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Bringing light to the inner life of synapses

Synapses: function and dynamic organization

The central nervous system (CNS) consists of a vast assembly of neurons which process information in order to react to external or internal stimuli and acquire and store information for future behavior regulation and fundamental body functions. This communication between nerve cells largely occurs by means of chemical "synapses". Charles Scott Sherrington first coined the term "synapse" as a physiological concept in 1897. Derived from the Greek words *syn* meaning"together" and *hapsis* meaning"joining" (to join together), synapses represent intercellular contact sites, at which neurons communicate with their partner cells (usually other neurons). At synapses, the rapid transmission of information is mediated by the flow of electrical current from the pre- to the postsynaptic cell. The current may flow directly from one cell to another (electrical synapse), or rely on an intermediate step involving the release and detection of chemical transmitter substances (chemical synapse). Importantly, the mechanism of chemical synaptic communication enables the strength of signal transmission to be adapted in a highly variable manner, and thereby chemical synapses (from here on synapses) filter, integrate or modify information, thus acting as key regulators of many complex brain processes.

The human brain harbors around 10^{11} neurons connected via 10^{15} chemical synaptic contacts of this kind. Complex genetic programs steer brain development by instructing the specification of neuronal identity, establishing proper connectivity by forming synapses in a spatiotemporally highly controlled manner. As if this complexity were not enough of a challenge to the researcher, once formed during development, synapses are in no way static entities. Particularly, the activity-dependent modulation of synaptic strength, termed 'synaptic plasticity', has received much attention in recent decades. The concept that learning involves plastic changes of synaptic connections and that memory storage requires the stabilization of such modulations was already proposed in 1894 by Santiago Ramón y Cajal and later refined by Donald Hebb. Systematic investigations of both the invertebrate nervous system and the mammalian hippocampus support the hypothesis that the plasticity of chemical synapses is fundamental for certain forms of learning. On the flip side, degenerative diseases of the nervous system, most importantly Parkinson's and Alzheimer's disease, might be the result of disturbances in synapse structure and function.

Thus a detailed understanding of how synapses form on the molecular level and how their molecular organization defines their function is critical [8, 14]. Particularly due to the improving sensitivity of biochemical ("proteomic") techniques, recent years have seen the identification of a bewildering list of proteins present at synapses. In methodological terms, challenges remain in the analysis of these questions.

On the one hand, to better understand the function of specific proteins' synapse structure and/or function, these proteins must be analyzed using genetic methods (i.e. their function must be eliminated or modified and the consequences thereof studied). Often, however, genetic analysis of synapse-relevant proteins is complicated by either "early lethality" (i.e. the genetic deletion of a specific protein causes death of the organism at an early, non-informative state) or "functional redundancy" between two or several proteins. These problems are particularly pressing in mammalian (typically rodent) model systems, while effective invertebrate genetic models such as the fly *Drosophila* and the nematode *Caenorhabditis elegans* are typically less affected by these complications.

On the other hand, due to their small size of only a few hundred nanometers (in more common terms, a fraction of a thousandth of a millimeter), synapse architectures traditionally could not be accessed with the arguably most powerful technique of cell biological analysis, fluorescence light microscopy. This is because light microscopy fundamentally suffers from a limitation in resolution also referred to as the diffraction barrier. Recent advances in physics (in particular stimulated emission depletion microscopy, STED) have "broken" the diffraction barrier and allowed our group deeper insights into synapse molecular architectures [3, 7]. Finally, our knowledge of how synapse architectures form under in vivo conditions is still very sparse. This is due to the fact that, to date, synapses could hardly be directly observed over time in an intact organism. Our group, however, has established protocols to do so in *Drosophila* (in vivo imaging).

While, as mentioned above, we have learned about the sheer protein compo-

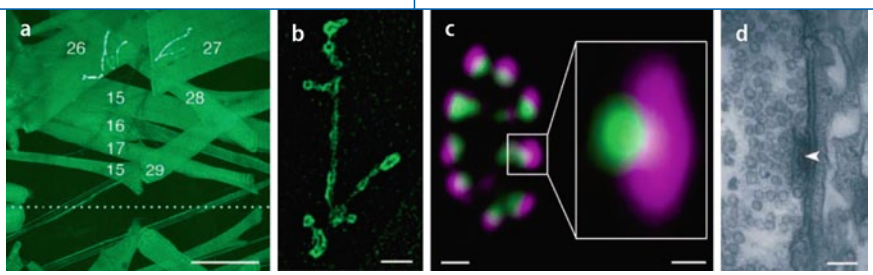

Fig. 1 ▲ The *Drosophila* neuromuscular junction (NMJ). **a** Repetitive muscle pattern of *Drosophila* larvae in an abdominal segment as seen from the exterior. The image was modified to highlight NMJs at muscles 26 and 27 (scale bar, 100 µm). **b** Morphological structure of a *Drosophila* larval NMJ at muscle 27 (scale bar, 10 µm). **c** Immunohistochemical stainings of a bouton of a larval NMJ and an individual synapse in lateral view (*right box*). *Green* monoclonal antibody Bruchpilot^{Nc82} [9], *magenta*: antibody against the glutamate receptor subunit DGluRIID. Scale bar bouton, 1 µm; scale bar synapse, 100 nm. **d** Ultrastructure of an active zone. The *arrowhead* points at the T-bar that typically clusters synaptic vesicles (scale bar, 100 nm). (Taken from [1])

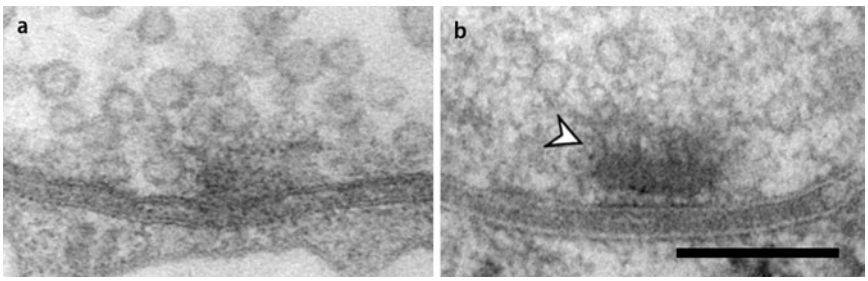

Fig. 2 ▲ The neuromuscular T-bar ultrastructure. **a** Depicted is a *Drosophila* neuromuscular T-bar after fixation for immobilization. The proximal part of the T-bar is covered by a typical "roof". **b** A T-bar which has been rapidly frozen for immobilization is depicted. Clearly, the "roof" is replaced by filaments (*arrowhead*), emerging from the proximal part of the T-bar. Scale bar, 100 nm

nents of synapses, how these different protein components integrate into scaffolds ("synapse molecular architecture") to drive synapse function in a highly controlled and effective manner still requires clarification. Fine deficits in how synapses establish their structural and functional architecture can contribute to the pathogenesis of schizophrenia and autism spectrum disorders. For this reason, our group seeks to contribute to the analysis of these synapse assembly processes.

Genetic or acquired deficits in these processes definitively contribute to the pathogenesis of schizophrenia and autism spectrum disorders. Moreover, deficits in synapse assembly/stability and consequently function appear to also play a role in the prevailing neurodegenerative diseases, Alzheimer's and Parkinson's.

In the following we describe the efforts our group has taken to address both in vivo synapse assembly as well as to better decipher the molecular architecture of synapses. We start, however, by depicting the model system we predominately use to study synapses: neuromuscular synapses of developing *Drosophila* larvae.

Model system: glutamatergic synapses allow for effective genetic analysis of synaptic protein function

Glutamate is the dominant excitatory neurotransmitter in the human brain. The primary models of our group are glutamatergic ("glutamate-using") synapses of the *Drosophila* neuromuscular junctions (NMJs). While similar to glutamatergic CNS synapses in mammals in terms of ultrastructure and molecular composition, the NMJ combines a comparatively simple overall morphology (◘ **Fig. 1**) with straightforward genetic accessibility.

An NMJ appears like beads on a string, with each bead representing a bouton, which can be as large as 5 µm in diameter (◘ **Fig. 1a, b**). Boutons consisting of glutamatergic synapses are designated as type I (◘ **Fig. 1c**). Type I boutons show stereotypic morphology and can be further subdivided into Ib and Is boutons. Type Ib are larger boutons that mainly contain clear synaptic vesicles (SV). Each synapse consists of a presynaptic active zone (the site of transmitter release), a postsynaptic density and a synaptic cleft separating both. The active zone (AZ) is defined as the presynaptic region where synaptic vesicles fuse with the presynaptic membrane in response to incoming activity (action potential) and release their neurotransmitter cargo into the synaptic cleft.

The ultrastructure of NMJ synapses is thoroughly described and they are easily accessible using electrophysiological methods, which have been applied in numerous studies. In addition, the so called GAL4-UAS system makes it possible to restrict expression of proteins in either the presynaptic (motoneuron) or postsynaptic (muscle) cell, allowing for the functional definition of the site of action of synaptic proteins by rescue experiments.

The T-bar: a role model for the analysis of presynaptic active zone cytomatrices

At synaptic active zone membranes, action potentials lead to the formation of Ca^{2+} microdomains at strategically localized clusters of voltage-gated Ca^{2+} channels. These Ca^{2+} microdomains trigger exocytosis of SVs, a process that requires tight physical coupling between vesicles and Ca^{2+} channels in order to operate efficiently. In addition to the major core machinery such as Ca^{2+} channels and the so-called SNARE complexes that mediate the actual fusion process, recent proteomic and genomic studies have suggested additional protein species that localize to synapses [2, 8]. Highly-ordered synaptic protein architectures are clearly seen by electron microscopy, which reveals electron-dense specializations (cytomatrix at the active zone [CAZ]) that cover the presynaptic plasma membrane at places where SV fusion occurs. By interacting with the core fusion machinery, the CAZ is a candidate structure to confer efficacy and controllability to the SV fusion process. However, while these CAZs have been observed for decades using electron microscopy, their molecular composition and functional roles, espe-

cially in the context of the SV cycle and Ca^{2+} channel function, are much less well understood [14, 16]

Fortunately, *Drosophila* synapses display prominent cytomatrices called T-bars (**Fig. 1d**), per se providing the opportunity to combine efficient genetics with ultrastructural and electrophysiological analyses. **Fig. 2** shows a detailed ultrastructural analysis of our group using the rather recent method of rapid freezing under high pressure as a new preparation method for transmission electron microscopy. Rapid freezing (**Fig. 2b**) conserves natural topology of structures better than "traditional protocols" using chemical fixation (**Fig. 2a**). With rapid freezing, we see that filaments emerge from the distal part of the T-bar that tether SVs [4, 16] (compare **Fig. 2a, b**).

A large scaffold protein—Bruchpilot—forms the T-bar

However, a biochemical approach—which we took in collaboration with the laboratory of Erich Buchner (Würzburg University)—was required to "molecularly" access the T-bar. In this way, we were able to identify a novel large protein as a detrimental component of the *Drosophila* T-bar [9, 15]. In initial experiments with Erich Buchner's group we recognized that a reduction (but not a full elimination) of the protein provoked collapse in flight. Thus, the name Bruchpilot (BRP) was coined for this factor. Noteworthy, however, is the fact that this protein is "not a fly specialty", but instead belongs to a family of proteins, the ELKS family, meanwhile known to be generically present at synaptic active zones.

Importantly, genetic elimination of the BRP eliminated the T-bars, allowing for an analysis of the cytomatrix function in our system. Animals lacking BRP died prematurely (while they still formed synapses and a nervous system) and showed a severe (approximately 70%) reduction in the number of SVs released per action potential. Furthermore, the localization of Ca^{2+} channels to active zones was clearly reduced at BRP-deficient NMJs. Taken together we could show that BRP is involved in concentrating Ca^{2+} channels near docking sites for SVs, thereby ensuring the appropriate probability for SVs to get released.

Super-resolution light microscopy for the analysis of synaptic protein architecture

Synapses are very fast, extremely controlled and effective communication devices. All available evidence suggests that the synaptic protein-based architectures (CAZ as e.g. *Drosophila* T-bars) evolved to support these features. In addition, the protein-based architectures might well define synapse-specific differences in the spatio-temporal profile of SV fusion (short term plasticity), a feature pivotal in the case of neuronal computing in the course of information processing, learning and memory.

Dissecting synaptic protein architecture requires imaging with nanometer-scale resolution as well as highly-specific and efficient molecular identification, a difficult task to accomplish by conventional imaging techniques. The size of a *Drosophila* T-bar is approximately 200 nm in diameter. Thus, in order to visualize its spatial architecture properly, the resolution during the image acquisition process needs to be correspondingly high. Under normal conditions conventional light microscopy offers a resolution capacity of 180–250 nm at the x, y orientation. Here the so-called numerical aperture of the objective and light diffraction set the limitations together with the wavelength properties of light.

Thus, the minimal size of a light spot is limited by diffraction to about half the wavelength used. For this reason, confocal laser-scanning microscopes are unable to further decrease the excitation spot to less than approximately 200 nm, half the wavelength of blue light. Due to this limited resolution, neither "normal" epifluorescence nor confocal microscopes are capable of adequately visualizing subsynaptic organization. Thus, the analysis of small structures of this kind was long reserved electron microscopy, since the latter provides sufficient resolution due to the short wavelength of electrons (**Fig. 2**). However, here the labeling efficiency of specific proteins is still not fully perfected and

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Abstract
Chemical synapses are pivotal for information transfer and storage within neuronal circuitry. At the same time, various diseases of the nervous system most likely originate from disturbances in synapse structure and function. Synapses are very fast, extremely controlled and effective communication devices, with synaptic vesicles fusing at specialized membrane domains associated with highly-ordered protein architectures (cytomatrices) traditionally seen using electron microscopy. *Drosophila* synapses with prominent cytomatrices called T-bars provide per se a highly suitable model system to apply genetic analysis to the roles of these protein architectures. Here we describe the principles behind these techniques as well as their application to the analysis of the molecular architecture of the synapse. In this context, the advent of super-resolution light microscopy methods yielding two- to 10-fold higher resolution than conventional microscopy has provided an efficient tool.

Keywords
Synapse · Active zone · Learning and memory · In vivo imaging · *Drosophila*

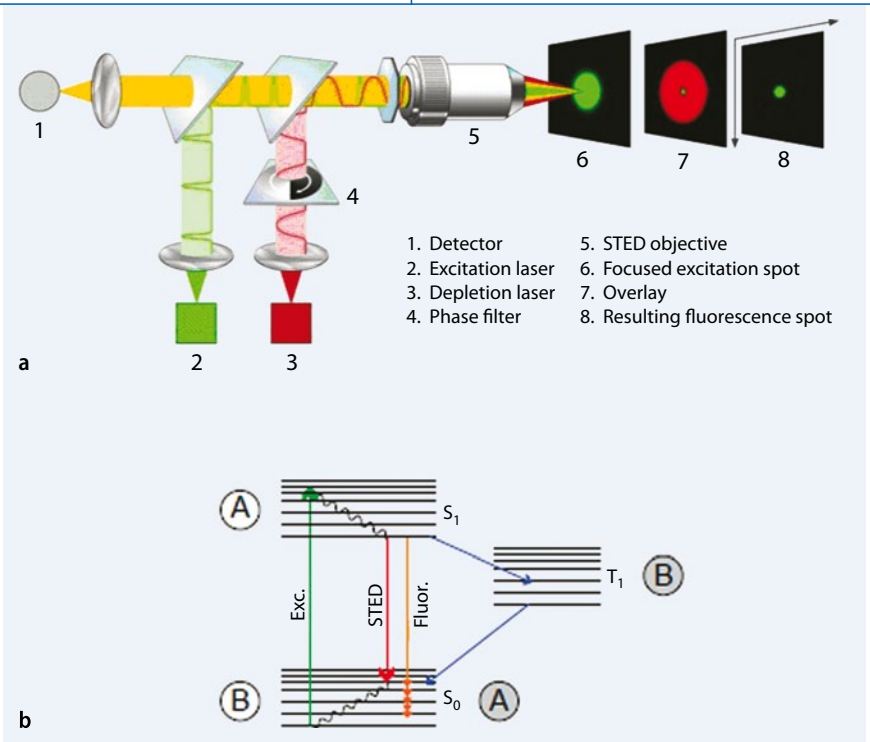

Fig. 3 ▲ The principle of stimulated emission depletion microscopy (STED). **a** The first laser (*2*, *green*) excites the fluorophores of the specimen, directly followed by the doughnut-shaped red-shifted depletion laser beam (*3*, *red*). Due to the doughnut shape of the depletion laser only fluorophores in the outer areas of the excitation spots are de-excited before they can emit fluorescence. This results in a small but sharply focused laser spot which overcomes the diffraction limits and allows for high resolution laser scanning. (**a** © Leica Microsystems.) **b** Energy diagram of an organic fluorophore. Molecules in the excited state S_1 return to the ground state S_0 by spontaneous fluorescence emission. Return to S_0 may also be enforced by light through stimulated emission, a phenomenon with the same cross section and intensity dependence as normal absorption. To prevail over the spontaneous return, STED requires intense light pulses lasting a fraction of the S_1 lifetime. Tuning the STED wavelength to the red edge of the emission spectrum prevents re-excitation by the same pulses. T1 is a dark triplet state that can be accessed through S_1 and then returns to S_0 within 1–104 µs. (**b** Modified with permission from [5])

well elaborated staining protocols are not yet as effective as desired.

To overcome the rigid diffraction barrier of far-field microscopy (elaborated by Ernst Abbe, published in the 1870s), Stephan Hell and colleagues developed an elegant technique based on a quantum mechanical phenomenon called the stimulated emission. Under natural conditions a fluorescent molecule is brought to an excited state by stimulation with light of a certain wavelength (**Fig. 3a**). From the excited state the molecule spontaneously falls back to the resting state, emitting fluorescent light of red-shifted wavelength. Stimulated emission now allows the targeted down-regulation of excited molecules to its ground state (**Fig. 3a**). Excited molecules are thus stimulated by light of a similar wavelength (red-shifted de-excitation beam) to the fluorescent emission light. They immediately fall back to the ground state by emitting photons of the same wavelength as the de-excitation beam. After stimulated emission of this kind the fluorescent molecule does not usually show any spontaneous emission of fluorescent photons. STED microscopy now uses this basic mechanism to reduce the resolution in light microscopy by partly depleting fluorophores located at the edge of the focal spot [6]. The essential characteristic of STED microscopy is that the excitation beam is overlapped by a donut-shaped depletion beam, which is capable of quenching fluorophores by stimulated emission (**Fig. 3b**). Given that both laser beams are perfectly aligned, the fluorescence signal is retained only in the very center of the excitation spot. The depletion laser beam is not present here (**Fig. 3b**). Thus scanning with such a narrowed spot across a sample already allows for scanning images with subdiffractional resolution.

STED (**Fig. 3**) proved an efficient technique to determine why BRP is that critical for CAZ (T-bar) formation. In particular, this enabled us to study the molecular orientation of BRP at the active zone in further detail (**Fig. 4**). We found that the C-terminal monoclonal antibody BRP^{Nc82} forms doughnut-shaped structures when visualized at "planar-imaged" active zones orientated vertical to the optical axis [9] **Fig. 4a, b, c**, (arrow)]. Other than BRP^{Nc82}, $BRP^{N\text{-}Term}$ does not show a doughnut-shaped distribution when imaged with STED. Instead, the $BRP^{N\text{-}Term}$ signal appears centered within the "doughnut hole" of the BRP Nc82 signal. Moreover, both signals appear segregated at "vertical-imaged" active zones with $BRP^{N\text{-}Term}$ facing to the active zone membrane (**Fig. 4c**). Overall, the combination of STED resolution for BRP^{Nc82} and confocal resolution for $BRP^{N\text{-}Term}$ suggested a polarized and funnel-like distribution of BRP epitopes (**Fig. 4c**). This suggests that individual BRP molecules can adopt an elongated conformation as shown in the model in **Fig. 4c** [4]. Thereby, the N-terminus of BRP was found at a distance of about 100 nm from the C-terminus, with the N-terminus superimposed on the Ca^{2+} channel clusters centered in the AZ membrane center. In these studies, STED provided ~80 nm xy resolution and the rather simple topology of *Drosophila* neuromuscular synaptic terminals allowed identification of whether individual AZs were imaged in a planar or vertical fashion. A model describing the STED-derived topology is seen in **Fig. 4d**. STED analysis further showed that other AZ-enriched proteins, such as DLiprin-α, localize to a distinct sub-compartment of the AZ.

In vivo imaging of synapse assembly

How do synaptic protein architectures actually form under in vivo conditions? Although a simple question at first glance, our understanding of this process is scant. While data acquired in cultivated neurons show that synapses can assemble quickly,

other studies analyzing mammalian tissue samples suggest that this process takes approximately one day. Thus, it is conceivable that the regulation of synapse formation differs between in vitro and in vivo models, and that synapse formation in fact might be a long and intricate process involving multiple interrelated steps with reciprocal induction as well as independent assembly of pre- and postsynaptic structures. At present, a lack of knowledge on the detailed spatio-temporal sequence of in vivo synaptic assembly remains a barrier to a comprehensive understanding of the development of synaptic circuits. This issue could be overcome by extended molecular intravital imaging of individual synaptic proteins at identified sets of synapses. However, such an approach requires an ideally suited model system, allowing effective imaging with effective possibilities for labeling synaptic proteins of interest.

Throughout *Drosophila* larval development, the number of synaptic boutons and individual synapses decorating these boutons increases significantly on a time scale of hours (**Fig. 5d**). Moreover, similar to central synapses in mammals, synapses at *Drosophila* NMJs are not static structures but rather undergo activity-dependent and experience-dependent changes regarding the number of individual boutons and the overall number of synapses per NMJ [11, 13]. *Drosophila* larval muscles are built and arranged in a stereotypical, repetitive manner, as are the motoneurons innervating them. Therefore, the same individual NMJs can be easily identified among different individual larvae as well as within one particular larva at several points in time.

Some years ago our group devised protocols to allow in vivo imaging of identified synapse populations over days of NMJs in living larvae [11]. Here, intact larvae are anesthetized and non-invasively imaged with confocal or two-photon microscopy (**Fig. 5**). In this way, the protein dynamics organizing the synapse assembly process could be directly visualized in living animals, e.g. by using in vivo fluorescence recovery experiments. Our in vivo experiments on developing NMJs revealed that individual synapses (consisting of the presynaptic AZ and the respective adjunct postsynaptic density, PSD) assemble on a time scale of several hours, with pre- and postsynaptic proteins joining in the assembly of synapses in a temporally defined order [10, 11, 12].

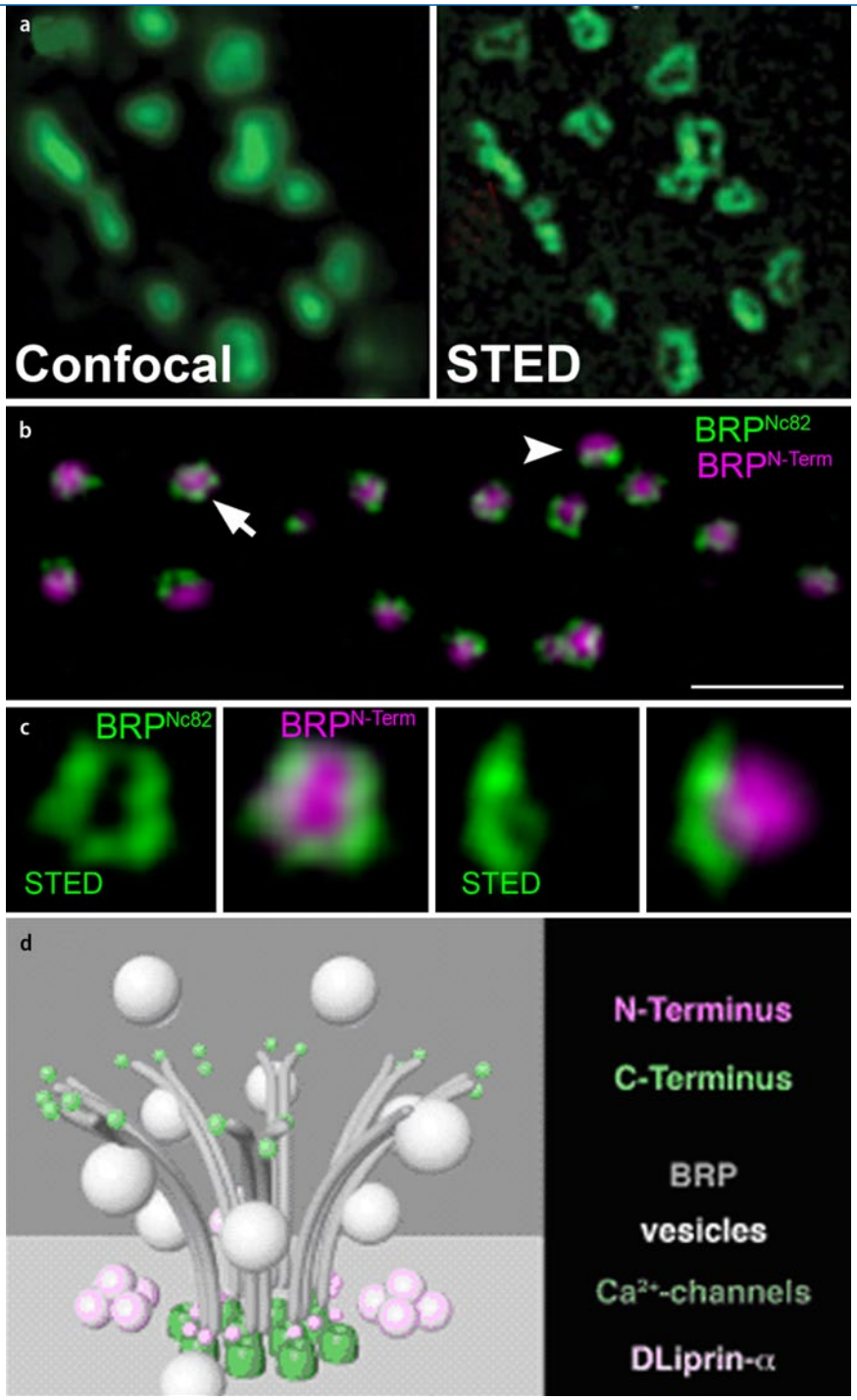

Fig. 4 ▲ STED analysis of synaptic protein architecture. **a–c** STED imaging of *Drosophila* neuromuscular synapses. **a** STED microscopy reveals doughnut-shaped structures recognized by the monoclonal antibody Nc82 against the protein Bruchpilot that are not resolvable by confocal microscopy. **b** Boutons stained for $BRP^{N\text{-}Term}$ (*magenta* confocal) and BRP^{Nc82} (*green* STED) showing planar (*arrow*) and vertical (*arrowhead*) active zones. **c** Magnifications of individual planar (*left*) and vertical (*right*) active zones stained for BRP^{Nc82} (STED) and $BRP^{N\text{-}Term}$ (confocal). (**d**) Spatio-temporal model of AZ assembly and organization at *Drosophila* NMJs. (**a** modified with permission from [9]; **b–d** taken and modified from [4])

In detail, we found that neuromuscular accumulations of glutamate receptors

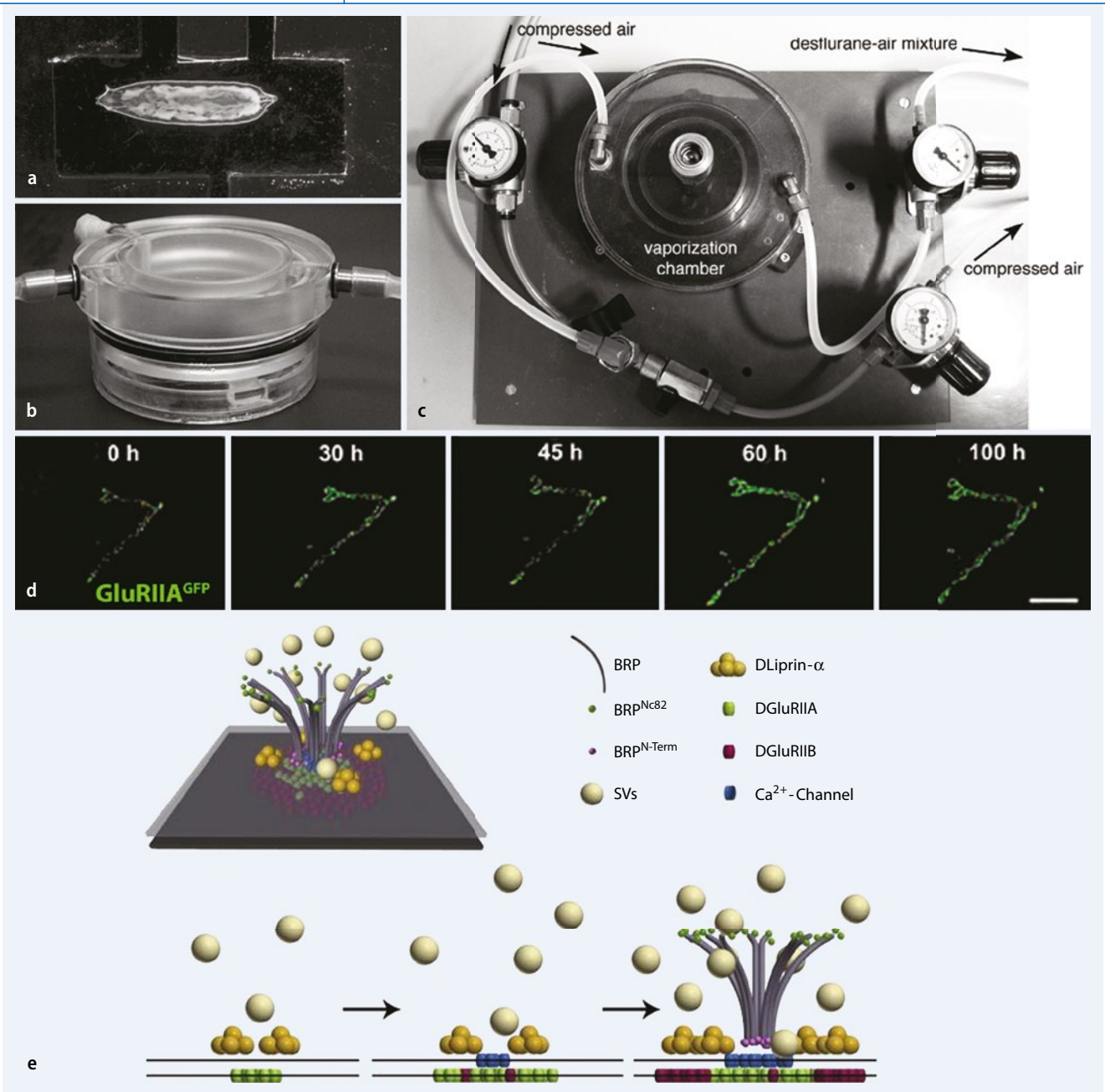

Fig. 5 ▲ In vivo imaging of *Drosophila* NMJs. **a** Larva immobilized in the imaging chamber. **b** In vivo imaging chamber. **c** Anesthetization is accomplished using desflurane. Via the depicted vaporization chamber a controlled desflurane-air mixture can be directed to the larva. **d** The development of an identified NMJ at muscle 27 tracked over 100 h at 16 °C visualized using the glutamate receptor subunit IIA tagged with GFP. **e** Schematic drawing of the development of *Drosophila* NMJ synapses. Scale bar in **d**, 10 µm. (Taken from [1] with permission; **e** taken from [4], JCB)

(as DGluRIIAs) in PSDs typically form at a distance from existing PSDs and then grow over several hours before reaching a final mature size [11, 12]. Thus, for the analysis of AZ assembly in vivo, DGluRIIA was co-imaged to serve as a reference point for our temporal analysis. Larvae co-expressing two fluorescently tagged synaptic proteins were imaged and quantitative data were obtained to analyze the temporal sequence of protein arrival at developing AZs. For a given larval NMJ, two in vivo images were acquired at particular time intervals such as, e.g. 12 h; during the intervening time period larvae were allowed to move freely. In this way, we were able to obtain a time series of proteins arriving at synapses as shown in **Fig. 5e**.

Outlook

Despite rapid advances in tools and techniques for studying synapse structure, function and assembly, many open questions remain. For example, it is unclear whether some synaptic components are trafficked down the axon in preassembled units, as indicated in mammals, or whether they traffic individually and as-

semble at the synapse. Additional areas under investigation include how the precise spatio-temporal organization of proteins takes place and how developing synapses are stabilized. By combining intravital imaging with super-resolution light microscopy (live-STED) we hope to contribute to a more comprehensive understanding of these processes.

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Stephan Sigrist studied chemistry and biochemistry in Berlin and Tübingen. He received his PhD from the University of Tübingen in 1997. After postdoctoral training in neurogenetics he led a Max-Planck Junior Research Group at the European Neuroscience Institute (ENI) in Göttingen. Since 2008 he has been a full professor of neurogenetics at the Freie Universitaet Berlin, and a director of the Neurocure Cluster of Excellence. His work focuses on mechanisms of synapse assembly and plasticity, particularly the question of how functional and structural differentiation of synapses is integrated. To this end, high resolution light microscopy and intravital imaging are combined with genetic approaches in Drosophila melanogaster.

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C. Wichmann Born 1973 in Brake, Germany, Dr. Carolin Wichmann studied biology at the Georg-August University of Göttingen, Germany, from 1993 to 1999. After receiving her PhD in 2002, for which she investigated the activity of liposome-bound enzymes, she changed from microbiology to the field of neurosciences. She worked on the morphology of wild-type and mutant synapses of the fruitfly Drosophila via transmission electron microscopy as a member of Prof. Dr. Stephan Sigrist's group at the European Neuroscience Institute Göttingen. She has been leading her own junior research group under Prof. Dr. Tobias Moser in the department of Otolaryngology at the UMG in Göttingen since July 2011. In this context, she is focusing on the molecular architecture and vesicle dynamics of mouse inner ear ribbon synapses, mainly using electron tomography and 3D serial reconstructions.

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