PÁZMÁNY PÉTER CATHOLIC UNIVERSITY

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Investigation of the Structure and Interactions of Globular and Intrinsically Disordered Postsynaptic Proteins

Theses of PhD Dissertation

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2026

1. Introduction

The neural network found in the mammalian brain is composed of billions of neurons. Between neurons, information transfer and communication occur through synapses. The formation and maintenance of synaptic connections, as well as synaptic plasticity, are fundamental factors for normal brain function and homeostasis (Grabrucker et al., 2011). The postsynaptic density (PSD) is responsible for receiving signals from neighboring neurons via neurotransmitters and transmitting them toward deeper layers (Suzuki et al., 2018). It plays a role in maintaining synaptic structure, and through its reorganization, it has a key function in the molecular-level processes of learning and memory.

According to recent research, the PSD is a plate-shaped protein network approximately 30–50 nm thick and 300–400 nm wide, with a mass of 1.1 ± 0.36 GDa (Boeckers, 2006; Suzuki et al., 2018; Feng & Zhang, 2009; Chen et al., 2005). However, its size and composition are not constant; they can change dynamically depending on the brain region in which it is located, the level of synaptic activity, and they may also vary across different species (Sheng & Hoogenraad, 2007).

The first layer of the PSD is the so-called “core” layer, located 30–50 nm from the plasma membrane, where most of the scaffold proteins are found (Suzuki et al., 2018). Scaffold proteins function as bridges within the PSD, linking glutamate receptors located at the upper levels to signaling complexes and the cytoskeleton found at the lower levels. The MAGUK (membrane-associated guanylate kinase) proteins also belong to the scaffold proteins; in mammalian organisms, they form the largest protein group within the PSD. One example is the PSD-95 protein (also known as DLG4, disks large homolog 4).

Below the “core” layer lies the “pallium” layer, situated 50–60 nm from the plasma membrane, where the Homer and Shank proteins investigated in this study are also found (Suzuki et al., 2018; Dosemeci et al., 2016). The protein GKAP connects the “core” and “pallium” layers by interacting, among others, with the Shank protein. The Shank protein interacts, among others, with the Homer protein. The PSD-95–GKAP–Shank–Homer interaction constitutes an important structural framework of the PSD (Hayashi et al., 2009; Romorini et al., 2004; Shin et al., 2012).

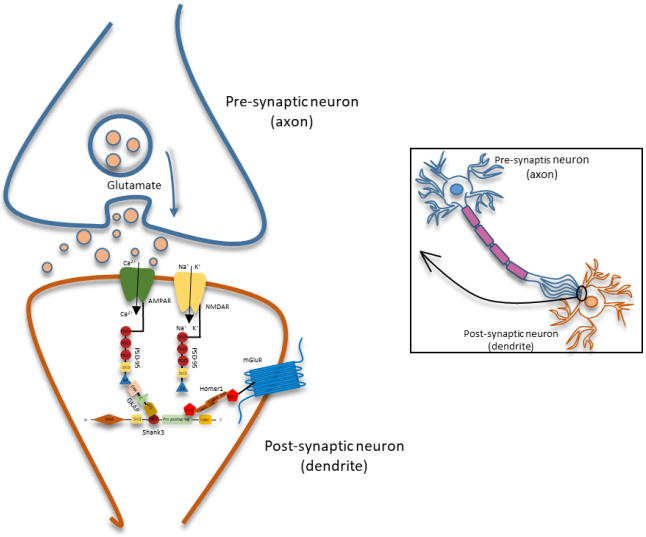

Figure 1. *Communication between neurons and the proteins that build up the postsynaptic density. The chemical synapse formed between the presynaptic neuron (axon) and the postsynaptic neuron (dendrite) operates through neurotransmitters, primarily glutamate. The postsynaptic density is a complex network located beneath the postsynaptic cell membrane, composed of various interacting proteins, including the Homer1, Shank3, and GKAP proteins investigated in this study.*

The Homer protein family, also known as VesI, Cupidin, or PSD-Zip45, is a key member of the postsynaptic scaffold proteins and plays an important role in synapse organization. In addition, it participates in

synaptic activity-induced plasticity (Hayashi et al., 2006), and thus contributes to the molecular mechanisms underlying learning and memory formation. Three members of the Homer protein family are known (Homer1, Homer2, and Homer3), encoded by three separate genes (Feng et al., 2002). Both short and long Homer proteins are present in the PSD, where they are involved in signal transduction and regulate the localization of target proteins (Luo et al., 2011).

In the long isoforms of Homer proteins (e.g., Homer1c, Homer2a, Homer2b, Homer3a, Homer3b), the amino-terminal region contains the EVH1/WH1 (Enabled/vasodilator-stimulated phosphoprotein homology 1) domain, which has ligand-binding function. The approximately 110 amino acids of the N-terminal EVH1 domain show about 80% sequence identity within the Homer family. The Homer1 protein is a key component of the PSD scaffold and one of the most extensively studied Homer proteins (Luo et al., 2012). The long isoform of Homer1 plays a role in linking PSD membrane proteins to the deeper layers of the cytoskeleton. The short isoform, Homer1a, contains an EVH1 domain and a short disordered C-terminal region. In contrast, the long isoforms (Homer1b and Homer1c) contain an EVH1 domain connected to a long C-terminal coiled-coil region.

The EVH1 domain consists of seven antiparallel β-sheets and a C-terminal α-helix. It has been shown that the binding partner of the EVH1 domain is a highly conserved proline-rich short linear motif

(SLiM), with a consensus sequence of PPxxF or FPxoP (where x represents any amino acid and o a hydrophobic amino acid). By recognizing such motifs, the EVH1 domain forms interactions with proteins including mGluR1, IP3R, Shank, Drebrin, Dynamin3, and the Ryanodine receptor (Ball et al., 2002; Feng et al., 2002; Shiraishi-Yamaguchi & Furuichi, 2007; Beneken et al., 2000).

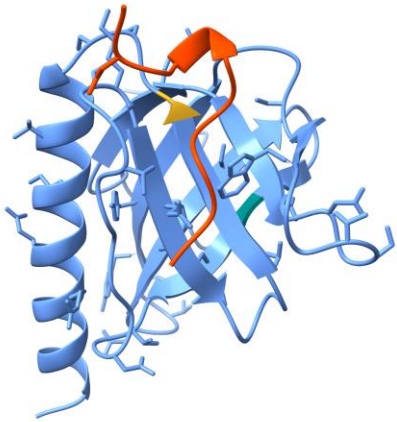

Figure 2. *Locations of the M65I (green) and S97L (yellow) mutations in the EVH1 domain structure, as well as the binding site of the Shank3 peptide (orange).*

The Shank protein family (also known as ProSAP, SSTRIP, Synamon, CortBP, or Spank) plays a role in the formation of the PSD scaffold structure in the central nervous system. It belongs to the scaffold proteins (Dosemeci et al., 2016; Luo et al., 2011), linking postsynaptic

membrane receptor complexes to cytoskeletal elements and thereby establishing the fundamental structure of the PSD (Grabrucker et al., 2011). Shank proteins are involved in the formation, development, and function of synapses (Sala et al., 2015).

GKAP (guanylate kinase-associated protein), also known as SAPAP (SAP90/PSD-95-associated protein) or DLGAP (Dlg-associated protein), is a scaffold protein present in relatively high abundance in the core region of the PSD (Verpelli et al., 2012). GKAP is an almost entirely functionally disordered protein and exhibits structural flexibility at its binding sites (Droogers & MacGillavry, 2023). Intrinsically disordered proteins are entire proteins or protein regions that lack a stable, well-defined three-dimensional structure under physiological conditions. Due to their structural disorder, they are capable of interacting with multiple proteins and can therefore perform diverse functions.

2. Objectives

During my work, I aimed to investigate the structural, dynamic, and partner-binding properties of the postsynaptic Homer1 protein EVH1 domain and the GBR region of the GKAP protein.

-Finalization of the solution-state structure of the Homer EVH1 domain and comparative analysis of its structure and dynamics

My goal was to complete the final steps of structure determination, finalize the evaluation of the related dynamic measurements, and perform comparative analysis and functional interpretation of the obtained results.

-Investigation of the structural effects of two point mutations in the Homer EVH1 domain by NMR spectroscopy

I aimed to express and purify the M65I and S97L point mutant EVH1 domains and to prepare isotope-labeled (^{15}N and ^{15}N-^{13}C) samples suitable for NMR measurements. The resulting spectra were evaluated, including resonance assignment and the model-free analysis of internal dynamics. Small-angle X-ray scattering (SAXS) and circular dichroism (CD) experiments were performed to characterize the global structure. The obtained results were compared with the wild type and with each other, and the experimental findings were complemented by computational comparative and dynamic analyses.

-Investigation of the functional effects of the two point mutations by Shank3 interaction measurements and NMR titrations

My aim was to examine the effect of the individual point mutations on the interaction between the EVH1 domain and the Shank3

peptide. I intended to prepare isotope-labeled samples suitable for NMR measurements, as well as unlabeled samples for biolayer interferometry and small-angle X-ray scattering (SAXS) experiments, followed by evaluation of the obtained data. I also aimed to examine the thermal stability of the domains and their complexes.

-Establishment and optimization of the purification protocol for the GBR1–3 protein and its NMR characterization

Another objective was to develop and optimize the expression and purification protocol of the functionally disordered GBR1–3 region of the GKAP protein, which, according to the literature, had not previously been produced and characterized in this form. I also aimed to prepare samples suitable for NMR studies, record NMR measurements of the GBR1–3 protein, evaluate the resulting spectra, perform resonance assignment, and characterize in detail the intrinsically disordered nature of the protein.

3. Methods

For the experiments, recombinant postsynaptic proteins and their mutant variants were produced in a bacterial expression system. The proteins were purified using affinity chromatography, ion-exchange chromatography, and size-exclusion chromatography. Their homogeneity and purity were verified by SDS–PAGE.

Solution-state structural investigations were performed using NMR spectroscopy, which enabled the determination of secondary structural elements and dynamic properties. The spectra were analyzed using the CCPNMR software package, and dynamic parameters were fitted with the Tensor2 program.

Small-angle X-ray scattering (SAXS) was used to analyze global structural parameters and conformational changes.

Protein–protein interactions were examined by biolayer interferometry (BLI), which allowed determination of binding affinity and kinetic parameters. For more detailed characterization of the interactions, NMR titration experiments were also performed. The thermal stability of the domains and their complexes was investigated using a thermal shift assay (TSA).

The general dynamic behavior characteristic of the EVH1 domain was analyzed using the Gaussian Network Model as implemented in the ProDy software package.

4. Theses of the Dissertation

1. I successfully produced constructs of postsynaptic proteins suitable for structural and functional investigations.

1/a The produced Homer1 EVH1 domain remains stable and free of precipitation even in ^{15}N–^{13}C labeled form and at high concentrations. - *I. Publication, P3, O3*

Expression of the Homer1 EVH1 protein in E. coli BL21 bacterial cells was efficient, as confirmed by SDS–PAGE analysis. For SAXS, BLI, and thermal shift assays, unlabeled samples were required; for NMR titration experiments, ^{15}N-labeled samples; and for NMR resonance assignment, ^{13}C–^{15}N double-labeled samples were prepared. In all cases, I successfully produced Homer1 EVH1 protein variants (wild type, M65I, and S97L) in high purity, stable, and precipitation-free form.

1/b The intrinsically disordered GBR1–3 region of the produced GKAP protein is prone to precipitation. - O4, P4

The GBR1–3 region of the GKAP protein was expressed in E. coli BL21 cells and purified using a multistep protocol. The disordered sample showed a strong tendency to precipitate and was stable only in the presence of guanidinium hydrochloride. However, this condition does not maintain the protein in its native state and is therefore

unsuitable for structural studies. The purification protocol underwent extensive optimization, including testing multiple buffers and additives, resulting in a relatively stable sample for a short period of time. NMR measurements were performed and spectral evaluation was initiated, but the spectra were not of sufficient quality for complete resonance assignment. Further optimization steps will therefore be required in the future.

2. The solution-state structure of the Homer1 EVH1 domain reveals functionally important features. - *I. Publication, II. Publication, P1, O2*

The structure of the EVH1 domain of the Homer1 protein corresponds in its main features to previously described structures in the literature, containing a β-sandwich composed of seven β-strands and a long C-terminal α-helix. Compared to available X-ray crystallographic structures, however, minor differences were observed, primarily in loop regions near the ligand-binding site (residues 17–29 and 40–43).

The indole ring of Trp24, which is directly involved in ligand binding, also shows a different orientation. This suggests that ligand binding induces subtle structural and dynamic rearrangements within the domain, in contrast to the previously available structures that implied a completely rigid conformation.

3. The investigated mutations do not substantially alter the global or local structure of the EVH1 domain. - *I. Publication, II. Publication, P1, O2*

Comparison of the HSQC spectrum of the M65I mutant with that of the wild type shows that nearly all chemical shifts are displaced, with peaks shifted but remaining close relative to one another. In contrast, the S97L mutation causes only minimal spectral changes. Thus, the two mutations differ fundamentally in their effects.

Based on resonance assignment and analysis of Cα and Hα chemical shifts, no significant differences in secondary structure were detected relative to the wild type. SAXS measurements further confirmed that the global structure remains similar to the wild type in both mutants. CD measurements supported these findings. Therefore, although the $^{1}H–^{15}N$ HSQC spectra clearly indicate different effects of the two mutations, neither mutation induces major structural rearrangements.

4. Ligand binding differs only minimally among the variants, whereas thermal stability shows more pronounced differences. - *I. Publication, II. Publication, P1, P2, O1, O2*

Biolayer interferometry (BLI) experiments were performed using a short, unlabeled peptide (LVPPPEEFANG) derived from the PRO region of Shank3. In the case of the M65I variant, binding affinity

decreased slightly, whereas in the S97L variant, it increased slightly compared to the wild type.

NMR titration experiments showed similar types of peak changes for all three variants (wild type, M65I, and S97L), supporting the reliability of the results. In all cases, the largest changes were observed in regions directly involved in binding, consistent with known structural data.

Thermal stability measurements indicated a partial, mild stabilizing effect for S97L, whereas M65I caused destabilization of the domain. Binding to the Shank3 peptide did not significantly influence denaturation kinetics in either case.

5. The investigated mutations in EVH1 induce rearrangements in the internal dynamics of the domain. - *I. Publication, II. Publication, P1*

Dynamic analysis of the mutant variants revealed subtle but likely relevant differences compared to the wild type. The S^2 order parameters, characterizing fast (ps–ns) timescale motions, were highly similar across all three variants. In contrast, the distribution of Rex parameters, reflecting slower µs–ms timescale motions, showed characteristic differences.

CEST experiments performed to further characterize these conformational rearrangements did not yield conclusive results,

suggesting that the relevant motions may occur within a narrower timescale window. Gaussian Network Model (GNM) analysis of the EVH1 domain indicated the presence of slow internal motions that may be affected by the mutations. Several residues predicted to function as hinge points—or located in their immediate environment—showed larger changes in chemical shifts and/or Rex parameters. For example, residue M65, affected by the M65I mutation, participates in the two most dominant GNM modes and also exhibits changes in NMR-derived parameters.

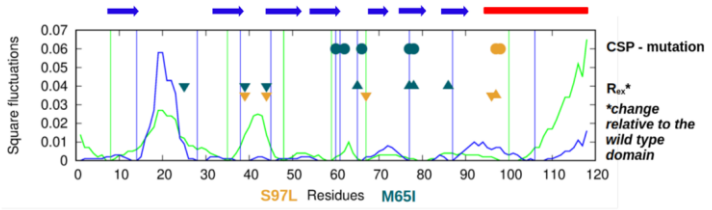

Figure 3. *Square fluctuations along the first and second modes shown along the sequence with green and blue lines, respectively. Vertical lines denote the positions of the hinge residues of the modes. Circles show the residues with substantial chemical shift perturbation relative to the wild type domain, the diamond the perturbation upon ligand binding that differs from the wild type. Triangles indicate sites with changes in*

substantial R_{ex} contributions relative to the wild type, symbols pointing downwards for decrease, those pointing upwards for increase in the R_{ex}. Sites are only shown where the change causes a transition to/from a large Rex (> 2,5 Hz) and the change itself exceeds 1 Hz. Symbols colored according to the mutants as shown in the key.

5. Discussion

In the course of my work, I determined the solution-state structure of the Homer1 EVH1 domain, which to my knowledge represents the first NMR structure of this domain. The structure indicates flexibility at the binding site, a finding further supported by my solution-state dynamic measurements. According to NMR data, the wild-type Homer1 EVH1 domain is rigid on the ps–ns timescale but exhibits more pronounced internal motions on the µs–ms timescale, as suggested by the observed Rex contributions. These motions affect the ligand-binding region as well as nearby positively charged surface loop regions identified in this study.

Results obtained for the two investigated mutants support this interpretation. SAXS and CD measurements clearly show that neither

S97L nor M65I significantly alters the overall fold of the domain. Similarly, interaction with a short Shank3 peptide containing only the minimal binding region does not show substantial changes as a result of the mutations.

However, analysis of dynamic properties reveals marked differences on a defined timescale. Notably, the presence of dynamic differences may explain the more pronounced changes observed in the HSQC spectrum of the M65I mutant, even if this initially appears surprising, and may provide insight into the functional significance of these mutations.

Although investigation of the GKAP guanylate kinase–binding region was also among my objectives, and some progress was achieved, I was ultimately unable to produce a construct suitable for detailed functional characterization within this project.

Overall, my results highlight the complementary nature of structural biology techniques and demonstrate that solution-state NMR studies can provide important new insights—even for a well-characterized protein family—particularly with regard to dynamic behavior.

6. Publications

Publications forming the basis of the theses:

I. Publication: Zs. E. Kálmán, A.Czajlik, B. Maruzs, **F. Farkas**, I. Pap, Cs. Homonnay, T. Klumpler, Gy. Batta, Z. Gáspári, B. Péterfia, "Structural modelling and dynamics of the full-length Homer1 multimer"
doi: https://doi.org/10.1101/2025.05.26.655084

II. Publication: **F. Farkas,** B. Maruzs**,** Zs. E. Kálmán-Dobson, T. Klumpler, Gy. Batta, B. Ferenc Péterfia, Z. Gáspári, "Modulation of the internal dynamics of the Homer1 EVH1 domain by putative autism-associated mutations"

doi:

<u>Poster and oral presentations related to the dissertation:</u>

P1 **F. Farkas**, Zs. E. Dobson-Kálmán, G. Batta, B.F. Péterfia, Z. Gáspári "Structural investigation of the wild-type and mutant EVH1 domain of the Homer1 protein in regards of Shank interaction" , 49th FEBS CONGRESS, Istanbul, Turkey, 05-09 July 2025.

P2 **F. Farkas**, Zs. E. Dobson-Kálmán, G. Batta, B.F. Péterfia, Z. Gáspári, „The Homer1-Shank3 interaction: NMR and SAXS studies on wild type and mutant Homer1 EVH1 domains" Magyar Molekuláris Élettudományi Konferencia, Eger, Magyarország, 28-30 March 2025.

P3 **F. Farkas**, Zs. E. Dobson-KálmánI G. Batta, B.F. Péterfia, Z. Gáspári, „Structural studies of the HOMER1 protein: combining

experiments and modeling", 48th FEBS CONGRESS, Milano, Olaszország, 29 Jun- 03 July 2024.

P4 **F. Farkas,** A. Czajlik, G. Batta, B. Péterfia, Z. Gaspari, **"**Atomiclevel investigations of the PSD-95 GK domain and its interaction with GKAP" July, 2021 – The 45th FEBS Congress, Ljubljana, Slovenia (online conference).

O1 **F.Farkas.** 'Investigation of the Homer1:Shank3 interaction." Jun, 2025 - PhD Proceedings, Budapest Hungary. Scientific talk.

O2 **F.Farkas. "**Structural and functional investigation of the wild type and mutant EVH1 domain of the HOMER1 protein." Jun, 2024 - PhD Proceedings, Budapest Hungary. Scientific talk.

O3 **F.Farkas. "**Structural and functional investigation of the postsynaptic Homer protein". Jun, 2023 - PhD Proceedings, Budapest Hungary. Scientific talk.

O4 **F.Farkas. "**Optimization of the expression and purification protocol of the GBR region of GKAP protein and initial NMR analysis." Jun, 2022 - PhD Proceedings, Budapest Hungary. Scientific talk.

<u>Other publications not related to the dissertation:</u>

Sz1 E. Nagy-Kanta, Zs. E. Kálmán, H. Tossavainen, T. Juhász, **F. Farkas**, J. Hegedüs, M. Keresztes, T. Beke-Somfai, Z.Gáspári, P. Permi, B.Péterfia "Residual flexibility in the topologically constrained multivalent complex between the GKAP scaffold and LC8 hub

proteins”

doi: https://doi.org/10.1111/febs.70219

References

Ball, L. J., Jarchau, T., Oschkinat, H., & Walter, U. (2002). EVH1 domains: Structure, function and interactions. *FEBS Letters*, *513*(1), 45–52. https://doi.org/10.1016/s0014-5793(01)03291-4

Beneken, J., Tu, J. C., Xiao, B., Nuriya, M., Yuan, J. P., Worley, P. F., & Leahy, D. J. (2000). Structure of the Homer EVH1 domain-peptide complex reveals a new twist in polyproline recognition. *Neuron*, *26*(1), 143–154. https://doi.org/10.1016/s0896-6273(00)81145-9

Boeckers, T. M. (2006). The postsynaptic density. *Cell and Tissue Research*, *326*(2), 409–422. https://doi.org/10.1007/s00441-006-0274-5

Chen, X., Vinade, L., Leapman, R. D., Petersen, J. D., Nakagawa, T., Phillips, T. M., Sheng, M., & Reese, T. S.

(2005). Mass of the postsynaptic density and enumeration of three key molecules. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(32), 11551–11556. https://doi.org/10.1073/pnas.0505359102

Dosemeci, A., Weinberg, R. J., Reese, T. S., & Tao-Cheng, J.-H. (2016). The Postsynaptic Density: There Is More than Meets the Eye. *Frontiers in Synaptic Neuroscience*, *8*, 23. https://doi.org/10.3389/fnsyn.2016.00023

Droogers, W. J., & MacGillavry, H. D. (2023). Plasticity of postsynaptic nanostructure. *Molecular and Cellular Neurosciences*, *124*, 103819. https://doi.org/10.1016/j.mcn.2023.103819

Feng, W., Tu, J., Yang, T., Vernon, P. S., Allen, P. D., Worley, P. F., & Pessah, I. N. (2002). Homer regulates gain of ryanodine receptor type 1 channel complex. *The Journal of Biological Chemistry*, *277*(47), 44722–44730. https://doi.org/10.1074/jbc.M207675200

Feng, W., & Zhang, M. (2009). Organization and dynamics of PDZ-domain-related supramodules in the postsynaptic density. *Nature Reviews. Neuroscience*, *10*(2), 87–99. https://doi.org/10.1038/nrn2540

Grabrucker, A. M., Schmeisser, M. J., Schoen, M., & Boeckers, T. M. (2011). Postsynaptic ProSAP/Shank scaffolds in the cross-hair of synaptopathies. *Trends in Cell Biology*, *21*(10), 594–603. https://doi.org/10.1016/j.tcb.2011.07.003

Hayashi, M. K., Ames, H. M., & Hayashi, Y. (2006). Tetrameric hub structure of postsynaptic scaffolding protein homer. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *26*(33), 8492–8501. https://doi.org/10.1523/JNEUROSCI.2731-06.2006

Hayashi, M. K., Tang, C., Verpelli, C., Narayanan, R., Stearns, M. H., Xu, R.-M., Li, H., Sala, C., & Hayashi, Y. (2009). The postsynaptic density proteins Homer and Shank form a polymeric network structure. *Cell*, *137*(1), 159–171. https://doi.org/10.1016/j.cell.2009.01.050

Luo, P., Fei, F., Zhang, L., Qu, Y., & Fei, Z. (2011). The role of glutamate receptors in traumatic brain injury: Implications for postsynaptic density in pathophysiology. *Brain Research Bulletin*, *85*(6), 313–320. https://doi.org/10.1016/j.brainresbull.2011.05.004

Luo, P., Li, X., Fei, Z., & Poon, W. (2012). Scaffold protein Homer 1: Implications for neurological diseases. *Neurochemistry International*, *61*(5), 731–738. https://doi.org/10.1016/j.neuint.2012.06.014

Romorini, S., Piccoli, G., Jiang, M., Grossano, P., Tonna, N., Passafaro, M., Zhang, M., & Sala, C. (2004). A functional role of postsynaptic density-95-guanylate kinase-associated protein complex in regulating Shank assembly and stability to synapses. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *24*(42), 9391–9404. https://doi.org/10.1523/JNEUROSCI.3314-04.2004

Sala, C., Vicidomini, C., Bigi, I., Mossa, A., & Verpelli, C. (2015). Shank synaptic scaffold proteins: Keys to understanding the pathogenesis of autism and other

synaptic disorders. *Journal of Neurochemistry*, *135*(5), 849–858. https://doi.org/10.1111/jnc.13232

Sheng, M., & Hoogenraad, C. C. (2007). The postsynaptic architecture of excitatory synapses: A more quantitative view. *Annual Review of Biochemistry*, *76*, 823–847. https://doi.org/10.1146/annurev.biochem.76.060805.160029

Shin, S. M., Zhang, N., Hansen, J., Gerges, N. Z., Pak, D. T. S., Sheng, M., & Lee, S. H. (2012). GKAP orchestrates activity-dependent postsynaptic protein remodeling and homeostatic scaling. *Nature Neuroscience*, *15*(12), 1655–1666. https://doi.org/10.1038/nn.3259

Shiraishi-Yamaguchi, Y., & Furuichi, T. (2007). The Homer family proteins. *Genome Biology*, *8*(2), 206. https://doi.org/10.1186/gb-2007-8-2-206

Suzuki, T., Kametani, K., Guo, W., & Li, W. (2018). Protein components of post-synaptic density lattice, a backbone structure for type I excitatory synapses. *Journal of*

Neurochemistry, *144*(4), 390–407. https://doi.org/10.1111/jnc.14254

Verpelli, C., Schmeisser, M. J., Sala, C., & Boeckers, T. M. (2012). Scaffold proteins at the postsynaptic density. *Advances in Experimental Medicine and Biology*, *970*, 29–61. https://doi.org/10.1007/978-3-7091-0932-8_2