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**JOINT GERMAN-ISRAELI RESEARCH PROJECTS**

# FINAL SCIENTIFIC REPORT

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Title of Project

**Maintaining the integrity of the postsynaptic density: A core mechanism to prevent synaptic dysfunction**

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Abbreviated Title: **Maintaining the postsynaptic density**

Principal Investigators: Noam Ziv, Eckart Gundelfinger, Michael R. Kreutz
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**Abstract**

This proposal was concerned with a fundamental neurobiological question: How CNS excitatory synapses maintain their integrity for long periods in spite of continuous protein turnover and additional erosive forces that act in neuronal tissue. This question is fundamental in two respects: First, recent studies indicate that the majority of excitatory synapses in the adult brain are remarkably persistent, surviving for weeks, months, perhaps lifetimes. On the other hand, synaptic loss seems to be a hallmark of a growing number of CNS diseases, ranging from neurodegenerative disease states to epilepsy and schizophrenia. Thus, understanding the mechanisms that allow the minute synapse to maintain its structural and functional integrity is of utmost importance both for understanding normal brain function and for uncovering the origins of major pathological states. Here we proposed to study processes involved in maintaining the molecular contents of the postsynaptic compartment. Specifically we proposed to study the targeting, loss, degradation and replenishment of a family of key postsynaptic scaffolding molecules known as ProSAPs/Shanks. We proposed to combine advanced imaging technologies with molecular and biochemical methods to study the dynamics and molecular mechanisms of ProSAP synthesis, trafficking from the soma, assimilation into individual postsynaptic sites, removal from such sites, and ultimately, its degradation, and how these processes are affected by synaptic activity, intracellular signaling pathways, and perturbations of protein synthesis and degradation pathways..

**Conclusions**

The structural basis for ProSAP1/Shank2 and ProSAP2/Shank3 synaptic targeting to synaptic sites was analyzed in detail utilizing electron microscopy. This work (Baron et al., 2006), carried out in collaboration with the lab of James Bowie revealed that the SAM-domains of ProSAP2/Shank3, previously shown to be essential for ProSAP targeting to synapses, form large sheets composed of helical fibers that are aligned in a side by side manner. A potential $Zn^{2+}$-binding site was identified in the SAM-domain, which was shown to dramatically improve sheet ordering upon $Zn^{2+}$-binding. Mutant forms of these proteins that disrupted sheet formation as well as those that prevented $Zn^{2+}$-binding failed to localize to synapses, indicating that sheet formation and $Zn^{2+}$-binding are essential for synaptic clustering of ProSAP2/Shank3. These findings may be taken to suggest that the assembly of the SAM-domain into macromolecular sheets provides a platform for the construction of the PSD-protein complex. Interestingly, however, our experiments also revealed (Tsuriel et al., 2006) that these putative platforms are dynamic in nature and that ProSAP2 molecules are in a state of flux, that is, continuously exchanged with extrasynaptic pools at considerable rates (near complete replacement within 1-3 hours). Counter to current views of synaptic maintenance our findings indicated that ProSAP2 molecules are not necessarily degraded when they dissociate from synaptic structures. Rather, it seems that most of these molecules are reincorporated back into the same synaptic structures, and more intriguingly, into adjacent synaptic sites, indicating that ProSAP2 molecules are continuously reused and exchanged among nearby synapses. Furthermore we found that these dynamics can be accelerated by activity, suggesting that some forms of activity can act as "dispersive" forces that destabilize rather than stabilize the postsynaptic structure. Additional analysis provide indications that these local dynamics may be strongly affected by binding to interacting proteins such as ProSAPiP, and by GTPase activating proteins such as SPAR2. (Wendholt et al., 2006). In comparison, our experiments indicated that the replenishment of remote synapses with ProSAP from somatic sources occurs at surprisingly slow rates, and that replenishment may be regulated by ProSAP2 binding partners involved in synapse-to-nucleus communication such as abelson interacting

protein-1 (Abi-1; Proepper et al., 2007) and Jacob (Dieterich et al, 2008). Altogether our experiments so far provide new insights on the molecular mechanisms and dynamic processes that generate, regulate and maintain postsynaptic structure.

**Publications:**


Baron MK, Boeckers TM, Vaida B, Faham S, Gingery M, Sawaya MR, Salyer D, Gundelfinger ED, Bowie JU (2006). An architectural framework that may lie at the core of the postsynaptic density. *Science*. **311**, 531-535.

Gundelfinger ED, Boeckers TM, Baron MK, Bowie JU. (2006) A role for zinc in postsynaptic density asSAMbly and plasticity? *Trends Biochem Sci*. **31**, 366-373.

Tsuriel S, Geva R, Zamorano P, Dresbach T, Boeckers T, Gundelfinger ED, Garner CC, Ziv NE (2006). Local sharing as a predominant determinant of synaptic matrix molecular dynamics. *PLoS Biol*. **4**, 1572-1587.

Proepper C, Johannsen S, Liebau S, Dahl J, Vaida B, Bockmann J, Kreutz MR, Gundelfinger ED, Boeckers TM. (2007) Abelson interacting protein 1 (Abi-1) is essential for dendrite morphogenesis and synapse formation. *EMBO J*. **26**, 1397-1409.

Wendholt D, Spilker C, Schmitt A, Dolnik A, Smalla KH, Pröpper C, Bockmann J, Sobue K, Gundelfinger ED, Kreutz MR, Böckers, TM. (2006) ProSAPiP1, a novel protein of the postsynaptic density that links SPAR to the PDZ domain of ProSAP2 / Shank 3. *J Biol. Chem*. **281**, 13805-13816.

Spilker C, Acuña Sanhueza GA, Böckers TM, Kreutz MR, Gundelfinger ED.SPAR2, a novel SPAR-related protein with GAP activity for Rap1 and Rap2 (2008). *J Neurochem*. **104**; 187-201.

Dieterich DC, Karpova A, Mikhaylova M, Zdobnova I, König I, Landwehr M, Kreutz M, Smalla KH, Richter K, Landgraf P, Reissner C, Boeckers TM, Zuschratter W, Spilker C, Seidenbecher CI, Garner CC, Gundelfinger ED, Kreutz MR. (2008) Caldendrin-Jacob: a protein liaison that couples NMDA receptor signalling to the nucleus. *PLoS Biol* **6**, 286-306.


Additional collaborative publications


Dresbach T, Torres V, Wittenmayer N, Altrock WD, Zamorano P, Zuschratter W, Nawrotzki R, Ziv NE, Garner CC, Gundelfinger ED. (2006) Assembly of active zone precursor vesicles: obligatory trafficking of presynaptic cytomatrix proteins Bassoon and Piccolo via a trans-Golgi compartment. *J Biol Chem*. **281**, 6038-6047.

Fejtova A, Davydova D, Bischof F, Lazarevic V, Altrock WD, Romorini S, Schöne C, Zuschratter W, Kreutz MR, Garner CC, Ziv NE, Gundelfinger ED (2009) Physical interaction with dynein light chain regulates axonal trafficking and synaptic levels of Bassoon. *J Cell Biol* (in press).

**Body of Report**

Loss, redistribution and replenishment dynamics of ProSAP2

We have studied the cellular dynamics of the postsynaptic molecule ProSAP2 in order to obtain new insights as to the nature of deconstructive and reconstructive forces acting at synapses. In the course of this work we have developed the methodologies required to evaluate these processes both qualitatively and quantitatively. These are mainly based on Fluorescence Recovery After Photobleaching (FRAP) of GFP-tagged ProSAP2 and spatially restricted Photoactivation of Photoactivatable-GFP (PA-GFP) tagged ProSAP2. These methods have allowed us to determine the residency times of ProSAP molecules at individual PSDs, determine the fates of molecules once they leave the postsynaptic density, the degree to which ProSAP2 molecules are exchanged among nearby synapses and the rates at which molecules from somatic sources are incorporated into remote postsynaptic sites. These experiments (Tsuriel et al., 2006) indicated that ProSAP2 and (as shown since by other groups) additional PSD molecules are in a state of flux, that is, continuously exchanged with extra synaptic pools at considerable rates (near complete replacement within 1-3 hours). Furthermore we found that ProSAP2 molecules that leave synapses are not necessarily degraded but can become reincorporated into adjacent synaptic sites, indicating that ProSAP2 molecules are continuously reused and exchanged among nearby synapses. Interestingly, we found that these dynamics can be accelerated by activity, suggesting that some forms of activity can act as "dispersive" forces that destabilize rather than stabilize the postsynaptic structure. In comparison, replenishment of remote synapses with ProSAP from somatic sources was observed to occur at surprisingly slow rates (as exemplified in Figure 1), indicating that at times scales of several hours the molecular dynamics of postsynaptic proteins may be dominated by local processes (loss, redistribution, reincorporation) rather than global forces (centralized protein synthesis and degradation mechanisms). This conclusion is further supported by further experiments in which we found that the dynamics described above are not affected by protein synthesis inhibitors

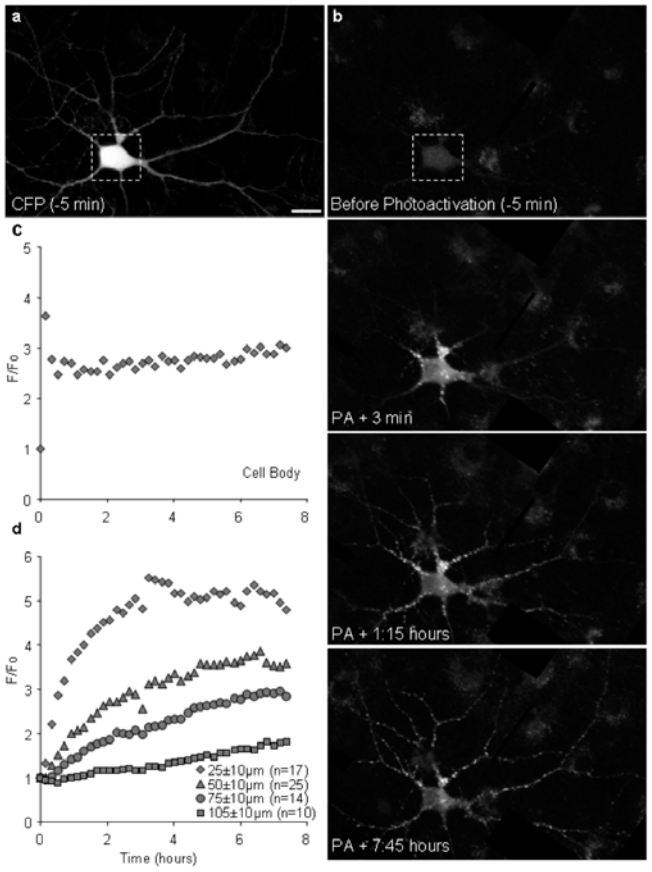


***Figure 1:*** *Incorporation rates of ProSAP2 from somatic sources into remote PSDs.* ***A)*** *Composite image of a neuron expressing Cyan Fluorecent Protein (CFP) and PA-GFP:ProSAP2. Only CFP fluorescence is shown here.* ***B)*** *PA-GFP:ProSAP2 fluorescence for same region as in A. At time t=0 PA-GFP:ProSAP2 within the cell body was photoactivated by selective illumination of the soma at 405nm. With time, photoactivated PA-GFP:ProSAP2 migrated to PSDs along the dendrites, initially to proximal PSDs and later on to more distal ones. During the time lapse session, recurrent, low-level photoactivation of the soma was performed, to maintain a constant level of somatic, photoactivated PA-GFP:ProSAP2.* ***C)*** *Quantification of fluorescence changes at the soma.* ***D)*** *Quantification of fluorescence changes at PSDs, grouped according to distance from the soma. Fluorescence data for each PSD was normalized to pre-photoactivation fluorescence levels for the same PSD. Bar- 20 µm.*

over times scales of several hours. Furthermore these dynamics were only marginally affected by pharmacologically blocking proteasome-mediated protein degradation.

Trafficking and molecular dynamics of ProSAP/Shank at the synapse

Our previous results indicate that the Sterile Alpha-Motif (SAM)-domain in ProSAP1/Shank2 and ProSAP2/Shank3 is essential for the synaptic targeting of both proteins. Therefore we analyzed in collaboration with the lab of James Bowie the structural basis of this targeting process in more detail. Utilizing electron microscopy we could show that the SAM-domain of ProSAP2/Shank3 forms large sheets composed of helical fibers that are aligned in a side by side manner (Baron et al., 2006). After bacterial overexpression we could solve the crystal structure of the domain at 2.1Å resolution and show that more than 25 fibers stack tightly side by side in a highly ordered manner (Baron et al., 2006). During the course of these studies we could also identify a potential $Zn^{2+}$-binding site in the SAM-domain. $Zn^{2+}$-binding dramatically improved the ordering of the sheets suggesting that it may play an important structural role in organizing huge ProSAP/Shank complexes (Baron et al., 2006). To determine whether sheet formation is important for trafficking and localization of ProSAP2/Shank3 to synapses we generated a variety of mutant constructs and followed up their localization after transfection in hippocampal primary neurons. Mutants that disrupted sheet formation as well as those that prevented $Zn^{2+}$-binding failed to localize to synapses (Baron et al., 2006). This indicates that sheet formation and $Zn^{2+}$-binding are essential for synaptic clustering of ProSAP2/Shank3. It is plausible to assume that the assembly of the SAM-domain into macromolecular sheets provides the platform for the construction of the PSD-protein complex. With the identification of this molecular target we want to address in our future studies which mechanisms could potentially be responsible for the stabilization and de-stabilization of ProSAP/Shank oligomerization via the SAM-Domain (Gundelfinger et al., 2006)

Of interest in this regard is also that PDZ-domain interactions of ProSAP/Shanks are quite different. It was shown previously that GKAP / Shank1 form insoluble intracellular aggregates that are subsequently degraded. However, the two proteins became organized in synaptic clusters when they were associated with SAP90/PSD-95. These experiments suggested that the two-dimensional organization provided by the SAP90/PSD-95-GKAP complex plays an important role for the assembly and the stability of Shank1 within the PSD protein network at synapses. With the identification of the ProSAPiP family of proteins we could show now that family members provide a highly specific PDZ-domain dependent binding network with ProSAP1/Shank2 and ProSAP2/Shank3 (Wendholt et al., 2006). ProSAPiP's bind the PDZ-domains of ProSAP 1/ Shank2 and ProSAP2/Shank3 with different affinities and can built up heterooligomers. In ongoing studies we found that they only exhibit weak interactions with Shank1. Importantly, we realized that they also interact with the PDZ domain of MAGUK's with some family members binding to a subset of MAGUK family members and vice versa. Probably due to their oligomerization ProSAPiPs frequently built up insoluble intracellular aggregates after overexpression. Thus, although PDZ-domain binding seems not to be essential for ProSAP1/Shank2 and ProSAP2/Shank3 synaptic targeting the presence of a MAGUK / ProSAPiP complex might be crucial for the stability of ProSAP1/Shank2 and ProSAP2/Shank3 in the PSD protein network. To substantiate this hypothesis we generated a PDZ-ProSAP2/Shank3 mutant that is incapable of binding to ProSAPiP. This mutant localizes efficiently to synapses but preliminary data show that its residing time at spines is much shorter than the wild-type construct.

In further work related to ProSAPiP and ProSAP2 we could identify SPAR2 as a novel GTPase activating protein (GAP) for the small GTPase Rap that shows significant sequence homology to SPAR, a synaptic RapGAP that was reported to regulate spine morphology in hippocampal neurons (Spilker et al., 2008). SPAR2, like SPAR, interacts with ProSAPiP which in turn binds to the PDZ domain of ProSAP/Shank postsynaptic density (PSD) proteins (Spilker et al., 2008). In subcellular fractionation experiments SPAR2 is enriched in synaptosomes and PSD fractions indicating that it is a synaptic protein. Furthermore, we could show using in vitro GAP assays that SPAR2 has GAP activity for Rap1 and Rap2. Expression in COS-7 cells, however, revealed different actin binding properties of SPAR2 and SPAR. Additionally, overexpression of SPAR2 in cultured hippocampal neurons did not affect spine morphology as it was reported for SPAR. In situ hybridization studies also revealed a different tissue distribution of SPAR and SPAR2 with SPAR2 transcripts being mainly expressed in cerebellar and hippocampal granule cells (Spilker et al., 2008). Moreover, in the cerebellum SPAR2 is developmentally regulated with a peak of expression around the period of synapse formation. Our results imply that SPAR2 is a new RapGAP with specific functions in cerebellar and hippocampal granule cells.

A surprising finding of the last two years was that many factors that control synaptic stability via synapse-to-nucleus communication are binding ProSAP2 binding partners. Thus we identified an important component for controlled actin assembly, abelson interacting protein-1 (Abi-1), as a ProSAP2 binding partner (Propper et al., 2007). During early neuronal development, Abi-1 is localized in neurites and growth cones; at later stages, the protein is enriched in dendritic spines and PSDs, as are components of a trimeric complex consisting of Abi-1, Eps8 and Sos-1. Abi-1 translocates upon NMDA application from PSDs to nuclei. Nuclear entry depends on abelson kinase activity. Abi-1 co-immunoprecipitates with the transcription factor complex of Myc/Max proteins and enhances E-box-regulated gene transcription. Down regulation of Abi-1 by small interfering RNA results in excessive dendrite branching, immature spine and synapse morphology and a reduction of synapses, whereas overexpression of Abi-1 has the opposite effect (Proepper et al., 2007). We therefore propose that Abi-1 can act as a specific synapto-nuclear messenger and is essentially involved in dendrite and synapse formation.

Another molecule that seems to play a pivotal in synapse-to-nucleus communication is Jacob (Dieterich et al., 2008). Jacob is like Abi-1 a ProSAP2-binidng partner. But its mechanism of action and nuclear translocation are completely different. N-methyl-D-aspartate (NMDA) receptors and calcium can exert multiple and very divergent effects within neuronal cells impacting opposing occurrences such as synaptic plasticity and neuronal degeneration. The neuronal $Ca^{2+}$-sensor Caldendrin is a postsynaptic density component with high similarity to Calmodulin and like ProSAP2 a Jacob binding partner. Strictly depending upon activation of NMDA-type glutamate receptors Jacob is recruited to neuronal nuclei, resulting in a rapid stripping of synaptic contacts and in a drastically altered morphology of the dendritic tree. Jacob's nuclear trafficking from distal dendrites crucially requires the classical Importin pathway. Caldendrin controls Jacob's extra-nuclear localization by $Ca^{2+}$-dependently competing with the binding of Importin-α to Jacob's nuclear localization signal. This competition requires sustained synapto-dendritic $Ca^{2+}$-levels, which presumably cannot be achieved by activation of extrasynaptic NMDA receptors, but are confined to $Ca^{2+}$-microdomains such as postsynaptic spines. Extrasynaptic NMDA receptors as opposed to their synaptic counterparts trigger the CREB shut-off pathway, and cell death. We found that nuclear knock-down of Jacob prevents CREB shut-off after extrasynaptic NMDA receptor activation while its nuclear overexpression induces CREB shut-off without NMDA receptor stimulation. Importantly, nuclear knock-down of Jacob attenuates

NMDA-induced loss of synaptic contacts, and neuronal degeneration. This defines a novel mechanism of synapse-to-nucleus communication via a synaptic $Ca^{2+}$-sensor protein, which links the activity of NMDA receptors to nuclear signaling events involved in modeling synapto-dendritic input and NMDA-receptor induced cellular degeneration. This work has now been submitted for publication.

In summary, we deciphered a number of novel mechanisms related to the scaffolding function of ProSAP2 that directly affect the stability of excitatory synapses. Our data not only show that the association of ProSAP2 to synapses is highly dynamic but that essential synapse-to nucleus signaling pathways for synapto-dendritic integrity are directly hooked up to the protein.

**Collaboration**

The collaboration during this project was based on regular discussions, mutual visits and "metagroup" meetings, leading to joint publications (see above), and to additional joint projects, some of which are currently being formulated as part of applications to other funding agencies.

Mutual visits:

- Drs. Gundelfinger and Kreutz visited the laboratory of Dr. Ziv in Haifa during the month of February 2006.
- Michal Stern, from the Ziv lab, spent a week in the lab of Dr. Gundelfinger during the month of September 2006 for training in techniques concerning transgenic mice.
- A "Metagroup meeting" was held in Germany involving most lab members of participating groups in Israel and Germany (Wanzleben Castle, February 7-10, 2007.)
- Dr. Ziv visited the labs of Drs. Gundelfinger and Kreutz in Germany in February 2007.
- Drs. Gundelfinger visited the laboratory of Dr. Ziv in Haifa during the month of August 2008.
- Anna Fejtova from the Gundelfinger lab spent 3 weeks in Jan 2008 in the lab of Dr. Ziv to perform experiments involving live imaging of synaptic proteins in neurons.

Additional aspects

The collaboration between the two groups has started long before this project (as early as 1999) and has been extended to future projects that concern, for example presynaptic protein trafficking, the roles of active zone proteins in presynaptic stability and the roles of protein synthesis in the maintenance and plasticity of synapses. Over the years, the collaboration resulted in six papers, all in major journals (Neuron, PLoS Biol., J. Neurosci., J Biol. Chem, J. Cell Biol) and one review article. Papers published independently by each group were often inspired or affected, in part, by this collaboration or by long discussions between us. Last but not least, this collaboration has led to strong personal ties among PIs, post docs and graduate students alike which had made this collaboration as enjoyable as it has been productive.