Neuroscience 171 (2010) 421–433

PROLINE-RICH SYNAPSE-ASSOCIATED PROTEIN-1 AND 2 (ProSAP1/Shank2 AND ProSAP2/Shank3)—SCAFFOLDING PROTEINS ARE ALSO PRESENT IN POSTSYNAPTIC SPECIALIZATIONS OF THE PERIPHERAL NERVOUS SYSTEM

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Abstract—Proline-rich synapse-associated protein-1 and 2 (ProSAP1/Shank2 and ProSAP2/Shank3) were originally found as synapse-associated protein 90/postsynaptic density protein-95-associated protein (SAPAP)/guanylate-kinase-associated protein (GKAP) interaction partners and also isolated from synaptic junctional protein preparations of rat brain. They are essential components of the postsynaptic density (PSD) and are specifically targeted to excitatory asymmetric type 1 synapses. Functionally, the members of the ProSAP/Shank family are one of the postsynaptic key elements since they link and attach the postsynaptic signaling apparatus, for example N-methyl-D-aspartic acid (NMDA)-receptors via direct and indirect protein interactions to the actin-based cytoskeleton. The functional significance of ProSAP1/2 for synaptic transmission and the paucity of data with respect to the molecular composition of PSDs of the peripheral nervous system (PNS) stimulated us to investigate neuromuscular junctions (NMJs), synapses of the superior cervical ganglion (SCG), and synapses in myenteric ganglia as representative synaptic junctions of the PNS. Confocal imaging revealed ProSAP1/2-immunoreactivity (-iry) in NMJs of rat and mouse sternomastoid and tibialis anterior muscles. In contrast, ProSAP1/2-iry was only negligibly found in motor endplates of striated esophageal muscle probably caused by antigen masking or a different postsynaptic molecular anatomy at these synapses. ProSAP1/2-iry was furthermore detected in cell bodies and dendrites of superior cervical ganglion neurons and myenteric neurons in esophagus and stomach. Ultrastructural analysis of ProSAP1/2 expression in myenteric ganglia demonstrated that ProSAP1 and ProSAP2 antibodies specifically labelled PSDs of myenteric neurons. Thus, scaffolding proteins ProSAP1/2 were found within the postsynaptic specializations of synapses within the PNS, indicating a similar molecular assembly of central and peripheral postsynapses.

Key words: postsynaptic density, peripheral nervous system, motor endplate, superior cervical ganglion, myenteric ganglion, intraganglionic laminar endings.

The postsynaptic density (PSD) is a cytoskeletal specialization close to the postsynaptic membrane and the active zone (AZ). It constitutes a structural matrix that anchors and clusters neurotransmitter receptors, synaptic cell adhesion molecules and components of intracellular signalling pathways exactly opposite to the site of neurotransmitter release, thus representing a general organizer of the postsynaptic signal transduction machinery (Ziff, 1997; O'Brien et al., 1998; Kennedy, 2000). Consequently, the identification of the protein components of the PSD is a main topic in synaptic research (Walsh and Kuruc, 1992; Langnaese et al., 1996; Kennedy, 1998).

ProSAP1 is one of the components of the PSD belonging to the ProSAP/Shank family (Sheng and Kim, 2000; Böckers, 2006). Originally isolated as a 180 kDa protein of synaptic junctional protein preparations from rat brain, it was ultrastructurally localized within the PSD (Langnaese et al., 1996; Böckers et al., 1999a). The protein is identical to Shank2 and CortBP1, which has been identified as an interaction partner of the actin-binding protein cortactin (Du et al., 1998), indicating that ProSAP1/CortBP1 is one of the molecules that link the postsynaptic signalling apparatus to the actin-based cytoskeleton (Böckers et al., 1999a). Immunohistochemical studies at the light and electron microscopic level revealed that ProSAP1 is widely expressed in neurons and located in the submembraneous matrix of the PSD primarily at excitatory asymmetric type 1 synapses (Böckers et al., 1999a,b).

ProSAP2/Shank3 is a very closely related multidomain protein that is coexpressed in many regions of the rat brain including cerebral cortex and hippocampus, whereas a complementary expression pattern has been observed in the cerebellum (Böckers et al., 1999b).

Functionally ProSAP/Shank proteins have been characterized as multi-domain scaffolding molecules that act as the molecular interface between glutamate receptor clusters, for example N-methyl-D-aspartic acid (NMDA)-

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Abbreviations: ANS, autonomic nervous system; AZ, active zone; BT, α-bungarotoxin; DAB, diaminobenzidine; ENS, enteric nervous system; GFAP, glial fibrillary acidic protein; GKAP, guanylate-kinase-associated protein; IGLE, intraganglionic laminar ending; -ir, immunoreactive; -iry, immunoreactivity; MaGuk, membrane-associated guanylate kinases; mGLUR, metabotropic glutamate receptor; nAChR, nicotinic acetylcholine receptor; NADPH-d, nicotinamide adenine dinucleotide phosphate – diaphorase; NF, neurofilament; NMDA, N-methyl-D-aspartic acid; NMJ, neuromuscular junction; PNS, peripheral nervous system; ProSAP1, proline-rich synapse-associated protein-1; ProSAP2, proline-rich synapse-associated protein-2; PSD, postsynaptic density; SAPAP, synapse-associated protein 90/postsynaptic density protein-95-associated protein; SC, synaptic cleft; SCG, superior cervical ganglion; SIB, single IGLE-bouton; SV, synaptic vesicles; SYN, synaptophysin; TH, tyrosine hydroxylase; VAChT, vesicular acetylcholine transporter; VGLUT, vesicular glutamate transporter.

0306-4522/10 $ - see front matter
doi:10.1016/j.neuroscience.2010.08.041

receptors and the actin-based cytoskeleton (Böckers et al., 2002, 2005; Sheng and Kim, 2002). Furthermore, ProSAP/Shank proteins bind to Homer, a metabotropic glutamate receptor (mGLUR) binding protein (Tu et al., 1999; Sheng and Kim, 2000). These interactions increase the complexity and regulatory capability of the postsynaptic complex (Kennedy, 2000) and the potential of these proteins has been impressively shown by their ability to induce spines in normally aspiny neurons (Roussignol et al., 2005).

Members of the ProSAP/Shank family have been identified in different parts of the CNS (Böckers et al., 2002, 2005; Brandstätter et al., 2004), but also in some non-neuronal tissue as thymus, endocrine cells and testicular germ cells (Redecker et al., 2001, 2003, 2006). However, there are no data on the ultrastructural localization and hitherto no investigations were performed within the peripheral nervous system (PNS). This lack of data prompted us to investigate the occurrence of ProSAP1/Shank2 and ProSAP2/Shank3 in neuromuscular junctions (NMJs), synapses in the superior cervical ganglion (SCG), and synapses in myenteric ganglia as representative synaptic junctions of the PNS.

The NMJ has served as a prototype for understanding synaptic transmission (Fagerlund and Eriksson, 2009). There is also evidence that glutamatergic NMDA-receptors, in addition to nicotinic acetylcholine receptors (nAChR), are located within the postsynaptic membrane of skeletal motor endplates (Berger et al., 1995; Urazaev et al., 1995, 1998; Grozdanovic and Gossrau, 1997). Autonomic synapses of the SCG are often used to investigate the synaptic transmission process (Forssmann, 1964; Kiyama et al., 1993; Hou and Dahlstrom, 2000; Ito et al., 2005; Gingras et al., 2006). A third group of synapses representative for the PNS is found in the enteric nervous system (ENS).

Parts of this study have been previously presented in abstract form (Raab et al., 2010).

EXPERIMENTAL PROCEDURES

Adult mice (n=9; C57Bl/6, The Jackson Laboratory, Bar Habor, USA; stock number 00664, inbred) and adult rats (n=4; Wistar, Charles River Laboratory, Kißlegg, Germany, 200–300 g) were used. The federal animal welfare legislation implemented by the local government was followed for all procedures.

Tissue preparation for immunohistochemistry

Mice were deeply anesthetized with an overdose of carbon dioxide, and euthanized by Thiopental (Trapanal; Byk Gulden, Konstanz, Germany; 250 mg/kg i.p.). When they were unresponsive to nociceptive stimuli, the abdomen and the thorax were opened, and the mice were perfused transcardially with 20 ml Ringer solution (Berlin-Chemie AG, Berlin, Germany) containing 1000 IE/100 ml heparin (ratiopharm GmbH, Ulm, Germany) followed by 80 ml 3% paraformaldehyde (Roth, Karlsruhe, Germany) and 10 ml 15% sucrose phosphate buffer (pH 7.4; Roth) (n=5). The esophagus from the level of the cricoid cartilage to the gastroesophageal junction, stomach, SCG, sternomastoid and tibialis anterior muscles were removed, transferred to 15% sucrose phosphate buffer (pH 7.4) at 4 °C, and freed from adhering connective tissue under a dissecting microscope.

Rats (n=4) were killed by an overdose of carbon dioxide and the esophagi were removed from level of the cricoid cartilage to the gastroesophageal junction. Esophagi were immersion fixed with 3% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4 °C and transferred to 15% sucrose phosphate buffer (pH 7.4).

The following day, mouse and rat esophagi were divided into three segments of similar length, which defined the cervical (= upper third), thoracic (=middle third) and abdominal (=lower third) parts, respectively. Together with stomach, ganglia and muscles, the segments of the esophagi were each mounted in Tissue-Tek (GSV 1, Slee Technik, Mainz, Germany), rapidly frozen in methylbutan (Roth) at −70 °C and stored at −20 °C.

Esophageal wholemount preparation for electron microscopy

For ultrastructural examinations (n=4) wholemount preparations were done as previously described (Raab and Neuhuber, 2005). Mice were transcardially perfused as described above. After the prewash a small polyethylene tube was inserted to distend the esophagus before fixation started. Three % paraformaldehyde and 0.05% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3; Merck, Darmstadt, Germany) were used as fixative. The removed esophagi were freed from adhering connective tissue including main vagal nerve trunks under a dissecting microscope and opened approximately along the ventral midline. Wholemounts of muscularis and mucosa were prepared by separating both layers along the submucosal plane using jeweller's forceps and stored in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C or immediately processed for nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry.

Antibodies and reagents

The antibody against ProSAP1 was raised in rabbit against the C-terminal part of ProSAP1 (encoding amino acids 355–509 and 826–1259) (Böckers et al., 1999a). The antibody against ProSAP2 was raised in rabbit against the C-terminal part of ProSAP2/Shank3 (amino acids 1055–1806) (Bockmann et al., 2002).

All primary and secondary antibodies used in this study are listed in Tables 1 and 2.

Double- and triple label immunohistochemistry

12 μm thick cryostat sections of mouse and rat esophagi were mounted on poly-L-lysine-coated slides and dried for 1 h at room temperature. Sections were preincubated with 5% normal donkey serum (DAKO, Hamburg, Germany) containing 0.5% Triton X-100 (Merck), 1% bovine 1% bovine serum albumin (BSA; Roth) in TRIS buffered saline (TBS, pH 7.4; Roth) for 1 h at room temperature. After a TBS buffer rinse, sections were incubated for double and triple immunolabeling. For double immunohistochemistry, primary antibodies against ProSAP1 and ProSAP2, respectively, and synaptophysin (SYN), vesicular glutamate transporter 2 (VGLUT2), vesicular acetylcholine transporter (VAChT), neuronal nitric oxide synthase (nNOS), anti-protein gene product 9.5 (PGP 9.5) and tyrosine hydroxylase (TH) were used. NMJ were specifically labelled with α-bungarotoxin (BT) and primary antibodies against ProSAP1/2 and VAChT/anti-neurofilament 200 (NF 200) and glial fibrillary acidic protein (GFAP), respectively. Antibodies against VAChT/NF 200 were used for specifically labelling cholinergic/presynaptic motor terminals and GFAP immunostaining was performed for displaying terminal Schwann-Cells (teloglia). Myenteric plexus were studied using triple staining for ProSAP1/2, VAChT, and nNOS and triple immunostaining for ProSAP1/2, VAChT and TH and PGP 9.5, respectively, was performed in the SCG. All primary antibodies were diluted in TBS containing 1% BSA, 0.5% Triton X-100 at room temperature overnight. After a TBS rinse, sections were incubated with donkey anti-goat secondary antibody coupled to Alexa Fluor® 647, donkey anti-mouse