PROJECT FINAL REPORT

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Name, title and organisation of the scientific representative of the project's coordinator:

Prof. Nils Brose

Max-Planck-Institute of Experimental Medicine

Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.

Tel:	+49-551-3899 725
Fax:	+49-551-3899 715
E-mail:	brose@em.mpg.de
Project website address:	http://eurospin.mpg.de

1. FINAL PUBLISHABLE SUMMARY REPORT

Executive Summary

By initially focusing on 20 disease relevant genes and by selecting three key levels of analytical complexity, EUROSPIN defined a very substantial 'parameter space' of projects, which was further extended by adding several additional disease genes and mutants to the EUROSPIN portfolio in the course of its activities. The EUROSPIN consortium tackled this challenge systematically and made excellent progress on all fronts.

The work of EUROSPIN led to the establishment of a large protein-protein-interaction (PPI) database, which was complemented with systematic data on high-throughput electrophysiological analyses and on behavioral phenotypes of disease-relevant mouse models, as well as with data on dozens of new antibodies generated by EUROSPIN (eurospindb.genes2cognition.org). The collated EUROSPIN database will serve as an invaluable resource for the neuroscience community worldwide. EUROSPIN work on small molecules that affect PPIs in synapses had pioneering character. The first lead structures that affect neurite outgrowth or Aβ42 aggregation will serve as the basis for new strategies to promote neuronal regeneration (e.g. after injury) or to treat Alzheimer's disease. Further, EUROSPIN partners developed multiple new mouse lines that allow for the targeted purification and/or imaging of disease relevant gene products as well as new disease models. These new mouse lines, along with the large number of mouse models that EUROSPIN partners brought into the consortium, represent one of the largest mutant mouse collections in academia and will serve - like the EUROSPIN database - as an extremely important resource in the neurosciences. Using some of these mouse lines as well as newly developed subcellular fractionation protocols, EUROSPIN partners have characterized key disease-relevant protein complexes of synapses and their protein components. Studies on disease-relevant gene products concentrated on a very wide array of proteins. In this regard, EUROSPIN work ranged from focused studies on single genes in specific experimental settings (e.g. NLGN1, N-Cadherin, Munc18-1, a.m.o.), via analyses of entire gene families and the effects of their dysfunction using selected experimental approaches (e.g. DLGs), to analyses of the role and disease-relevant dysfunction of individual genes at all envisioned analysis levels - from molecular and cellular studies, via behavioral analyses, to studies in neuronal networks (e.g. NLGN2, SNAP25). Finally, EUROSPIN studies on neuronal networks in vitro and in vivo defined new principles of network function in cerebellum and amygdala and led to a first characterization of the role of defined disease gene products in amygdala function and related behavioral outputs.

On aggregate, EUROSPIN work on a large number of disease gene products has yielded a wealth of insights into the mechanism by which the respective mutation or loss of such proteins might cause disease phenotypes. Many of the corresponding proteins represent potential targets for intervention, so that the information generated by EUROSPIN can be used in the future to develop new diagnostic or therapeutic strategies for neuropsychiatric disorders, ranging from ADHD, via schizophrenia, to autism spectrum disorders, to and mental retardation.

Project Context and Objectives

Signalling at nerve cell synapses - a key determinant of all aspects of brain function - depends on hundreds of synaptic proteins and their interactions, and a wide range of neurological and psychiatric disorders are synapse disorders or 'synaptopathies', whose onset and progression are due to mutations of synaptic proteins and subsequent synaptic dysfunctions. The EUROSPIN consortium was initiated with the aim of pursuing a multilevel systems biology approach to determine mechanistic relationships between mutations of synaptic proteins and neurological and psychiatric diseases, and to develop new diagnostic tools and therapies. In this regard, the EUROSPIN concept was based on three complexity levels of analysis, at which the key challenges of the synaptopathy model were tackled (Figure 1):

1. Synaptic Systems Biology - Analyses of the synaptic protein network by using a combination of single-gene/protein and multi-gene/protein approaches. Here, EUROSPIN groups in WP1-4 analysed the synaptic proteome and protein interactome, modeled synaptic protein sub-networks of disease relevance, developed small molecules to interfere with synapse protein function, and generated new mouse models that express tagged target proteins of disease-relevant protein complexes. **2. Disease-Relevant Mouse Lines or Gene Products** - Analyses of disease relevant mouse mutants and gene products at several levels of complexity. Here, work in WP5-9 focused on 'medium-to-high throughput' behavioural studies, studies on synaptic function and plasticity using MEA technology, RNAi studies on disease gene products, and imaging and electrophysiological studies on the trafficking and function of disease gene products. **3. Disease Relevant Dysfunctions at the Level of Neuronal Circuits** - Analyses of disease relevant dysfunctions at the level of neuronal

circuits in vitro and in the intact brain. In this context, studies in WP10 involved electrophysiological recordings in vitro and in vivo, complemented by behavioral analyses, imaging, and modelling of normal network function and its perturbation.

SYSTEMS BIOLOGY OF SYNAPTIC PROTEIN NETWORKS (WP1-4)

Work in WP1 was concerned with the generation of new mouse lines expressing tagged proteins for affinity purification or imaging of disease gene products, with the purification of synaptic protein complexes involving disease gene products, and with the production of new antibodies. Regarding new mouse models suitable for TAP tag based affinity purification or for imaging (WP1), we had the main objective to generate six new mouse lines expressing tagged glutamate receptors and scaffold proteins of excitatory postsynapses, to develop protocols for the purification of protein complexes from these mice, and to characterize these complexes biochemically. Further, the TAP tag mutations were to be bred into defined disease model mouse lines with the aim of analyzing disease-related changes in synaptic protein complexes. An additional objective was to generate mouse lines expressing fluorescently tagged synaptic proteins for use in life imaging. As to tag-independent protein purification approaches, we planned to standardise and characterise a preparation of synaptic vesicle docking complexes. This preparation was to be analyzed using proteomics, and to be used for the characterization of novel proteins present in these fractions. The plan was further to complement these studies by experiments aiming at the isolation of new protein complexes from presynaptic vesicle docking complex preparations. The protein chemical work outlined above as well as all other projects were to be supported by a systematic antibody production pipeline (WP1). Synaptic Systems received input in the form of antibody requests from the other scientific partners and developed immunization strategies, produced antigens, and tested the resulting antibodies in close cooperation with the scientific partners.

Regarding synapse interactomics (WP2), our main objective was to conduct automated yeast-2-hybrid screens to generate a protein-protein interaction network of synapses. Synapse informatics (WP3) was to be a key focus of EUROSPIN. Objectives were to integrate the new yeast-2-hybrid data with interaction data from the literature and public databases and with functional data from consortium members in order to generate a high-confidence protein-protein interaction database for the consortium as a basis for network modelling. LUMIER based validation assays were to follow in order to validate the protein-protein interaction data. Along with a protein-protein interaction database, databases for electrophysiological and behavioural data were to be generated for the consortium - and for ultimate release to the public. In addition, we planned to develop statistical approaches to test if grouping genes according to synaptic function, pathway, or interactome results in a combined effect that is more strongly associated with the trait than the combined effect of all other genes expressed in the brain, to initiate grouping of synaptic genes according to their cellular function, and to start using functional gene groups in genome wide association studies.

Regarding chemical biology studies on synapse proteins (WP4), we planned to identify the first lead compounds that interfere with defined protein-protein interactions and with defined and disease-relevant neuronal characteristics. Further, the currently available natural product based small molecule libraries were to be expanded by the development of novel chemical synthesis strategies.

DISEASE-RELEVANT MOUSE LINES OR GENE PRODUCTS (WP5-9)

A main focus of EUROSPIN was to be on the mouse genetics pipeline of synaptopathy models (WP5). Here, our main objectives were to produce novel mutant mice for EUROSPIN based on current knowledge of synaptopathies, and to search for and obtain mouse models with mutations in synaptic genes that cause behavioural phenotypic changes.

Regarding the functional analysis of synaptopathy models (WP6), we planned to conduct systematic behavioural profiling in test batteries, focusing initially on 20 selected EUROSPIN mutants. In parallel, we planned to analyze synaptic properties of mice carrying mutations in synaptic proteins, including glutamate receptor subunits and their interacting proteins. Corresponding phenotpye analysis data were to be annotated and deposited in a database.

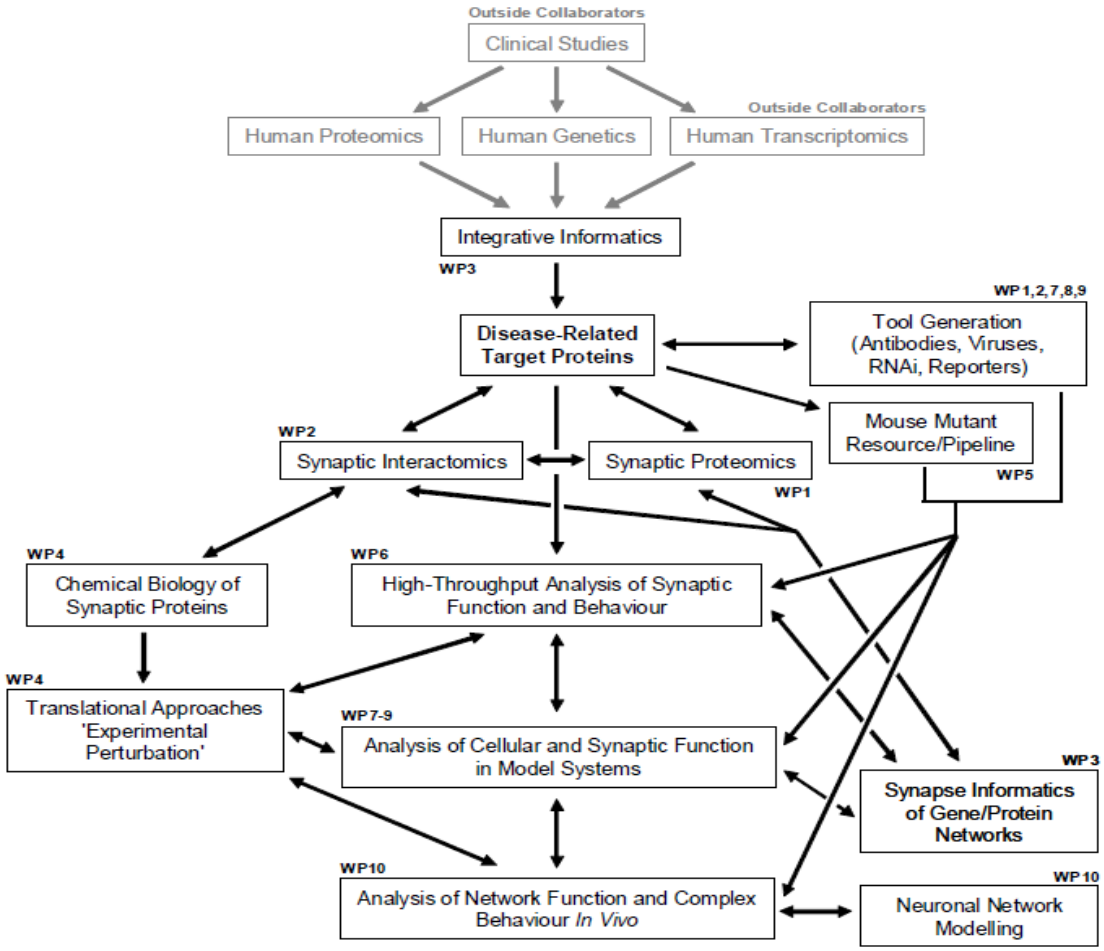

Figure 1. The EUROSPIN Concept. *The consortium was set up to allow for the synergistic analysis of normal brain function and synaptopathic dysfunctions at multiple levels of analytical complexity. Nine corresponding work packages were defined.*

Complementing mouse genetic approaches, we planned parallel functional analyses of synaptopathy genes using lentivirus delivery of shRNAs (WP7). The objectives were to produce and test a series of shRNAs, to clone these into viral vectors, and to test these on cell lines and neuron cultures, and ultimately in intact animals, which were to be analyzed with regard to behaviour. The electrophysiological consequences of shRNA treatment were to be examined in neuronal networks in vitro. Regarding electrophysiological in vitro studies, we decided to focus on slice preparations instead of cultured neurons.

Regarding the analysis of synaptopathy models using model synapses (WP8), we initially wanted to produce new antibodies directed against the extracellular domains of GluR1 and GluR2, which can be used for live labelling of postsynapses and as a readout of synaptic strength. In addition, we planned to study synaptic weight distributions in different parts of neurons, to examine the role of N-cadherin in synapse maturation, and to study short-term plasticity and homeostatic synaptic plasticity in cultured neurons. A further objective was to establish hippocampal cultures from synaptopathy model mice on cover slips and multi-electrode arrays, and to use these preparations for the analysis of SNAP25 mutant mice, with a focus on synaptic vesicle and Ca2+ dynamics. In addition, we planned to study transmitter release characteristics at the calyx of Held of control and disease model mice, and to examine the ultrastructural correlates of functional changes. Here we extended our initial focus to include mutants with altered synaptic vesicle priming characteristics.

Regarding the cell biology of synaptopathy gene products (WP9), we planned to study the stability and trafficking of SNAP25, to analyze the effects of loss or mutation of synaptopathy genes on steady-state levels of synaptic proteins, synaptic tenacity, and protein trafficking. Here, the focus was to be put on Neuroligin 1 first in order to establish a proof-of-principle preparation. Additional studies were to focus on other synaptopathy gene products. These experiments were

to be combined with FRET assays, where FRET pairs within synapses were to be defined by the EUROSPIN consortium. Here the focus was initially on establishing the microscopy setup and on the refinement of FRET measurements.

DISEASE-RELEVANT DYSFUNCTIONS AT THE LEVEL OF NEURONAL CIRCUITS (WP10)

The final set of experiments planned for EUROSPIN concerned the analysis of synaptopathy genes in intact neuronal networks (WP10). Here, the objectives were to perform a first behavioral characterization of selected synaptopathy mice using amygdala-dependent paradigms, and to begin with the characterization of deficits in inhibitory synaptic transmission in these synaptopathy mice using whole-cell recordings in acute amygdala brain slices. Further, we planned parallel behavioral analyses of cerebellum-dependent paradigms. Once available, we planned to use the electrophysiological data from amygdala and cerebellum to develop neuroinformatics approaches for probing disruptions in network function in synaptopathy models.

In preparation of more detailed analyses of social behaviour, we were to develop novel protocols for social behaviour assays and to use these for the analysis of mutant mice, with a focus on autism models.

Main Results

By initially focusing on 20 disease relevant genes and by selecting three key levels of analytical complexity, EUROSPIN defined a very substantial 'parameter space' of projects, which was further extended by adding several additional disease genes and mutants to the EUROSPIN portfolio in the course of its activities. The EUROSPIN consortium tackled this challenge systematically and made excellent progress on all fronts.

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BIBLIOMETRY

The EUROSPIN consortium has published a total of 99 papers so far. Importantly, 30 of these were published in journals with impact factors above 10, and 66 in total in journals with impact factors above 6. This represents a quantitatively and qualitatively excellent publication track record of the consortium.

SYSTEMS BIOLOGY - PPIS

EUROSPIN partners systematically characterized PPIs between a defined set of major synaptic proteins. We selected and annotated a set of 1,500 synapse-associated proteins, of which 940 were then selected for detailed analyses based on their known presence in human chemical synapses. For high-throughput pairwise interaction tests in YTH screens (e.g. Suter et al., 2013), the genes were cloned into classic or split-Ub YTH vectors, validated, and then tested. For the analysis of corresponding data, we developed a confidence scoring procedure and performed a bioinformatically inferred annotation regarding localization and function. Detected interactions were then validated using the LUMIER assay system. 11,693 PPIs were detected by YTH and then retested with the LUMIER assay. The resulting PPI network was combined with data on known interactions from the HIPPIE database to yield a network of over 16,722 PPIs, one of the largest and best validated worldwide (Figure 2).

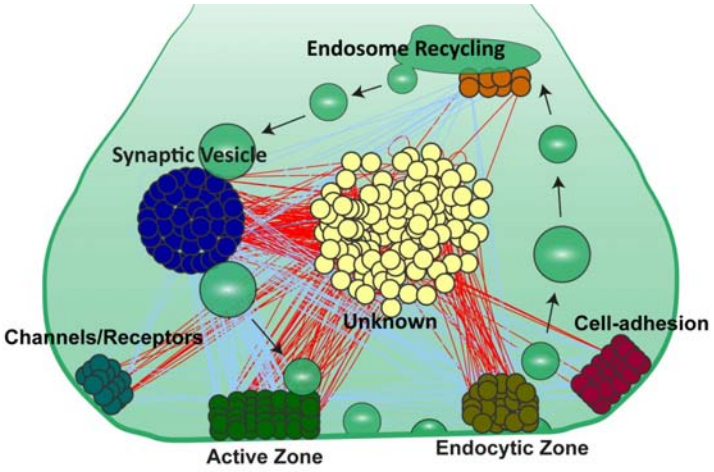

Figure 2. Presynaptic PPIs. *Example of PPIs (blue, 318 PPIs; red, 498 PPIs) identified between 274 presynaptic proteins, sorted by function and subsynaptic localization.*

In addition, we developed networks and databases for integrating data on synapse biology. The EUROSPIN database (eurospindb.genes2cognition.org) was created by extending the G2Cdb database model previously developed by consortium members. The system in its entirety is available under open source licenses to the scientific community. EUROSPIN partners defined a data standardisation between the partner labs and submitted data in such standardised formats, using approved gene nomenclature. This allows integration and presentation of the EUROSPIN consortium data on proteomics, interactomics, electrophysiological, and behavioural phenotyping of mouse disease models at the gene level.

Modelling work (Sorokina et al., 2011; Armstrong and Sorokina, 2012; Cohen et al., 2013; Sorokina et al., 2013) interlinked closely with the database work (Figure 3). Here, we mined protein-protein interaction information from a variety of publicly available databases, linked this information to the data of the EUROSPIN interactomics groups (WP2), and added to the database a wide variety of annotations from within the consortium (e.g. protein turnover) or from external databases (e.g. disease association). Throughout EUROSPIN we have used Cytoscape as a tool to visualise and manipulate simple graphical models. Cyctoscape format models have been available to those in the consortium interested in network visualisation and analysis throughout the programme of work. To address the problem that many types of PPI models cannot be used to provide information on stoichiometry or binding properties within elements of the network, we developed the first Kappa based model of the post-synaptic density focused on EUROSPIN proteins, and in particular proteins where there are mouse models and phenotypic data could be expected. As regards tool development, we generated several new software packages that are open source and the directly available from public repositories in advance of publication.

The EUROSPIN database was placed online in mid 2011 and has been continuously available to the whole world since then. Throughout the project the EUROSPIN consortium data have been loaded in batches (approximately 30) continuously since 2011 and currently contains information on over 18,300 PPIs, 92 datasets on the behavioral phynotyping of mouse models, data on the characterization of 70 new antibodies, 22 datatsets on the electrophysiological

phenotyping of mouse models, and 7 software tools. The majority of these data are still escrowed, pending publication, but it is anticipated that the majority of the data will be made public during 2014.

We now cover a total of 7623 genes from over 100 overlapping studies in our database. The overlap between related studies is high, with a very high degree of overlap between pre-synaptic and post-synaptic studies, and there is a very high degree of enrichment within the datasets of genes associated with neuropsychiatric disorders in humans. We built and clustered models from these data, e.g. in the context of protein turnover and its relation to PPI networks. Significant models in preparation include an analysis of new presynaptic and postsynaptic pull-down data sets, and YTH interaction datasets and their impact on synapse models and the emerging disease networks. In terms of next generation models, our first Kappa models of synaptic biology were published in 2011-2013, with more advanced models and methods forthcoming.

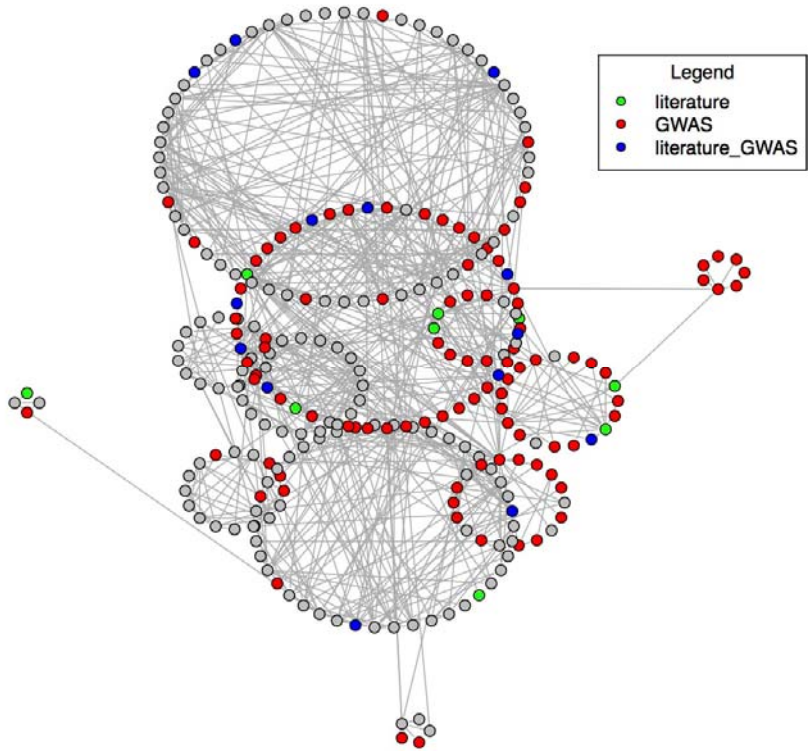

Figure 3. Model of Presynaptic PPIs - Disease Relevance. *Presynaptic proteins closely associated with vesicle cycling. The PPIs derive from a combination of YTH data from within the consortium. The proteins are clustered based on their interactions alone. Subsequently the network is overlaid with Parkinson's disease association. Green indicates well-known Parkinson's disease related genes from the literature. Red indicates genes with a significant (although normally small) enrichment in GWAS studies, and blue indicates an overlap between both. The network as a whole is significantly enriched for Parkinson's disease related proteins. More interestingly, the central cluster is massively enriched with GWAS hits filling in the gaps between previously known disease genes.*

The PPI and bioinformatics datasets and the tools developed by EUROSPIN will be of extreme value to the neuroscience community, e.g. for the identification of disease relevant PPIs or of 'hubs' in such networks and their use as targets for diagnosis and treatment strategies.

SMALL MOLECULES AND LEAD STRUCTURES FOR THE TREATMENT OF NEUROPSYCHATRIC DISEASES

A core part of the EUROSPIN strategy was to include medicinal chemistry and chemical biology approaches with the aim of interfering with defined PPis and of developing lead structures for future intervention in neuropsychiatric disorders. In this regard, EUROSPIN partners developed novel synthesis strategies for natural product inspired smart molecule libraries.

Core-structures or scaffolds of neurobiologically relevant natural products were used as targets for the generation of smart libraries. Chemical synthesis access, including asymmetric synthesis, was developed for different small focused

compound libraries (Figure 4). For instance, iridoid natural products inspired the synthesis of a library that may provide small bioactive molecules with neuroenhancing properties. About 4,000 molecules were tested in a primary screening to identify - in a fluorescence-based assay - molecules that induce increases in the neuronal membrane over 2-5 days of treatment. Hits obtained in these screens, followed by synthesis of further derivatives and a repetition of the screen led to a selection of 50 molecules, which were analyzed for their effects. Out of these molecules, the ones showing relevant dose-response characteristics were analyzed for further in-depth analysis to see the individual morphological features modified by the small molecules, e.g. neurite length or increase in the number of neurites per cell. In these studies, which included assays on cultured hippocampal neurons and neurons derived from, we identified several lead structures that had positive effects on neurite growth and synaptogenesis (Antonchick et al., 2013; Dakas et al., 2013).

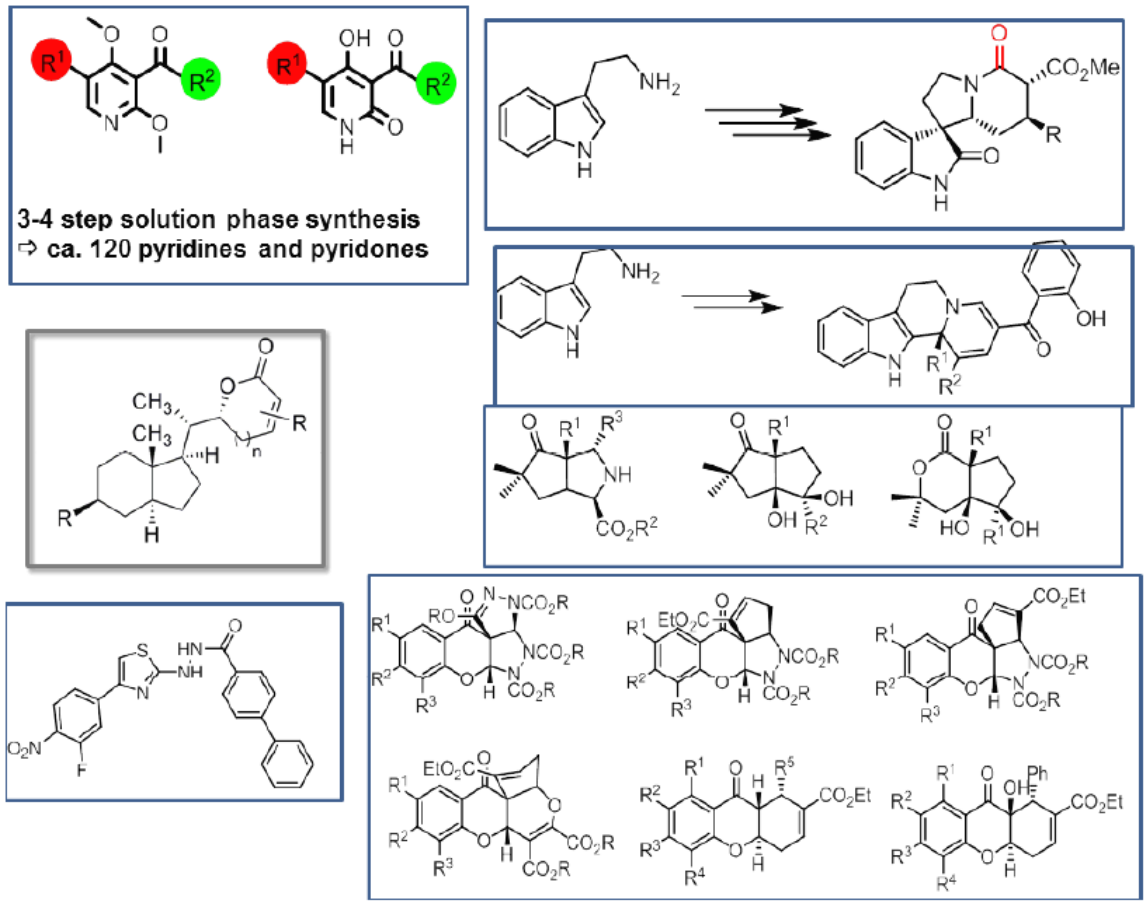

Figure 4. Representative Examples of Smart Libraries Synthesized. *See also Antonchick et al. (2013) and Dakas et al. (2013).*

In parallel to these studies, a novel screen was established to identify small molecule modifiers of PPIs between Aβ42 peptides in Aβ aggregates. Intra- and extracellular Aβ aggregates are a pathological hallmark of Alzheimer's disease (AD). Several lines of evidence indicate that Aβ aggregation is associated with neuronal dysfunction and toxicity in AD. Thus, stimulation of Aβ aggregate clearance with small molecules is a promising therapeutic strategy against AD. EUROSPIN partners established a high content screening assay that allows for the identification of small molecules that promote Aβ42 aggregate clearance. Utilizing this assay, we screened a library of ~1,000 compounds for their effects on Aβ42 aggregates and identified the small molecule 1H11 as a promising hit compound. 1H11 stimulates Aβ42 aggregate degradation and reduces aggregate toxicity in mammalian cells in a concentration dependent manner. Furthermore, 1H11 promotes the degradation of other amyloidogenic proteins such as Huntingtin, α-synuclein, IAPP, and Tau, indicating that it influences the abundance of disease-causing protein aggregates more generally. We subsequently screened a set of 1H11 derivatives to reveal structure activity relationships, and identified several related compounds that are active in various cell model systems. This part of the EUROSPIN activities indicates that lead compounds based on 1H11 could open up novel therapeutic avenues for the treatment of AD (Bieschke et al., 2011).

Our work on small molecules that affect PPIs in synapses had pioneering character. The first lead structures that affect neurite outgrowth or Aβ42 aggregation will serve as the basis for new strategies to promote neuronal regeneration (e.g. after injury) or to treat Alzheimer's disease.

MOUSE LINES - DISEASE MODELS - REAGENTS

A major focus of EUROSPIN work was on the generation of new mouse, with particular emphasis on lines expressing tagged proteins for affinity purification or imaging of disease gene products, and on new disease models. New TTAPT mouse lines produced by EUROSPIN partners include, for example, modified mice expressing FLAG- or His-tagged Grin1, PSD-95, Arc/Arg3.1, or TNIK, or FP-tagged SAP102 or PSD93. Further, several mouse lines expressing fluorescently tagged proteins were generated. These include PSD95-EGFP, PSD95-mEOS2, PSD95-GCaMP3, SAP102-mKOrange2, PSD93-mCBlue, Munc18-1-Venus, and Arc/Arg3.1-Venus. Several of these mouse lines have been bred into disease models to assess disease-related changes in synapse composition. Using transgenic mice expressing tagged proteins, we purified novel assemblies containing NMDA receptors and discovered a tripartite genetic mechanism confining receptors within a hierarchy of 840 kDa tetramers and families of 1.5 MDa supercomplexes with specific ion channels, signaling, and activity-regulated proteins. The principal protein constituents were identified by peptide mass fingerprinting as GluN2A/GluN2B, TAP-GluN1, and two scaffold proteins from the Dlg/MAGUK family, PSD93 (SAP102), and PSD95, respectively. Much of the corresponding work is still in progress, but it is clear already that the many new mouse lines of EUROSPIN, along with the dozens of lines brought into the consortium by various partners, represent a resource of major importance for the fields of cellular neuroscience and translational neuroscience alike.

A similarly important resource has been generated by the antibody production pipeline of EUROSPIN, which was driven by the company SynapticSystems. Here, EUROSPIN generated and valdiated 59 new polyclonal and 15 new monoclonal antibodies, with 31 antibody projects pending.

Complementing the work on TTAPT mouse lines, EUROSPIN partners developed highly effective methods for the separation of subcellular neuronal fractions. A new method to purifiy the presynaptic compartment led to deep insights into the protein composition of presynaptic active zones, which had been impossible before. Using iTRAQ to compare the glutamatergic and GABAergic active zones, it was revealed that only a few proteins showing preferences for one type of complex (Figure 5) (Boyken et al., 2013).

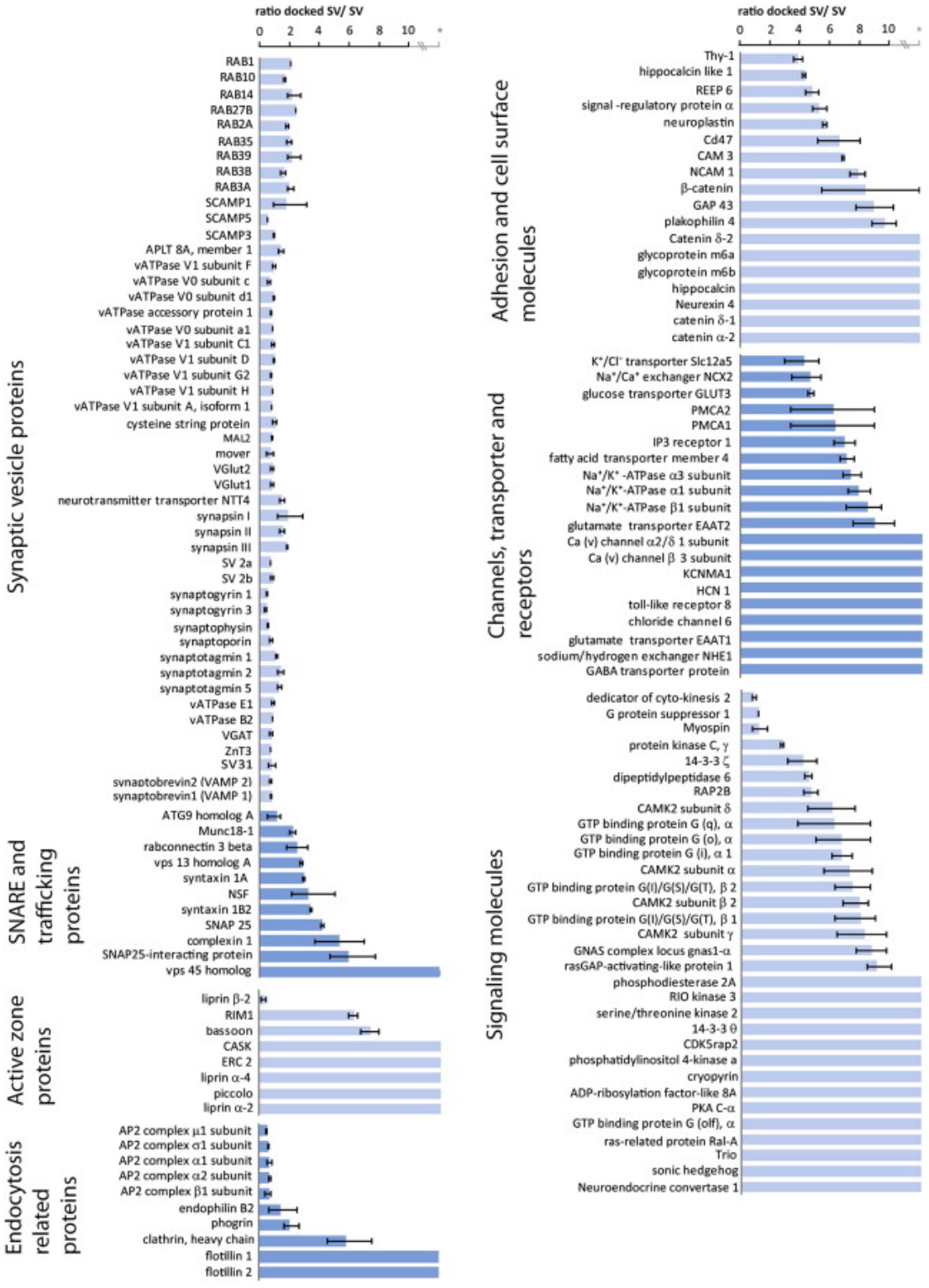

Figure 5. iTRAQ Quantification of Proteins Identified from Free and Docked Synaptic Vesicle Fractions by Mass Spectrometry. *Data show the average of three independent biological replicates. Only proteins identified in at least two of the independent experiments are listed. Error bars indicate the range of data points (lowest-highest). Proteins with an iTRAQ ratio of more than 2.5 are considered to be significantly enriched in the docked synaptic vesicle fraction. Data are from Boyken et al. (2013).*

The new mouse lines and antibodies produced by EUROSPIN represent tools of key importance to the fields of basic and translational neuroscience, e.g. for the assessment of biochemical and cell biological consequences of synaptopathies or for the analysis of defined synaptopathy gene products in health and disease.

CELL BIOLOGY, PHYSIOLOGY, AND BEHAVIORAL FUNCTIONS OF SYNAPTOPATHY GENE PRODUCTS

A key goal of EUROSPIN was to study network properties of neurons and behavioral profiles in synaptopathy models in mutant mice using highly standardized and quantitative tests. This approach was based on the notion that a comparison of phenotypic data between mutants allows one to identify genes with similar functions and thus genetically dissect pathways in the synapse proteome, and on the notion that the specific phenotypic findings are of interest and can be further examined by in-depth analysis. A wide range of behavioral tests were conducted that assess different psychological dimensions (exploration, novelty, motor function, multiple forms of learning). Behavioral screening of selected mouse mutants in a high throughput (HTP) and several low throughput behavioral assays were performed, screening of mouse behavior using well-validated batteries of stand-alone tests used for mouse mutant phenotyping. These tests comprised different behavioral domains, such as anxiety, cognition, motor and sensory function. In parallel to the behavioral studies, we assessed altered basal synaptic responses and two forms of synaptic plasticity (paired-pulse facilitation and long-term potentiation) using MEAs.

Electrophysiological analyses focused on 22 mutant mouse lines. Altered input-output relationships were seen in slices from 4 mutants (PSD-95 KO; GKAP1 KO; PSD-95-eGFP; TNIK KO). Changes in short-term plasticity were observed in 8 mutants (PSD-95 KO; PSD-93 KO; GKAP1 KO; TNIK KO; MAGUIN KO; eEF2k-KI; NSF KO and Tjp1 KO), while long-term potentiation was affected in 4 mutants (PSD-95 KO, GKAP1 KO, MUPP-1 KO and Maguin KO). In two mutants (PSD-95 KO and GKAP1 KO) all three measured parameters were altered.

As regards mouse behavior, the effects of synaptic mutations were profiled by observing behavioral parameters of relevance to cognition, motivation, fear and anxiety, and motor function. At the outset of the EUROSPIN project, the company Synaptologics profiled 20 selected EUROSPIN mutants. Significant investment was placed on developing and validating new protocols that can be of particular interest for the behavioral characterization of the mutant lines generated by the consortium. Ultimately, EUROSPIN partners profiled the behavior of more than 90 lines covering almost 70 genes, greatly exceeding the original objective of the task.

A key finding of our activities was that proteins of high abundance in the PSD, such as PSD-95, alpha-CaMKII, and SynGAP, have some of the highest effect sizes. We therefore looked more carefully at the relationship between protein abundance and found that the overall behavioral defect is correlated with protein abundance in the PSD. This result indicates that protein stoichiometry in synapses has a fundamental impact on the strength of a behavioral effect (Figure 6). Similar approaches will be applied to slice electrophysiology data for final publication (e.g. Bayés et al., 2012; Emes and Grant, 2011 and 2012; Grant, 2012; Kirov et al., 2012; McMahon et al., 2012; Nithianantharajah et al., 2013; Ryan et al., 2013).

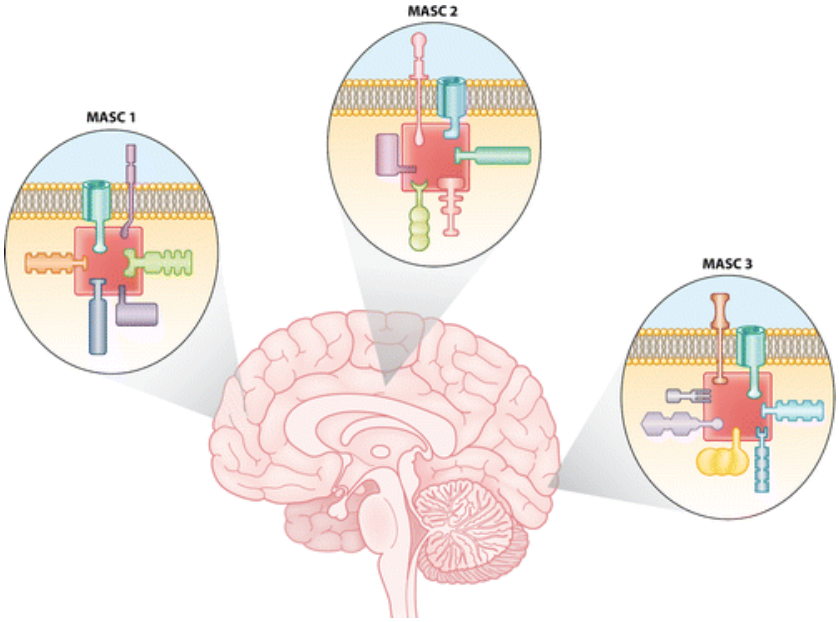

Figure 6. Synapse Diversity in the Mammalian Brain is Generated by Combinations of Protosynapse and MASC Proteins. *Three varieties of MASC complexes (labeled MASC1, 2, 3) comprising central scaffold proteins bound to receptors and adhesion protein, enzymes, and cytoskeleton are shown. The variation in shapes of the components between the three complexes indicates that they are paralogs in expanded vertebrate gene families arising from duplication of the ancestral genes. The paralogs arising in early vertebrate evolution played a major role in diversifying neuroanatomical function. From Emes and Grant (2012).*

All data from the behavioral and electrophysiological profiling are available in the EUROSPIN database (eurospindb.genes2cognition.org) and will go public soon - pending publication of the key data. Phenotyping data were integrated into synaptic models to predict synaptic physiology outcomes based on known molecular interactions and processes. A comprehensive model of the PPI interactome using the EUROSPIN database was assembled that overlays proteomics and PPI data with behavioral, electrophysiological, and human disease data. To date, the model contains unpublished data and release is on a negotiated basis at present. Overall, the datasets created by EUROSPIN and the corresponding database information will be extremely useful tools for future studies of the EUROSPIN partners and the worldwide neuroscience community alike.

In another systematic analysis of disease-relevant gene products, EUROSPIN partners performed knock-down and overexpression studies using over 40 viral vectors to assess the biochemical, electrophysiological, and behavioral effects of manipulating the expression of synapthopathy genes in specific brain areas. We found that dopaminergic and glutamatergic transmission converge on NMDA receptors (i.e. at the Y1472 site of the NR2B subunit) and that this convergence is essential for ERK1/2 activation in the mature brain and for processing new sensory information in the cortex. Further, we discovered that translation regulation factors can serve as novel targets for cognitive enhancement in the normal population and as therapy for neurodegenerative diseases. Different brain areas can be affected differentially by manipulating the function of translation regulation factors, which applies with regard to both, translation initiation (e.g. PERK) and elongation (eEF2). The differential effect on different cell types in the brain is yet to be determined. Finally, we found that gene expression regulation in the brain is affected by general physiological conditions, such as hydration levels, and by the information encoded by the brain. The complex interaction between the two regulatory principles may explain normal transcription-dependent memory consolidation as well as disease states such as epilepsy (e.g. Chinnakkaruppan et al., 2014; Edry et al., 2011; Gal-Ben-Ari and Rosenblum, 2011; Inberg et al., 2013; Taha et al., 2013).

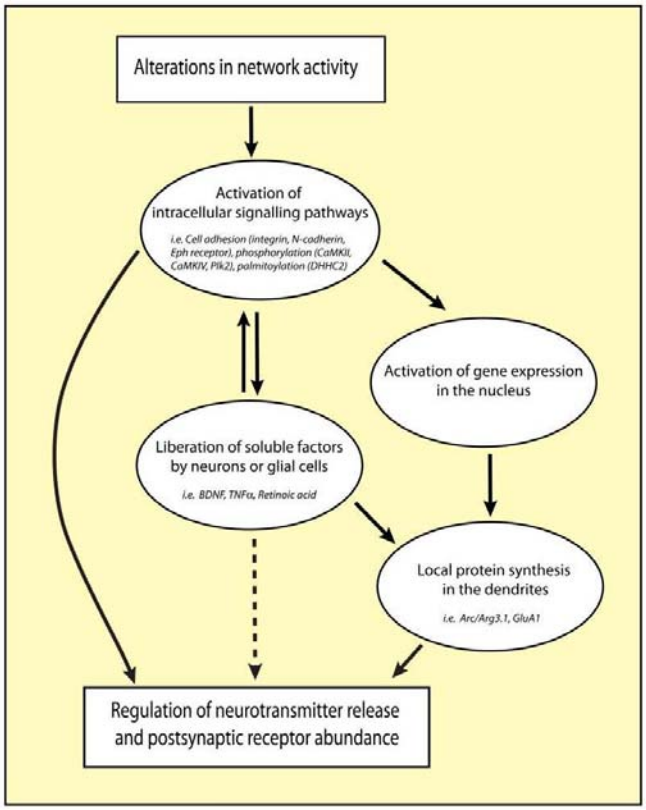

Figure 7. Molecular Mechanisms underlying homeostatic synaptic plasticity. *Changes in network activity are detected by an unknown mechanism by neurons or glial cells and activate intracellular mechanisms to modify presynaptic release and/or the abundance of functional postsynaptic receptors. This can involve activation of gene expression in neurons and triggering of local dendritic protein synthesis (e.g. Arc/Arg3.1, AMPA receptors) as well as the release of soluble factors such as BDNF or TNFα from neurons and glial cells that then engage additional signaling pathways.*

Detailed analyses of disease-relevant genes and their products focused on a selected group of genes. N-cadherin, for example, links directly or indirectly to several key postsynaptic synaptopathy gene products, such as Nlgns and GluA2. We found that postsynaptic N-cadherin can control presynaptic release retrogradely by affecting endocytosis as well as basal release probability. This ability of postsynaptic N-cadherin to modulate presynaptic function is dependent upon GluA2 AMPARs, and observed under basal conditions but not during homeostatic compensation of presynaptic release probability following activity manipulation (e.g. Pozo and Goda, 2010; Vituerira et al., 2011; Vituerira and Goda, 2013; Pozo et al., 2012) (Figure 7). A second example to be discussed here is Munc13-1, a presynaptic priming protein that represents a potential target of modulation in synaptopathies. Calyx of Held synapses of knock-in mice carrying a Munc13-1 mutant deficient in calmodulin binding were examined for defects in short-term plasticity. Compared to control synapses, mutant synapses showed altered short-term depression, delayed recovery from depression induced by high frequency stimulation, and a decrease in the rate of synaptic vesicle replenishment. Therefore, Munc13-1 is a target of Ca2+-calmodulin signaling and consequently a key determinant of Ca2+-dependent short-term plasticity (Lipstein et al., 2013). Finally, a key aspect of EUROSPIN work was focused on proteins linked to neuropsychiatric disorders as regards their capacity to preserve their properties over behavioral time scales (Figure 8). We found that this capacity is inherently limited and affected by disease-related mutations, indicating that the dynamic nature of synaptic molecular assemblies and the limited life span of their constituents may play a key role in synaptopathies (e.g Cohen et al., 2013; Fisher-Lavie et al., 2011; Fisher-Lavie and Ziv, 2013; Zeidan and Ziv, 2012; Ziv and Fisher-Lavie, 2014).

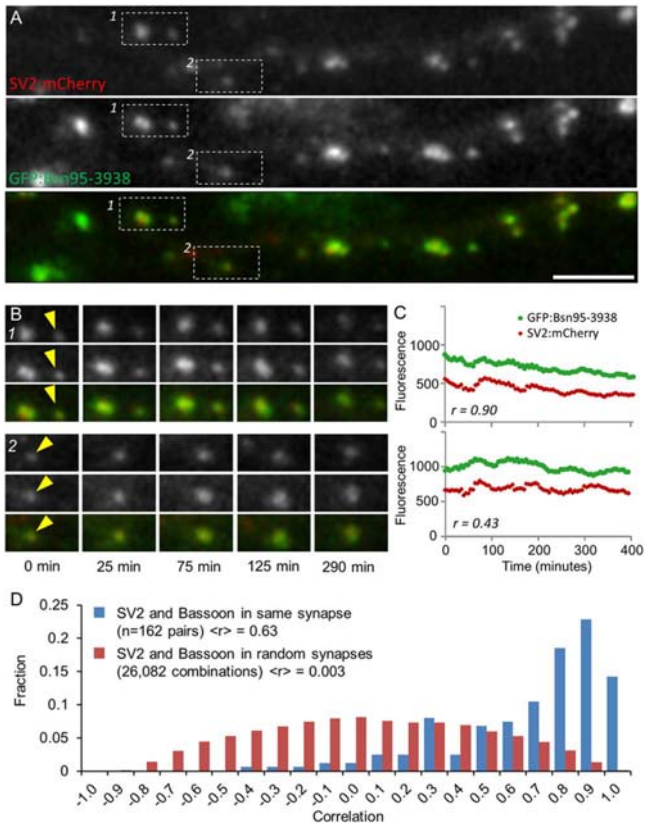

Figure 8. Changes in the Synaptic Contents of the Active Zone Molecule Bassoon and Synaptic Vesicles (SVs) are Temporally Correlated. *(A) An axon of a neuron expressing a variant of Bassoon tagged with a green fluorescent protein and the SV molecule SV2A tagged with a red fluorescent protein (mCherry). Bar = 10 µm. (B) Changes in Bassoon and SV2A contents measured over several hours in two presynaptic boutons. (C) Changes in Bassoon and SV2A over time. Note that in one case, the correlation (Pearson correlation) between the temporal changes in the fluorescence of the two molecules is very high (0.90), whereas in the other, it is lower (0.43). (D) Distribution of temporal correlation values for all boutons followed in these experiments compared to the correlations measured for all possible combinations in which the data for Bassoon and SV2A were taken from different boutons. Data shown here were corrected for ongoing photobleaching. Mean correlations are shown as r. Data from Ziv and Fisher-Lavie (2014).*

On aggregate, EUROSPIN work on defined disease gene products, of which only selected ones were discussed here, has yielded a wealth of insights into the mechanisms by which the mutation or loss of such proteins might cause disease phenotypes. We trust that this information can be used in the future to develop new diagnostic or therapeutic strategies.

SYNAPTOPATHIES AND NEURONAL CIRCUITS

A major aim of EUROSPIN work was to study disease models at the level of intact neuronal circuits. Starting from detailed behavioral analyses, we have characterised the role of defined neuronal circuits in the wild-type cerebellum and amygdala, e.g. in the context of fear learning and extinction or cerebellum-dependent behaviors.

As regards the cerebellum, we used voltage clamp analysis in cerebellar granule cells to quantify the contribution of tonic inhibition and phasic inhibition (mediated by Golgi cell spiking activity) in controlling sensory transmission in the cerebellar granule cell layer. This has been complemented by modelling of granule cells based on our experimental measurements, which can later be used to interpret data from synaptopathy mutants, both for single-cell recordings, and for analysis of network activity. Further, we used quadruple simultaneous patch-clamp recordings to investigate connectivity patterns in the inhibitory circuitry of the molecular layer of the cerebellar cortex. We identified specific, non-random connectivity patterns in both GABAergic chemical and electrical interneuron networks in the molecular layer. Both networks contain clustered motifs and show specific overlap between them. Chemical connections exhibit a preference for transitive patterns, such as feed-forward triplet motifs. This structured connectivity is supported by a characteristic spatial organization, where transitivity of chemical connectivity is directed vertically in the sagittal plane, and electrical synapses appear strictly confined to the sagittal plane. The specific, highly structured connectivity rules

indicate that these motifs are essential for the function of the cerebellar network. Moreover, we succeeded in making targeted in vivo patch-clamp recordings from the two inhibitory neuron populations in the granule cell layer, the Golgi cells and Lugaro cells. In both cell types, we made voltage clamp recordings, which enabled us to separate between excitatory and inhibitory synaptic inputs. This puts us in the position to dissect changes in inhibitory synaptic inputs in synaptopathy mutants. We also succeeded in making simultaneous dual patch-clamp recordings from Golgi cells in vivo, and demonstrated millisecond synchrony among neighbouring Golgi cells. This synchrony is crucial for orchestrating the timing of synaptic inhibition in the input layer of the cerebellar cortex, and results from an interaction between electrical coupling of the Golgi cell network, and the synaptic input driven by sensory-motor integration. Finally, we made substantial progress in making whole-cell recordings from cerebellar granule cells in awake, head fixed mice locomoting on a styrofoam ball. These recordings enable us to link synaptic events, and spike output, with movement. Our experiments show that locomotion can be directly read out from mossy fiber synaptic input and spike output in single granule cells, which is enhanced by glutamate spillover currents recruited during movement. The step cycle can be predicted from input EPSCs and output spikes of a single granule cell, indicating that a robust gait code is present already at the cerebellar input layer and transmitted via the granule cell pathway to downstream Purkinje cells (e.g. Rieubland et al., 2014) (Figure 9).

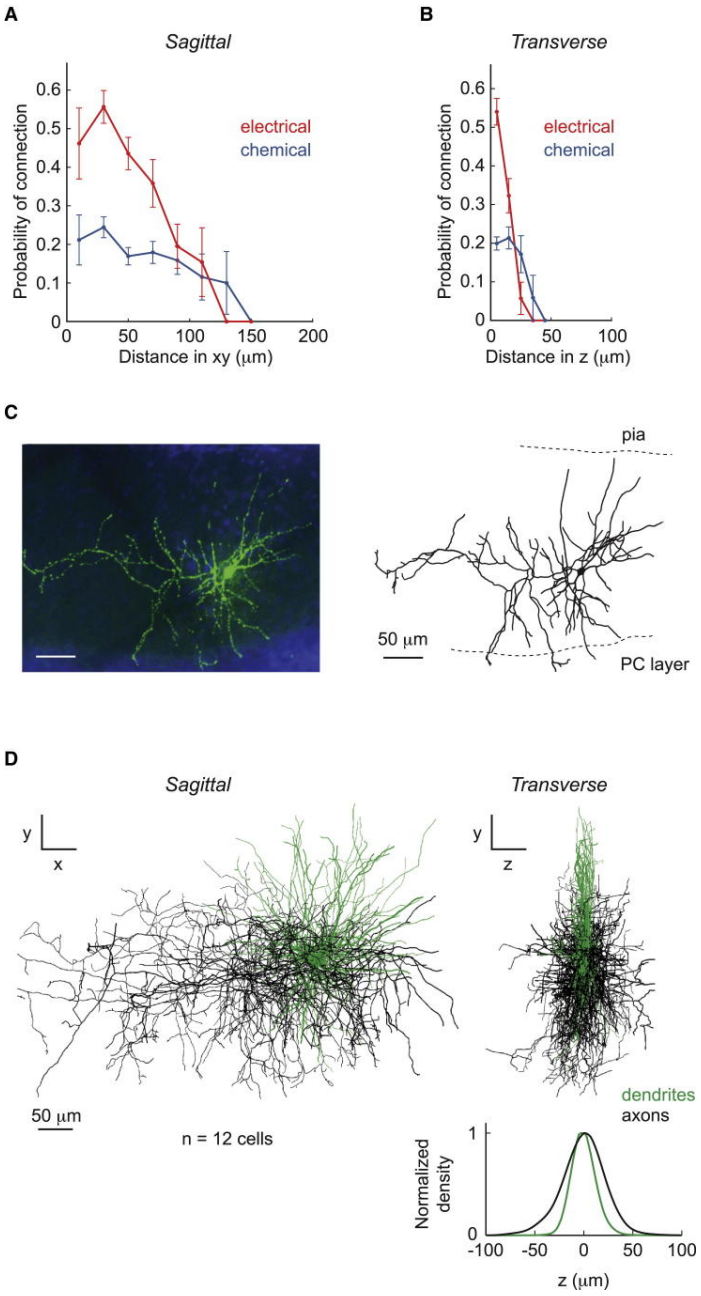

Figure 9. Distance Dependence of Electrical and Chemical Connection Probability. *(A) Probability of electrical and chemical connections versus intersomatic distance in xy (sagittal plane) between recorded pairs. (B) Probability of electrical and chemical connections versus intersomatic distance in z (transverse axis) between recorded pairs. Error bars indicate SD based on bootstrap analysis. (C) MLI filled with biocytin and imaged using confocal microscopy after streptavidin-conjugated Alexa 488 histochemistry (left; blue, DAPI), and its reconstructed morphology (right). (D) Superposition of 12 reconstructed MLI morphologies in xy view (left) and yz view (right). Bottom right, normalized density profile along the z axis. Dendrites (green) are more strongly confined to the sagittal plane than axons (black). Data from Riebland et al. (2014).*

The main goal of our work in the amygdala was to investigate the role of defined inhibitory circuits in classical fear conditioning in wild-type mice. In the basolateral amygdala, interneurons account for about 20% of all neurons. They comprise several subtypes, which differ in their morphology, electrophysiological properties, expression of genetic markers, and likely in their physiological role during behavior. The most abundant type are fast-spiking, parvalbumin expressing basket cells, which form strong, perisomatic synapses with their target cells to control their firing. In contrast, somatostatin expressing interneurons mainly target distal dendrites and thereby control the processing of inputs, dendritic processes, and burst firing. These differences in the targeted subcellular compartments indicate also functional differences in plasticity and learning processes. Interestingly, these interneuron types have been recently shown to interact in the hippocampus, thereby fine-tuning pyramidal cell output and network function. To dissect the differential roles and interactions of these interneuron subclasses in the amygdala network, we applied an approach combining optogenetics and electrophysiological recordings during fear learning. This allowed us to manipulate the activity of defined interneurons, and to determine the physiological activity of unambiguously identified interneurons during behavior. Our results show a differential, opposite involvement of parvalbumin expressing and somatostatin expressing interneurons during fear acquisition. Furthermore, these different interneurons interact to form a microcircuit that can open disinhibitory gates both during fear conditioning, allowing for plasticity and learning in the amygdala network during fear acquisition (e.g. Ciocchi et al., 2010; Johansen et al., 2012; Senn et al., 2014).

Our studies on the basic circuitry principles in cerebellum and amygdala formed the essential basis for studies on disease models (see below).

GENES - PROTEINS - SYNAPSES - CELLS - CIRUITS - MICE - BEHAVIOR

The ultimate aim of EUROSPIN was to characterize disease models and disease-related proteins at multiple levels of analytical complexity (Figure 1). We report here a selection of three important cases of disease-relevant synaptic proteins where this goal was achieved in a particularly nice way, SNAP25, Munc18-1, and Neuroligin-2, which play roles in ADHD, mental retardation, and schizophrenia/ASD.

SNAP25 Function - A Shared Biological Pathway in Different Psychiatric Diseases

SNAP25 is a component of the SNARE complex, which is central to synaptic vesicle exocytosis. EUROSPIN partners demonstrated previously that SNAP25 also regulates intracellular calcium dynamics by negatively modulating neuronal voltage-gated calcium channels. The SNAP25 gene has been associated with different psychiatric diseases, including ADHD and schizophrenia. Consistently, SNAP-25 levels are lower in the hippocampus and in the frontal lobe of patients with schizophrenia. Also, DNA variants of the SNAP25 gene that associate with ADHD are associated with reduced expression level of the transcript in prefrontal cortex. The mechanisms by which reduced SNAP25 expression causes psychiatric diseases are still undefined, although alterations in neurotransmitter release have been implicated as potential causative processes.

In the EUROSPIN project, we used heterozygous SNAP25 KO mice to investigate to which extent selective reduction of SNAP25 levels affects mouse behavior. As interactions of genotype with the specific laboratory conditions may affect behavioral results, the study was performed through a cooperative study in which behavioral tests were replicated in at least 2 of 3 distinct EUROSPIN laboratories. We found that reductions of SNAP25 levels were associated with a moderate hyperactivity, which disappeared in the adult animals, and with impaired associative learning and memory. Electroencephalographic recordings revealed the occurrence of frequent spikes, indicating a diffuse network hyperexcitability. Consistently, heterozygous SNAP25 KO mice displayed higher susceptibility to kainate-induced seizures, paralleled by degeneration of hilar neurons. Notably, we found that not only the EEG profile but also the cognitive defects were improved by antiepileptic drugs, and in particular valproic acid (Corradini et al., 2012).

Given that reduced SNAP25 levels result in traits typical of psychiatric defects (comorbidity with seizures, hyperactivity, impaired learning), we studied the underlying cellular mechanisms. We found, unexpectedly, that reduced SNAP25 levels in neurons does not impair glutamatergic synaptic transmission, but instead enhances it. This effect was dependent on presynaptic voltage-gated calcium channel activity and was not accompanied by changes in spontaneous quantal events or in the pool of readily releasable synaptic vesicles (Antonucci et al., 2013). Notably, synapses of 13-14 DIV neurons with reduced SNAP25 expression showed paired-pulse depression as opposed to paired-pulse facilitation occurring in their wild-type counterparts (Antonucci et al., 2013). This phenomenon, which is commonly linked to defects in information processing, might contribute to cognitive impairments in intellectual disability syndromes. Based on these results, a dual role of SNAP25 not only as a carrier but also as a guardian of synaptic transmission can therefore be envisaged (Figure 10).

Notably, we found that the phenotype of increased evoked glutamatergic transmission and switch in paired pulse ratio disappeared with synapse maturation, most likely due to the constant increase in SNAP25 expression during neuronal maturation, which might partly counteract the defects caused by heterozygosity. The transience of the functional phenotype in vitro is paralleled by the disappearance of the hyperactive phenotype, which occurs in young SNAP25 heterozygous mice and disappears in adult mice.

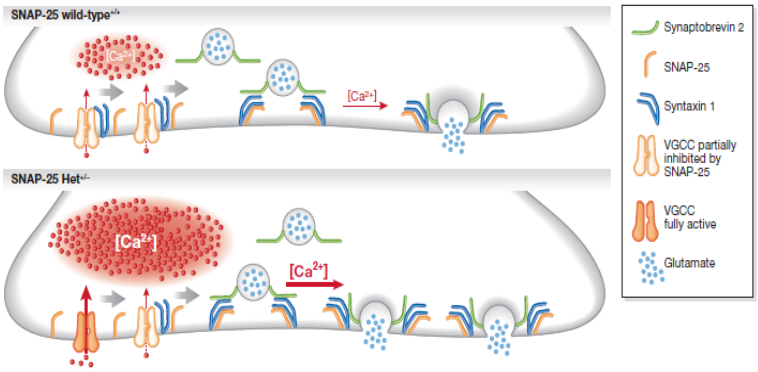

Figure 10. SNAP25 as Carrier and Guardian of Synaptic Transmission. *In wild-type neurons, SNARE-mediated, calcium-triggered synaptic vesicle fusion is negatively regulated by complex formation between SNAP25 and VGCCs. Reduced SNAP25 expression in heterozygotes partly releases VGCCs from SNAP25-mediated clamping, resulting in elevated calcium influx through VGCCs and increased glutamate release through SNARE-mediated calcium-triggered synaptic vesicle fusion. Based on Antonucci et al. (2013).*

In parallel with the further maturation of the synapse in vitro, a defect in postsynaptic function was noticed in SNAP25 heterozygous cultures (Antonucci et al., 2013). Different lines of evidence have indicated an unexpected postsynaptic role of SNAP25. For example, the protein was shown to control NMDA receptor trafficking, while acute SNAP25 downregulation results in LTP impairment, indicating that besides a presynaptic effect, chronic reduction of SNAP25 levels may impair the structure and/or function of the postsynaptic compartment, which would provide an interesting explanation for the involvement of SNAP25 perturbations in psychiatric diseases, such as schizophrenia, which are known to be characterized by defects at the postsynaptic compartment. The postsynaptic role of SNAP25 was further confirmed by our studies, which showed that acute reduction of SNAP25 expression in primary hippocampal cultures leads to an immature phenotype of dendritic spines. Conversely, overexpression of the protein resulted in an increase in the density of mature, PSD-95-positive spines (Tomasoni et al., 2013). In a more recent study, we found that acute down-regulation of SNAP25 in CA1 hippocampal region by lentiviral expression also reduced spine density and caused an immature spine morphology in vivo. Thus, acute reduction of SNAP25 levels acutely affects spine density and synapse function. As regards chronic reductions of SNAP25 expression, as they likely occur in psychiatric diseases, these also caused reduced densities of dendritic spines and defective PSD-95 dynamics.

Interestingly, the cleavage of SNAP-25 by BoNT/E, which prevents the protein from engaging in SNARE complexes, does not affect spine density or PSD size, which excludes the possibility that SNAP25 controls PSD-95 recruitment through its SNARE function and rather indicates a protein scaffolding role of SNAP25 in postsynaptic spines. Given that we previously found that the acute regulation of spine morphogenesis by SNAP25 depends on SNAP25 binding to the plasmamembrane and to the adaptor protein p140Cap, a key regulator of the actin cytoskeleton and spine formation (Tomasoni et al., 2013), we tested the possibility that SNAP25 acts in a protein network comprising p140Cap and PSD95. To test this possibility, we performed Proximity Ligation Assays in rat cultured neurons and evaluated complex

formation in situ, and subsequently used LUMIER assays to test different PPIs. The results indicated that SNAP25 and PSD95 are localized in close proximity but do not interact directly. Conversely, we found that p140Cap specifically interacts with PSD95. Thus, SNAP25 is part of a molecular complex including PSD95 and p140Cap, with p140Cap acting as a bivalent interaction partner.

Altogether, our data on SNAP25 show that neurons developing in the chronic presence of low levels of SNAP25 are affected in their presynaptic function at early developmental stages, while subsequently even the postsynaptic compartment becomes defective, with neurons showing reduced spine density, altered glutamatergic transmission, and perturbed plasticity. It is possible that reduced levels of SNAP25 might be partially responsible for specific behavioural traits in neuropsychiatric diseases, even in diseases to which the gene has not been directly linked yet. For example, SNAP25 gene polymorphisms analyzed in a group of children affected by ASD revealed a significant association between SNAP25 SNP rs363043 and hyperactivity trait (Guerini et al., 2011) and of SNAP25 SNPs rs363050 and rs363039 defective cognitive abilities. A first analysis of transcriptional activity using luciferase reporter gene assays showed that SNP rs363050 contains a regulatory element leading to a decrease in SNAP25 protein expression. Thus, the possibility that reduced SNAP25 levels may contribute to specific traits, such as hyperactivity, in multiple psychiatric diseases remains a fascinating possibility to be tested in the future.

The Role of Munc18-1 in Synaptic Transmission

Synaptic vesicle exocytosis is executed by a multi-subunit protein machinery between the vesicle and target membranes. The central components of the machinery are the SNARE proteins Syntaxin-1, SNAP25 and Synaptobrevin-2/VAMP2 and the Sec1p/Munc18 (SM) protein Munc18-1. Deletion of Munc18-1 in mice leads to the complete loss of neurotransmitter secretion. In vivo studies show that Munc18-1 is involved in the initial docking step of secretory vesicles with the plasma membrane, but it has also been suggested to have an additional post-docking role, and was proposed to promote or regulate the final fusion reaction.

To dissect the role of Munc18 in synaptic transmission we conducted a number of experiments during the course of EUROSPIN. We generated a novel mutant mouse line with which we investigated the molecular principles that underlie Munc18-1 recruitment to synaptic terminals and the consequence of altering synaptic Munc18 levels for synaptic transmission. Using re-expression of Munc18-1 with carefully designed mutations in Munc18-1 KO neurons we tested the importance of SNARE-complex binding of Munc18-1 for synaptic transmission. In addition, we also analyzed the functional consequences of disease-causing mutations in Munc18-1 in supporting synaptic transmission. Here, we found that de novo point mutations in STXBP1/Munc18 that cause early onset epilepsy (Othahara syndrome) result in protein instability and reduced synaptic transmission when re-expressed in Munc18-1 KO neurons.

To investigate the dynamics of endogenous Munc18-1 in neurons, we created a mouse model expressing fluorescently tagged Munc18-1 from the endogenous Munc18-1 locus. We show using fluorescence recovery after photobleaching in hippocampal neurons that the majority of Munc18-1 is trafficked through axons and targeted to synapses via lateral diffusion together with syntaxin-1. Munc18-1 is strongly expressed at presynaptic terminals, with individual synapses showing a large variation in expression. Axon-synapse exchange rates of Munc18-1 are high. During stimulation, Munc18-1 rapidly disperses from synapses and reclusters within minutes. Munc18-1 reclustering is independent of Syntaxin-1, but requires Ca2+ influx and PKC activity. Importantly, a PKC-insensitive Munc18-1 mutant does not recluster. We found further that synaptic Munc18-1 levels correlate with synaptic strength, and that synapses that recruit more Munc18-1 after stimulation have a larger releasable vesicle pool. Hence, PKC-dependent dynamic control of Munc18-1 levels enables individual synapses to tune their output during periods of activity (Cijsouw et al., 2014) (Figure 11).

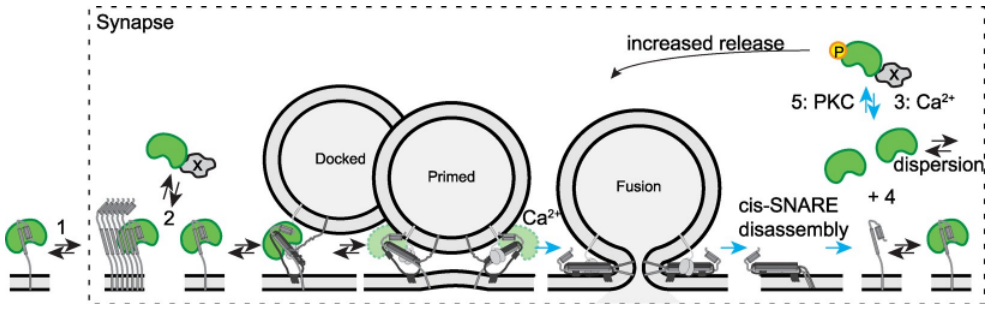

Figure 11. Munc18-1 Transport to and Behavior in the Synapse*. In mature neurons, Munc18-1 (shown in green) is transported to synapses via lateral diffusion with membrane-bound Syntaxin-1 (shown in light gray, 1). At the synapse, Munc18-1 interacts with Syntaxin-1 (individually or in microdomains) or dissociates and interacts with other binding partners (X; 2). Ca2+ influx triggers dissociation from binding partners (3) and temporarily increases free Munc18-1 concentrations in the synapse (4). After stimulation, PKC controls reclustering of Munc18-1 (5). Figure from Cijsouw et al. (2014).*

Synaptic transmission depends critically on the Sec1p/Munc18 protein Munc18-1, but it is unclear whether Munc18-1 primarily operates as a integral part of the fusion machinery or has a more upstream role in fusion complex assembly. We found that point mutations in Munc18-1 that interfere with binding to the free Syntaxin-1a N-terminus and strongly impair binding to assembled SNARE complexes all support normal docking, priming and fusion of synaptic vesicles, and normal synaptic plasticity in Munc18-1 null mutant neurons. These data support a prevailing role of Munc18-1 before/during SNARE-complex assembly, while its continued association to assembled SNARE complexes is dispensable for synaptic transmission (Meijer et al., 2012).

Neuroligins at Inhibitory Synapses - From Synaptogenesis to ASD

Apart from work on other ASD-related proteins, such as Neurobeachin (Nair et al., 2013) and LRRTM4 (Siddiqui et al., 2013), a substantial part of EUROSPIN activities focused on Neuroligin-2 and Neuroligin-4, which are cell adhesion proteins at inhibitory synapses and are mutated in certain forms of schizophrenia and ASD, respectively (Krueger et al., 2012).

We characterized the homomeric and heteromeric dimerization of the four Neuroligin isoforms (Poulopoulos et al., 2012) and found that Neuroligin-4 controls inhibitory synapse maturation. Loss-of-function mutations in Neuroligin 4 are linked to rare forms of monogenic heritable autism, but its localization and function have long been unknown. Using the retina as a model system, we found that Neuroligin 4 is preferentially localized to glycinergic postsynapses and that the loss of Neuroligin 4 is accompanied by a reduced number of glycine receptors mediating fast glycinergic transmission. Accordingly, Neuroligin 4 deficient ganglion cells exhibit slower glycinergic miniature postsynaptic currents and subtle alterations in their stimulus-coding efficacy, and inhibition within the Neuroligin 4 deficient retinal network is altered as assessed by electroretinogram recordings. These data indicate that Neuroligin 4 shapes network activity and information processing in the retina by modulating glycinergic inhibition. Importantly, Neuroligin 4 is also targeted to inhibitory synapses in other areas of the CNS, such as the thalamus, colliculi, brainstem, and spinal cord, and forms complexes with the inhibitory postsynapse proteins Gephyrin and Collybistin in vivo, indicating that Neuroligin 4 is an important component of glycinergic postsynapses (Hoon et al., 2011).

Given that Neuroligin 2 and Neuroligin 4 interact similarly with the inhibitory synapse proteins Gephyrin and Collybistin, we studied their molecular mode of action, using Neuroligin 2 as the model. The formation of neuronal synapses and the dynamic regulation of their efficacy depend on the assembly of the postsynaptic neurotransmitter receptor apparatus. Receptor recruitment to inhibitory GABAergic and glycinergic synapses is controlled by the scaffold protein Gephyrin and the adaptor protein Collybistin. We derived new insights into the structure of Collybistin and used these to design biochemical, cell biological, and genetic analyses of Collybistin function. Our data define a Collybistin-based protein interaction network that controls the gephyrin content of inhibitory postsynapses. Within this network, Collybistin can adopt open/active and closed/inactive conformations to act as a switchable adaptor that links Gephyrin to plasma membrane phosphoinositides. This function of Collybistin is regulated by binding of the adhesion proteins Neuroligin 2 and Neuroligin 4, which stabilize the open/active conformation of Collybistin at the postsynaptic plasma

membrane by competing with an intramolecular interaction in Collybistin that favors the closed/inactive conformation. By linking trans-synaptic Neuroligin-dependent adhesion and phosphoinositide signaling with Gephyrin recruitment, the Collybistin-based regulatory switch mechanism represents an integrating regulatory node in the formation and function of inhibitory postsynapses (Soykan et al., 2014) (Figure 12).

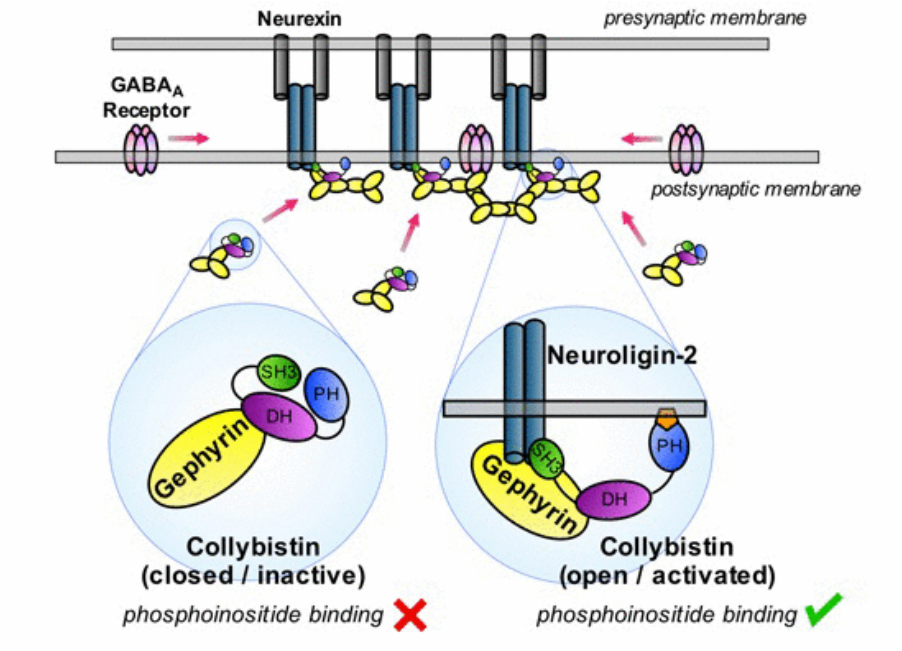

Figure 12. Neuroligin 2 (and neuroligin 4) at inhibitory synapses. *The postsynaptic cell adhesion protein Neuroligin-2 stabilizes the open/active conformation of Collybistin to regulate Gephyrin and GABAA receptor clustering at postsynaptic plasma membranes. Collybistin regulates the recruitment of the postsynaptic scaffold protein Gephyrin to inhibitory synapses in the brain, which, in turn, leads to the postsynaptic clustering of GABAA (and glycine) receptors. Collybistin exhibits a dynamic equilibrium between closed/inactive and open/active conformations and requires a conformational activation for its phosphoinositide-binding and Gephyrin-clustering activity. At synapses, the active conformation of collybistin is selected by interactions with the postsynaptic cell adhesion proteins Neuroligin-2 and Neuroligin-4 and is further stabilized by the subsequent formation of a Neuroligin/Gephyrin/Collybistin holocomplex. From Soykan et al. (2014).*

In subsequent studies, EUROSPIN partners analysed the consequences of Neuroligin deficiency in the amygdala. We performed an analysis of inhibitory synaptic transmission in the basolateral and central amygdala nuclei in Neuroligin 2 and Neuroligin 4 deficient mice. Consistent with the behavioral phenotypes in fear conditioning, which was perturbed only in the absence of Neuroligin 2, we observed that inhibitory synaptic transmission is massively reduced in Neuroligin 2 but not in Neuroligin 4 deficient mice mice. Interestingly, this effect was specific for the basolateral amygdala, a cortex-like, feed-forward structure containing similar interneuron subtypes as those described in cortex and hippocampus. The most parsimonious explanation of this phenotype that would be consistent with the concomitant deficit in fear learning and inhibitory transmission would be if loss of Neuroligin 2 would selectively affect inhibitory synapses made by parvalbumin expressing basket cells onto the perisomatic region of principal neurons and onto somatostatin expressing interneurons. In particular, such a loss of disinhibition could explain why activity-dependent synaptic plasticity onto dendrites of projection neurons, and hence fear conditioning, could be impaired. We therefore crossed Neuroligin 2 KO animals with both PV-Cre and SOM-Cre animals. These animals were then injected with conditional AAV vectors expressing the red fluorescent marker mCherry in a Cre-dependent manner, thus allowing for identification of parvalbumin or somatostatin positive interneurons in Neuroligin 2 KO animals. We completed mIPSC recordings from parvalbumin positive interneurons and found that, like in principal neurons, lack of Neuroligin 2 was associated with a massive reduction in mIPSC frequency. This indicates that Neuroligin 2 is not only necessary for the formation and maintenance of perisomatic inhibitory synapses onto principal cells, but also regulates inhibitory interactions between interneurons. We are currently recording from somatostatin positive interneurons, and we expect that these experiments will reveal a more detailed picture of how loss of Neuroligin 2 impacts amygdala circuit function and thereby leads to behavioral abnormalities, e.g. in schizophrenia.

Altogether, our combined biochemcial, structural, cell biological, and circuit data on Neuroligin 2 and Neuroligin 4 identify inhibitory GABAergic synapses as possible targets for intervention in ASD and schizophrenia.

Potential Impact

The output of EUROSPIN in the form of PPI databases, software tools and PPI models, antibody reagents, mouse lines, and pathophysiological pathway discoveries will have profound effects and impact at various levels, from academic research via translational research to medicine. Four selected key aspects are summarized below.

PPIs - Databases - Models

As outlined in detail in the report on the Main Results of EUROSPIN research, we established a very large EUROSPIN database (eurospindb.genes2cognition.org) that will go public in the very near future. The database mainly contains an extremely complex dataset on PPIs between synaptic proteins. The EUROSPIN PPI dataset is one of the largest of its kind and very well validated and cross-checked. It has already served the EUROSPIN consortium for the prediction and validation of PPIs in vivo, and for the characterization of disease relevant PPI networks and their 'hubs'. The EUROSPIN PPI database will be of similarly extreme importance for the basic and translational research communities in the field of neuroscience outside of EUROSPIN. The PPI data along with new modeling tools will allow researchers to identify key protein networks and 'hubs' of functional relevance in health and disease.

In addition, the EUROSPIN database contains electrophysiological and behaviroal data on a large number of disease-relevant mouse lines. This will be made available to the research community for in-depth comparative data mining, which will support the identification of commonalities between disease phenotypes and the selection of processes to be targeted by experimental intervention strategies.

MOUSE LINES AND ANTIBODY REAGENTS

As outlined in detail in the report on the Main Results of EUROSPIN research, EUROSPIN provides a huge number of mouse lines, which will be made available to the basic and translational neuroscience research communities. In a few selected cases (e.g. with NMDA receptors or SUMO1-conjugated proteins), EUROSPIN partners have demonstrated that the new KI mouse lines expressing TAP tagged disease-relevant proteins can be used to characterize key protein complexes in synapses and to assess changes in synaptic protein composition in disease models - with unprecedented specificity. In essence, the TAP-tag KI lines surpass all other models and methods for the purification of native protein complexes with regard to specificity and minimal perturbation of the native state. Similarly, the EUROSPIN KI mouse lines expressing fluorescently tagged disease-relevant proteins will allow researchers to assess dynamics of synaptic proteins under physiological and pathophysiological conditions in a minimally perturbed mammalian organism. In the case of Munc18-1, the extreme usefulness of such mouse lines has been demonstrated by EUROSPIN partners.

The antibody pipeline of EUROSPIN generated a plethora of antibodies to disease gene products. Information on these antibodies is available in the EUROSPIN database (eurospindb.genes2cognition.org). Given that these new antibodies are very well validated, they will be extremely useful for basic and translational neuroscience research alike.

SMALL MOLECULES AND LEAD STRUCTURES FOR THERAPY

As outlined in detail in the report on the Main Results of EUROSPIN research, chemical biology research had a key position within the EUROSPIN consortium. EUROSPIN partners developed novel synthesis strategies for natural-product inspired smart small-molecule libraries that will be of great value to researchers in the areas of medicinal chemistry, chemical biology, and translational medicine.

In two cases, EUROSPIN researchers identified and optimised lead structures that have the clear potential for future drug discovery. In one case, EUROSPIN partners identified drug lead structures that inhibit Aβ aggregation. In the future, these structures can be developed into drugs to treat Alzheimer's disease. In a second case, EUROSPIN partners identified lead structures that promote neurite growth. In the future, these structures can be used to develop drugs to promote neuroregeneration. These two examples of lead structure discovery represent a particularly fascinating EUROSPIN perspective as regards the development of new medicines for some of the most deleterious nervous system diseases - a perspective that is likely to involve interactions with the pharma industry in the near future.

PATHOPHYSIOLOGICAL PROCESSES AND PATHWAYS

As outlined in detail in the report on the Main Results of EUROSPIN research, the EUROSPIN research portfolio contained a large number of disease-relevant genes and gene products. Regarding several of these (e.g. DLGs, Munc13s, N-Cadherin a.o.), EUROSPIN partners made substantial progress in characterizing the physiological function of the corresponding proteins and their pathophysiological dysfunction in the disease context. This work, in general, will boost future research on the corresponding gene products, which has the ultimate potential to identify new targets for therapeutic intervention.

In two cases, work in the EUROSPIN consortium has gone much further by characterizing selected disease gene products in substantial detail and at several levels of analytical complexity. For example, EUROSPIN work identified the SNARE protein SNAP25 as a likely central player in several disease contexts, leading to the notion that SNAP25 and its expression levels represent interesting targets for therapeutic intervention in various neuropsychiatric disorders, including ADHD and ASDs. Similarly, EUROSPIN work on Neuroligins related to ASDs and schizophrenia has identified dysfunctions of defined inhibitory synapses to be at the core of the disease phenotypes. These findings can now be used to guide future intervention strategies. Interestingly, clinical studies to manipulate GABAergic transmission in ASDs are already under way in different parts of the world, but currently seem to have little promise or traction. With the EUROSPIN data on Neuroligins, it will be possible to guide future clinical studies to specific subsets of inhibitory synapses, e.g. by selecting drugs that only affect selected GABA receptor types at selected synapses. Interactions with the pharma industry will be needed here, and, correspondingly, EUROSPIN partners are deeply involved with an EU-IMI on ASDs (EU-AIMS) to further develop the link between Neuroligins and ASDs.

Contact

Coordinator: Prof. Nils Brose

Max-Planck-Institut für Experimentelle Medizin
Molecular Neurobiology
Hermann-Rein-Straße 3
37075 Göttingen
Germany

+49 (0) 551 38 99 – 725
brose@em.mpg.de

Project Manager: Dr. Kerstin Mosch

Max Planck Institute for Biophysical Chemistry
Am Fassberg 11
37077 Goettingen
Germany

+49-(0)551-201 1157
kmosch@gwdg.de

Website

http://www.eurospin.mpg.de