ice and put into ice-cold HBSS (with phenol red).
The following steps were performed under a sterile bench. The hippocampal tissue specimens were washed five times with 10 to 15 ml cold HBSS (with phenol red) each. 200 µl trypsin-EDTA for enzymatic digestion was added to a rest volume of 1,8 ml HBSS (final concentration of the enzyme was 0,25 %) and incubated at 37 °C for approx. 1 min per embryonic brain. The enzymatic reaction was inactivated by two further washing steps minimum 3 ml each with DMEM + (room temperature (RT) to 37 °C). Finally, a rest volume of approx. 1,8 ml remained. 10 to 200 µl of deoxyribonuclease I solution were added, which contained 1 to 20 milliunits (mU) of deoxyribonuclease I. For dSTORM experiments, this step was skipped. By pipetting the cells approx. ten times up and down through a plastic pipette tip (tip volume 1000 µl), hippocampal cells were mechanically dissociated until the suspension was turbid, which was then poured over a sterile cell sieve. The suspension was filled up with 10 to 20 ml DMEM +++ (37 °C). Subsequently, cell density was determined with a Neubauer counting chamber under a light microscope (Nikon Eclipse TS.100). Cells were finally seeded out on PLL-coated and heat-sterilized glass coverslips in cell culture plates. Plates were cultivated for several weeks in an incubator (37 °C, 5 % carbon dioxide, 95 % humidity). High-precision coverslips (12 and 18 mm) were specifically applied for acquisitions with super-resolution microscopy. Coating with PLL solution was performed before the preparation and lasted minimum 30 min at 37 °C, followed by two to three washing steps with deionized water. To achieve appropriate cell concentrations, the suspension was diluted to 60.000 cells/ml, and, finally, 0,5 and 1 ml were applied to 24 and 12 wells cell culture plates, respectively. Thereby, similar cell densities could be achieved for both sizes of plates and coverslips. After 1 to 2 h or at the next day, the medium was substituted with 37 °C heated Neurobasal ++++. Of note, hippocampal cells cannot be passaged but remain stable for several weeks with ongoing maturation.

2.2.1.2 Fixation and processing of mouse neuronal tissue for immunostaining

A 6 weeks old male mouse was anesthetized with ketamine / xylazine. For the intraperitoneal (i.p.) injection, the animal was held in a slightly upside down position in order to let the inner organs move toward the diaphragm to avoid unintentional injections into organs. The injection was set on the left side paramedian to the linea alba in a low angle to the body surface in order to spare the abdominal organs. The depth of narcosis was closely monitored by observing the motor control of the mouse,
and as soon as narcosis was sufficient – after approx. 5 min – the mouse was fixed with clamps onto a metal fixation plate. Analgesia and hypnosis were verified by checking corneal and interdigital reflexes and also by the observation of a flattening respiration. The peritoneal cave and the thorax were opened with a scissor. The hollow needle connected to the perfusor was pricked into the left ventricle while the right atrium was incised by a small cut with a fine surgical scissor. Immediately after that, bleeding and concomitant rinsing of the vascular system started with 25 ml ice-cold PBS at a rate of approx. 5 ml/min. Without interruption, perfusion was continued with ice-cold PFA fixation solution (4 %, 50 ml). Fixation success was controlled by the observation of a stiffening of the inner organs and the tail.

The brain was removed by dissecting the skull in the midsagittal line from caudal to rostral, starting at the foramen magnum and ending at the frontal bone. This was followed by perpendicular cuts starting from the initial cut toward each side (medial to lateral) and the subsequent removal of the cranial vault. Succeedingly, inner organs were removed and the spinal cord was extracted by cutting open the spinal column from cranial to caudal by dissection of the arcus vertebrae and the removal of the corpora vertebrae from the ventral side. The brain and spinal cord were subsequently immersed in the PFA fixation solution (4 %) overnight at 4 °C. Before freezing, the samples were immersed in sucrose solution for another 24 h at 4 °C in order to shelter tissue from damage while freezing. Finally, the spinal cord was cut into three equally long pieces: cervical, thoracic, and lumbar parts. Orientations were the thickenings in the cervical and lumbar spinal cord (intumescentiae cervicalis et lumbalis, respectively). The brain and the parts of the spinal cord were finally placed into embedding molds filled with liquid Tissue-Tek O.C.T. Compound and were slowly frozen from bottom to top on dry ice for storage at -80 °C.

The mouse brain and lumbar spinal cord tissues were transferred from -80 °C to -20 °C freezers and then into the cryotome chamber (cryotome CM3050 S) the day and 1 h before cutting, respectively. Tissue was cut into 40 µm thick sections and collected with a brush in a PBS-filled cell culture plate (24 wells). After another washing step in PBS, sections were processed in the same way as were the coverslips from primary culture for immunocytochemistry.
2.2.1.3 Immunocytochemistry for conventional and super-resolved imaging

Parts of the contents of the following passage were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). The primary neurons were evaluated after approx. 7, 10, and 14 days in vitro (DIV) under a light microscope (Nikon Eclipse TS.100) to determine the suitability for further processing. Included criteria were comparable densities, the ratio of neurons to glial cells, and the homogenous outgrowth of spine-bearing dendritic protrusions. The hippocampal neurons were fixated at the indicated DIV as follows. The cells were rinsed four times with 37 °C heated PBS. Then, chilled 100 % methanol was added, and the cells were kept 5 min at -20 °C, followed by 3 washing steps 5 min each with PBS. Occasionally, PBS + glycine was used for the washing after fixation to reduce autofluorescence. At this point, the experiments were sometimes paused while keeping the cells at 4 °C.

In the following, both the coverslips and the tissue sections were treated in the same manner. Subsequent steps were all performed with the samples on a shaker. The coverslips and tissue were incubated in blocking / permeabilization solution for 2 to 3,5 h at RT, before the primary antibodies (see 2.1.4.2, table 1) were added for 2 to 3 d at 4 °C. After three washing steps 30 min each at RT, the samples were incubated for 2 to 3,5 h with the secondary antibodies at RT. Both the primary and secondary antibodies were diluted in the blocking / permeabilization solution (see specific dilutions of the antibodies in 2.1.4.2). The samples were washed again three times for 30 min each.

For conventional imaging, the coverslips were shortly dipped into deionized water, while the tissue sections were directly dried on the microscope slide surface and mounted under microscope cover glasses. The mounting was performed with a drop of VectaMount + DAPI on the microscope slide. If dSTORM samples had to be kept for longer times, another fixation step followed with PFA fixation solution (3 %, EM grade) for 20 min at 37 °C, which was followed by three washing steps 5 min each. To the contrary, the post-fixation was omitted when the imaging followed immediately after the staining. For STED microscopy, the mounting was conducted with a drop of TDE. Eventually, the coverslips were sealed with nail polish. For storage, the samples were kept at 4°C. The samples for dSTORM imaging were kept in PBS and stored at 4 °C. Further processing of the samples is explained in the following sections.
2.2.2 Conventional fluorescence microscopy and image analysis

Both epifluorescence and confocal microscopy were employed. The acquired images were processed with image softwares or further analyzed for statistical evaluations if necessary.

2.2.2.1 Epifluorescence microscopy – image acquisition

Immunostained hippocampal neurons were acquired with two upright, table-top fluorescence microscopes, Zeiss Axioskop 2 and Zeiss Imager.Z1. Both microscopes were controlled with AxioVision software. For all images used for statistical analysis, exposure time was identical within each preparation and staining condition. A 40x magnification (Plan-Apochromat 40x / 1.0 Oil Iris, Zeiss, Oberkochen, Germany) was chosen for all samples.

2.2.2.2 Confocal fluorescence microscopy – image acquisition

Confocal microscopy was conducted with a table-top, inverted laser scanning microscope (Zeiss LSM 710, kindly provided by the Institute of Molecular Virology, Ulm University). Up to four individual lasers were applied and sequentially acquired, including 405 nm, 488 nm, 561 nm, and 633 nm. The laser powers and digital gains were adjusted in order to obtain sufficient excitation with minimum photobleaching of samples, while the offset remained in the default setting during all acquisitions.

For all types of specimens, the images were acquired with a 63x magnification objective (Plan-Apochromat 63x / 1.40 Oil DIC M27; Zeiss, Oberkochen, Germany). Acquisition parameters were set in ZEN software, which controlled the setup. For hippocampal neurons, the image quality settings were set to 8 bit with a frame size of 134,95 µm for both the x- and y-axes. The pixel size was 30 nm (pinhole 51 µm in channel 1 = 405 nm, 41 µm in channel 2 / 3 = 488 / 561 nm, 90 µm in channel 4 = 633 nm). The channels were acquired sequentially.

For slices, the pixel size was set to 70 nm but 130 nm (pinhole 60 µm) when stacks were additionally acquired (stack size approx. 2,5 µm with 0,5 µm steps). The image quality settings were set to 8 bit with a frame size of 134,95 µm for both the x- and y-axes. For the display of stacked images (acquisitions from cornu ammonis, sector 1 (CA1); and partially from spinal cord sections), one section of a stack was chosen for further analysis. All other CNS areas were acquired in one plane. The analyzed CNS
regions were identified by typical nuclei patterns of the cortical layers and spinal cord, which were identified in channel 1 (DAPI) (see 3.1.1, figure 7).

2.2.2.3 Conventional fluorescence microscopy – image analysis
The images from conventional microscopy were either processed for the display in figures, or the raw data were further statistically analyzed.

Image processing
For the application in figures, the images were processed in AxioVision software (images from Zeiss Axioskop 2 and Imager.Z1 microscopes), whereas for the confocal images, LSM Image Browser was employed to evaluate the staining quality. The confocal images of the mouse brain sections, which were selected for the figures, were deconvoluted with AutoQuant X3 software (5 iterations based on a theoretical calculation of the PSF) and further processed with Imaris software to adjust the channel colors, brightness, and contrast to finally obtain tagged image file format (TIFF) images. The final editing of the images for the figures was performed with ImageJ (confocal microscopy of primary neurons), Adobe Photoshop, PowerPoint, and Word softwares.

Statistical evaluation
The contents of the following passage were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). The co-localization analysis, which underlies the graphs in figures 13A and B was conducted with Imaris software. 10 cells were included, which were acquired from three independent hippocampal cell cultures fixated at DIV 14. The viability of these cells was estimated by the evaluation of the DAPI signals. A round, homogenous nuclear stain was indicative for healthy neurons. Cells were excluded in case of irregularly shaped nuclei or in case of increased local cell density with a strong overlap of dendrites. Another hint for appropriate quality was indicated by an intact, continuous dendritic MAP2 signal, which had to be absent in spines. The analyzed neurons also had to be representative for the particular preparation to be included in the analysis.

In Imaris software, MAP2-labeled dendrites were randomly sought and then selected within a rectangular area. Supported by a program tool, a so called surface was generated at the borders of the MAP2 signals, which approx. represents the dendritic cell membrane surface. In a second step, a spot analysis was conducted in this region of
interest for synaptic FUS puncta (Alexa Fluor 568 nm) as well as for synaptic markers (Alexa Fluor 488 nm). The underlying parameters for the spot detection were based on roundness, size, and intensity of the signals with lower and upper thresholds. Parameters were kept stable within a condition (antibody combination) and preparation. Spots farther away than 3 µm of the generated surface were excluded, while all puncta within this area or the surface were processed in a subsequent co-localization analysis, which refers to the reported maximum length of spines (Hering and Sheng 2001). Spots co-localization was only then considered as positive when the centers of two spots exhibited a distance of 700 nm or less. Total values of non-co-localized and co-localized FUS with synaptic marker spots were transferred to Excel software for further statistical evaluation. Calculated parameters included the mean values of the respective percentages of isolated FUS and marker spots and the co-localization events of FUS with synaptic markers. Indicated error values represent standard errors of the mean (SEM) and standard deviations (SD).

2.2.3 Super-resolution fluorescence microscopy and image analysis
Initial acquisitions were performed with STED microscopy and, later on, with the SMLM technique dSTORM.

2.2.3.1 STED – image acquisition and processing
Primary hippocampal neurons from mainly DIV 14 (more detailed indications in figure 14) were used for the acquisitions. Different primary and secondary antibodies were tested in respective dilution series. ATTO fluorescent dyes seemed to be superior to Star dyes, and a dilution ranging from 1:750 – 1:1000 seemed to be the best compromise between sufficient labeling and limited unspecific binding.

Image acquisitions were conducted together with Dr. Christian Osseforth, Institute of Biophysics. An acquisition started with the search for a dendritic area in confocal settings. An optimal z-plane was adjusted at putatively synaptic signals. After taking a TIFF image in confocal mode, a second super-resolved acquisition followed. Therefore, a STED depletion laser beam with a doughnut shape was applied with wavelengths of 720 and 750 nm for the 591 nm and 647 nm excitation lasers, respectively. The pixel dwell time was set to 400 µs for high quality acquisitions. Eventually, the images were rendered with 20 nm pixel size (Osseforth et al. 2014). Images were post hoc evaluated,
adjusted in brightness and contrast in ImageJ, and further edited in PowerPoint and Word softwares.

**2.2.3.2 SMLM with dSTORM – image acquisition and processing**

A general description of SMLM imaging is presented in 1.5.3 but will be recapitulated in the following paragraph.

**Background**

This paragraph is based on a publication from Hess et al., which addresses a different but comparable SMLM technique (Hess et al. 2009). dSTORM makes use of two lasers, an activation laser bringing fluorophores in an active state, from which they can be excited to fluoresce by a so called excitation (also read-out) laser. In concert, these lasers effect the blinking of the fluorophores. The laser intensities correlate with the blinking frequencies, which implicates that the intensities should be always kept at a state where fluorophores and their blinking events can still be distinguished. During the acquisition, a rather constant activation can be achieved by short pulses of the activation laser (less than 1.5 s) while the read-out laser usually remains at a constant intensity, which is also referred to as asynchronous acquisition. If this is not enough, the activation laser can be kept active at a low but increasing power level to keep fluorophores blinking on a constant level (Hess et al. 2009). In the present study, the excitation lasers were also employed as activation lasers, while the UV laser was only used to boost activation of the fluorophores if required.

Photoswitching or blinking of fluorophores relies upon photoinduced electron transfer. The process can be controlled by adjusting the intensity of the excitation beam and by the amount of beta-mercaptoethylamine (synonym: cysteamine) in the so called oxygen scavenger buffer, which is also referred to as the switching buffer. The oxygen scavenger buffer additionally contains an oxygen scavenger system, which consists of glucose, glucose oxidase, and catalase (Heilemann et al. 2008, Sanders 2015). This specific buffer for dSTORM imaging enhances the blinking behavior of Alexa Fluor dyes by reducing the oxygen levels in the buffer.

As applied in this study, dSTORM can be combined with total internal reflection fluorescence (TIRF) illumination, which refers to a technique to image the surface of a specimen at the border of two media with differing refractive indices. In this setting, the
excitation laser beam hits the sample beyond the critical angle and becomes reflected. Thereby, background signals are minimalized (Fernández-Suárez and Ting 2008).

Subtraction of background signals is an important step in the post-processing. Usually, rendering includes weighted plots, which result from the signal intensities (photon counts acquired) and the number of localizations (corresponds to the number of blinking events from one fluorophore). To sum up, in dSTORM it is the goal to acquire signals from as many fluorophores as possible with a density to still be capable to discern the signals (Hess et al. 2009).

Image acquisition
The contents of the following passage were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). Most super-resolution images in this study were acquired with an SMLM technique also referred to as dSTORM. The following experiments were conducted together with Jochen M. Reichel and Dhruva Deshpande, both from the Institute of Biophysics, Ulm University. To start with, the coverslips were rinsed with deionized water. The water was then substituted by the switching buffer, which was freshly prepared prior to the administration. After an incubation time of ca. 2 min at RT, a drop of the buffer was placed in the middle of a microscope slide, and the coverslip was put inversely on top. The coverslip was eventually sealed with a two-component adhesive or a nail polish. The imaging started as soon as the nail polish or the adhesive became completely solid. The stability of the freshly prepared buffer is optimal for approx. 120 min.

The microscope slides were fixed on the object table with two magnets, and the xy-movements of the stage were controlled with custom-written software (programmed by members of the Institute of Biophysics, Ulm University). Laser intensities for the green (532 nm) and red (640 nm) channels were manually adjusted with an acousto-optical tunable filter (AOTF) device. The UV activation laser (402 nm) was also manually modulated, which was occasionally used when required. If necessary, the powers of the excitation lasers rose stepwise in the course of the image acquisitions. For optimal excitation and reduced signal noise, a TIRF configuration of the oil-immersion objective (APO TIRF 60x, NA 1.49 Oil; Nikon, Düsseldorf, Germany) was employed. After identifying a synaptic area, red and green channel images were consecutively acquired with a
series of at least 30,000 TIFF images each in a 900 µm² area. For most imaged areas, a diffraction-limited image preceded the super-resolved acquisitions.

**Image processing**

The contents of the following passage were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). The image processing to obtain calculated super-resolved images was conducted by Jochen M. Reichel and in later experiments also by Dhruva Deshpande (both Institute of Biophysics, Ulm University) with custom-written software based on Matlab software. In brief, raw data were fed into a computational process, in which centers of single fluorophores were located by identifying the COM of their underlying PSFs. Eventually, reconstructions were performed with a pixel raster of 10 nm. According to a process called weighted rendering, every pixel received a brightness value corresponding to the acquired signal intensities (photon counts). The processed images of both channels were then superimposed onto each other based on a transformation function, which was generated by the acquisition of TetraSpeck Microspheres in both channels for achieving a so called beadmap in order to correct for chromatic aberrations. Further processing algorithms were applied for signal noise reduction. Drift was mostly negligible (Schoen et al. 2016). If needed be, specific algorithms, which include cross-correlation, were used for the correction of drift in xy-directions (Wang et al. 2014). Drift in the z-direction was automatically corrected by a built-in autofocus system.

For the display in figures, images were selected and initially adjusted in ImageJ software with a Gaussian blur of 10 nm and were occasionally further processed with Adobe Photoshop software. Finally, images were arranged and provided with symbols, scale bars, and captions in PowerPoint and Word softwares.

**2.2.3.3 SMLM with dSTORM – image analysis**

The contents of the following passage were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). The distance analysis of the axial distributions of synaptic proteins was performed under blinded conditions using ImageJ software on the raw image data. Synapses were initially identified as spot- or bar-like structures of the overlapping synaptic markers. The presumable trans-synaptic axis was then determined based on several morphological aspects of the labeled synaptic proteins, which refers to a recent STORM study, which
MATERIALS and METHODS

has briefly been described in 1.5.4.2 (Dani et al. 2010). Due to the fact that synaptic proteins of the PAZ and PSD display a disk-like distribution, they appear in two extreme variants in 2D images – as well as in all other imaginable three-dimensional orientations. The imaging plane was always parallel to the coverslip surface in the employed setting. When the synaptic cleft is parallel to the imaging plane, in a so called face view, these proteins appear in a circularly shaped pattern. In turn, when the synaptic cleft is perpendicular to the imaging plane, which represents a side view, synaptic scaffolding proteins display as bar-like structures. In this viewing angle, a cleft between pre- and postsynaptic proteins can become obvious. Of note, the trans-synaptic axis is always perpendicular to the plane of the synaptic cleft. Only those synapses were considered for statistical analysis whose trans-synaptic axes were determinable.

In a subsequent step, a line with a defined width of 300 nm was manually drawn along the trans-synaptic axis. The lengths of these lines were adjusted to the extensions of the respective protein populations. For illustration of the method see figure 6, in which the white rectangles represent the aforementioned lines.

Figure 6: Analysis of the trans-synaptic distance of synaptic proteins
MATERIALS and METHODS

In the overview, a dendritic area of primary hippocampal neurons from DIV 14 is displayed with adjacent FUS- and PSD-95-positive spots, which are indicative for synaptic contacts. The magnification in the lower left displays a series of synapses with immunolabeled FUS (red) and PSD-95 (green). White rectangles mark exemplary lines of measurement along a trans-synaptic axis (long sides) with a line thickness of 300 nm (short sides). The lengths of the lines were adjusted individually according to the extension of the synaptic markers. DIV = days in vitro, FUS = fused in sarcoma, PSD-95 = postsynaptic density protein 95. Scale bars represent 1 µm. Adapted from Schoen et al. (Schoen et al. 2016), open access article: CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

Intensity profiles were then generated resulting from the sum intensity values for each pixel (10 nm) along the axis of the 300 nm thick lines. Subsequently, the centers of mass (COM) of the obtained intensity profiles were calculated. The arithmetic means of the distances between the COM of the two synaptic markers were chosen to determine the positions of the proteins. The peak intensities as values to determine the position of a protein accumulation proved to be too ambiguous (data not shown). A macro for ImageJ software, written by Jochen M. Reichel (Institute of Biophysics, Ulm University), helped to order the values and to calculate the distances for each synapse. Mean values with error calculations (SD and SEM) were computed with Origin software by Jochen M. Reichel. These values underlie the graphs in the figures 19, 20, and 22. Statistical graphs of these calculations were mainly obtained with GraphPad Prism. Intensity profiles in figure 19 were generated with Origin software together with Jochen M. Reichel.

If the orientation of a synapse was indeterminate, another approach followed, in which the COM were determined in 2D (2D-COM). The combination of the two markers FUS and SYP, which turned out to be in same synaptic compartment with a strong overlap and a rather spherical distribution, rendered it impossible to use the 1D-COM analysis along a line, as described above. Along these lines, a pair of co-localizing spots was selected within a manually drawn rectangular area, and a COM in two dimensions was ascertained. The following computational process was supported by another custom-written macro by Jochen M. Reichel. A subsequent Euclidean distance calculation was based on the distributions of the signals in the xy-plane and on the underlying intensities of each pixel.
3 RESULTS

3.1 Analysis of synaptic FUS in mouse brain and spinal cord tissue

To expand the knowledge of synaptic FUS localization in the CNS, immunocytochemistry in combination with confocal microscopy was employed for the investigation of several brain and spinal cord regions. The choice of regions was a priori reasoned with regard to their importance in ALS and FTD as well as to the reported high expression levels of FUS in these CNS regions (see explanations in 4.2).

3.1.1 Description of the analyzed mouse CNS structures

In order to investigate FUS at different classes of synapses in the mouse CNS, extensive immunostainings were performed, which included markers to distinguish between pre- and postsynaptic compartments as well as between excitatory and inhibitory synapses. Some staining combinations even allowed a distinction of synaptic subtypes according to their neurotransmitter usage.

The following anatomical descriptions of mouse CNS structures, which are displayed in figure 7, are partly based on the Allen Brain Atlas (Allen Institute for Brain Science 2011) and a study, in which synaptic contacts of the mouse hippocampus were characterized with immunostainings (Heise et al. 2016). The dark signals in the exemplary tissue images in figure 7 display DAPI-positive nuclei of mouse tissue sections, which will be further described in the following paragraphs. Neocortical, archicortical (hippocampal), and cerebellar sections represent coronal sections; the lumbar spinal cord is cut horizontally.

Figure 7 (upper part) shows an overview of the mouse hippocampus in a coronal section. The depicted DAPI-positive nuclei are concentrated in the two major somatic belts: the cornu ammonis (CA; lateral) and the dentate gyrus (DG, also gyrus dentatus; medial). The CA layer comprises the sectors CA1, 2, and 3 with an outer and lateral dendritic area named stratum oriens (SO), the adjacent stratum pyramidale (SP), which contains the pyramidal cell somata, and an inner and medial dendritic layer called stratum radiatum (SR). The innermost layer between the SP and the SR is also referred to as the stratum lucidum and represents a dendritic area where mossy fibers terminate with mossy fiber boutons. They represent a class of oddly giant presynapses, which embower specialized spines at thorny excrescences of CA3 neurons. Several PSDs
RESULTS

(labeled with postsynaptic density protein 95 (PSD-95) antibodies) of a thorny excrescence can be observed in figure 8B (third row). The granule cells in the stratum granulare (SG) of the DG are the origin of the axonal mossy fibers, which project into the hilum of the dentate gyrus. The hilum of the DG encloses the polymorph layer (PO). Axons from the SP of CA3, in turn, terminate as Schaffer collaterals in the SR of CA1. A major input of the hippocampus is the so called tractus perforans, which conveys signals from the entorhinal cortex to the stratum moleculare (SM) of the DG. Thus, a trisynaptic pathway is postulated with the following network-antegrade sequence: entorhinal cortex – via tractus perforans – 1st synapse in DG (SM) – via mossy fibers – 2nd synapse in CA3 (stratum lucidum) – via Schaffer collaterals – 3rd synapse in CA1 (SR).

Further analyzed CNS structures are displayed in the lower part of figure 7. The mouse neocortex comprises 6 layers of differently shaped and sized neuronal cells. The neocortex ranges from the outermost layer 1 (I) to layer 6 (VI). The layer IV is not existent within the analyzed parasagittal cortical zone; the layer VI is not shown. In the present study, only neurons from the layers II / III were analyzed.

The cerebellar cortex consists of the following layers from exterior to interior: the molecular layer (ML), the Purkinje cell layer (PC), and the granule cell layer (GCL). The cerebellar cortex is followed by the medulla, which corresponds to the cerebellar white matter. The only area chosen for analysis was the ML, which harbors synapses from climbing and parallel fibers, which both originate from the brain stem and the GCL, respectively (figure 7, lower right).

In the grey matter of the lumbar spinal cord (figure 7, lower left), the neuronal cell somata can be found in the center of each half of the displayed section. The two anterior horns on both sides of a midsagittal center line form convex cell clusters toward the anterior side in horizontal sections and are adjacent to the funiculi anteriores, which represent the white matter of the spinal cord. Within the anterior horns, the alpha-motoneurons can be identified mainly by the sizes of their nuclei and somata as well as by their typical location.
**Figure 7**: Histoanatomy of the analyzed mouse CNS regions

Analyzed mouse CNS areas are exemplarily displayed in order to delineate the anatomy and connectivity. In the upper part of the figure, the somatic belts within the hippocampus are highlighted by DAPI-labeled nuclei, namely, the cornu ammonis (CA) and the dentate gyrus (DG). Major axonal fiber tracts are part of the trisynaptic intrahippocampal circuitry, which includes the tractus perforans, the mossy fibers, and the Schaffer collaterals (tracts are indicated as colored arrows). A section of the neocortex with indicated layers, the anterior part of the lumbar spinal cord, and a folium of the triple-layered cerebellum are depicted in the lower part of the figure. Hippocampus, neocortex, and cerebellar cortex sections were coronally sliced; spinal cord sections were cut horizontally. CA = cornu ammonis, CNS = central nervous system, DAPI = 4’6-diamidino-2-phenylindole, DG = dentate gyrus, GCL = granule cell layer, ML = molecular layer, PC = Purkinje cell layer, PO = polymorph layer, SG = stratum granulare, SM = stratum moleculare, SO = stratum oriens, SP = stratum pyramidale, SR = stratum radiatum.

### 3.1.2 FUS with synaptic markers in mouse hippocampus

The hippocampus was analyzed in several dendritic areas, including crucial parts of the trisynaptic circuit. The following data is presented in the sequence of anterograde neuroaxis within the hippocampus, commencing with the mossy fiber tract and ending with the SO of CA1, which is located already beyond the intrahippocampal trisynaptic relay.

FUS and the PAZ protein bassoon appear to significantly overlap at mossy fiber boutons in the dendritic areas of the PO and the stratum lucidum and also at smaller synapses in the SR of CA1 (*figure 8*, second row). Consistent with the larger bassoon signals in synapses of mossy fiber boutons (*figure 8A* and *B*, second row), also the sizes of synaptic FUS spots are relatively larger in these synapses as compared to the synapses in CA1 (*figure 8C* and *D*, second row). A similar distribution pattern was observed with markers of excitatory synapses, as can be seen in *figure 8* (rows 3 to 5). More precisely, FUS strongly co-localizes with proteins specific for excitatory, glutamatergic synapses, namely, with the postsynaptic proteins PSD-95, the glutamate ionotropic receptor AMPA type subunit 1 (GluA1), and the presynaptic vesicle protein vesicular glutamate transporter 1 (VGLUT1). The sizes of FUS signals again seemed to correlate with the extensions of synaptic proteins. Moreover, the co-localization of FUS with all markers seemed to be less in the SO of CA1 (*figure 8D*, rows 3 to 5).

On the contrary, a co-staining of FUS with gephyrin, a scaffolding protein of inhibitory synapses, unveils an almost independent distribution of the two proteins (*figure 8*, lowest row).
Figure 8: FUS at synapses in dendritic layers of the mouse hippocampus
RESULTS

Immunohistochemistry of FUS (red) with synaptic markers (indicated with colored captions in A) from defined dendritic areas of the mouse hippocampus is displayed. The rectangles in the schematic overviews of the hippocampus in the first row refer to the magnifications in the columns below (A to D). Each row under the overview images displays a similar marker combination.Magnifications represent the following hippocampal areas: (A) the polymorph layer of the dentate gyrus with mossy fiber boutons, (B) the stratum lucidum of CA3 with mossy fiber boutons at thorny excrescences (circular-shaped PSD-95-positive structure in the third row), (C) the stratum radiatum of CA1, and (D) the stratum oriens of CA1. Arrowheads display double or triple co-localizations of FUS with synaptic markers, stars indicate isolated FUS spots, and circles represent areas with FUS-negative synaptic marker spots. CA1–3 = cornu ammonis, sectors 1–3, FUS = fused in sarcoma, GluA1 = glutamate ionotropic receptor AMPA type subunit 1, MAP2 = microtubule associated protein 2, PSD-95 = postsynaptic density protein 95, SYP = synaptophysin, VGLUT1 = vesicular glutamate transporter 1. Scale bars represent 1 µm.

FUS seems to have a larger topical overlap with bassoon than with PSD-95 spots in mossy fiber boutons (figure 8, rows 2 and 3, respectively). It is remarkable that markers of the PSD, such as PSD-95 and GluA1, appear to rarely overlap with FUS on a submicrometer distance, but are rather found in a close-by position. In turn, VGLUT1 strongly matches with FUS signals (figure 8, row 5, third image from left to right).

3.1.3 FUS with synaptic markers in mouse neocortex and cerebellar cortex

In neocortical sections (figure 9A), FUS largely overlaps with the presynaptic markers bassoon and SYP. FUS is also detected in PSD-95-, GluA1-, and VGLUT1-positive clusters, as was mostly observed in hippocampal dendritic layers. Also in accordance with the situation in hippocampus, the inhibitory synapse marker gephyrin is hardly associated with FUS accumulations.

Consistent with the aforementioned findings in the archi- and neocortex, cerebellar synapses of the ML exhibit a strong overlap of FUS with most of the synaptic markers, namely, bassoon, PSD-95, SYP, GluA1, and VGLUT1 but almost no co-distribution with gephyrin (figure 9B). Only few gephyrin clusters occasionally seem to be contiguous to FUS clusters; a direct association at synapses is beyond the resolution capacities of a confocal microscope.

It is noteworthy that FUS seems to overlap to a greater extent with markers of the presynaptic compartment (bassoon, VGLUT1) as compared to those of the postsynaptic side (GluA1) (compare first and third images from left to right in each row of figure 9). This observation was less obvious than in the mossy fiber boutons.
Figure 9: FUS at synapses in the mouse neocortex and cerebellar cortex

Both rows display confocal images from synapses, which are immunolabeled with FUS (red) and synaptic markers (indicated with colored captions) in the neocortex (A) and in the molecular layer of the cerebellum (B). Arrowheads display double or triple co-localizations of FUS with synaptic markers, stars indicate isolated FUS spots, while circles represent areas with FUS-negative synaptic marker spots. FUS = fused in sarcoma, GluA1 = glutamate ionotropic receptor AMPA type subunit 1, PSD-95 = postsynaptic density protein 95, SYP = synaptophysin, VGLUT1 = vesicular glutamate transporter 1. Scale bars represent 1 µm.

3.1.4 FUS with synaptic markers in mouse lumbar spinal cord

Besides a nuclear localization, FUS in the lumbar spinal cord is abundantly present in large granular structures engulfing motoneuron somata as well as in the surrounding neuropil alongside MAP2-positive dendrites (figure 10). Alpha-motoneurons were identified according to their soma and nucleus sizes, which exceed those of the surrounding neuronal cells in the anterior horn.

In line with the findings in the above-described brain regions, FUS and the presynaptic markers bassoon and SYP demonstrate a strong overlap at the displayed neuronal structures of the anterior horns of the lumbar spinal cord (figure 10, first and second column). In particular, the somatic membrane appears to be almost completely covered with plentiful amounts of FUS, which distributes in broad, flame-shaped spots at axosomatic synapses of alpha-motoneurons (see figure 10, overview images of the first and third column). FUS at these synapses frequently appears farther away from the cell surface than bassoon, which seems to be closer to MAP2-labeled somata and dendrites (see figure 10, second row, first image from left to right).
Spinal cord tissue was immunolabeled with FUS (red) and synaptic markers (indicated with colored captions). Alpha-motoneurons of the lumbar spinal cord exhibit nuclear (indicated with asterisks) and also synaptic FUS (overview images). Magnifications from the white rectangles in the overview images (upper row) are depicted in the second row to highlight FUS-positive axosomatic synapses. The lower row displays a magnification of a random area in the surrounding neuropil with FUS-positive synapses. Arrowheads display double or triple co-localizations of FUS with synaptic markers, stars indicate isolated FUS spots, while circles represent areas with FUS-negative synaptic marker spots. DAPI = 4’6-diamidino-2-phenylindole, FUS = fused in sarcoma, GluA1 = glutamate ionotropic receptor AMPA type subunit 1, MAP2 = microtubule associated protein 2, PSD-95 = postsynaptic density protein 95, SYP = synaptophysin, VGLUT1 = vesicular glutamate transporter 1. Scale bars represent 5 µm (overview images) and 1 µm (magnifications).

FUS and the excitatory synapse marker PSD-95 strongly co-localize at axosomatic synapses of motoneurons as well as in the contiguous neuropil (figure 10, second column). The distribution of FUS with GluA1 and VGLUT1 is in derogation from observations in the hippocampus, the neocortex, and the cerebellum. GluA1 is almost absent in the analyzed areas. In turn, VGLUT1 partially co-localizes with FUS in large spots in the neuropil adjacent to FUS, however, VGLUT1 is mostly present in synaptic spots remote from FUS puncta at axosomatic synapses of alpha-motoneurons (figure 10, third column).

As was the case in the other CNS areas, FUS spots at motoneuron somata distribute mostly independent of the inhibitory synapse marker gephyrin. Only few and weak gephyrin-positive accumulations can be found alongside the surface of alpha-motoneuron somata. As opposed to this, FUS appears amply co-distributed with gephyrin in the neighboring dendrites (figure 10, last column).
3.2 FUS in primary rat hippocampal neurons

Taking the results from immunohistochemistry into consideration, further analyses were conducted with cultivated hippocampal neurons from embryonic rats. The aim was to verify and more thoroughly scrutinize synaptic FUS in a much defined and readily comprehensible model system, which has also advantageous properties with regard to fluorescence imaging. Some of the contents of this chapter were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016).

3.2.1 FUS expression in primary neuronal and glial cells

Primary hippocampal cells were fixated and processed 15 days after seeding. Immunostainings for FUS in combinations with specific markers for neurons and glial cells are displayed in figure 11. FUS demonstrates a strong nuclear presence in all present cell types according to the overlap with DAPI-positive nuclei. Additionally, FUS can also be found as spots alongside MAP2-positive dendritic protrusions, which most likely represent synaptic contacts. Co-immunolabelings of FUS with two different astrocyte cell markers, namely, glial fibrillary acidic protein (GFAP) and S100 calcium binding protein, beta (neural) (S100B), reveal an exclusively nuclear localization of FUS in these cells (see figure 11, asterisks).

![Figure 11: FUS distribution in primary hippocampal neurons and glial cells](image)

FUS (red) is found in nuclei (DAPI, white) and at spots alongside MAP2-positive (blue) dendrites of primary hippocampal neurons from DIV 15. Astrocytes are labeled with GFAP (A, green) and S100B (B, green). Asterisks indicate FUS-positive glial cell nuclei. DAPI = 4',6-diamidino-2-phenylindole, DIV = days in vitro, FUS = fused in sarcoma, GFAP = glial fibrillary acidic protein, MAP2 = microtubule associated protein 2,
3.2.2 FUS localization at synapse subtypes of primary hippocampal neurons

Taking the granular pattern of FUS at dendrites into account, a more precise localization analysis of FUS co-immunolabeled with selective synaptic markers followed (figure 12).

FUS amply co-localizes with the PAZ protein bassoon (figure 12, left column). To distinguish between excitatory and inhibitory synapses, stainings for FUS in combinations with PSD-95 and gephyrin were performed, respectively. Thereby, a predominant localization of FUS at excitatory, glutamatergic synaptic markers is observable (figure 12, middle column). In marked contrast, FUS only sparsely overlaps with gephyrin spots (figure 12, right column).

Figure 12: FUS with specific synaptic markers in primary hippocampal neurons

Primary hippocampal neurons at DIV 14 are shown after immunolabeling with FUS (red) in combinations with the presynaptic active zone marker bassoon (left column, green), the postsynaptic scaffolding marker of glutamatergic synapses PSD-95 (middle column, green), and the postsynaptic scaffolding marker of inhibitory synapses gephyrin (right column, green). Dendritic areas within the white rectangles in the overview images (upper row) are shown in the lower rows as merge and single channels in magnifications. Single channels are displayed in black and white. While synaptic FUS strongly overlaps with bassoon and PSD-95 signals, it rarely does with gephyrin (see co-localizations indicated with arrowheads). The dendrite marker MAP2 is displayed in blue. DIV = days in vitro, FUS = fused in sarcoma, MAP2 = microtubule associated protein 2, PSD-95 = postsynaptic density protein 95. Scale bars represent
To further substantiate these observations, co-localizations were evaluated statistically (figure 13A and B). In brief, in a defined dendritic area, FUS and synaptic marker spots were defined as co-localizing when undercutting a certain distance threshold (see 2.2.2.3 for a detailed description of the analysis parameters). In the combination FUS plus PSD-95, a minority of both markers existed as separate spots: approx. 30% (on average (Ø) 28,3 % +/- 11,3 % SD) FUS spots and 20% (Ø 21,5 % +/- 10,5 % SD) PSD-95 puncta (figure 13A). As a consequence of this, about 50% (Ø 50,2 % +/- 8,1 % SD) of all spots (co-localizations were counted as one spot; single spots plus co-localizations sum up to 100%) are found in co-localization. Regarding the contribution of the markers to co-localization, two thirds of all FUS puncta overlap with PSD-95 (Ø 65 % +/- 11,6 % SD), and even more PSD-95 spots (Ø 70,8 % +/- 11,4 % SD) co-localize with FUS. In contrast to these results, FUS co-distributed less with the postsynaptic, inhibitory scaffolding protein gephyrin (figure 13B). In this combination, 70% (Ø 70,0 % +/- 10,7 % SD) of all spots (isolated spots and co-localization sites) appear exclusively FUS-positive, whereas approx. 10% (Ø 11,6 % +/- 4,5 % SD) exist in co-localizations. 20% (Ø 18,4 % +/- 11,7 % SD) of all spots are exclusively gephyrin-positive. About every sixth FUS spot (Ø 14,2 % +/- 4,8 % SD) co-localizes with a gephyrin spot. In turn, approx. 40% (Ø 42,4 % +/- 17,8 % SD) of gephyrin puncta are found in close-by position to FUS. In sum, a majority of all FUS puncta co-localized with PSD-95 but to a much lesser degree with gephyrin (65% versus 14%, respectively).

In line with the aforementioned observations, large FUS synaptic clusters co-localize with VGLUT1 and GluA1, which are regarded as pre- and postsynaptic markers of glutamatergic synapses, respectively (figure 13C, left panel). As opposed to this, FUS co-localizes to a much lesser extent with another inhibitory synaptic marker, which specifically represents a protein of gamma-aminobutyric acid transmitting (GABAergic) synapses (figure 13C, right panel). A portion of these glutamate decarboxylase 2 (65 kilodalton) (GAD65) spots co-localizes with FUS spots. To differentiate between a spatial co-localization of FUS with inhibitory synapses or a random close-by position as a result of limited resolution, confocal microscopy in high quality settings was subsequently employed (figure 13D). As already indicated by epifluorescence microscopy (figure 12,
right column), synaptic FUS mainly exists in spots remote from gephyrin (figure 13D, star symbols). Only a few co-localizations with gephyrin can be observed (figure 13D, arrowheads). Of note, the co-localizing FUS spots usually appear in smaller sizes than the isolated FUS spots and are closer to the dendrite surface. In turn, larger synaptic FUS clusters seem to be rather detached along the dendrite in a micrometer-scale distance where spines are located (figure 13D, star symbols). This is in accordance with the observations from epifluorescence microscopy with regard to the ample co-localization of FUS with a PSD marker (figure 12, middle column).

Figure 13: FUS at excitatory and inhibitory synapses of primary hippocampal neurons

An analysis of the distribution of FUS at excitatory and inhibitory synapses is demonstrated in a statistical evaluation (A and B) and in immunostainings of primary hippocampal neurons (C and D). Extranuclear, spot-like FUS is statistically analyzed in terms of co-localization with markers for excitatory, glutamatergic (PSD-95, A) and inhibitory synapses (gephyrin, B). Values on the y-axes represent the percentages of isolated or co-localizing spots of FUS and synaptic markers. The error bars are calculated as SEM. In C, synaptic FUS (red) is displayed with further synaptic markers for glutamatergic (left column, in green and blue as indicated in the captions) and inhibitory, GABAergic (right column, in green) synapses. The latter marker is co-stained with the dendrite marker MAP2 (blue). The stainings are displayed as merged and single channels in black and white. Arrowheads indicate co-localizations of FUS with synaptic markers. A weak topical association of an inhibitory marker with FUS is demonstrated in D by employing confocal
resolution, which confirms the observations indicated in B and C. The insert in D represents a magnification of the dendritic area within the white rectangle of the overview image. Arrowheads mark co-localizations of FUS (red) with gephrin (green), stars indicate isolated FUS spots, while circles represent areas with FUS-negative gephrin puncta. The hippocampal neurons in A and B were processed after 14 DIV, in C and D after 15 DIV. DIV = days in vitro, FUS = fused in sarcoma, GABAergic = gamma-aminobutyric acid transmitting, GAD65 = glutamate decarboxylase 2 (65 kilodalton), gephr. = gephrin, GluA1 = glutamate ionotropic receptor AMPA type subunit 1, MAP2 = microtubule associated protein 2, PSD-95 = postsynaptic density protein 95, SEM = standard error(s) of the mean, VGLUT1 = vesicular glutamate transporter 1. Scale bars represent 20 µm (D, overview) and 5 µm (magnifications).

Modified from Schoen et al. (Schoen et al. 2016), open access article: CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

But even with maximum resolution settings in confocal microscopy, further localization analyses were elusive, especially with respect to the exact localization of FUS within the synaptic compartments. Therefore, super-resolution microscopy techniques were employed for more meticulous investigations. This type of distribution within the pre- and postsynaptic compartments is, hereinafter, also referred to as sub-synaptic localization.

3.3 Sub-synaptic localization of FUS with super-resolution microscopy
Two super-resolution microscopy techniques were employed in this study to investigate synaptic contacts from primary hippocampal neurons with respect to synaptic FUS localization. Initial experiments were conducted with STED microscopy, whereas a more substantial analysis of FUS localization followed with the single molecule localization microscopy technique dSTORM.

3.3.1 FUS and synaptic markers in STED microscopy
Super-resolution microscopy was employed in order to determine the sub-synaptic localization of FUS, namely, a pre- or a postsynaptic localization. First experiments were performed with a STED microscope, which was home-made by Dr. Christian Osseforth from the Institute of Biophysics, Ulm University. Initial acquisitions exhibit insufficient labeling of synaptic structures for super-resolution microscopy (figure 14A). Significant improvement could be achieved by testing several primary and especially secondary antibodies in varying combinations (figure 14B and C). For instance, ATTO dyes proved
to be superior to Star and Alexa Fluor dyes with respect to a better signal-to-noise ratio (data not shown).

**Figure 14:** STED microscopy of FUS and synaptic markers in hippocampal neurons

Primary hippocampal neurons are displayed with immunolabeled FUS and synaptic markers after acquisitions with a STED microscope. In A, inadequate signal-to-noise ratio is demonstrated as a result of the insufficient labeling of FUS and bassoon. Hippocampal neurons were processed after 15 DIV. (B) After optimization of the staining procedure, classical pre- (red) and postsynaptic (green) proteins demonstrate an expected bar-like shape in STED acquisitions with an apparent cleft between the markers (right image). The left image shows the same area in conventional confocal mode with hardly distinguishable, coarse signals, which appear yellow in the overlap area of the red and green signals. The hippocampal neurons were processed after 14 DIV. (C) FUS can be detected either in the nucleus (asterisk in the overview image, left) or at synaptic structures adjacent to and partly overlapping with the presynaptic marker bassoon (right image, magnification of the white rectangle in the left overview image). The hippocampal neurons were processed after 14 DIV. Bassoon(N) = N-terminal epitope of bassoon, DIV = days *in vitro*, FUS = fused in sarcoma, STED = stimulated emission depletion. Scale bars represent either 1
µm (A, C, left image) or 100 nm (B, C, right image). Pixel sizes are 20 nm in STED images and 100 nm in the confocal image.

As was previously described in 1.5.4.2, scaffolding proteins of the pre- or postsynapse exhibit a disk-like shape in three dimensions (Dani et al. 2010). Thus, in a side view on a synapse, these disks appear as bar-like structures with distinguishable localizations of pre- and postsynaptic markers (figure 14B, right image; and 14C, magnification, synapse at top left). However, in a face view (along the trans-synaptic axis), signals of pre- and postsynaptic markers overlap and demonstrate a rather circular shape (figure 14C, magnification, synapse at bottom right). Besides a nuclear localization (asterisk), FUS is detectable in co-localizations with Bassoon-positive synaptic structures (figure 14C).

In order to demonstrate the superior resolution of STED microscopy, figure 14B displays a synapse, which was initially acquired in confocal mode (left image) and, subsequently, with STED (right image). In contrast to the super-resolved acquisition, the diffraction blobs of fluorophores largely overlap in the confocally acquired image, which results in hardly distinguishable pre- or postsynaptic localizations (see yellow merge of signals from pre- and postsynaptically bound fluorophores).

3.3.2 Optimization of the dSTORM staining protocol
Since the spatial resolution in the xy-plane is significantly better with dSTORM in comparison to the STED setup, further analyses were conducted with this microscope. Before a more thorough analysis of FUS localization could be accomplished, optimization of the staining protocol was required. Initial experiments with STED and also dSTORM super-resolution microscopy were often not successful. The labeling obtained with a standardized immunocytochemistry protocol for conventional fluorescence microscopy usually resulted in a high background signal noise and an inappropriate labeling of proteins (figure 15, left image). Hippocampal neurons from the same preparations but acquired with conventional epifluorescence microscopy were sufficiently labeled for this specific imaging technique (data not shown).

Reliable labeling of synaptic structures, which was sufficient for precise analyses, was achieved in a stepwise optimization of the staining protocol (figure 15, middle to right images). Two aspects seemed to be prevailing: 1.) The use of high-precision coverslips, the substitution of paraformaldehyde with methanol fixation, and intensified
RESULTS

washing steps, occasionally with the autofluorescence quencher glycine before fixation, reduced the signal noise significantly. 2.) Less diluted antibodies, longer antibody incubation times, followed by prolonged washing steps on a shaker, and a final post-fixation of the samples with EM-grade paraformaldehyde seemed to increase the signal-to-noise ratio.

**Figure 15**: Synapse imaging of hippocampal neurons before and after optimization of the dSTORM staining protocol

Optimizations of the dSTORM staining protocol are displayed from left (before optimization) to right (after optimization) after immunolabeling of primary hippocampal neurons. Immunolabeled synaptic proteins are indicated with colored captions. The areas within the small white rectangles are magnified in the inserts. The left image represents an acquisition with a staining protocol for epifluorescence microscopy. Synaptic structures are not discriminable. Bulky, bright structures most likely represent background fluorescence or unspecifically bound antibodies. The middle image shows first improvements of labeling quality with partially adjacent pre- (green) and postsynaptic (red) markers but significant signal noise (see green signals in magnification). The right image displays the finally optimized staining protocol with clear-cut and confronted pre- (green) and postsynaptic (red) markers as bar-like structures. The signal noise is extraneous. The primary neurons were cultivated for 38, 22, and 17 DIV in the left, middle, and right images, respectively. Bassoon(C) = C-terminal epitope of bassoon, bassoon(N) = N-terminal epitope of bassoon, DIV = days in vitro, dSTORM = direct stochastic optical reconstruction microscopy, PSD-95 = postsynaptic density protein 95. Scale bars represent 1 µm (overview images) and 200 nm (magnifications).

**3.3.3 Super-resolved imaging of reference synaptic markers with dSTORM**

In order to validate the microscopy setup and to obtain reference values, classical pre- and postsynaptic markers of 14 DIV old primary hippocampal neurons were immunolabeled and acquired with dSTORM (**figure 16**).
RESULTS

**Figure 16**: Super-resolved reference synaptic markers in dSTORM

Primary hippocampal neurons were double-immunostained for synaptic markers with known synaptic localizations (as indicated in colored captions) and were acquired with dSTORM. The synapses are rotated in order to obtain aligned horizontal trans-synaptic axes (red channel always left). The following combinations are displayed from left to right: two presynaptic markers (left image), two postsynaptic markers (middle image), and a combination of pre- and postsynaptic markers (right image). The hippocampal neurons were processed after 14 DIV. Bassoon(C) = C-terminal epitope of bassoon, bassoon(N) = N-terminal epitope of bassoon, DIV = days in vitro, dSTORM = direct stochastic optical reconstruction microscopy, PSD-95 = postsynaptic density protein 95. Scale bars represent 100 nm. Pixel size is 10 nm.

Markers of the same compartment commonly demonstrate a strong overlap of their signals but are still distinguishable (figure 16, left and middle images). As expected, scaffolding markers of the presynaptic active zone (bassoon) and those from postsynaptic densities (HOMER-1B/C and PSD-95) appear in bar-like shapes, which is indicative for a side view on the acquired synapses displayed in figure 16. At the same time, a membrane marker of synaptic vesicles (SYP) uniformly demonstrates a bulky distribution. The orientation with such markers is oftentimes more difficult to determine (figure 16, left image, red marker). Pre- and a postsynaptic markers in combination regularly result in a visible cleft between the two detached protein accumulations, which is consistent with the greater spatial distance (figure 16, right image).

### 3.3.4 Super-resolved FUS with pre- and postsynaptic markers in dSTORM

Acquisitions from reference stainings proved a remarkably powerful resolution of the home-made dSTORM setup. Along these lines, first stainings with FUS followed. FUS is displayed in figure 17 in combinations with scaffolding markers of the pre- and postsynaptic protein densities of hippocampal neurons after 14 DIV. FUS regularly exhibits a bulky distribution similar to SYP. As was the case with SYP (figure 16, left
image), a tapering of the shape is oftentimes observed on the side, which is remote of the co-localizing synaptic proteins bassoon or PSD-95 (figure 17, left borders of the red signals). On the contrary, the FUS signals usually align with the bar-like shapes of the synaptic scaffolding proteins on the facing sides (figure 17, right borders of the red signals).

Figure 17: Super-resolved FUS with synaptic markers in dSTORM
FUS-containing (red) synapses from primary hippocampal neurons were acquired with dSTORM after co-immunolabelings with pre- and postsynaptic scaffolding markers (green). The synapses are rotated in order to obtain aligned horizontal trans-synaptic axes (red channel always left). The following combinations are displayed from left to right: FUS with the presynaptic active zone marker bassoon and FUS with the postsynaptic scaffolding protein PSD-95. The hippocampal neurons were processed after 14 DIV. Bassoon(N) = N-terminal epitope of bassoon, DIV = days in vitro, dSTORM = direct stochastic optical reconstruction microscopy, FUS = fused in sarcoma, PSD-95 = postsynaptic density protein 95. Scale bars represent 100 nm. Pixel size is 10 nm.

As was already indicated with conventional fluorescence imaging (figures 12 and 13), FUS in dSTORM acquisitions only occasionally co-localizes with the postsynaptic scaffolding marker gephyrin, which labels inhibitory synapses in primary hippocampal neurons. The arrowheads in figure 18 highlight few co-localizing FUS and gephyrin signals in a dendritic area.

RESULTS
Figure 18: Super-resolved FUS with the inhibitory synaptic protein gephyrin in dSTORM

Co-immunolabeled FUS with gephyrin is displayed in a dendritic area from primary hippocampal neurons after acquisition with dSTORM. FUS (red) only sparsely co-localizes (arrowheads) with gephyrin (green), a postsynaptic scaffolding marker of inhibitory synapses. Arrowheads display co-localizations of FUS with gephyrin, stars indicate isolated FUS spots, while circles represent areas with FUS-negative gephyrin clusters. Hippocampal neurons were processed after 14 DIV. DIV = days in vitro, dSTORM = direct stochastic optical reconstruction microscopy, FUS = fused in sarcoma. Scale bar represents 1 µm.

To sum up, synaptic FUS in primary hippocampal neurons seemed to predominantly be present at excitatory synapses, which is highlighted by the pronounced co-localizations with PSD-95, GluA1, and VGLUT1, and stands in contrast to the mostly absence at gephyrin and GAD65 puncta. Since synaptic FUS often shaped in bulky blobs and was not preferably overlapping with either a pre- or a postsynaptic marker in dSTORM images, a statistical approach was employed to determine the position of FUS in relationship to these markers. This analysis is topic of the next section.

3.3.5 Axial synaptic distributions of FUS and reference synaptic markers in dSTORM

Some of the contents of this section were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). Localization of FUS and synaptic markers in primary hippocampal neurons at DIV 14 was determined by measuring the axial distances between immunolabeled synaptic protein populations. The distances resulted from the calculated centers of mass of each protein
accumulation along a trans-synaptically drawn line (for detailed description see 2.2.3.3 and figure 6). Of all analyzed synapses, those with the highest distance values (upper 65 %) were preferably taken into account in order to exclude those synapses whose trans-synaptic axes were tilted out of the imaging plane (detailed explanation and analysis later in the next but two paragraphs with the corresponding figure 20).

In order to determine reference positions, synaptic markers from the pre- and postsynaptic compartments were analyzed. The axial distributions of some of these markers (N- and C-terminal epitopes of bassoon (bassoon(N) and bassoon(C), respectively), PSD-95, HOMER-1B/C) have already been reported in a similar approach (Dani et al. 2010). For each of the three combinations an exemplary synapse is presented in the left column of figure 19A, B, and C. The underlying intensity profiles of both markers of a combination are displayed with the calculated COM indicated as upright bars on the x-axis (figure 19, middle column). The right columns in figure 19 visualize the distance values of all measured synaptic co-localizations in a scatter plot with mean values as upright colored lines. The following values are based on the cutoff values, which rather exclude acquisitions other than in side views, as mentioned above.

SYP and bassoon strongly overlap with an average COM distance of 43 nm (+/- 2 nm SEM, +/- 25 nm SD, n = 150 synapses) (figure 19A). A combination of two postsynaptic scaffolding proteins (HOMER-1B/C and PSD-95) leads to similar results with an average distance of 54 nm (+/- 2 nm SEM, +/- 16 nm SD, n = 106 synapses) (figure 19B). Pre- and postsynaptic markers in combination (bassoon and PSD-95) result in 91 nm on average (+/- 2 nm SEM, +/- 29 nm SD, n = 190 synapses) (figure 19C) with an expectedly greater distance as compared to combinations with markers from the same compartment.

The following two combinations included stainings for FUS with either a pre- or a postsynaptic marker. The mean distance of 109 nm (+/- 2 nm SEM, +/- 42 nm SD, n = 301 synapses) between FUS and bassoon (figure 19D) is slightly higher than the one between bassoon and PSD-95 (compare figure 19C and D). In synopsis with the aforementioned values, the combination of FUS and PSD-95 (figure 19E) gave information about the localization of FUS in relationship to bassoon and PSD-95. Since the average value of 206 nm (+/- 3 nm SEM, +/- 56 nm SD, n = 294 synapses) is approx. the sum of FUS–bassoon plus bassoon–PSD-95, the conclusion was that the localization of FUS is adjacent to bassoon in the direction of the axon (see figure 24 in 3.3.7).
Figure 19: Trans-synaptic distances between synaptic markers and FUS in dSTORM

The axial synaptic distributions of synaptic proteins and FUS in hippocampal neurons were determined with distance measurements between synaptic protein accumulations, including FUS. Double-
immunostainings are indicated with colored captions in the images of the left column. These images depict representative dSTORM-acquired synapses. The displayed synapses are rotated in order to obtain aligned horizontal trans-synaptic axes from pre- (red marker) to postsynapse (green marker). The combinations are (A) two presynaptic markers, (B) two postsynaptic markers, (C) a pre- and a postsynaptic marker, (D) FUS and a presynaptic marker, and (E) FUS with a postsynaptic marker. The middle column demonstrates the underlying intensity profiles of the marker signals along the trans-synaptic axis of the representative synapses in the left column. The upright bars denote centers of mass of the red and green signals. The left bar corresponds to the red channel and the right bar to the green channel. Intensity values are presented in arbitrary units (a.u.), in which the respective peak values of each channel are normalized to 1. The right column provides scatter plots, which display every measured synapse as triangles (no cutoff applied), and the colored lines indicate the mean distance values for the respective combinations. The hippocampal neurons were processed after 14 DIV. a.u. = arbitrary units, bassoon(C) = C-terminal epitope of bassoon, bassoon(N) = N-terminal epitope of bassoon, COM = center(s) of mass, DIV = days in vitro, dSTORM = direct stochastic optical reconstruction microscopy, FUS = fused in sarcoma, PSD-95 = postsynaptic density protein 95, SYP = synaptophysin. Scale bars represent 100 nm. Pixel size is 10 nm. Modified from Schoen et al. (Schoen et al. 2016), open access article: CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

The employed 2D dSTORM setup allows for an analysis of three-dimensional synaptic structures in only two dimensions. Assuming that a synapse can be built up in every thinkable spatial orientation, certain considerations and, hence, calculations were necessary to approximate the true distance values between synaptic protein accumulations along a trans-synaptic axis. All orientations of a synapse except of synapses acquired in the side view (trans-synaptic axes parallel to the imaging plane) would, consequently, lead to false too short distance calculations. Thus, the obtained distances rather underestimate the real distance values. Along these lines, a cutoff was chosen to exclude those 35 % of all measured synapses with the shortest distances in order to select for synapses acquired in side views (values presented in the previous paragraphs). These values then fitted best with a reference publication from Dani et al., in which relative positions of bassoon, PSD-95, HOMER-1B/C, i.a., have been determined with STORM in mouse brains (Dani et al. 2010). Figure 20 displays a stepwise cutoff selecting for 100 to 5 % of those synapses with the largest distances between the COM. The afore-presented values excluded 35 % of all synapses with the shortest COM distances, and only the upper 65 % were taken into account (figure 20, grey dashed line).
RESULTS

**Figure 20:** Distances between synaptic markers and FUS as a function of exclusion of the shortest distance measurements

As result of the indeterminable 3D orientation of synapses acquired with a 2D dSTORM setup, average trans-synaptic distances between synaptic protein populations are calculated by excluding the shortest distance values, ranging from 0% exclusion (all synapses included) to 95% exclusion (5% of the synapses with the largest distance measurements included). The thicker colored lines represent the average values with indicated SEM (thinner accompanying lines). The colors of the graphs represent antibody combinations, as indicated in the upper left. The values crossed by the grey dashed line fit best with reference values for standard synaptic markers (lower three lines), which have been reported previously (Dani et al. 2010). Bassoon(C) = C-terminal epitope of bassoon, bassoon(N) = N-terminal epitope of bassoon, dSTORM = direct stochastic optical reconstruction microscopy, FUS = fused in sarcoma, PSD-95 = postsynaptic density protein 95, SEM = standard error(s) of the mean, SYP = synaptophysin. Modified from Schoen et al. (Schoen et al. 2016), open access article: CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

Beyond these theoretical considerations, the main statement of FUS as a presynaptically localized protein is independent of these calculations, even if including all measured synapses (**figure 20**, curves touching the y-axis). The average distance values then are as follows (also displayed in **figure 19**, scatter plots in the right
columns): SYP – bassoon Ø (average distance) 31 nm (+/- 2 nm SEM, +/- 26 nm SD, n = 230 synapses), HOMER-1B/C – PSD-95 Ø 41 nm (+/- 2 nm SEM, +/- 23 nm SD, n = 163 synapses), bassoon – PSD-95 Ø 71 nm (+/- 2 nm SEM, +/- 38 nm SD, n = 291 synapses), FUS – bassoon Ø 82 nm (+/- 2 nm SEM, +/- 51 nm SD, n = 462 synapses), and FUS – PSD-95 Ø 167 nm (+/- 3 nm SEM, +/- 73 nm, n = 452 synapses).

3.3.6 Distribution analysis of FUS at synaptic vesicles with dSTORM

Besides relative positions of proteins at synapses, dSTORM imaging enables the evaluation of the distribution of a protein, even if acquired in only two dimensions. Scaffolding proteins of the synapse display a discoid shape, whereas membrane proteins of synaptic vesicles tend to have a broader distribution and fill up the presynaptic bouton, as can be observed for SYP (figure 16, left image; and figure 19A, left image). Similarly, FUS also seems to distribute broader than the scaffolding markers bassoon, PSD-95, or HOMER-1B/C (figure 17; and figure 19, left images). Moreover, FUS resides adjacent to bassoon on the side remote of the synaptic cleft where vesicles are located. Taking these observations into consideration, it was hypothesized that there may be a stronger association between FUS and synaptic vesicles on the level of localization.

In order to further elucidate the association of FUS with synaptic vesicles, further staining rounds and acquisitions of DIV 14 old primary hippocampal neurons were performed with double-immunostainings of FUS and markers of synaptic vesicles. These proteins exhibit a bulky distribution and localize adjacent to the presynaptic active zone, as does FUS. FUS is found to be widely co-localized with VGLUT1, a glutamatergic vesicle marker (figure 21, left image). In turn, FUS only rarely co-distributes with the vesicular glutamate transporter 2 (VGLUT2), a marker of hippocampal synapses with excitatory and also inhibitory properties (figure 21, right image). The distribution pattern in the latter combination was reminiscent of FUS in co-stainings with gephyrin (figure 18).
Figure 21: Super-resolved FUS with vesicular glutamate transporters in dSTORM

Synaptic FUS in primary hippocampal neurons is displayed in combinations with two markers of glutamatergic vesicles after acquisitions with dSTORM. FUS (green) greatly overlaps with VGLUT1 (red, left image) but sparsely (indicated by arrowhead) with VGLUT2 (red, right image). The arrowhead displays a co-localization of FUS with VGLUT2, stars indicate isolated FUS spots, while circles represent areas with FUS-negative VGLUT2 clusters. Hippocampal neurons were processed after 14 DIV. DIV = days in vitro, dSTORM = direct stochastic optical reconstruction microscopy, FUS = fused in sarcoma, VGLUT1 / 2 = vesicular glutamate transporter 1 / 2. Scale bars represent 100 nm (left image) and 1 µm (right image). Pixel size is 10 nm (left image).

The contents of this paragraph were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). In another approach, a co-staining of FUS with the synaptic vesicle membrane marker SYP was conducted (figure 22). A dendritic area of primary hippocampal neurons 14 days after seeding is displayed in figure 22A. Apparently, these two proteins distribute very similarly. FUS in this combination exhibits the strongest topical association with a tested synaptic marker (for comparisons see figures 17, 18, and 19). On a closer look (figure 22A, insert with an exemplary magnification of a synapse), SYP frequently seems to enclose FUS signals. Moreover, at a scale of a few dozens of nanometers (see scale bars in figure 22A), these two proteins appear to be separated within FUS- and SYP-positive synaptic clusters. To corroborate the observation of a close topical distribution between FUS and SYP, the distances between these two proteins were statistically analyzed.
Figure 22: Analysis of the topical association between FUS and SYP with dSTORM.

dSTORM-acquired synapses from primary hippocampal neurons and a statistical evaluation show a strong overlap of FUS and SYP. The overview image in A shows a dendritic area of a double-immunolabeling of FUS and SYP. Large SYP (red) synaptic clusters regularly overlap with FUS (green) signals (co-localizations indicated with arrowheads). The insert shows a magnification from the synapse enclosed with a white rectangle, which is displayed as merge, SYP channel, and FUS channel from left to right. (B) The distances between synaptic protein populations are based on distance measurements between the 2D-COM, which underlie the red and green signals. The scatter plots display every measured synapse as triangles (no cutoff applied), and the colored lines indicate the mean distance values for the combinations.
RESULTS

Hippocampal neurons were processed after 14 DIV. 2D-COM = center(s) of mass in a two-dimensional area, Bassoon(N) = N-terminal epitope of bassoon, DIV = days in vitro, dSTORM = direct stochastic optical reconstruction microscopy, FUS = fused in sarcoma, PSD-95 = postsynaptic density protein 95, SYP = synaptophysin. Scale bars represent 1 µm (overview) and 100 nm (magnifications). Pixel size in magnifications is 10 nm. Modified from Schoen et al. (Schoen et al. 2016), open access article: CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.

Due to the broad distributions of both proteins and their close neighborhood, the trans-synaptic axes were indeterminable. Therefore, a determination of the COM in two dimensions (2D-COM) was necessary (for more detailed description of the analysis approach see 2.2.3.3). To better evaluate the obtained values, FUS was also analyzed in combinations with both bassoon and PSD-95 with the same method (figure 22B). After including all measured synapses in the analysis (see figure 22B), the combination FUS and SYP results in an average distance of 34 nm (+/− 1 nm SEM, +/− 18 nm SD, n = 303 synapses), but Ø 44 nm (+/− 1 nm SEM, +/− 15 nm SD, n = 197 synapses) when setting the same cutoff as in 3.3.5. FUS and bassoon exhibit an average distance of 87 nm (+/− 2 nm SEM, +/− 47 nm SD, n = 419 synapses), however, Ø 112 nm (+/− 2 nm SEM, +/− 39 nm SD, n = 273 synapses) when setting the same cutoff as in 3.3.5. In turn, FUS and PSD-95 demonstrate an average distance of 176 nm (+/− 4 nm SEM, +/− 73 nm SD, n = 409 synapses), but Ø 216 nm (+/− 3 nm SEM, +/− 55 nm SD, n = 266 synapses) when setting the same cutoff as in 3.3.5. The latter two results, namely, for the combinations FUS and bassoon as well as FUS and PSD-95, were higher than the ones obtained with the one-dimensional approach presented in 3.3.5. This results from taking into account more spatial information than along the trans-synaptic axis (for detailed explanation see the discussion in 4.4).

Remarkably, FUS and the synaptic vesicle marker SYP in combination demonstrate a different distribution pattern as compared to combinations of these markers with other synaptic markers. This observation is depicted in figure 23. FUS and SYP usually exhibit a homogenous, bulky distribution in combinations with synaptic scaffolding markers (see figures 16, 17, and 19). However, in a co-immunolabeling of FUS and SYP, both proteins distribute in a scattered and patchy pattern (see figure 23). Moreover, FUS spots commonly seem to be engulfed by SYP signals (see figure 23, magnifications). White arrowheads in the overview image demonstrate further synapses with similar distribution patterns.
Figure 23: dSTORM reveals a putative interference between FUS and SYP antibodies

A possible interference of antibodies for FUS (green) with the synaptic vesicle marker SYP (red) can be observed in dSTORM-acquired images from synapses of primary hippocampal neurons. From left to right are displayed: a merge image of both channels, followed by single channels in black and white. The inserts represent magnifications of the synapse within the white rectangle. FUS signals appear smaller in size than the SYP signals (magnifications). Both markers co-distribute in a patchy and scattered pattern (magnifications in the middle and right images). SYP engulfs the FUS signals (magnification in the left image) and leaves a central recess for FUS in its distribution (magnification in the middle image). Arrowheads indicate synapses with the same described pattern. The hippocampal neurons were processed after 14 DIV. DIV = days in vitro, dSTORM = direct stochastic optical reconstruction microscopy, FUS = fused in sarcoma, SYP = synaptophysin. Scale bars represent 1 µm (overview) and 100 nm (magnifications). Pixel size in magnifications is 10 nm.

3.3.7 FUS distribution in glutamatergic hippocampal synapses

To sum up, a visualization of the proposed model of FUS distribution at excitatory, glutamatergic synapses from primary hippocampal neurons is depicted in figure 24. In this sketch of a synapse, the trans-synaptic axis is a straight line from the pre- to the postsynaptic compartment (figure 24, white rectangle with a dashed frame). In this side view on a synapse, the presynaptic active zone marker bassoon and the postsynaptic PSD protein PSD-95 shape bar-like structures in acquisitions with super-resolution microscopy (figure 24, red and green spots). On the contrary, FUS and SYP form rather bulky structures, which are juxtaposed on the side of the PAZ, which is remote from the synaptic cleft (figure 24, blue and yellow puncta).

The relative position of FUS was determined by a series of double-stainings and acquisitions with dSTORM. Highest overlap was seen with SYP, while the distances between synaptic markers and FUS increased from bassoon to PSD-95. FUS mimicked the distribution of the synaptic vesicle markers SYP and VGLUT1, which is indicative for an abundant presence in the presynaptic bouton of excitatory, glutamatergic synapses of hippocampal neurons.
Figure 24: A model of FUS distribution in glutamatergic hippocampal synapses

The presynaptic and postsynaptic compartments of a glutamatergic hippocampal synapse are displayed with known (SYP, Bassoon, PSD-95) and novel (FUS) localizations of synaptic proteins. FUS (blue) exhibits a bulky distribution adjacent to the presynaptic scaffolding protein bassoon (red) but largely overlaps with the synaptic vesicle marker SYP (yellow). The farthest average distance was measured between FUS and PSD-95 (green). The positions were determined by measuring the distances between the protein populations (axial distributions) according to their centers of mass (COM) (crosshairs). The trans-synaptic axis is displayed as a white rectangle with a dashed frame and can be defined by either bar-shaped appearances of scaffolding molecules or an obvious cleft between the distributions. Ø = average distance, COM = center(s) of mass, FUS = fused in sarcoma, PSD-95 = postsynaptic density protein 95, SYP = synaptophysin. Modified from Schoen et al. (Schoen et al. 2016), open access article: CC BY 4.0, https://creativecommons.org/licenses/by/4.0/.
4 DISCUSSION

4.1 Main findings

On the basis of the presented results, the following main findings can be postulated:

1.) The ALS- and FTD-associated RNA-binding protein FUS is abundantly found at excitatory, glutamatergic synapses of the mouse CNS, including the cortex, the hippocampus, and the cerebellum.

2.) FUS signals at axosomatic synapses of alpha-motoneurons appear in large and bulky formations, which are negative for VGLUT1 and other markers of glutamatergic synapses.

3.) FUS is largely confined to glutamatergic synapses in primary hippocampal neurons.

4.) Super-resolution microscopy demonstrates a presynaptic localization of FUS in hippocampal neurons adjacent to the presynaptic active zone.

5.) FUS overlaps with the distribution of synaptic vesicles on a scale of a few dozens of nanometers.

6.) Super-resolved fluorescence imaging of synaptic proteins from the PAZ, the PSD, and from synaptic vesicles in primary neurons could be demonstrated in unprecedented quality and resolution.

4.2 FUS localizes to excitatory synapses in various regions of the mouse CNS

Several studies have been undertaken to investigate synaptic FUS. To date, distribution and functions have only been detected and analyzed with regard to the postsynaptic side of synapses (Fujii et al. 2005, Fujii and Takumi 2005, Aoki et al. 2012). Furthermore, an association of FUS to the N-methyl-D-aspartate receptor (NMDAR) complex is demonstrated, which is a main constituent of glutamatergic synapses in postsynaptic spines (Husi et al. 2000, Belly et al. 2005). Studies from Stefan Putz, presented in his doctoral thesis in the Institute for Anatomy and Cell Biology, Ulm University, reveal an abundant expression of FUS in the CNS of rats and a putatively synaptic localization in rat hippocampi. Furthermore, FUS and the closely related RNA-binding protein TDP-43 co-distribute in various brain regions, including the cortex, the hippocampus, the cerebellum, and the spinal cord (Putz 2013).
Having these findings in mind, the first aim was to further characterize the synaptic distribution of FUS in the mouse CNS. Therefore, analyses were focused on certain brain areas of the mouse brain and spinal cord tissue of an adolescent male mouse with state-of-the-art laser scanning confocal microscopy. The analyzed areas included the neocortex near the parasagittal cortical zone, the hippocampus (archicortex) with the dendritic layers around CA1, CA3, and DG, the cerebellar cortex, and, further on, the anterior horns of the lumbar spinal cord. This focus on the mentioned CNS regions was also reasoned by their significance in ALS and FTD. It has been repeatedly reported that FTD-FUS features FUS-positive inclusions most extensively in the cortex, the hippocampus, and in the anterior horns of the spinal cord (Munoz et al. 2009, Neumann et al. 2009b, 2009a, Armstrong et al. 2011, Mackenzie et al. 2011b, Lashley et al. 2011, Baborie et al. 2012, Lee et al. 2012, Mori et al. 2012). Furthermore, ALS-FUS compromises upper and lower motor neurons with deposited FUS pathological inclusions in the motor cortex and in the anterior horns of the spinal cord, respectively (Kwiatkowski et al. 2009, Suzuki et al. 2010, 2012, Rademakers et al. 2010, Mackenzie et al. 2011a, Mochizuki et al. 2012). In ALS-FUS, a predominant affection of the lower motoneurons has been reported several times based on clinical evaluations (Blair et al. 2010, Rademakers et al. 2010, DeJesus-Hernandez et al. 2011). In accordance with the disease course, FUS histopathology seems to initially reside at lower motoneuron areas (Rademakers et al. 2010, Mackenzie et al. 2011a). This is also reflected in vertebrate (Kabashi et al. 2011) and invertebrate animal models of FUSopathies (Wang et al. 2011). The cerebellum was chosen due to the significance in many central brain circuits. In marked contrast to the other brain regions, the cerebellar cortex attracts attention for being almost completely resilient to FUS pathology in FTLD (Neumann et al. 2009b, Mackenzie et al. 2011b, Baborie et al. 2012, Braak et al. 2013).

Taking these findings into consideration, FUS was labeled in combinations with marker antibodies for the following synapse proteins: markers of excitatory, glutamatergic synapses (GluA1 as a receptor marker and PSD-95 for PSDs), VGLUT1 for the corresponding presynaptic vesicles, and gephyrin as a postsynaptic scaffolding protein of inhibitory synapses. The pan-synaptic markers bassoon and SYP were used as PAZ and synaptic vesicle markers, respectively. In spinal cord sections, MAP2 indicated in some combinations the association of the markers to a dendritic area. To recapitulate the main findings, synaptic FUS widely overlapped with all tested antibodies, which

**DISCUSSION**
labeled excitatory, glutamatergic synapses, and FUS was found alongside MAP2-labeled dendrites in the spinal cord. While a synaptic FUS localization was present in all selected CNS areas, the amount of proteins per synaptic FUS spot varied. Prominent FUS spots were observed in the anterior horns of the spinal cord, in which particularly large FUS-positive synapses engulfed the somata of alpha-motoneurons. Furthermore, an ample synaptic stain was also present in the neocortex and cerebellar cortex. With respect to the archicortex, several sites within the intrahippocampal trisynaptic relay have been further analyzed. This circuit anatomically begins with the perforant tract, which ascends from the entorhinal cortex and targets the granule cells of the dentate gyrus (1st synapse). The granule cells, in turn, give rise to the mossy fibers with extraordinarily large mossy fiber boutons, which contact mossy cells along the tract. Finally, this axonal bundle terminates with mossy fiber boutons at thorny excrescences, which represent postsynaptic specializations of CA3 pyramidal neurons (2nd synapse). In the following, CA3 pyramidal cells project via Schaffer collaterals to the stratum radiatum of CA1 (3rd synapse). This in mammals conserved intrahippocampal circuit is pivotal for memory formation and cognitive processes. Within archicortical areas of the hippocampus, large FUS accumulations co-localized with mossy fiber boutons. These unique synapses are recognizable due to their large dimensions, which often exceed a size of more than 1 µm in diameter, and due to their engulfing distributions around PSD markers from thorny excrescences of CA3 pyramidal neurons. Following the Schaffer collaterals within the intrahippocampal circuits, synaptic FUS signals co-distributed with excitatory markers in the stratum radiatum of CA1. Synaptic FUS signals were not restricted to the trisynaptic relay within the hippocampus but were also detectable in the stratum oriens adjacent to CA1. Maccaferri et al. described that a certain fraction of excitatory synapses in this region originates from pyramidal neuron collaterals, which target interneurons for negative feedback loops (Maccaferri 2005). The association of FUS to glutamatergic synapses was tendentially less prominent in this region as compared to the synapses from the trisynaptic circuitry. Intriguingly, only a minor fraction of FUS was found at inhibitory synapses in all analyzed areas.

Another remarkable finding was that synaptic FUS resided in VGLUT1- as well as GluA1-negative spots at the somata of lower motoneurons. This observation stands in stark contrast to the abundant presence of FUS at glutamatergic, VGLUT1-positive synapses in all analyzed cortical areas. The affiliation of these axosomatic synapses from
alpha-motoneurons to a certain synapse subtype remains unclear. Co-immunolabelings targeting further synapse subtypes would be required to determine the nature of these synapses (author note: experiments are currently being addressed on my part). VGLUT1 is a marker for direct afferents from muscle spindles, which terminate at alpha-motoneurons (Basaldella et al. 2015). These synapses are predominantly affected in SMA, an ALS-cognate neurodegenerative MND (Martinez et al. 2012). A recent publication from Shang and Huang has presented an indication on the FUS-positive synapse subtype by showing an immunostaining on mouse lumbar spinal cord tissue. Therein, FUS is co-distributed with CHAT (choline O-acetyltransferase) at axosomatic synapses of alpha-motoneurons (Shang and Huang 2016). This enzyme is present in synapses, which originate from cholinergic interneurons acting as modulators of the excitability of alpha-motoneurons (Miles et al. 2007). These observations have not been thoroughly corroborated and require further analyses. Future studies will address the localization and role of FUS at specific axosomatic synapses of alpha-motoneurons, which might be linked to the pathology in ALS-FUS and might distinguish ALS-FUS from the synaptic pathomechanisms in SMA. Moreover, the deviating specificities of FUS at different types of synapses in the cortex versus the spinal cord might contribute to the two major FUSopathies: FTD-FUS (cortex) and ALS-FUS (spinal cord).

The presented data on synaptic FUS provide a comprehensive overview of the abundance of FUS at synaptic contacts in the mouse CNS. FUS seems to be uniformly present at synapses in the neocortex, the cerebellar cortex, and the phylogenetically older archicortex, which hints toward conserved functions of FUS at synapses. The results are in accordance with previous studies, in which synaptic localization of FUS is demonstrated in some of the investigated brain areas. Belly et al. report a synaptic localization of FUS in the rat hippocampus (Belly et al. 2005). Furthermore, a thorough comparison of different brain areas of human and mouse CNS unveils a prominent dendritic localization of FUS in the neocortex, hippocampus, cerebellar cortex, and subcortical areas in both species (Aoki et al. 2012). After I have critically revised figures from several publications, including immunohistochemistry on human tissue, a synaptic presence of FUS in both human and murine CNS tissues can be observed. This is particularly obvious in the hippocampus, the cerebellum, the spinal cord, and also in the neocortex, although this was not mentioned in the manuscripts (Deng et al. 2010, Armstrong et al. 2011, Mori et al. 2012, Kobayashi et al. 2013a, Lim et al. 2016). This
might have been overseen due to the focus on the presence and functions of FUS in the nucleus. Synapse dimensions are at the diffraction limit of fluorescence light microscopy. Therefore, these structures appear in granular, blurry distributions within dendritic areas after commonly applied non-fluorescence-based, immunohistochemical procedures, which highlight proteins with chromogens like 3,3’-diaminobenzidine (DAB). Thus, these favored techniques in immunostainings on human tissue lack the possibility to clearly distinguish subcellular structures like synapses. Moreover, immunohistochemistry with chromogens is widely restricted to the use of one antibody per sample. However, the evidence for synaptic presence of a protein requires confirmation by a co-immunolabeling with a known synaptic or dendritic protein. This is due to the small size of synapses and, hence, the lack of information on morphological aspects in acquisitions with conventional light microscopy.

It is noteworthy that the amount of synaptic FUS varied greatly dependent on the analyzed CNS areas. The sizes of FUS-immunoreactive spots seemed to correlate with the dimensions of the signals from the co-localizing synaptic markers. This observation might give an indication on dynamic properties of the amounts of synaptic FUS according to the needs of differently sized synapses. More precisely, FUS exhibited bulky, large distributions at axosomatic synapses of lower motoneurons and at synapses from mossy fiber boutons. Medium-sized synaptic FUS puncta were present in the neocortex and cerebellar cortex, whereas dendritic areas adjacent to CA1 exhibited rather smaller FUS spots. One explanation could be the sheer size of presynaptic boutons in different CNS areas and a putative correlation with the dimensions of the detected FUS signals at synapses.

These observations are partly in line with previous results. Nuclear and dendritic distributions of the FUS proteins purportedly vary between brain areas in human and mouse cortical and subcortical areas. For instance, Aoki et al. report an only faint granular distribution of FUS in the cortex and the DG but a pronounced synaptic FUS signal in the CA1 sector of human brain samples (Aoki et al. 2012). These findings are in conflict with the present results, although the authors employed the same FUS antibody. Explanations for these deviating results might be interspecies differences in FUS expression or age-dependent dynamics; both aspects remain widely unexplored to date. Future studies might shed light on the different properties of synapses in correlation to the presence of synaptic FUS. The diversity of synaptic contacts is higher
in the neocortex as compared to the archicortex. In future investigations, it might be of interest to include further antibodies in co-immunostainings with FUS in order to label other than the described synapse subtypes. Additionally, it could well be that synaptic FUS undergoes development-dependent changes with regard to the intensity and distribution, as was highlighted with developmental studies on FUS expression. The chosen age of analysis (40 days after birth) coincides with a dynamical phase of FUS expression in murine brains (Huang et al. 2010b, Putz 2013). Of note, the present data are derived from only one animal (adolescent male mouse) and, therefore, require further confirmation (author note: experiments are currently being addressed on my part).

Although the immunolabeled tissue sections were acquired in high quality settings with a confocal laser scanning microscope, a determination of a clear-cut synaptic sub-localization to either the pre- or postsynaptic compartment was impossible (further indications are discussed in synopsis with the super-resolution data in 4.4). This is due the limited resolution of subdiffraction-scaled structures, including synapses, with conventional fluorescence microscopy. As a result from the random three-dimensional orientations of synapses in tissue and the turbidity of tissue due to light scattering, it is challenging to differentiate the pre- and postsynaptic compartments of imaged synapses.

4.3 Glutamatergic synapses of primary rat hippocampal neurons contain FUS

The focus is, further on, set on hippocampal neurons due to the relevance of the hippocampus in human FUS pathology. Moreover, the model system is switched to primary embryonic rat hippocampal neurons, which offer optimal conditions to localize proteins in a highly uniform cell population. Therefore, these cells are regarded as a well-defined model system to study dendritic branching and synapse formation as well as the maturation of these structures in vitro. In order to isolate and cultivate hippocampal cells, hippocampi from rat embryos are dissected in their final in utero stage, and an enzymatic and mechanic separation of cells follows. Eventually, the cell suspension is seeded out in defined cell numbers on glass coverslips (Banker and Cowan 1977, Kaech and Banker 2006, Grabrucker et al. 2009). After the outgrowth of their protrusions, neurons initially contact each other by establishing synapses after 7 DIV. In the present investigations, 14 DIV old neurons were mainly chosen with well
maturated synapses but a still ongoing synaptogenesis (Grabrucker et al. 2009). The homogeneity of the cell population in primary hippocampal neurons is unique in comparison to other primary neuronal cell models with approx. 50 % of neuronal cells demonstrating a typical pyramidal neuron shape (Banker and Cowan 1979) and with only 6 % GABAergic neurons (Benson et al. 1994).

FUS was co-labeled in combinations with various synaptic markers in a similar approach as was conducted in immunostainings on mouse CNS tissue sections (see 4.2). A co-localization evaluation is usually facilitated due to the restriction to two dimensions in cultivated neurons in comparison to tissue stainings. It is unknown whether neuronal cells in primary cell cultures predominantly establish synaptic contacts with their trans-synaptic axes parallel to the coverslip surface. In theory, it is plausible since the initial encounter of an outgrowing axon with a dendrite, conceivably, is a side-to-side contact in a 2D culture. Such an orientation (side view on a synapse) would improve the evaluation of the distances between synaptic proteins along a trans-synaptic axis because the resolution in fluorescence microscopy is best within the imaging plane (xy-axes). These considerations fostered the plan to further set a focus on this neuronal model system. This was especially relevant for the investigations on synaptic FUS localization with dSTORM, which will be addressed in the following section (see 4.4). Consistent with the aforementioned data from tissue stainings, FUS strongly co-localized in a punctuated pattern along dendrites with the pan-synaptic marker bassoon and with a variety of markers indicating glutamatergic synapses: PSD-95, GluA1, and VGLUT1. Also in line with findings in vivo, FUS co-distributed less with the inhibitory synapse markers gephyrin and GAD65. The latter protein indicates presynaptic terminals of GABAergic synapses. Nonetheless, a certain fraction of gephyrin puncta was found in close proximity to FUS puncta, but the ratio of co-localizations was lower than with PSD-95. Exemplary confocal acquisitions demonstrated only little overlap of FUS with gephyrin. FUS spots in proximity to gephyrin puncta appeared small in size. This facet will be further addressed in the following section with data from super-resolution microscopy (see 4.4).

In summary, FUS is mainly confined to excitatory, glutamatergic synapses in murine model systems (mouse CNS and rat hippocampal neurons in culture). The association to excitatory synapses in mouse CNS tissue and in primary hippocampal neurons is in line with results from several publications (Husi et al. 2000, Belly et al. 2005, Fujii et al. 2005,
DISCUSSION

Fujii and Takumi 2005, Aoki et al. 2012). This work is the first approach to determine the association of FUS with markers for both excitatory and inhibitory markers.

4.4 SMLM reveals presynaptic FUS localization at glutamatergic synaptic vesicles

Some of the contents of this section referring to dSTORM data were briefly described in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). A distinction between pre- or postsynaptic distributions of FUS was so far indeterminable due to the resolution limits of conventional fluorescence microscopy. Single molecule localization microscopy was employed to further investigate the precise distribution of FUS at synapses on a nanometer-scale. First experiments were conducted with a home-made STED microscopy setup from the Institute of Biophysics, Ulm University (Osseforth et al. 2014). Thus, synaptic FUS was found in close-by positions to bassoon signals. FUS exhibited bulky signals in contrast to the oftentimes bar-like shapes of bassoon or HOMER-1B/C. The bar-like distributions were expected from synaptic scaffolding proteins imaged in a side view (Dani et al. 2010). Initial problems with an insufficient signal-to-noise ratio and the inferior resolution of the STED setup in the $xy$-plane (35 nm FWHM), prompted the switch to a home-made 2D-dSTORM setup with an $xy$-resolution of approx. 25 nm. This was required due to the following obstacles: the pre- and postsynaptic compartments are separated by an interjacent cleft, which spans approx. 23 nm (Boeckers 2006). Protein densities on both sides of the synaptic cleft are attached to the respective membranes, and, as a consequence of this, mean distances between protein populations of the PAZ and the PSD along the trans-synaptic axis are in a range of few hundreds of nanometers at maximum. Additionally, the trans-synaptic distance is only at maximum if a synapse is acquired in a side view. Otherwise, distances appear false too short, which implicates an even higher necessity for optimal resolution (see further explanations in 4.3, penultimate paragraph).

In accordance with the experiences in STED microscopy, similar obstacles occurred during the initial establishments of a staining protocol for SMLM. Methodological challenges included inadequate labeling and high signal noise, which most likely resulted from autofluorescence and unspecific binding of the applied antibodies. Both phenomena can arise from fluorophores with low signal-to-noise ratios, autofluorescence from the coverslip or the fixative chemical compound (for instance
DISCUSSION

PFA), and out-of-focus fluorescence, which can be counteracted by applying TIRF (Hess et al. 2009). Also cells themselves are considerable sources of autofluorescence if remnants of serum or phenol-containing media are still attached to the sample after the washing. Super-resolution microscopy requires higher labeling efficiencies of the antibodies and a reduced signal noise as compared to conventional fluorescence imaging (Hess et al. 2009, Betzig 2015). With reference to the Nyquist criterion, labeling densities in SMLM are recommended to be at least twice as high as the maximum resolution (Betzig 2015). A customary staining protocol optimized for conventional microscopy was initially applied and included paraformaldehyde as fixation chemical with short antibody incubation times (approx. 60 min per antibody incubation step). Immunostainings with this protocol resulted in an appropriate quality for conventional epifluorescence microscopy, however, an insufficient signal-to-noise ratio in super-resolution microscopy. Two major problems were identified, in a stepwise procedure further approached, and finally solved: 1.) Signal noise could be increased by paraformaldehyde, which is known to establish autofluorescing precipitates and unspecific binding of antibodies. Moreover, washing steps after the primary and secondary antibody incubations have to be sufficiently long enough to avoid unspecific antibody binding. 2.) Labeling could be incommensurate to the requirements of the Nyquist criterion as a consequence of too short antibody incubation times. The first issue was addressed by using high-precision coverslips, by changing the fixative compound to methanol, and by the introduction of longer washing steps with supplemented glycine as an autofluorescence quencher. Moreover, imaging in TIRF mode was applied in order to reduce the excited volume. Thus, out-of-focus fluorescence is diminished. Increased amounts of antibodies, extended antibody incubation times, and final post-fixation of labeled cells considerably improved the issues of the second point. The eventually applied protocol was inspired by an immunolabeling protocol, which was optimized for mouse brain tissue. Of note, initial experiments were conducted with a different FUS antibody, which demonstrated insufficient synaptic FUS labeling (data not shown). It also has to be taken into account that commercially available antibodies, which target different epitopes of FUS, display a high variability in the labeling intensities of nuclear or dendritic FUS (Aoki et al. 2012, Kobayashi et al. 2013b). Possible explanations for these distinct immunoreactivities are different phases, in which FUS resides either in the nucleus or at extranuclear sites.
Moreover, different binding partners of FUS in the nucleus or in the dendritic compartment might also contribute to a shielding of antigenic determinants (Aoki et al. 2012). Taking previous publications into account, a FUS antibody was employed, which more readily detects synaptic FUS (Aoki et al. 2012).

A co-localization of synaptic FUS with pan-synapse markers (SYP, bassoon) and markers for excitatory synapses (VGLUT1, PSD-95) could be underpinned in initial acquisitions, which was already indicated by conventional imaging. There was only sparse co-localization with markers for inhibitory synapses (gephyrin) or synapses with reported excitatory and also inhibitory properties (VGLUT2) (Heise et al. 2016). These findings are particularly interesting with regard to the affiliations of these different synapse subtypes within intra- and extrahippocampal circuits. The following passage also discusses facets of synaptic FUS in synopsis with the data obtained in mouse hippocampal tissue (see 4.2, third paragraph). VGLUT1-positive synapses are regarded as classical excitatory, glutamatergic synapses, also positive for scaffolding proteins of postsynaptic densities, for instance, SHANK1 to 3, PSD-95, and HOMER-1B/C. Synapses from this subtype usually originate from cortical neurons, both from the archi- and neocortex (Heise et al. 2016). A fraction of VGLUT1-positive synapses represents synaptic contacts of the so called trisynaptic circuit (see more detailed explanations in 4.2., third paragraph). However, VGLUT2-positive synapses are negative for PSD scaffolding proteins like SHANKs, despite the fact that these synapses use glutamate as neurotransmitter. Remarkably, VGLUT2-positive terminals are also positive for GABAergic markers. They originate from intrahippocampal (mossy cells) and extrahippocampal (diencephalic) inputs. The latter are assumed to have specific functions in motor learning and sleep regulation. Mossy cells are supposed to interlink single – in medio-lateral direction orientated – trisynaptic circuits along the rostro-caudal extension of the hippocampus (Heise et al. 2016). VGLUT2-positive synapses in primary hippocampal neurons conceivably represent inputs from mossy cells. Future investigations on murine tissue might include immunostainings for VGLUT2-positive synapses in order to corroborate the purported specificity of FUS toward VGLUT1-positive synapses in the hippocampus.

After the establishment of a working protocol, a method to measure the distances between protein distributions had to be developed. As a first step, it was necessary to validate the precision of the setup with immunolabelings for classical markers of the
pre- and postsynaptic compartments. A study from Dani et al. was the most significant reference literature (Dani et al. 2010), which is comprehensively described in 1.5.4.2. Although the applied measurement methods in the present investigations were developed independently, subtleties were adjusted according to this study. Dani et al. were the first to extensively explore the positions of synaptic proteins in axial (along the trans-synaptic axis) or lateral (perpendicular to the trans-synaptic axis, parallel to the synaptic cleft) distributions with single-molecule localization microscopy. They could demonstrate that their results are in line with data from previous studies, which are based on EM imaging (Dani et al. 2010). The capabilities of the setup to image in only two dimensions and a maximum of two available channels in one labeling round rendered it impossible to immediately determine a pre- or postsynaptic localization of FUS in the present study. This is a common restriction in super-resolution microscopy (Huang et al. 2010a). Only synapses with a determinable trans-synaptic axis were included in the further analysis. Two criteria were decisive: firstly, the appearance of scaffolding markers as bar-shaped structures and/or an apparent gap between two adjacent proteins, and secondly, the shortest distance between two distinct protein accumulations. Moreover, it was taken for granted that the membrane-bound protein densities of synapses are roughly mirror-symmetric with a center line along their trans-synaptic axes. The trans-synaptic axis was then determined as perpendicular to the bar-like distributions of the pre- and postsynaptic scaffolding proteins, including a sometimes observable gap. This axis was usually identical with the shortest distance between two immunolabeled proteins due to the symmetry of synaptic structures. A line was drawn along this axis with a defined width (300 nm), and the underlying intensity profiles along this line were measured for each channel. The chosen width of the line is underpinned by values, which were determined with EM acquisitions of primary hippocampal neurons at DIV 14, reported in a previous study. Thereby, an average width of a PSD is approx. 250 nm (Grabrucker et al. 2009). In the present study, most protein densities appeared to span more than 250 nm, therefore, a width of 300 nm was chosen. The distances between the respective centers of mass (COM) were considered as the mean distances between the two protein populations, which can be regarded as the axial distributions of synaptic proteins.

Stainings with either two pre- or two postsynaptic markers in combination or a pre- and a postsynaptic marker in a double-staining resulted in distinguishable protein
populations for all conditions. These analyses served as a reference and corroborated a resolution powerful enough to discern between two protein populations within the same compartment, which are known to be apart from each other on a sub-wavelength scale (Boeckers 2006, Grabrucker et al. 2009, Dani et al. 2010). Scaffolding proteins of the pre- (bassoon) and the postsynapse (HOMER-1B/C and PSD-95) frequently appeared as bar-like structures, which indicated an orientation of the trans-synaptic axes of these synapses in the imaging plane (side view). In turn, a vesicle marker (SYP) of the presynaptic compartment exhibited a broad and bulky appearance, as was expected with regard to the distribution of synaptic vesicles in a presynaptic bouton (Boeckers 2006, Grabrucker et al. 2009).

A large amount of synapses was measured with the abovementioned method, which finally resulted in mean distances lower than expected. Dani et al. report a distance of 91 nm on average between bassoon(C) and PSD-95 along the trans-synaptic axis, whereas a combination of PSD-95 and HOMER-1B/C results in a 33 nm distance. These results were + 20 nm for bassoon(C)-PSD-95 and - 8 nm for HOMER-1B/C different from the values obtained in the present investigations, respectively. It can be almost ruled out that the discrepancies are due to the applied primary antibodies since antibodies in both studies were identical, except of the HOMER-1B/C antibodies (Dani et al. 2010). In the following, several possible explanations will be argued for the marginally discrepant results between this study and the results from Dani et al.: 1.) It cannot be ruled out that a minor fraction of measured distributions represent no synaptic contacts. This could be due two labeled spots in a close-by position, albeit independent in terms of synaptic localization. This possibility is rather unlikely since proteins oftentimes align with the orientations of their distributions, which indicates an anatomical dependency. 2.) Despite the fact that synapses in primary hippocampal cell culture represent rather defined and similar subcellular structures, there is a certain diversity of synaptic contacts in terms of the transmitter use and developmental state. Synaptic protein distributions could be different dependent on the specific synapse subtype or the different developmental stages. This would give rise to a greater heterogeneity of the distance values. However, such a contribution is rather unlikely with regard to the low standard deviations. In line with this, Dani et al. noted that the diversity of lateral distributions is much more variable than differences of the axial distributions of scaffolding proteins and receptors (Dani et al. 2010). Thus, it can be
concluded that many synaptic proteins exhibit a defined affiliation to one compartment. Whether this holds also true for synaptic proteins with no association to the membrane-adherent protein densities like, for example (e.g.), synaptic vesicle proteins remains unknown. 3.) Furthermore, it is also thinkable that discrepancies to the values from Dani et al. result from analyzing synaptic contacts in different species and model systems. It is rather unlikely that highly conserved proteins from the PAZ and PSD markedly differ in terms of localization between mice and rats or between tissue and primary hippocampal neurons. 4.) Although the analysis methods were similar, Dani et al. apply peak-to-peak measurements of intensities of protein populations to determine the relative positions of proteins. Moreover, acquisitions in their study are also performed in the z-axis, which results in less resolved images (Dani et al. 2010). 5.) Imaging with SMLM is prone to drift artefacts, which particularly occur during the long-term acquisitions. This might have also contributed to erroneous distances between the protein clusters, however, this possibility is rather unlikely since drift was mostly very low, and a corrective algorithm was otherwise applied. 6.) The preferred explanation is the ambiguity of the orientations of the trans-synaptic axes. Every orientation, in which the trans-synaptic axis tilts out of the imaging plane, would result in false too low determinations of the distances. The analysis most likely profited from a preferable orientation of the trans-synaptic axes parallel to the imaging plane due to the restricted outgrowth of cells and their protrusions on the surface of a coverslip. As a consequence from these considerations, only the longest distance measurements were taken into account, which restricted the analysis to those synapses with trans-synaptic axes orientated almost in – or completely in – the imaging plane. After the exclusion of 35% of synapses with the shortest measured distances, the results were in accordance with the published data for the distance between bassoon(C)-PSD-95 (Dani et al. 2010). This combination was favored as a reference value in comparison to the combination HOMER-1B/C and PSD-95 since Dani et al. employed the same antibodies for bassoon(C) and PSD-95 but not for HOMER-1B/C. On this view, two synaptic proteins, which belong to the same compartment exhibited distances of approx. 50 nm, while opposed scaffolding proteins of the pre- and postsynapse displayed a distance of ca. 90 nm.

Hereinafter, FUS was included in the analysis, which was co-stained with scaffolding markers of the PAZ (bassoon) and PSD (PSD-95). The idea was that the distances to the two reference markers would help to determine the affiliation of FUS to either the pre-
or the postsynaptic compartment. Surprisingly, FUS mostly overlapped with neither of them in complete, which is indicative for a localization of FUS outside of the synaptic protein densities. More precisely, the combination of FUS with bassoon frequently resulted in only peripherally overlapping signals. The mean distance of 109 nm was similar to the distance between bassoon and PSD-95. Thus, two possible localizations were possible: a distribution proximal to the PAZ toward the axon or beyond the synaptic cleft in the postsynaptic compartment. The latter option seemed less favorable due to the apparently even lower overlap of FUS with PSD-95, which would be predicted in that case with regard to the almost equal average distances of bassoon-FUS and bassoon-PSD-95. The statistical analysis of the distances from many FUS- and PSD-95-positive synapses disclosed an average distance of 206 nm. Hence, a localization of FUS adjacent to bassoon on the side remote of the synaptic cleft can be postulated, which almost perfectly results from adding PSD-95-bassoon (91 nm) and bassoon-FUS (109 nm).

It is noteworthy that due to the restrictions in combining antibodies from the same species, a C-terminal antibody for bassoon (bassoon(C)) was used in combination with PSD-95. In contrast, FUS was analyzed in a counterstain with an N-terminal bassoon antibody (bassoon(N)). Bassoon is orientated to a high degree within the PAZ with a head-to-tail orientation almost perpendicular to the PAZ. Thereby, the molecules present their C-terminus toward the cleft. Axial distributions analysis for bassoon with both C- and N-terminal antibodies, thereby, results in a distance of approx. 30 nm along the trans-synaptic axis (Dani et al. 2010), which is due to the enormous size of bassoon (Richter et al. 1999, Dresbach et al. 2001). These considerations might, in part, explain the small discrepancy between the sum FUS-bassoon(N) plus bassoon(C)-PSD-95 (200 nm) in comparison to FUS-PSD-95 (206 nm). Of note, while distance measurements between markers of the PAZ and the PSD resulted in very similar values between synapses, the combinations of these markers with FUS oftentimes led to significantly higher variations (expressed in the higher standard deviations, see 3.3.5.) between each measured synapse. This was regardless of the even higher number of measured synapses in the combinations with FUS. One possible explanation could be the broader distribution of FUS, which might result in a higher ambiguity to determine the centroid positions of FUS accumulations. Due to the co-distribution of FUS with synaptic vesicles, the distribution of FUS might be more dependent on the individual shape of a
presynaptic bouton as compared to the scaffolding markers. This could, hence, also contribute to more variability of the COM positions of FUS spots relative to PAZ and PSD proteins.

Although the evidence of FUS localization within the presynaptic compartment is high with regard to the presented results, several aspects have to be carefully discussed. A weakness in the present approach is the availability of only two channels. Therefore, it was difficult to clearly label only excitatory synapses in the combinations with FUS. While FUS with PSD-95 reliably indicates excitatory synapses, FUS in combination with bassoon is less confined to this synapse subtype. Markers of the presynaptic active zone are less specific toward a certain synapse class than those of the postsynaptic side and can, thus, also be found at inhibitory synapses (Richter et al. 1999). Along these lines, the minor fraction of FUS at gephyrin-positive spots could have contributed to the distance analysis between FUS and bassoon. The proportion is presumably low with regard to the results from the co-localization analysis, which was performed with conventional imaging (see 4.3). Whether FUS is also confined to presynaptic terminals at inhibitory synapses remains unknown. Another consequence of the setup is the impossibility to easily rule out a fraction of FUS proteins localized to the postsynaptic compartment. FUS sometimes strongly overlapped with PSD-95, which might be explained by synapses acquired in face views. If FUS were also postsynaptically localized, a second intensity peak would be observable at synapses, which were acquired in side views. In this hypothetical situation, a part of the FUS signal would strongly overlap with PSD-95, which would represent the FUS protein accumulation in the spine. But this was not the case after thoroughly evaluating every measured synapse manually. On the contrary, the observation of only one FUS spot per synapse is unambiguous, which is also in conformity with the rather moderate variations of the distance values expressed in the standard deviations.

Where FUS localizes in hippocampal synapses in vivo is not known, although the present data suggests a presynaptic localization, at least for some synapse subtypes (see also 4.2). FUS greatly overlapped with presynaptic markers at mossy fiber boutons but with a distinct distance to the postsynaptic markers. The same observation was made in other cortical areas (neocortex, cerebellar cortex). Also the FUS spots at axosomatic synapses at lower motoneurons are most likely of presynaptic nature since the signals seemed to be outside of the soma. This observation is more consistent than
for cortical synapses as a result from the imaging of the axosomatic synapses in the spinal cord in no other orientation than in side views. This facilitates the evaluation of the axial distributions of synaptic proteins. For all other synapse subtypes in the different analyzed CNS regions, the resolution is inadequate to deduce an unambiguous pre- or postsynaptic localization. This might also be promoted by the higher heterogeneity of trans-synaptic axes orientations in \textit{in vivo} situations as compared to primary neuronal cell culture. In addition, imaging conditions are more challenging in tissue as compared to 2D culture samples due to the increased light scattering and the less specific binding of antibodies, which results in a worse signal-to-noise ratio. Moreover, the heterogeneity and the protein dynamics of synapse subtypes might be considerably higher in CNS tissue as compared to primary neurons.

Since FUS distributed similar to the vesicle membrane marker SYP, its distribution is reminiscent of the morphology of a presynaptic bouton in 2D. Both proteins appeared in bulky and sometimes triangular shapes, and the signals often tapered at the side facing away from the synaptic cleft. To investigate this observation in more detail, a further analysis was conducted by a double-immunostaining of FUS in combination with SYP. As was expected, the overlap was vast. As a consequence from the unavailable information about the orientation of the synaptic axes in this combination – both markers oftentimes appeared with no specific orientations and overlapped strongly – a COM analysis in two dimensions was employed according to the distributions and intensities of both markers in the \textit{xy}-plane. The average distance between FUS and SYP (34 nm) with this method was considerably lower in comparison to the combinations of FUS with bassoon (87 nm) or with PSD-95 (176 nm). The results of the latter two combinations are slightly higher than the distances measured with the 1D-COM method along a trans-synaptic line. This is a consequence of taking into account more information in two dimensions, which include differences in the distributions other than along the trans-synaptic axis. In addition, the inaccuracy to localize the exact position of a fluorophore increases in two dimensions. These ambiguities to determine photon emitter positons are, in general, a consequence of signal noise, insufficient abilities of different fluorophores to switch between on and off states, close proximity to other fluorophores, and inherent drift of biological samples (Hell and Wichmann 1994, Thompson et al. 2002, Rust et al. 2006, Fernández-Suárez and Ting 2008, Heilemann et al. 2008, Huang et al. 2010a, Nägerl and Bonhoeffer 2010, Betzig 2015). Another
conclusion can also be drawn with reference to the abundance of FUS proteins. Assuming that the efficiencies of both FUS and SYP antibodies were comparable, FUS would demonstrate a similar abundance in presynaptic terminals. Synaptic vesicle proteins are common proteins in the central nervous system due to the abundance of synaptic vesicles (Sudhof 2004), and SYP, in particular, is the most common synaptic vesicle protein (Takamori et al. 2006). This might imply that FUS is an ample protein at synapses. Whether this is also reflected in the functional importance of the protein at synapses might be addressed in studies to come.

Astonishingly, on a scale of a few nanometers, FUS and SYP proteins frequently did not overlap, which indicates a localization of FUS around vesicles in the presynaptic plasma. The staining pattern of both markers was conspicuous referring to the frequently demonstrated patchy distributions, in which the small areas between the scattered signals appeared to be filled up by the respective other marker. Moreover, FUS regularly demonstrated smaller extensions than SYP in this combination. A conceivable explanation is an interference of FUS and SYP antibodies, which might result from a steric hindrance of the primary and secondary antibodies due to the close neighborhood of both proteins with only little space between the boundaries of the presynaptic vesicles. Such a phenomenon has not been described before in super-resolution microscopy. Furthermore, the exact localization of FUS in or around vesicles cannot finally be answered with this approach. The present data rather supports localization around vesicles, which is reasonable with respect to the otherwise nuclear or cytoplasmic localization of FUS as a part of RNA granules and the putative role germane to local translation. These implications might be subject to future studies and will be addressed in the next section.

4.5 Outlook: Putative roles of presynaptic FUS in physiological and in diseased states

The affiliation of a protein to the pre- or postsynaptic compartment – although separated by only a few nanometers – implies tremendous consequences for its function with regard to the high polarity of synapse assemblies and functions. In the following, implications will be presented, which arise from the presynaptic localization of the FUS RNA-binding protein. These considerations are grouped in putative functions of FUS in the presynaptic compartment and, at the same time, possible detrimental
mechanisms in pathological situations. A schematic illustration of these reflections is sketched in **Figure 25**.

**Figure 25**: A model of presynaptic FUS in physiological and pathological states

An exemplary delineation of FUS functions and distributions is displayed of confirmed (left side, including the nucleus, soma, and dendrites) and novel or hypothetical aspects (right side, including putative roles of FUS in the presynaptic bouton). The sketch is, further on, divided in putative roles of FUS in physiological (upper neuron) and pathological conditions (lower neuron). In physiological states (upper neuron, left side), nuclear FUS orchestrates multiple DNA- and RNA-associated processes in the nucleus and possibly regulates local translation at dendritic sites. On the contrary, in pathological situations (lower neuron, left side) the nucleus is often devoid of FUS proteins, while accumulations of the protein can be found in the cytoplasm. Local RNA processing in dendrites is compromised. The present study introduces presynaptic FUS, which might be involved in the presynaptic local translation (upper neuron, right side). In pathological conditions, FUS depletion or dysfunctionality in the bouton (lower neuron, right side, upper part of the synapse, separated by a dashed line) may lead to a loss-of-function-induced local translation failure. Also a local gain-of-function via active FUS accumulations is thinkable (lower neuron, right side, lower part of the synapse). Thereby, local translation might be excessively driven. Propagation via the synaptic cleft or other mechanisms, for instance, exosomes, might explain the trans-
synaptic spreading behavior of FUS pathology. DNA = deoxyribonucleic acid, FUS = fused in sarcoma, RNA = ribonucleic acid.

4.5.1 Possible functions of FUS at the presynapse

Several previous studies give indications in how FUS might be involved in presynaptic processes. Moreover, the presented findings give also rise to novel speculations, which will be discussed in the following paragraphs.

Developmental and regional facets of synaptic FUS localization

Aoki et al., Huang et al., and also Putz in his doctoral thesis give insights in the abundant expression of FUS in various tissues and organ systems, including the CNS of rodents (Huang et al. 2010b, Aoki et al. 2012, Putz 2013) and humans (Aoki et al. 2012). To get an idea of the temporal expression, the amount, and distribution of FUS, Huang et al. conducted a longitudinal expression analysis in rodents. FUS is ample in all organs in early development, whereas adult animals exhibit a decreased expression in most tissues. Intriguingly, the expression in the CNS is sustained throughout lifetime, while motoneurons demonstrate an even increasing amount of cytoplasmic FUS in the course of development (Huang et al. 2010b).

To date, synaptic FUS localization was unisonously postulated as dendritic or postsynaptic, although applied methods were insufficient to resolve pre- and postsynaptic compartments (Husi et al. 2000, Belly et al. 2005, Fujii et al. 2005, Fujii and Takumi 2005, Zhang et al. 2012, Aoki et al. 2012). The veracity of the previously published microscope-based data can be challenged due to the exceptionless use of diffraction-limited devices. Moreover, whole mouse brain tissue subfractionation resulted in an enrichment of FUS in the PSD fraction (Aoki et al. 2012). Certain aspects have to be considered in comparison to the presented dSTORM data. The species, which were investigated in both studies, are closely related but different. While the present analysis focused on hippocampal neurons derived from rat embryonic tissue, Aoki et al. investigated a whole adult mouse brain. Developmental aspects of FUS expression and distribution at synapses in different brain regions also might have contributed to the discrepant results. Furthermore, it has to be taken into account that tissue subfractionation can be deceiving since the purity of each fraction is not always warranted.
DISCUSSION

It might be a matter of future studies whether a presynaptic localization of FUS is conserved across species, and whether FUS might exhibit a development- or brain region-specific expression pattern and distribution (author note: experiments are currently being addressed on my part). Moreover, it would be most intriguing to explore to what extent FUS-positive synapses in different brain regions share structural and functional commonalities. Some of these facets will be further discussed in the next but one paragraph.

What is known about functions of FUS in the axonal compartment and in synaptic transmission?
To date, axonal phenotypes in FUSopathies, which are observed in humans as well as in model organisms of FUS pathology, have been assigned to failures in the nuclear RNA metabolism. FUS regulates gene expression and splicing of axonally relevant genes. It is hypothesized that disturbances of this regulatory function or the sequestration of axonal proteins in cytoplasmic FUS accumulations, as for instance SMN, are responsible for the axonal decay in FUSopathies (Bowden and Dormann 2016). The FUS-associated gene-regulatory processes take place in the nucleus and include the transcription control of genes and further mRNA processing, which range from splicing to nuclear export. Thereby, FUS regulates the expression of genes, which are relevant in axon guidance and axonogenesis, such as MAPT, which codes for the tau protein (Orozco et al. 2012, Rogelj et al. 2012, Bowden and Dormann 2016). Furthermore, nuclear loss-of-function in FUSopathies rarefies microRNA production, which predominantly affects those contributing to neuronal activity, development, and synaptogenesis (Morlando et al. 2012). Nuclear FUS also influences the expression of proteins involved in the synaptic transmission, such as GluA1 (Bowden and Dormann 2016, Masuda et al. 2016). Most strikingly, FUS orchestrates the splicing of thousands of RNAs, including a large fraction of mRNAs, which encode for presynaptically (synaptic vesicle proteins, neurexins) and postsynaptically localized proteins (SHANKs, HOMER, glutamate receptors, neureligins) (Lagier-Tourenne et al. 2012, Nakaya et al. 2013, Honda et al. 2014). Intriguingly, these FUS-regulated gene products share a great overlap with the novel molecular neighborhood of FUS in presynaptic terminals of excitatory, glutamatergic synapses. A regulatory effect of the FUS RNA-binding protein on several steps within the processing of a specific set of mRNAs might be amplified by the multilocality of FUS in the nucleus.
and at synaptic sites. This might be particularly relevant for those mRNAs, which encode for proteins at excitatory synapses.

A putative role of FUS in the presynaptic local translation
Some of the contents of this section, referring to the putative roles of FUS in local translation, were briefly discussed in the publication, in which parts of the thesis have already been published (Schoen et al. 2016). It is a central dogma in neuroscience that synapses represent a core structure of memory consolidation, and it has been repeatedly demonstrated that it is indeed the strength of synaptic contacts, which serves as a prerequisite for memory. In response to electrochemical activity, neuronal cells produce certain proteins, which, in turn, influence synapse development. A variety of studies implicate not only a canonical mode of protein expression in the soma but an additional local translation machinery at synaptic contact sites (Kiebler and DesGroseillers 2000).

Local translation at synapses has long been accepted. Initial studies postulated a restriction to the postsynaptic compartment. In the 1980s, mRNA species were found in dendrites, which encode for a variety of cytoskeletal proteins and neurotransmitter receptors. Moreover, polyribosomes could be detected in dendritic spines (Kiebler and DesGroseillers 2000). The structural equivalents of this local protein production machinery are so called membrane-independent proteinaceous organelles or, more specifically, synapse-associated polyribosome complexes (Steward and Schuman 2001, 2003). For a long time, local translation with an influence on axonal development was believed to be confined to the growth cones of immature neurons. Researchers were puzzled to find mRNAs in synaptic endings, however, polyribosomes were seemingly absent (Steward and Schuman 2003). Later, synapse-associated polyribosome complexes have been identified in the axon and in presynaptic boutons (Giuditta et al. 2002, Younts et al. 2016). Local translation in presynapses has been shown to occur at those synapses, which are enabled to elicit LTP. In line with this, a multiplicity of mRNAs encoding for presynaptic proteins is identified in these presynaptic compartments (Akins et al. 2009). If regulators of translation, including FUS, are also involved in local protein production sites at synapses may be addressed in future studies. The assembly and functions of local translational units in the postsynaptic compartment have been more thoroughly deciphered than the processes of the presynaptic local translation.
Along these lines, this study might add a novel, essential regulator of RNA processing and local translation to the presynaptic compartment, which is sketched in figure 25 (upper neuron, synapse).

Interestingly, FUS is not the first RNA-binding protein, which was found in the axonal compartment but may act in concert with others, such as TDP-43 (Feiler et al. 2015), SMN (Jung et al. 2012), and FMR1 (Antar et al. 2006). The same holds true for the postsynaptic side, which purportedly harbors a variety of RBPs, including FUS (Belly et al. 2005), TDP-43 (Bowden and Dormann 2016), and FMR1 (Antar et al. 2006). FUS has been repeatedly described as a part of the postsynaptic translation machinery, thereby, regulating the local actin cytoskeleton arrangements in the spine compartment (Fujii et al. 2005, Fujii and Takumi 2005, Bowden and Dormann 2016). All of the mentioned RBPs have been associated to neurological disorders with a putatively disturbed RNA metabolism, which is maybe also relevant to synaptic sites (in brackets responsible protein with assumed physiological functions): SMA (SMN; RNA splicing), fragile X syndrome (FMR1; mRNA transport, local translation), ALS-FUS (FUS; splicing, regulation of translation, DNA repair), and ALS10 (TDP-43; splicing, regulation of translation) (Liu-Yesucevitz et al. 2011, Hanson et al. 2012). Future studies will show whether the aforementioned RBPs mutually regulate the same genes and their transcripts or whether they have more exclusive and, therefore, essential roles in regulating RNA metabolism. The latter possibility might be favorable with regard to the present data, which postulates a predominant distribution of FUS at excitatory synapses and a high specificity of FUS at lower motoneuron axosomatic synapses at Bassoon- and PSD-95-positive but VGLUT1-negative synapses.

Contradictory results from literature, which locate FUS to the spine compartment, possibly reflect the high diversity of synapses both in different brain regions and throughout development. Not only could one synapse subtype contain a specific subset of RBPs, but it could well be differently composed in the pre- and postsynaptic compartments. In addition, the distribution of RBPs at synapses might vary throughout the development according to the specific needs for the regulation, maturation, and plasticity of synaptic contacts. The present investigations, in synopsis with previous studies, provide an indication that the regulation of local translation is more intricate than expected with specific sets of RBPs, which might orchestrate gene translation at synapses dependent on the developmental and activity state of the synapse, the type of
synapse, and the affiliation of the RBPs to the pre- or postsynaptic compartments. Moreover, it is of interest whether a greater amount of FUS at synapses is paralleled by an increased necessity of local protein production. This scenario would, further on, implicate that exceedingly plastic synapses harbor higher amounts of FUS. This would be in line with the abundance of FUS at mossy fiber boutons, which are highly dynamic in size and function during learning and aging (Galimberti et al. 2006). The low amounts of FUS at inhibitory synapses might correspond to their comparably low complexity (Gray 1959, Sheng and Kim 2011).

It is not the intention to rebut the previous postulations, which state a distribution of FUS in the postsynaptic compartment. Moreover, a presynaptic presence is not contradictory to a postsynaptic localization in parallel, as was discussed above.

Where exactly is FUS in the presynaptic bouton?
Despite the application of state-of-the-art super-resolution microscopy in the present study, it was not unambiguously possible to determine the localization of FUS within or outside of synaptic vesicles. This uncertainty might have resulted from the sheer abundance of synaptic vesicle proteins and presynaptic FUS. Moreover, FUS and SYP molecules appear to be in extreme proximity to each other that interference of antibodies may have occurred. Most likely, the physiological distribution of FUS is in the axoplasma around vesicles. This is supported by the present dSTORM data, which showed a distinct distribution of FUS and SYP on a distance of a few nanometers. Moreover, the purported functions of FUS as a regulator of local translation would be in accordance with the distribution in the presynaptic cytoplasm (see figure 25, upper neuron, synapse). In studies to come, time-lapse super-resolution microscopy could also be of use to analyze FUS distribution and dynamics in the presynaptic compartment. This approach may be powerful enough to answer the question from where presynapses receive FUS and under which conditions, e.g., in response to synaptic activity.

4.5.2 Putative roles of presynaptic FUS in pathological states
Dysfunctional FUS at presynapses might be tightly correlated to the abovementioned putative physiological functions of FUS as a regulator of local translation (see also figure 25, lower neuron, synapse).
Possible implications of presynaptic FUS in ALS and FTD

Although FUS is an abundantly expressed protein, neurons exhibit a remarkable vulnerability to pathological alterations of FUS distribution. It remains elusive whether neuronal cells have a higher propensity to trigger pathological protein inclusions or whether they are more sensitive to detrimental effects of FUS mis-localization. It is also conceivable that neurons are worse in coping with FUS pathologies due their usual inability to divide. A peculiar property of FUS in neurons is the additional presence at synapses, which stands in contrast to the almost exclusively nuclear distribution in other body cells. This association of FUS to synapses might also contribute to the sensitivity of neurons for FUS pathology.

There seems to be a general commonality in human individuals suffering from ALS and in ALS mouse models that motoneurons exhibit a selective vulnerability to cell death, maybe due to their enormously long and heavily myelinated axon. They rank among the most asymmetric cells. Axonal degeneration of motoneurons is one of the initial events in ALS pathogenesis. Mouse models of ALS uniformly show a retraction of the presynaptic junction before the occurrence of initial symptoms (Boillée et al. 2006). In line with this, iPSCs-derived motoneurons from ALS-FUS patients receive less synaptic contacts in comparison to control cells (Naujock et al. 2016). Remarkably, a variety of FUSopathy model organisms (see detailed information in 1.3.4.3) have demonstrated a strong, but so far neglected, axonal and presynaptic phenotype in motoneurons. For instance, ALS models of the non-vertebrate organism Drosophila show reduced axonal branching, disturbed formation of neuromuscular junctions, dysfunctional synaptic development, decreased number and altered sizes of boutons and PAZs, disturbance of the presynaptic bruchpilot protein with aberrant presynaptic terminal architecture, impaired synaptic transmission, and altered synaptic currents (Chen et al. 2011, Wang et al. 2011, Sasayama et al. 2012, Shahidullah et al. 2013, Machamer et al. 2014, Baldwin et al. 2016). This is in accordance with observations in C. elegans (Therrien et al. 2016). A similar phenotype is observed in zebrafish FUSopathy models, which exhibit a reduced axonal length (Kabashi et al. 2011), perturbed synaptic transmission in terms of reduced quantal transmission but an increased excitability of motoneurons (Armstrong and Drapeau 2013). In rat ALS-FUS models, axons of the upper and also lower motoneurons display signs of degeneration with a decreased number of terminal branches of the lower motoneurons (Huang et al. 2011). A more general observation in rat models of
DISCUSSION

FUSopathies is the major affection of the cortex, the hippocampus, and the lower motoneurons with regard to inclusions and cell loss, whereas synaptic alterations were mainly detected in the cortex and spinal cord (Nolan et al. 2016). Axonal retraction has also been observed in mice mimicking FUS pathology (Shelkovnikova et al. 2013, Sharma et al. 2016). Most of these observations from animal models were conducted under FUS-mutated conditions. It is unknown how FUS pathology is triggered under non-mutated conditions, as is mostly the case in FTD-FUS. It might also be that non-mutated FUS in FTD-FUS behaves differently at presynaptic terminals in comparison to mutated FUS in ALS-FUS.

Only little is known about the alterations of synaptic contacts in individuals suffering from FUSopathies. This is connected to the low availability of fresh human post mortem specimens. Moreover, inadequate fixation conditions oftentimes result in bad tissue quality, which is due to the different causes of death, hypoxia, high temperature, and, most strikingly, the post mortem delay. Aoki et al. found immunoreactive, likely synaptic, FUS granules massively increased in number in post mortem FTLD-FUS material (Aoki et al. 2012). Whether this observation can be generalized to all mutated or non-mutated FUSopathies remains open for future research, and this finding is especially intriguing with regard to a conceivable gain-of-function mechanism of FUS at presynapses (figure 25, lower neuron, lower part of the synapse). In line with the observations from Aoki et al., Shang and Huang depict EM images of spinal cord motoneurons of a mutant FUS mouse model. They describe alterations in the morphology of presynaptic terminals and the PSDs in the anterior horn (Shang and Huang 2016). In my opinion, the presynaptic terminals appear more bulky and enlarged as compared to wildtype motoneurons.

The role of FUS as a regulator of local translation in physiological and pathological situations has to be further determined with regard to the presynaptic compartment. Even if FUS resides at presynaptic sites in pathological situations, the question arises whether its functions remain unaltered (author note: experiments are currently being addressed on my part). In many neurodegenerative diseases, two commonalities in the pathomechanisms are outstanding: 1.) the affection of highly translationally active neurons and 2.) the involvement of proteins in quality control. In this regard, it is not surprising that particularly long-living, highly metabolizing cells like neurons are among the first cells to show signs of decay (Monahan et al. 2016). Referring to the first point,
Bowden and Dormann state that RBPs regulate post-transcriptional steps of RNA processing, which comprises splicing, mRNA transport, and local translation (Bowden and Dormann 2016). Classical examples for RBPs affected in neurodegenerative diseases are FUS and TDP-43. Both proteins participate directly in various steps of RNA processing and shuttle between the cytoplasm and the nucleus. Furthermore, they can be found in stress granules and carry transcripts as cargo to axons or dendrites and even into spines in order to orchestrate local translation (Lagier-Tourenne et al. 2010). The postulated model in physiological conditions is that RBPs organize the assemblies of dynamic mRNPs (RNA granules, transport granules, stress granules, i.a.) and the release of mRNA species to polyribosomal sites for local translation. It is unknown whether these granules may become solidified under mutated conditions. Thereby, these mutated FUS-containing granules might become undynamic, which might, succeeding, hinder local translation with a decay of structures in the synaptic compartment (Bowden and Dormann 2016). Such a situation with a loss-of-function of FUS at presynaptic terminals is sketched in figure 25 (lower neuron, upper part of the synapse). A paucity of FUS at synaptic sites, caused by either the absence or by segregation of dysfunctional FUS aggregates, might trigger translational shortage of pivotal proteins. However, it is shown that granules containing mutant FUS can be translationally active (Yasuda et al. 2013). Moreover, it will also be intriguing whether other proteins from the FET protein family exhibit a presynaptic localization. This might, then, also be linked to FTD-FUS, in which all members of the FET protein family co-aggregate in the cytoplasm (Neumann et al. 2011, Dormann and Haass 2013). A putatively crucial role of FUS in the local presynaptic translation control may have tremendous consequences in FUSopathies by either losing FUS functions at the presynaptic terminals or by the exaggerated local translation in a toxic gain-of-function manner (see figure 25, lower neuron, synapse).

Future studies will shed light on how the molecular setup of presynaptic sites is altered under FUS-pathologic conditions, for instance, by determining the synaptic proteome in animal models under wildtype and mutated conditions (author note: experiments in the process of planning on my part). Further studies, which will address the contribution of a presynaptic pathology in ALS and FTD, are required to not only better understand the role of FUS in FUSopathies, but this research might be relevant to MNDs and even neurodegenerative diseases in general. This is due to the many
intersections of their pathologies. For instance, TDP-43 might be in a signaling cascade upstream of FUS, which is indicated by findings in animal models of FUS-associated diseases (Kabashi et al. 2011, Wang et al. 2011). Moreover, TDP-43 has recently been detected in the axonal and even presynaptic compartments (Feiler et al. 2015). TDP-43 is the most common inclusion protein in ALS. If and how this might affect presynaptic FUS in proteinopathies with TDP-43 inclusions is unclear. Moreover, alterations in the presynaptic local translation might not be restricted to the pathologies affecting FUS or TDP-43. This is supported by an increased number of axonal ribosomes in early stages of disease in a genetic ALS model with a SOD1 mutation (Verheijen et al. 2014).

To further elucidate the role of presynaptic FUS in neurodegeneration, it will be necessary to seek for correlations between the amount of FUS at a certain synapse subtype and the contribution of these possibly defective FUS-containing synapses to the clinical phenotype. To date, the synaptic role of RBPs in neurodegenerative diseases was rather marginally taken into account. The present findings may promote further research on that topic.

Is FUS able to surmount the synaptic cleft in physiological and pathological states?
Proteins involved in conformational diseases share several characteristic traits, as was described in the introduction in 1.1.1. The prion-like FUS RNA-binding protein has the ability to homomerize and, thereby, induces protein accumulations, which serve for specific biochemical processes (Altmeyer et al. 2015, Kai 2016). However, this intrinsic property of FUS comes at a cost as a consequence of changing into a hydrogel phase via an amyloid-similar β-sheet structure. In pathological states, a liquid droplet can turn into an unresolvable gel (March et al. 2016). Proteins with prion-like properties accumulate, and disease pathology seizes more and more areas of the CNS during the disease course. Several studies have implicated a spreading of self-aggregating proteins in an infection-like propagation (Hawkes et al. 2007, Aguzzi and Rajendran 2009, Costanzo and Zurzolo 2013, Jucker and Walker 2013, Braak et al. 2013, Brettschneider et al. 2015). Frequently, the propagation follows the axonal projections in a network-anterograde manner (Braak and Braak 1991, Braak et al. 2002, 2013, Brettschneider et al. 2015, Pokrishevsky et al. 2016), which is also shown in FTLD-FUS (Armstrong and Cairns 2012). In accordance with this, several animal studies have demonstrated a spreading of protein misfolding pathology mainly via synaptic contacts rather than directly to the
DISCUSSION

contiguous cells (Aguzzi and Rajendran 2009, Kfoury et al. 2012, Brettschneider et al. 2015). In line with this, an uptake or release of the cognate protein TDP-43 via the axon is demonstrated in cultured neurons (Feiler et al. 2015). Intriguing consistent features also exist with Parkinson’s disease. The misfolded protein synuclein alpha is presynaptically localized (Brettschneider et al. 2015) and propagates via synapses in an anterograde direction (Costanzo and Zurzolo 2013, Dunning et al. 2013). Similar observations have been made for the tau protein, which underlies Alzheimer’s disease and a sub-fraction of FTD (Kfoury et al. 2012).

FUS as a novel presynaptic protein and in close association to synaptic vesicles opens new avenues to investigate the mechanisms of propagation in the course of neurodegenerative diseases. More precisely, the proximity of FUS to a highly efficacious exocytosis machinery at the PAZ might elicit future studies to scrutinize a putative trans-neuronal propagation of aggregated FUS in a dying forward manner (see figure 25, lower neuron, lower part of the synapse). This might exemplarily be transferable to other conformational diseases. On the contrary, it might be likewise interesting whether trans-neuronal transfer of synaptic proteins via the synaptic cleft occurs physiologically. From a theoretical point of view, it might be reasonable to consider this possibility with regard to the high necessity to fine-tune both compartments for an optimal interplay of the pre- and postsynapse. This would also be in accordance to the functions of FUS in RNA metabolism, in which the expression of both pre- and postsynaptic proteins are nuclearly regulated by FUS (Lagier-Tourenne et al. 2012, Nakaya et al. 2013, Honda et al. 2014).

**Therapeutical implications**

To date, both ALS-FUS and FTD-FUS are deemed to be incurable. It will be tremendously important for future therapeutic interventions to determine whether a loss-of-function or a toxic gain-of-function of the FUS protein is responsible for the decay of certain neurons or for the local alterations of synapses. Therefore, it would be the goal to either increase the local translation or restrain excessive protein amounts via, e.g., promoting the protein degradation machinery of neuronal cells.

A spreading of FUS pathology via or at synaptic contacts has to be further investigated. This might facilitate to find solutions to counteract this process, for instance, via the application of antibodies targeted against extracellular FUS. A similar
strategy has in part been successfully administered in Alzheimer’s disease (Sevigny et al. 2016).

4.6 Conclusion

The RNA-binding protein FUS has pivotal functions in RNA metabolism in the nucleus and has recently attracted attention for the additional localization at synapses. The clinical importance of FUS arises from its involvement in both ALS and FTD.

An analysis with high-resolution microscopy was employed with the aim to scrutinize the specificity and distribution of synaptic FUS localization in defined regions of the mouse CNS (neocortex, archicortex, cerebellar cortex, and lumbar spinal cord). Moreover, super-resolution microscopy could comprehensively determine the distribution of FUS within synaptic compartments.

In synopsis, this study concisely demonstrates an abundant synaptic presence of FUS in all analyzed cortical areas of the mouse brain and also at axosomatic synapses of lower motoneuron somata. FUS signals appeared to be largely restricted to excitatory, glutamatergic synapses, but a markedly lower overlap was observed with inhibitory synaptic markers. Astonishingly, only axosomatic synapses of the lumbar spinal cord were negative for glutamatergic markers of all analyzed synapses in the mouse CNS. These synapses presumably represent a certain subtype of synapses, which originate from cholinergic interneurons (Shang and Huang 2016). Future studies might address this facet, in particular with regard to the distinct but overlapping major FUS pathologies: FTD-FUS, which mainly affects cortical areas and ALS-FUS with a decay of motor neurons. The staining pattern in mouse hippocampal sections was reflected in primary hippocampal neurons from embryonic rats.

Super-resolution microscopy from synapses of hippocampal neurons revealed a clear presynaptic and vesicle-associated localization of FUS, which is surprising due to the previously reported postsynaptic localization in neurons (Husi et al. 2000, Belly et al. 2005, Fujii et al. 2005, Fujii and Takumi 2005, Zhang et al. 2012, Aoki et al. 2012). This study demonstrates a localization analysis of synaptic proteins with super-resolution microscopy in an unprecedented grade of detail in cultured neurons. Thereby, the present super-resolution data exhibit a precision comparable to the investigations from Dani et al. who analyzed synapses in mouse brain tissue (Dani et al. 2010). Furthermore, two conclusions can be drawn: 1.) Super-resolution microscopy requires optimal quality
of immunolabeling, and 2.) this super-resolution microscopy study demonstrates for the first time a possible interference of antibodies targeted against proteins with a distance of a few nanometers.

These findings might have tremendous implications with regard to the functions of the RNA-binding protein FUS, which allude to a putative role as a novel regulator of translation in presynaptic boutons. A presynaptic compartment fully equipped with ribosomal units and RBPs, such as FUS, might confer unexpected abilities of self-regulation to the presynaptic compartment. These results dovetail with several recent reports, which support the importance of local translation at presynaptic sites (Giuditta et al. 2002, Younts et al. 2016). Possible disturbances of the local function of FUS in the presynaptic compartment would be in accordance with presynaptic phenotypes in animal models mimicking FUSopathies (Huang et al. 2011, Chen et al. 2011, Kabashi et al. 2011, Wang et al. 2011, Sasayama et al. 2012, Armstrong and Drapeau 2013, Shahidullah et al. 2013, Machamer et al. 2014, Therrien et al. 2016, Baldwin et al. 2016). The two major hypotheses to explain the detrimental effects in FUS pathologies are a loss-of-function of FUS in the nucleus or a toxic gain-of-function in the cytoplasm. With the present study, another possibility has to be taken into account: a loss- or a gain-of-function pathomechanism in the presynaptic boutons of FUS-containing synapses. Furthermore, the abundance of presynaptic FUS might be regarded as an anatomical prerequisite for the observed anterograde trans-synaptic propagation of the inclusion pathology in FUSopathies and might be transferable to conformational diseases in general (Braak and Braak 1991, Braak et al. 2002, Hawkes et al. 2007, Aguzzi and Rajendran 2009, Armstrong and Cairns 2012, Costanzo and Zurzolo 2013, Jucker and Walker 2013, Brettschneider et al. 2015, Pokrishevsky et al. 2016). Both the functions in the presynaptic compartment and the intertwined consequences for FUS-associated diseases deserve further interest. A more synapse-based approach to assess physiological functions and pathomechanistic processes with an involvement of FUS may expedite our understanding what causes the fatal decay of motoneurons.

Furthermore, the present study adverts to the future need to employ most precise methods to determine the localization of proteins at synapses. Along these lines, the knowledge about the molecular anatomy of neuronal contact sites is the basis to decipher the intricate functions of the eminently dynamic processes at synapses.
5 SUMMARY

Conformational diseases are devastating disorders and are accompanied by an irrevocable decay of neurons. Albeit the phenotypes vary in form of dementia, movement disorders, and behavioral abnormalities, the core histopathological finding in these diseases is the self-propagating accumulation of proteins in neuronal cells. Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) share the same underlying genes and properties of the protein accumulations. Along these lines, both diseases are currently considered as phenotypical variants of the same disorder. The majority of the involved accumulation-prone proteins exhibit two significant characteristics: 1.) they are part of the ribonucleic acid (RNA) processing machinery, and 2.) they form large protein accumulations in diseased states.

Among these proteins, fused in sarcoma (FUS) is an abundantly expressed RNA-binding protein with an ample list of known and in part assumed functions. FUS is integral in several steps of the nuclear and extranuclear RNA metabolism. It is also believed that FUS is able to orchestrate local translation. In ALS, most FUS mutations lead to a cytoplasmic mis-localization of the otherwise preponderantly nuclear protein. Two major, but contradictory mechanisms are favored to explain the pathological effects of FUS mis-localization: 1.) a loss-of-function mechanism in the nucleus and / or 2.) a toxic gain-of-function in the cytoplasm. To date, it is an unanswered question, which of these processes is dominant in the pathogenesis. Previous studies have shown that FUS is not confined to the nuclei in neurons but can also be found at synaptic sites. The most likely distribution was consistently assumed to be in dendrites and in postsynaptic compartments.

The aim of the study is to further scrutinize synaptic FUS in the central nervous system of mice with state-of-the-art imaging technology. By employing laser-scanning confocal microscopy, histological sections of various areas of the mouse central nervous system were analyzed. After immunolabeling of FUS in combinations with synaptic markers, it was demonstrated that FUS is abundantly expressed at synapses and localizes to excitatory, glutamatergic synapses in all investigated brain regions, namely, the cortex, the archicortex, and the molecular layer of the cerebellar cortex. Of note, mossy fiber boutons in the hippocampus and axosomatic non-glutamatergic synapses at alpha-motoneurons exhibited vast amounts of synaptic FUS. In contrast, FUS-
containing axosomatic synapses in the spinal cord are negative for classic markers of glutamatergic synapses. This specificity toward different synapse subtypes in the brain versus the spinal cord might be interlinked with the distinct but overlapping phenotypes in FUS-related ALS and FTD.

A further thorough investigation followed in cultured neuronal cells derived from rat embryonic hippocampi. By co-immunolabelings of different synaptic markers, FUS was found to be predominantly affiliated to excitatory, glutamatergic synapses with less association to inhibitory synaptic contacts. In order to unravel the precise localization of FUS at synapses, super-resolution microscopy was employed. This approach was required due to the proximity between the pre- and postsynaptic compartments, which are below the resolution limit of conventional fluorescence light microscopy. Initially, stimulated emission depletion (STED) microscopy was employed, while an in-depth analysis was conducted with direct stochastic optical reconstruction microscopy (dSTORM). Initially, a staining protocol could successfully be established. In a statistical approach, the axial distributions of FUS and of classical pre- and postsynaptic markers were determined. Thus, FUS could be localized to presynaptic terminals adjacent to the presynaptic active zone and largely overlapping with excitatory synaptic vesicles on a nanometer-scale. Thereby, immunolabeled synaptic proteins in primary hippocampal neurons could be displayed in unprecedented resolution.

The present study displays a comprehensive description of the synaptic distribution of FUS in crucial areas of the mouse central nervous system. Furthermore, by employing state-of-the-art super-resolution microscopy techniques, a precise anatomical localization of FUS at presynaptic terminals could be determined. The results pose pivotal implications for future investigations. It will be most intriguing whether FUS regulates local translation in presynaptic terminals, and whether this function is affected in diseased states. FUS might be in the cross-hair of local protein production at excitatory synapses, which might be connected to the vulnerability of certain synapses in pathological states of FUS. Finally, a presynaptic localization might be connected to the postulated mechanism of trans-synaptic propagation of protein accumulations in FUSopathies, which might explain the observed spreading of the disease pathology along neuronal circuits.

SUMMARY
REFERENCES

(6 REFERENCES

6. Allen Institute for Brain Science: http://mouse.brain-map.org/static/atlas (information was not downloaded; last update 2011; 14.11.2016)
REFERENCES


REFERENCES


REFERENCES


41. Crozat, A., P. Aman, N. Mandahl, and D. Ron. 1993. Fusion of CHOP to a novel RNA-


50. Dormann, D., T. Madl, C. F. Valori, E. Bentmann, S. Tahirowic, C. Abou-Ajram, E.
REFERENCES


REFERENCES

Neurosciences 25:400–404.


REFERENCES


REFERENCES


Loss of fused in sarcoma (FUS) promotes pathological Tau splicing. EMBO reports 13:759–764.


REFERENCES


REFERENCES


Acknowledgments

I want to express my gratitude to several important individuals who supported me beyond expectations in my scientific work.

To start with, I want to thank my mentor and supervisor Prof. Dr. Tobias M. Böckers for giving me the opportunity to conduct this study. Moreover, I appreciate all tips and inspiring discussions of professional and personal nature. He always supported me. I consider him as a brother in spirit keeping up the idealism and creativity in science. I am looking forward to our mutual work in ongoing and future projects.

Special thanks are due to Juniorprof. Dr. Andreas M. Grabrucker for introducing me into many aspects of laboratory and literature work. He is one of the most gifted scientists I have met. For discussions, inspirations and useful tips, I wish to thank Dr. Stefan Putz, Dr. Christopher Heise, Dr. Stefanie Pfänder, Dr. Maria Demestre, Dr. Dominik Reim, Jan Philipp Delling, and further colleagues of the Institute for Anatomy and Cell Biology. PD Dr. Francesco Roselli and his team (Florian olde Heuvel) gave useful inspirations and help for the processing and analyses of the nerve tissue. Anne-Kathrin Lutz, Dr. Christopher Heise, Alberto Catanese, and Dhruva Desphande deserve a big thank you for a critical look on parts of the manuscript. Moreover, I want to acknowledge that Dr. Stefan Putz’s previous studies laid the foundation for my work.

It was a pleasure and very fruitful to collaborate with the Institute of Biophysics headed by Prof. Dr. Jens Michaelis. The intense discussions and endeavors with Jochen M. Reichel and Dr. Christian Osseforth and the following seminal work with Dhruva Desphande eventually resulted in a concise data set, which became part of a publication. Further investigations with super-resolution microscopy are currently being carried out. I would also like to express my gratitude to Prof. Dr. Kirchhoff (Institute of Molecular Virology, Ulm University) for kindly providing access to their confocal microscope.

I owe a special thank you to the laboratory technicians Ursula Pika-Hartlaub and Renate Zienecker for most competent support in laboratory.
Curriculum vitae

Personal information
Name: Michael Schön
Place of birth: Wasserburg am Inn, Bavaria, Germany
Citizenship: EU citizen, German
Address (work): Institute for Anatomy and Cell Biology
Albert-Einstein-Allee 11
89081 Ulm
Contact: telephone (work): +49731-50023223
mail: michael.schoen@uni-ulm.de

Development and career
School: elementary school, Rott am Inn
secondary school, Luitpold-Gymnasium Wasserburg
Graduation: final secondary school qualifications
Study: medicine, Ulm University
University degree: First Part of the Medical Exam (Physikum), grade: magna cum laude (1,0)
Second Part of the Medical Exam, grade: very good (1,5)
Clinical traineeship: nursing internship (Schön Klinik Vogtareuth)
clinical traineeships in internal medicine (Universitätsklinikum Ulm),
anesthesia and intensive-care medicine (Donauklinik Neu-Ulm), pediatrics (Westküstenklinikum Heide), neurological surgery (Bezirkskrankenhaus Günzburg)
Practical year: pediatrics, surgery, and internal medicine (Klinikverbund Kempten-Oberallgäu)
Doctoral thesis: 2017: submission of the doctoral thesis performed in the Institute for Anatomy and Cell Biology, Ulm University; doctorate supervisor Prof. Dr. Tobias M. Böckers
title: “Analysis of the synaptic localization of the FUS RNA-binding
protein with high- and super-resolution microscopy” supported by fellowships and grants of the Studienstiftung des deutschen Volkes; Promotionsprogramm Experimentelle Medizin, Ulm University; and Helmholtz Virtuelle Institut RNA Dysmetabolismus bei ALS und FTD

Professional career:
research fellow in the Institute for Anatomy and Cell Biology, in postgraduate training (Facharzt für Anatomie)

Main research topics:
1. Super-resolution imaging of synapses in SHANK mouse models of autism-spectrum disorders
2. MRI imaging of SHANK mutant and non-genetic autism mouse models
3. Novel interaction partners of SHANK proteins
4. Clinic for Phelan-McDermid syndrome patients
5. Analyses on the SHANK distribution in human brain tissue
6. Anatomical and developmental analyses of the human entorhinal cortex
7. Novel immunohistochemical methods on long-term fixated human brain tissue
8. Analysis of the synapse loss in Alzheimer’s disease
9. Investigations on the role of FUS at synapses

Qualifications:
- 2013: advanced training in Good Clinical Practice (GCP), Ulm University
- 2014: advanced training in animal handling, certified by GV-SOLAS (level FELASA, category B)
- 2016: CLARITY workshop in Karl Deisseroth’s lab, Stanford University, California, USA

Talks, conferences, and posters:
- 2009: poster Annual Meeting of the Anatomische Gesellschaft in Würzburg, Germany
- 2009: poster on the Society for Neuroscience annual meeting in Chicago, Illinois, USA
- 2010: talk Annual Meeting of the Anatomische Gesellschaft in Würzburg, Germany
- 2010: poster on the Society for Neuroscience annual meeting in San Diego, California, USA
- 2011: First International Phelan-McDermid Syndrome (PMS)
Symposium in New York, New York, USA

- 2016: Phelan-McDermid Syndrome International Family Conference in Orlando, Florida, USA (initiation of meetings in order to set consensus guidelines for the diagnosis and treatment of PMS)

- 2016: talk Annual Meeting of the Anatomische Gesellschaft in the Young Investigator Award Session in Würzburg, Germany

Awards:

Certificate of Excellence of the Landrat of Rosenheim for excellent secondary school qualifications; Certificate of excellence of the school rector

Certificate of Excellence of the Institute for Anatomy and Cell Biology for finishing the histological course among the top 4%

Certificate of Excellence of the Institute for Anatomy and Cell Biology for finishing the gross anatomy course among the top 3%

Certificate of Excellence for being one of four students of the cohort with grade 1.0 in the First Part of the Medical Exam)

presented by the dean of studies (in my cohort among the top 25 students of about 9000 medical students in Germany)

Certificates of Excellence in pathology exams

2017: Eva Luise Köhler Forschungspreis 2017 (in a team of researchers and physicians for my contribution in the establishment of a clinic for Phelan-McDermid syndrome patients)

Scholarships:

2006 – 2010: fellowship of the Studienstiftung des deutschen Volkes

fellowship of the Promotionsprogramm Experimentelle Medizin, Ulm University

2009: travel grant for the Society for Neuroscience annual meeting in Chicago awarded by the GlaxoSmithKline Stiftung

2010: travel grant for the Annual Meeting of the Society for Neuroscience in San Diego awarded by the Phelan-McDermid Syndrome Foundation (PMSF)
Publications:
(in alphabetical order according to the last names of the first authors)


* these authors contributed equally to this work


