

Mass Spectrometric Analysis of Synapsins in *Drosophila melanogaster* and Identification of Novel Phosphorylation Sites

Tulip Nuwal,^{†,§} Seok Heo,^{‡,§} Gert Lubec,[‡] and Erich Buchner^{*,†}

Department of Neurobiology and Genetics, Biozentrum, University of Wuerzburg, Am Hubland, 97074 Wuerzburg, Germany, and Department of Pediatrics, Medical University of Vienna, Waehringer Guertel 18, 1090 Vienna, Austria

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Synapsins are synaptic vesicle-associated phosphoproteins that play a major role in the fine regulation of neurotransmitter release. In *Drosophila*, synapsins are required for complex behavior including learning and memory. Synapsin isoforms were immunoprecipitated from homogenates of wild-type *Drosophila* heads using monoclonal antibody 3C11. Synapsin null mutants (*Syn*⁹⁷) served as negative controls. The eluted proteins were separated by SDS-PAGE and visualized by silver staining. Gel pieces picked from five bands specific for wild type were analyzed by nano-LC–ESI–MS/MS following multienzyme digestion (trypsin, chymotrypsin, AspN, subtilisin, pepsin, and proteinase K). The protein was unambiguously identified with high sequence coverage (90.83%). A number of sequence conflicts were observed and the N-terminal amino acid was identified as methionine rather than leucine expected from the cDNA sequence. Several peptides from the larger isoform demonstrated that the in-frame UAG stop codon at position 582 which separates two large open reading frames is read through by tRNAs for lysine. Seven novel phosphorylation sites in *Drosophila* synapsin were identified at Thr-86, Ser-87, Ser-464, Thr-466, Ser-538, Ser-961, and Tyr-982 and verified by phosphatase treatment. No phosphorylation was observed at the conserved PKA/CaM kinase-I/IV site (RRFS, edited to RGFS) in domain A or a potential PKA site near domain E.

Keywords: *Drosophila melanogaster* • synapsin • immunoprecipitation • mass spectrometry • phosphorylation • UAG suppression • post-translational modification

Introduction

Neurotransmitter release from presynaptic nerve terminals must be delicately regulated if the brain is to produce adapted behavior modulated by experience. Among the large number of proteins involved in the fine regulation of transmitter release from presynaptic terminals, synapsin phosphoproteins constitute the most abundant family. Information on the structure and function of synapsins in mammals has been extensively reviewed.^{1–7} Briefly, mammalian synapsins are encoded by three genes with alternate splicing leading to at least 10 isoforms.^{3,7,8} Common to all known synapsins is an N-terminal A-domain and a large, highly conserved central C-domain, whereas B- and D- through I-domains are more variable and define specific isoforms.⁹ Mammalian synapsins have been shown to bind with domains C and E to each other and to lipid and protein components of synaptic vesicles in a phosphorylation-dependent manner.^{10–12} They also bind to microtubules and various cytoskeletal proteins, including actin and spectrin.^{13–18}

Mammalian synapsins can be phosphorylated in domain A by cAMP-dependent protein kinase (PKA) and Ca²⁺/calmodulin-dependent protein kinases (CaM kinases) I and IV,^{19,20} in domain B and D by mitogen-activated protein kinase/Erk 1/2 (MAPK/Erk),²¹ in domain C by the tyrosine kinase Src,²² in domain D by CaM kinase II²³ and Cdk1/5.²¹ Recently, a novel phosphorylation site at S-546 has been discovered.²⁴ Present views propose that synapsins tether synaptic vesicles (SVs) to actin filaments of the presynaptic cytoskeleton thereby establishing a reserve pool of SVs. When synapsins are phosphorylated by various kinases at specific sites, SVs detach from the cytoskeleton and move to the sites of exocytosis at the active zone to supplement the readily releasable pool of SVs. However, activity-induced increase of SV mobility is independent of synapsins in mouse motor nerve terminals,²⁵ and differential functions in development have been described for several isoforms. In addition, properties of mammalian synapsins like ATP binding,²⁶ structural similarity to ATPases,²⁷ and the functions of the various phosphorylation sites are not fully understood. Synapsin I knockout mice show increased propensity for epileptic seizures.²⁸ In humans, synapsins are involved in neurological diseases like Alzheimer's,^{29,30} bipolar disorder, and schizophrenia,³¹ and a family has been described in which a nonsense mutation in synapsin I is linked to epilepsy, learning difficulties, and aggressive behavior.³² Re-

* Corresponding author: Erich Buchner, Department of Neurobiology and Genetics, BioCenter, University of Wuerzburg, Am Hubland, 97074 Wuerzburg, Germany. Tel: +49-931-31-84478. Fax: +49-931-31-84452. E-mail: buchner@biozentrum.uni-wuerzburg.de.

† University of Wuerzburg.

‡ Both authors contributed equally to this work.

§ Medical University of Vienna.

cently it was reported that polymorphisms in the synapsin III gene might be linked to multiple sclerosis in an Italian population.^{33,34}

In the *Drosophila* genome, a single synapsin gene (*Syn*, CG3985) encodes protein isoforms with high homology to mammalian synapsins, showing 50% amino acid identity in the central vesicle- and actin-binding C domain and less pronounced similarity in the A and E domains.^{3,35} Two splice variants RA (identical to RD, RE, RF) and RC, differing by 4 bases, have been described.^{35,36} All predicted proteins contain the conserved C domain but differ at the N- and C-terminus. For prediction of the N-terminus of the PA, PC, and PF isoforms, the canonical first ATG codon was used.³⁶ However, since the conserved A-domain lies upstream of this ATG, the usage of the first CTG as a possible translation start was assumed for isoforms PD and PE.^{35,36} Edman degradation of immunoprecipitated synapsin has identified the second CTG as the likely start codon which lies immediately upstream of the conserved PKA recognition motif in domain A.³⁷ In Western blots, three small isoforms of 70, 74, and 80 kDa and two large isoforms of about 140 kDa have been observed.³⁵ The RA splice variant codes for two large open reading frames (ORFs) separated by a single UAG stop codon. Western blots had shown that the polypeptides encoded by the two ORFs are connected to generate the large isoforms.³⁵ Two possible molecular mechanisms have been proposed to explain the linkage of the two ORFs, UAG read-through³⁵ or removal of the UAG by splicing.³⁷ In the A domain of most *Syn* transcripts of larvae and adults, the conserved PKA consensus sequence RR_xS is edited by the ADAR enzyme to RGxS.³⁸ An N-terminal undecapeptide containing the genomic RRFS sequence was shown to be an excellent substrate for bovine PKA, whereas the undecapeptide with the edited RGFS sequence is not significantly phosphorylated by bovine PKA.³⁸ Phosphorylation of synapsins in *Drosophila* embryos has been studied by a large-scale phosphoproteome analysis using phosphopeptide enrichment and mass spectrometry. Three phosphorylated serines have been found in the nonconserved C-terminal domain of the short isoform.³⁹

To obtain information on the systemic function of synapsins in *Drosophila*, the expression pattern of the *Syn* gene and the phenotype of null mutants have been analyzed. Immunohistochemistry using monoclonal antibody anti-Synorf1 (3C11) which recognizes all known *Drosophila* synapsin isoforms revealed specific staining in all neuropil regions and in type I boutons of larval neuromuscular preparations.³⁷ Characterization of synapsin null mutants (*Syn*⁹⁷) which suffered a deletion of the promoter region and the first exon demonstrated that the development of basic nervous system structure and function does not require synapsins.³⁷ No striking qualitative defects in synaptic morphology or synaptic transmission at the larval neuromuscular junction (NMJ) were observed.³⁷ A recent FM1-43 dye uptake study on third instar larval NMJs of *Drosophila* synapsin null mutants (*Syn*⁹⁷) has revealed that the localization of vesicles in the recycling pool at presynaptic boutons is modified in the absence of synapsin.⁴⁰ It was also shown that the number of vesicles in the reserve pool is diminished and basal transmitter release after intense stimulation is increased in the null mutants.^{40,41} Quantitative comparison of mutant and wild-type behavior revealed minor differences in locomotion and certain visual responses as well as defects in several learning and memory paradigms such as adult courtship conditioning³⁷ and olfactory associative conditioning of both

larvae⁴² and adults.^{37,43} Here, we use multienzyme digestion and nano-LC–ESI–MS/MS of immunoprecipitated *Drosophila* synapsin to determine the N-terminus and phosphorylation sites of this protein and investigate the mechanism by which the large isoforms are generated.

Materials and Methods

A. Animals. We used the wild-type strain Canton S (CS) of *Drosophila melanogaster* and the “cantonized” synapsin null mutant *Syn*⁹⁷ for this study.³⁷ The flies were approximately 3–5 days old and were grown in large vials containing standard cornmeal food medium. The vials were maintained at 25 °C, 60–70% relative humidity, with a 14/10-h light/dark cycle. The flies were collected in a 100 mL Falcon tube and immediately frozen in liquid nitrogen. The tube was vortexed vigorously to dissociate the various body parts. Heads were collected using a 500 μm sieve and kept in liquid nitrogen until used for further analysis.

B. Sample Preparation. A total of 375 mg of *Drosophila* heads (approximately 2500 heads) was homogenized using precooled glass homogenizers (Hartenstein, Germany) in homogenization buffer consisting of 150 mM NaCl (chemicals from AppliChem, Germany, unless otherwise noted), 0.1% nonidet P-40 (NP-40), 20 mM Tris-HCl, pH 7.6, 10 mM sodium fluoride (NaF), 10 mM β-glycerol phosphate, and EDTA-free protease inhibitors (“complete, mini”, Roche, Switzerland), 2 tablets in 10 mL of buffer. The homogenate was cleared by centrifugation at 16 000g at 4 °C. The cleared homogenate was transferred to a fresh tube and ultracentrifuged (L8–70M, Beckman, Irvine, CA) at 100 000g for 60 min at 4 °C. The final cleared lysate was transferred to fresh 15 mL Falcon tubes and kept on ice until used for further analysis.

C. Immunoprecipitation. A total of 50 μL of protein G-agarose beads (Roche, Switzerland) was added to 2 mL of 3C11 hybridoma supernatant in two polyethylene filter attached centrifuge columns (pore size 30 μm) and incubated on a rotator for 4 h at 4 °C. The columns were placed in a 15 mL Falcon tube and centrifuged at 300g for 1 min to remove supernatant components not bound to the beads. The columns were washed 3 times with wash buffer containing 500 mM NaCl, 0.1% NP-40, 20 mM Tris-HCl, pH 7.6, 10 mM NaF, 10 mM β-glycerol phosphate, and “complete, mini” EDTA-free protease inhibitors, 2 tablets in 10 mL of buffer. The wash buffer was completely removed from the columns by centrifugation at 300g for 5 min. The cleared lysates from CS and *Syn*⁹⁷ were added to the antibody-coupled beads in two columns and incubated overnight (16–18 h) on a rotator at 4 °C. The columns were centrifuged at 300g for 1 min and the flow-through was discarded. The columns were washed 7 times with 2 mL of wash buffer each. The columns were centrifuged at 300g for 1 min after each wash to remove the wash buffer and the centrifugation after the last wash was carried out for 5 min to remove any traces of the buffer. The beads were incubated in 50 μL of 4× LDS sample buffer (Invitrogen, Germany) for 30 min. The columns were centrifuged at 500g and the flow-through was collected in fresh Eppendorf tubes. To the tubes, 6 μL of 0.5 M dithiothreitol (DTT; Sigma, Germany) was added and the tubes were incubated in a heat block at 70 °C for 10 min. The tubes were cooled on ice and 15 μL per lane was

loaded on a 10% precast Bis-Tris gel (Invitrogen, Germany) for further analysis.

D. SDS-PAGE and Detection by MS Compatible Silver Staining. SDS-PAGE was performed using 10% precast Bis-Tris gels at constant voltage of 200 V in a MOPS running buffer (Invitrogen, Germany). Upon completion, the gel was transferred to a clean and dry glass chamber for silver staining according to modified Blum silver staining protocol for mass spectrometric compatibility.⁴⁴ Briefly, the gel was fixed in a solution containing 40% ethanol, 10% acetic acid at room temperature for 1 h on a shaker. The gel was washed with 30% ethanol for 20 min on a shaker and this step was repeated two more times with the final wash being with ultra pure water. Sensitization of the gel was performed by incubation in 0.02% sodium thiosulfate (Sigma, Germany) for 1 min and the gel was immediately washed 3 times with ultra pure water for a total of 1 min. Cold staining solution containing 0.2% silver nitrate (Sigma, Germany) and 0.007% formalin (Sigma, Germany) was added and the gel was incubated at 4 °C for 20 min on a shaker. The gel was washed with ultra pure water 4 times for a total of 1 min and then transferred to a new glass chamber. The gel was again washed for 1 min with ultra pure water. For visualization of the protein bands, the gel was incubated with developer solution containing 3% sodium carbonate and 0.05% formalin until sufficient signal to background contrast was obtained. The reaction was terminated by addition of 5% acetic acid. The gel was washed and stored in 1% acetic acid until further analysis.

E. In-Gel Multienzyme Digestion of Immunoprecipitated *D. melanogaster* Synapsin. Gel pieces from SDS-PAGE gels were cut into small pieces to increase surface and placed into a 1.5 mL Eppendorf tube. They were washed with 100 μL of destaining solution (50 mM potassium hexacyanoferrate, 300 mM sodium thiosulfate) for 10 min with vortexing. Destained gel pieces were then washed 4 times with washing solution (50% methanol, 40% water, and 10% glacial acetic acid) for 5 min each with vortexing. An aliquot of 100 μL of 100% acetonitrile (ACN) was added to the tube to cover the gel pieces completely. Gel pieces were subsequently incubated for 10 min and dried completely using a SpeedVac concentrator. Reduction of cysteine residues was carried out with a 10 mM DTT solution in 100 mM ammonium bicarbonate, pH 8.6, for 60 min at 56 °C. After discarding the DTT solution, the same volume of a 55 mM iodoacetamide (IAA) solution in 100 mM ammonium bicarbonate buffer, pH 8.6, was added and the gel pieces were incubated in darkness for 45 min at 25 °C to achieve alkylation of cysteine residues. The IAA solution was replaced by washing buffer (50% 100 mM ammonium bicarbonate, 50% ACN) and washing was done twice for 15 min each with vortexing. Gel pieces were washed and dried in 100% ACN followed by drying using a SpeedVac.

The dried gel pieces were reswollen with 12.5 ng/μL trypsin (Promega, Germany) solution reconstituted with 25 mM ammonium bicarbonate. Gel pieces were incubated for 16 h (overnight) at 37 °C. Endoproteinase AspN (Roche Diagnostics) digestion was performed in 25 mM ammonium bicarbonate at 37 °C for overnight. For subtilisin digestion, the gel pieces were covered with 30 μL of 10 ng/μL subtilisin (proteinase from *Bacillus subtilis* var. *biotescus* A. Sigma) in a digestion buffer consisting of 6 M urea and 1 M Tris, pH 8.5, then rehydrated for 10 min at 4 °C. The excess enzyme was removed and replaced by 50 mM ammonium bicarbonate. Gel pieces were incubated for 1 h at 37 °C. The enzymatic reaction was stopped

by adding 10% formic acid to achieve a final concentration of 1% formic acid). Proteinase K (Sigma) digestion was carried out in 50 mM ammonium bicarbonate and kept at 37 °C for 1 h. Pepsin (Sigma) digestion was performed in 0.1 M HCl (pH 1.0) and kept at 37 °C for 1 h.

After the incubation, the supernatant was transferred to new 0.5 mL tubes, and the peptides were extracted with 50 μL of 0.5% formic acid, 20% ACN for 20 min in a sonication bath. This step was repeated three times. Samples in extraction buffer were pooled in 0.5 mL tubes and evaporated in a SpeedVac concentrator. The volume was reduced to approximately 15 μL and then 15 μL of HPLC grade water (Sigma) was added for nano-LC–ESI–(CID/ETD)–MS/MS analysis via high capacity ion trap (HCT; Bruker, Germany).

F. Phosphatase Treatment of *Drosophila* Synapsin. *Drosophila* synapsin gel pieces were destained, reduced, alkylated, and dried as described above. The dried gel pieces were incubated in a solution of 0.5 μL of calf intestine alkaline phosphatase (New England Biolabs, Ipswich, MA) in the presence of 100 mM ammonium bicarbonate for 1 h at 37 °C. The gel pieces were then washed with washing solution (50% 100 mM ammonium bicarbonate, 50% ACN), shrunk in 100% ACN, and dried in a SpeedVac followed by in-gel digestion and extraction for nano-LC–ESI–(CID/ETD)–MS/MS analysis via high capacity ion trap (HCT; Bruker, Germany).

G. Peptide Analysis by Nano-LC–ESI–(CID/ETD)–MS/MS HCT. Trypsin digested peptides were separated by bio-compatible Ultimate 3000 nano-LC system (Dionex, Sunnyvale, CA) equipped with a PepMap100 C-18 trap column (300 μm i.d. × 5 mm long cartridge, from Dionex) and PepMap100 C-18 analytic column (75 μm i.d. × 150 mm long, from Dionex). The gradient consisted of (A) 0.1% formic acid in water, (B) 0.08% formic acid in ACN: 8–25% B from 0 to 195 min, 80% B from 195 to 200 min and 8% B from 200 to 205 min. An HCT ultra-PTM discovery system (Bruker Daltonics, Bremen, Germany) was used to record peptide spectra over the mass range of *m/z* 350–1500, and MS/MS spectra in information-dependent data acquisition over the mass range of *m/z* 100–2800. Repeatedly, MS spectra were recorded followed by three data-dependent collision induced dissociation (CID) MS/MS spectra and three electron transfer dissociation (ETD) MS/MS spectra generated from three highest intensity precursor ions. The voltage between ion spray tip and spray shield was set to 1500 V. Drying nitrogen gas was heated to 150 °C and the flow rate was 10 L/min. The collision energy was set automatically according to the mass and charge state of the peptides chosen for fragmentation. Multiple charged peptides were chosen for MS/MS experiments due to their good fragmentation characteristics. MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 4.0 (Bruker Daltonics).

Searches were performed using the MASCOT v2.2.06 (Matrix Science, London, U.K.) against latest UniProtKB database for protein identification. Searching parameters were set as follows (i) MASCOT: enzyme selected as used with four maximum missing cleavage sites, species limited to *Drosophila*, a mass tolerance of 0.2 Da for peptide tolerance, 0.2 Da for MS/MS tolerance, fixed modification of carbamidomethyl (C) and variable modification of methionine oxidation (M) and phosphorylation (S, T, Y). Positive protein identifications were based on significant MOWSE scores. After protein identification, an error-tolerant search was performed to detect unspecific cleavage and unassigned modifications. Protein identification and modification information returned from MASCOT were manu-

MS/MS synapsin	MKRGFSSGDLSSEVDDV
Edman degrad	?KRGFSSGD
Melanog flybase Syn-PD	MNFSSFKSSFTSNVNFLKRRFSSGDLSSEVDDV
Melanog cDNA translated	*LSTTVNISAIATAGCGRICAPPPEPKSSTLNFSFKSSFTSNVNFLKRGFSSGDLSSEVDDV
Virilis	*TQANCSPSLSTVNISAIATAGCGRICAPPPEPKSSTLNFSFKSSFTSNVNFLKRRFSSGDLSSEVDDV
Pseud	*ESESELQRASEAHCSTPLSTTVNISAIATAGCGRICAPPPEAKSSTLNFSFKTSFTSNVNFLKRRFSSGDLSSEVDDV
Willistoni	*LKDKYQLYFVYFMCVQEPKSSTLNFSFKSSFTSNVNFLKRRFSSGDLSSEVDDV
Anopheles	MSPSKGGITNDPQAGTLPASFKSSFTSNVNFLKRRFSSGDLSSECDV
Loligo syn	TGVGFLKRRFSSGDLLQGELRDA
Mus, rattus, homo syn-I	MNYLRLSDSNFMANLPNG
Xenopus I	MMNYLRLSDSNFMANLPNG
Mus, rattus, homo syn-II	MMNFLRRRLSDSSFIANLPNG
Xenopus syn-II	MNYLKRLSDSGFLGSLPSC
Mus, rattus, syn-III	MNFLRRRLSDSSFMANLPNG
Homo syn-III	MNFLRRRLSDSSFVANLPNG
Xenopus syn-III	MNFLRRRLSDSSFVANLPNG

Figure 1. N-terminal amino acid sequence alignments of synapsins from various species. The N-terminal sequence of *D. melanogaster* synapsin obtained by MS/MS (present results) matches the peptide sequence obtained by Edman degradation. Translation of cDNAs from various *Drosophilidae* and *Anopheles* shows that high homology among dipterans extends beyond this N-terminus. The amino acid exchanged due to pre-mRNA editing is marked in red; amino acids identical to the genomic or edited N-terminus of *D. melanogaster* are in bold.

ally inspected and filtered to obtain confirmed protein identification and modification lists of CID MS/MS and ETD MS/MS.

Post-translational modification searches were done using Modiro v1.1 software (Protagen AG, Germany) with the following parameters: enzyme selected as used with four maximum missing cleavage sites, species limited to *Drosophila*, a peptide mass tolerance of 0.2 Da for peptide tolerance, 0.2 Da for fragment mass tolerance, modification 1 of carbamidomethyl (C) and modification 2 of methionine oxidation. Searches for unknown mass shifts, amino acid substitution and calculation of significance were selected on advanced PTM explorer search strategies. A list of 172 common modifications including phosphorylation, methylation, and hydroxylation was selected and added to virtually cleaved and fragmented peptides searched against experimentally obtained MS/MS spectra. Positive protein identification was first achieved by spectra view and subsequently each identified peptide was considered significant based on the 0.2 Da delta value, ion-charge status of peptide, b- and y- ion fragmentation quality, ion score (>200), and significant scores (>80). The Modiro software is complementary to the MASCOT software, using already identified sequences, and has the advantage that also unknown mass shifts can be handled. Protein identification and modification information returned were manually inspected and filtered to obtain confirmed protein identification and modification lists.⁴⁵

Results

A. The N-Terminus of *Drosophila* Synapsin. The N-terminus of vertebrate synapsins is highly conserved (Figure 1). Earlier we had sequenced the N-terminus of immunoprecipitated *D. melanogaster* synapsin by Edman degradation³⁷ and obtained the sequence: ? K R/G D/G F/L S/A S/A G/E R/D L (first amino acid undetermined, two amino acids detected at most positions). This sequence contains ?KRGFSSGD which is encoded by all known cDNAs (after editing, cf. Discussion) and resembles the N-terminus of mammalian synapsins (Figure 1). In the present study, mass spectrometric analysis of synapsin peptides after AspN digestion and CID (90 sig.) or ETD (50 sig.) revealed a peptide MKRGFSSGD which contains methionine as the first amino acid instead of a leucine encoded by the second in-frame CTG of the cDNA (cf. Discussion). Sequence comparison among cDNAs of various diptera showed that 16 triplets upstream of this CTG (including a potential CTG

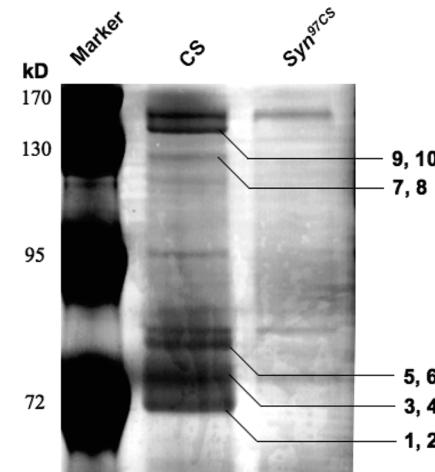


Figure 2. Silver-stained SDS-PAGE gel of proteins immunoprecipitated from head homogenate of *D. melanogaster* wild-type Canton-S (CS) and synapsin null mutant *Syn*⁹⁷CS (in CS background) using anti-synapsin monoclonal antibody Synorf1 (3C11). Indicated bands were excised and in-gel digested with various enzymes. Peptides were analyzed by nano-LC–MS/MS (high-capacity ion trap). Peptides identified via MASCOT and Modiro are listed in Supplementary Tables 1 and 2, respectively.

start) are highly conserved suggesting either coding or regulatory functionality (cf. Discussion). A canonical ATG in-frame start codon is observed 45 codons downstream of the second CTG. However, a peptide having the corresponding methionine as the N-terminal amino acid has not been observed in any of the protein bands analyzed (cf. Discussion).

B. Mass Spectrometric Characterization of *Drosophila* Synapsin. To obtain further information on the primary structure of *Drosophila* synapsins, in particular post-translational modifications (PTMs), and on the mechanism to circumvent the stop codon mentioned above, proteins immunoprecipitated with anti-synapsin antibody 3C11 from wild-type and synapsin null mutant head homogenate (~2500 heads, ~180 mg of brain tissue) were separated by SDS-PAGE and visualized by MS-compatible silver staining (Figure 2). Gel pieces from several regions indicating the presence of proteins in wild type but missing in the mutant (as marked in Figure 2) were cut out from both the wild-type and the mutant lanes and processed for nano-LC–ESI–MS/MS (cf. Materials and Methods). All proteins reliably identified in wild-type but

Table 1. Mascot and Modiro Analysis of MS/MS Data Obtained from Proteins Precipitated with Anti-Synapsin Antibody 3C11^a

enzyme	incubation conditions	identified protein (Swiss-Prot number)	Mascot v2.2.06 (%)		Modiro v1.1 [min. sig. 90/80] (%)	
			CID	ETD	CID	ETD
Trypsin	12.5 ng/μL trypsin solution in 25 mM NH ₄ HCO ₃ (pH 7.8). 37 °C overnight.	Synapsin (Q24546)	80.00	71.22	73.07/78.63	62.24/70.15
Chymotrypsin	12.5 ng/μL chymotrypsin solution in 25 mM NH ₄ HCO ₃ (pH 7.8). 25 °C overnight.		52.78	40.78	40.88/43.70	22.92/25.85
AspN	25 ng/μL AspN solution in 50 mM NH ₄ HCO ₃ (pH 7.8). 37 °C overnight.		52.39	26.83	10.83/22.73	6.82/12.29
Subtilisin	10 ng/μL subtilisin solution in 5.4 M urea and 100 mM Tris (pH 8.5). 37 °C for 1 h.		26.44	25.46	27.80/30.24	1.95/2.44
Proteinase K	50 ng/μL Proteinase K solution in 50 mM NH ₄ HCO ₃ (pH 7.8). 37 °C for 1 h.		21.27	Not identified	8.49/11.22	1.46/1.46
Pepsin	50 ng/μL pepsin solution in 100 mM HCl. 37 °C for 4 h.		33.85	Not identified	29.07/34.93	3.02/10.24

enzyme	identified protein (Swiss-Prot number)	Mascot v2.2.06 (%)		Modiro v1.1 [min. sig. 90/80] (%)		
		CID	ETD	CID	ETD	
Trypsin	Chymotrypsin	Synapsin (Q24546)	85.17	75.02	77.27/83.71	71.12/78.83
Trypsin	AspN		90.83	76.49	74.54/85.66	63.41/72.20
Trypsin	Subtilisin		84.29	78.73	78.83/84.59	62.24/70.15
Chymotrypsin	AspN		77.17	47.32	45.85/56.68	27.90/35.61
Chymotrypsin	Subtilisin		63.41	58.83	58.05/66.05	24.00/28.10

identified protein (Swiss-Prot number)	enzyme	analyzing condition	sequence coverage (%)
Synapsin (Q24546)	Trypsin	MASCOT CID + Modiro CID (min. sig. 90)	84.68
		MASCOT CID + Modiro CID (min. sig. 80)	85.95
	Chymotrypsin	MASCOT CID + Modiro CID (min. sig. 90)	58.34
		MASCOT CID + Modiro CID (min. sig. 80)	58.93
	AspN	MASCOT CID + Modiro CID (min. sig. 90)	52.49
		MASCOT CID + Modiro CID (min. sig. 80)	57.46
Subtilisin		MASCOT CID + Modiro CID (min. sig. 90)	45.56
		MASCOT CID + Modiro CID (min. sig. 80)	47.22

^a Incubation conditions and sequence coverages (%) for all enzymes used in this study are listed. The sequence coverage was evaluated by combining sequence coverages of all spots from individual protein bands.

missing in the mutant samples at corresponding positions in the gel were unambiguously identified as *Drosophila* synapsins (cf. Discussion). All MS/MS peptides generated via MASCOT v2.2.06 and Modiro v1.1 are provided in Supplementary Tables 1 and 2, respectively.

Table 1 shows the sequence coverage of synapsin from different enzyme digestions, conditions, and different search engines. By combining results from different enzymes and bioinformatic tools, high sequence coverage (maximum 90.83%) was obtained. The majority of peptides were identified from trypsin, chymotrypsin, and AspN digestion, proteases widely used for mass spectrometric analysis. Other enzymes, such as subtilisin, Proteinase K, and pepsin, contributed to obtain more peptides which were not identified by the above-mentioned digestions. Identified amino acid conflicts of *D. melanogaster* synapsin are listed in Supplementary Table 3.

C. Post-Translational Modifications on *Drosophila* Synapsin. A series of PTMs was observed. Various PTMs detected by Modiro were consistent and had an ion score above 200 and significance higher than 80, including methylation, deamidation, oxidative modifications like hydroxylation, methionine dioxygenation, tryptophan oxidations expressed by kynurenin, and pyrophosphorylation (Supplementary Figure 1, pink color, additional PTMs detected at lower ion score or significance: green color). However, seven phosphorylation sites were identified and verified by phosphatase treatment. The phosphorylated amino acids were T86, S87, S464, T466, S538, S961,

and Y982 (cf. Table 2). These phosphorylation sites were either identified by MASCOT v2.2.06 or Modiro v1.1, and representative MS/MS spectra of seven phosphorylation sites with a-, b-, y-, b-Pi, and y-Pi ion series that were identified either by MASCOT v2.2.06 or Modiro v1.1 are shown in Figure 3 and Supplementary Figure 2. The use of two different search algorithm, MASCOT v2.2.06 and Modiro v1.1, for detecting and interpreting all phosphorylations from our raw MS/MS data resulted in the unambiguous identification of five phosphorylation sites (Figure 3) and indicated two additional phosphorylation sites in the C-terminus at significance 76.6 (Supplementary Figure 2). Representative mass spectra of the phosphatase-treated peptides for verification of phosphorylation are shown in Supplementary Figure 2.

D. Mass Spectrometric Sequence Analysis of the Stop Codon at X582. The *Drosophila* synapsin cDNA of splice variant RA contains an in-frame TAG stop codon separating two large open reading frames both of which are represented in the large isoforms (cf. Introduction). The amino acid in the large isoforms at the position of the stop codon (X582) is not known. From our mass spectrometric analysis, it is now concluded that the UAG stop codon at position X582 can be read through to synthesize the long isoforms. Lysine was unambiguously identified via MASCOT as shown in Figure 4A. Modiro search was performed by replacing the stop codon (designated as X in the Swiss-Prot database) by each amino acid determined by MASCOT. In this way lysine again was identified from

Table 2. *Drosophila* Synapsin Phosphorylation Sites^a

residue	position	NetPhos 2.0 prediction	NetPhosK 1.0 prediction
Peptide [max Modiro ion score] with phosphorylation site (bold) identified in adults by LC–MS/MS (verified)			
RGVSA P TSPAKS [289]	T-86	0.125	0.79 (PKC), 0.50 (Cdc2)
RGVSA P T ²⁷² PAKS [272]	S-87	0.970	0.53 (GSK3), 0.58(MAPK), 0.7 (Cdk5)
RAES P TDEGVAPT ²⁷² PPLPAGPRP [445]	S-464	0.998	0.55 (RSK), 0.51(GSK3), 0.65 (Cdk5)
RAE SPT *DEGVAPT ²⁷² PPLPAGPRP [278]	T-466	0.031	0.52 (CKII)
RRDSQT ²⁷² SQS*STI ²⁷² SSVRA [286]	S-538	0.397	0.55 (Cdc2)
KS*MSMTSGGVGSGNGSGSGLGGYKI [242]	S-961	0.883	0.50 (Cdc2)
K S MSMTSGGVGSGNGSGSGLGGY*KI [242]	Y-982	0.118	-
Sequence with phosphorylation sites (bold) identified in embryos by LC–MS/MS, from Zhai et al.³⁹ (unverified)			
LSSRS* S ISE	S-509	0.974	-
SSR S *ISEV	S-510	0.996	0.60 (CKI), 0.67(PKA)
TSQSS* T ISS	S-539	0.915	0.58 (PKC)
Predicted PKA phosphorylation sites (bold, unverified) not observed in the present experiments			
KRRFSSGDL	S-6	0.998	0.66 (RSK), 0.85(PKA)
VRRDQS ⁵³³ T ⁵³³ SQ	S-533	0.998	0.60(RSK), 0.81(PKA), 0.61(DNAPK)

^a Several phosphorylation sites are suggested by in silico analysis of *Drosophila* synapsin using Web based prediction tools NetPhos and NetPhosK. Higher scores correlate to higher confidence in prediction (NetPhos and NetPhosK have a threshold of 0.5). ‘-’ in the table indicates that the enzymes (PKA, PKC, PKG, CKII, Cdc2, CaM-II, ATM, DNA PK, Cdk5, p38 MAPK, GSK3, CKI, PKB, RSK, INSR, EGFR, and Src) used in the prediction software (NetPhosK 1.0) do not phosphorylate the indicated residue.

Modiro with high significance and score (Figure 4B). MS/MS spectra of all amino acids which can be coded at X582 are listed in Supplementary Figure 3.

Discussion

Drosophila synapsin is involved in neurotransmission^{40,41} and plays a significant role for learning and memory formation at both larval and adult stages.^{37,42,43} While different phosphorylation sites of vertebrate synapsins and functional consequences of their modification have been extensively studied (reviewed in ref 7), there was so far only limited information on post-translational modification (PTMs) of *Drosophila* synapsin.³⁹ As a first step to investigating the relevance of PTMs for *Drosophila* synapsin function, we here identified and verified seven unique phosphorylation sites. The availability of a null mutant (*Syn*⁹⁷CS) and a monoclonal antibody (MAB 3C11) for *Drosophila* synapsin provided the basis for the immunochemical purification of synapsin isoforms and, potentially, tightly bound interaction partners for characterization by nano-LC–ESI–MS/MS. Mass spectrometric analysis of polypeptides picked at five positions from silver stained SDS gel lanes of immunoprecipitated proteins present in wild type but lacking in the null mutant (Figure 2) exclusively revealed synapsins. No interacting proteins were identified, indicating that under the homogenization and washing conditions used (150–500 mM NaCl, 0.1% NP-40) protein complexes containing stoichiometric amounts of synapsins were not precipitated. This was surprising because it is known that vertebrate synapsins interact with various cytoskeletal and synaptic vesicle associated proteins.⁷ Our results do not exclude the formation of homo- or hetero-multimers of synapsin isoforms under our conditions, an interaction that has been observed *in vitro* for vertebrate synapsins.⁴⁶

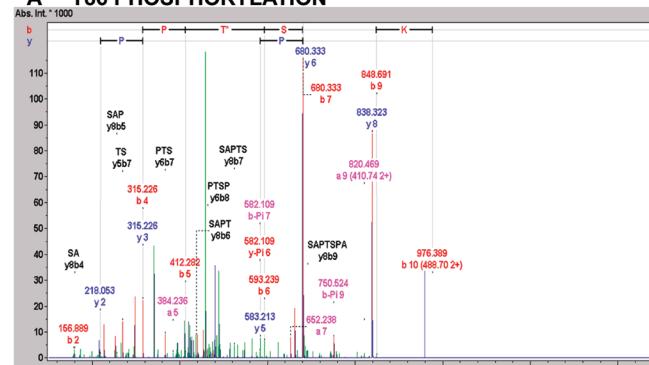
The identification of novel splice isoforms of a protein, its post-translational modifications, amino acid substitutions due to polymorphisms or RNA editing by MS/MS all require high sequence coverage. To achieve this, we applied multienzyme digestion and used different analyzing tools, MASCOT v2.2.06 and Modiro v1.1, to complement each other. Most standard database search algorithms enable users to search for matched peptides, PTMs, and enzyme nonspecificity, but they still have

the disadvantage of less significant results caused by a great variety of generated peptides which have to be analyzed. Two different analyzing softwares were used to get higher sequence coverage and more PTMs. MASCOT v2.2.06 was used as a standard search algorithm and Modiro v1.1 was used to analyze both, the identified and unidentified spectra from Mascot searching for different PTMs, unknown mass shifts,^{47,48} unspecific cleavage and sequence errors. Because Modiro v1.1 cannot search complete protein databases, it uses protein sequences from users' input and evaluates all spectra against this set of protein sequence. By combining all peptides identified from different enzyme application and analyzing tools, the total sequence coverage was 90.83% (Table 1). A series of sequence conflicts were identified (Supplementary Table 3) by the two different search engines. Even though differences exist between MASCOT and Modiro search results, some of these sequence conflicts may be due to polymorphism.

Earlier Western blots with MAB 3C11 had identified 3 small isoforms at 70, 74, and 80 kDa and 2 isoforms at about 143 kDa.³⁵ We isolated and unequivocally detected the shortest (~70 kDa) and a read-through isoform (~143 kDa). A few peptides of the longer isoform detected in the protein band at 70 kDa could either be accidental matches or possibly belong to degraded products of the longer isoforms. The synapsin isoform SYN-2³⁵ (SYN-PC³⁶) was not unequivocally identified.

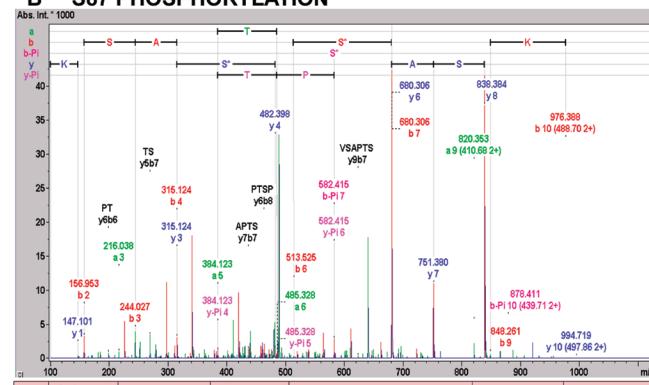
The N-terminus of *Drosophila* synapsin was controversial due to the lack of a canonical AUG start codon upstream of the conserved A domain. Edman sequencing had identified amino acids 2–10 leaving the first amino acid encoded by CUG undetermined (Figure 1). The high conservation of 16 amino acids further upstream (also starting with a CUG codon) could indicate functional relevance of a hypothetical 1.8 kDa larger isoform. In several cases, start codons with one base different from AUG have been shown to base pair with the initiation Met^{tRNA_i} anticodon.⁴⁹ Use of such non-AUG start codon is controlled on the basis of context and the presence of certain sequence features⁴⁹ or by a leaky-scanning process^{50,51} for generating several isoforms of a given protein with an N-terminus encoded upstream of the first in-frame AUG codon. This process could also be responsible for generating different isoforms of *Drosophila* synapsin with possibly different func-

A T86 PHOSPHORYLATION



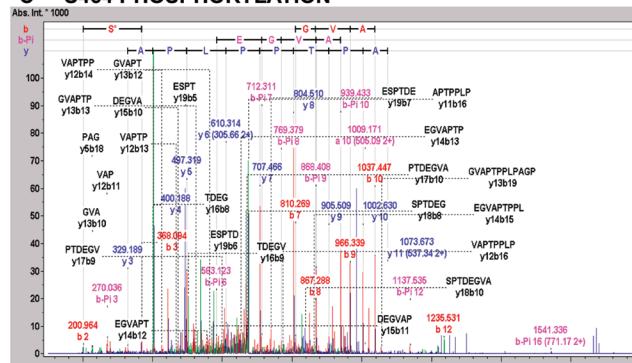
	200	400	600	800	1000	1200	1400	m/z
Spot Nr.	Swissprot Acc. Nr.	Protein name	Enzyme	Peptide sequence		Position	Identified PTMs	
4	Q24546	Synapsin	Trypsin	R.GVSAPT'SPAK.S		81-91	Phosphorylation	
m/z meas. [Da]	m/z theor. [Da]	Error [Da]	z	Spectrum type		Score	Significance	
497.69	497.739	-0.1439	2	CID		229	99.1	

B S87 PHOSPHORYLATION



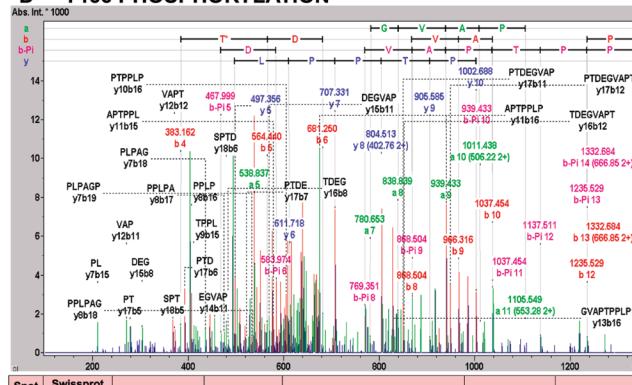
Spot Nr.	Swissprot Acc. Nr.	Protein name	Enzyme	Peptide sequence		Position	Identified PTMs
m/z meas.	[Da]	m/z theor. [Da]	Error [Da]	z	Spectrum type	Score	Significance
1	C24546	Synapsin	Trypsin	R.GVSAPTS'PAK.S		81-90	Phosphorylation
497.76	497.739	0.0261	2	CID		272	92.2

C S464 PHOSPHORYLATION



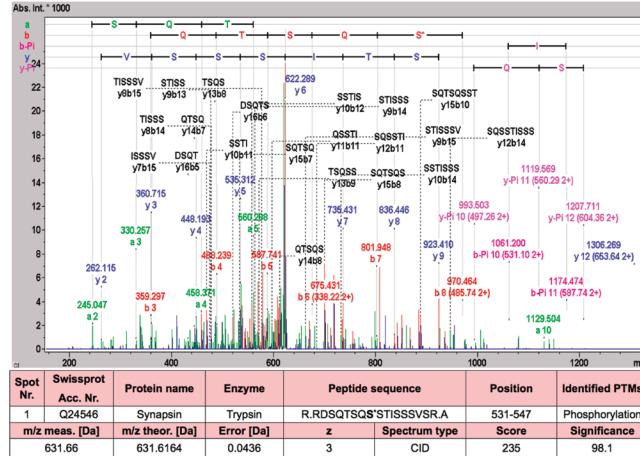
	200	400	600	800	1000	1200	1400	1600	n
Spot Nr.	Swissprot Acc. Nr.	Protein name	Enzyme	Peptide sequence			Position	Identified Protein	
10	Q24546	Synapsin	Trypsin	R.AE.SPTDEGAVTPPLPAGR.P			462-481	Phosphorylation	
Observed [Da]		Experimental [Da]	Calculated [Da]	Delta [Da]	z	Spectrum type		Score	
680.37		2038.09	2037.94	0.1476	3	CID		42	

D T466 PHOSPHORYLATION



Sob. Nr.	UniProt Nr.	Protein name	Enzyme	Peptide sequence			Position	Identified PTM
				m/z meas. [Da]		m/z theor. [Da]		
Z	Q24546	Synapsin	Trypsin	R.AEESP ^T DEGVAPTPPLPAGPR.P		462-481		Phosphorylation
m/z meas. [Da]	680.37	680.3209	Error [Da]	0.0491	z	CID	278	Significance

E S538 PHOSPHORYLATION



Spot Nr.	Swissprot Acc. Nr.	Protein name	Enzyme	Peptide sequence		Position	Identified PTMs
m/z meas. [Da]	m/z theor. [Da]	Error [Da]		z	Spectrum type	Score	Significance
1	Q24546	Synapsin	Trypsin	R.RDSQTQS ST SISSVSR.A		531-547	Phosphorylation
631.66	631.6164	0.0436		3	CID	235	98.1

Figure 3. Representative mass spectra demonstrating five phosphorylation sites (A–E) of *Drosophila* synapsin. Proteins from silver stained SDS-PAGE bands marked in Figure 2 were digested by the indicated enzymes and extracted peptides were analyzed by nano-LC–ESI–MS/MS (high capacity ion trap). CID and ETD MS/MS spectra were interpreted and peak lists were generated by DataAnalysis 4.0. Data searches were performed via MASCOT v2.2.06 and Modiro v1.1 against latest UniProtKB database for protein identification and PTM search. An asterisk marks the phosphorylated amino acid residue. All phosphorylations have been shown to be removed by phosphatase treatment (spectra are shown in Supplementary Figure 2).

tions. Our MS/MS data verified the N-terminus identified by Edman degradation and revealed methionine as the first amino acid, but did not provide evidence for alternative translation initiation at the first CUG 16 triplets upstream, or the first AUG downstream, of the conserved domain A. Isoforms initiated at this downstream methionine would lack the conserved PKA site.

of domain A which has been shown to be of high functional relevance for regulation of mammalian synapsin function.⁷ Bioinformatic analysis of alternative translation initiation sites in mammalian mRNAs has identified the relative abundance of nucleotides surrounding the non-AUG start codon and typical secondary structural features of the 5' UTR regions of

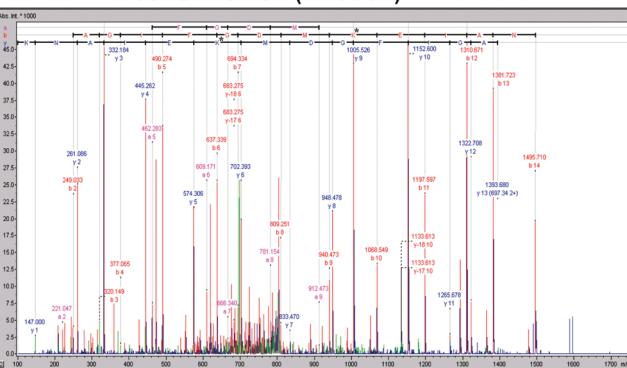
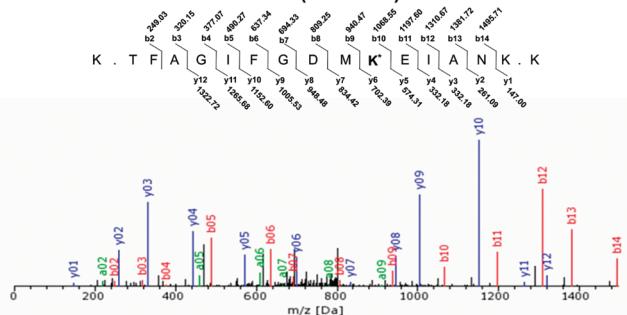
A X → K SUBSTITUTION (MASCOT)**B X → K SUBSTITUTION (MODIRO)**

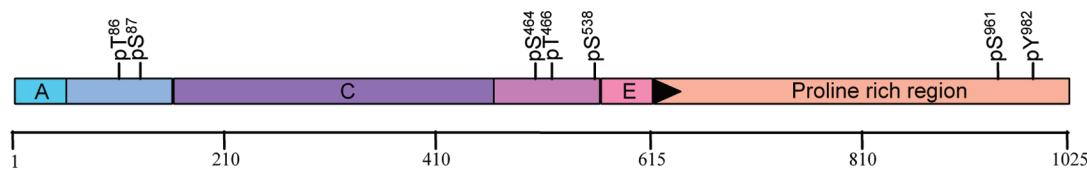
Figure 4. Amino acid substitution of UAG stop codon (X582) by lysine. For mass spectrometric analysis of *Drosophila* synapsin, immunoprecipitated synapsin spots were excised from the MS-compatible silver stained gel and subjected to multienzyme digestion, as summarized in Table 1. The resulting peptides were then analyzed using nano-LC–ESI–MS/MS high capacity ion trap. Data searches were performed via MASCOT v2.2.06 (A) and Modiro v1.1 against latest UniProtKB database. For Modiro search, the stop codon indicated as 'X' in Swiss-Prot database in *Drosophila* synapsin was replaced by each candidate amino acid according to the MASCOT results. An asterisk marks the amino acid which contains the corresponding substitution.

these mRNAs.⁵² Comparison of the 5' region of the *Drosophila* synapsin mRNA with these mammalian predictions reveals no significant sequence similarity to the mammalian consensus around either the two potential CUG start sites or the first AUG codon, but secondary structure analysis predicts a stem-loop containing the second CUG (shown here to encode the N-

terminal methionine) highly similar to the representative secondary structure of mammalian alternative translation initiation sites.⁵² To our knowledge, no bioinformatic analysis of insect alternative translation initiation sites has been reported so far.

A puzzling feature of the *Drosophila* synapsin gene is the in-frame amber (UAG) stop codon separating two large open reading frames. Earlier experiments using domain-specific antibodies have shown that the large synapsin isoforms contain domains translated from both reading frames. Two alternative mechanisms have been hypothesized to generate the large isoforms, UAG read-through or alternative splicing.^{35,37} Using the BDGP *Drosophila* splice site prediction tool, no high-score splice site candidates can be found to remove the UAG stop codon. We have, however, previously demonstrated that GST-synapsin fusion proteins in sup⁻ and sup⁺ *Escherichia coli* strains have different read-through efficiencies of the in-frame stop codon (X582).³⁵ The present mass spectrometric analysis reveals that this amber stop codon is translated to lysine with high ion score. The corresponding MS/MS spectrum is shown in Figure 4. The present finding supports the hypothesis of tRNA specific stop codon read-through which may be controlled by the presence of secondary structure in the vicinity, upstream or downstream. Such structures can directly interfere with the ribosome, release factors, and tRNAs and facilitate suppression of the termination process.⁵³ Other amino acids, such as asparagine, glutamine, glutamic acid, histidine, threonine, and serine, were also occasionally identified at the position X582 but with lower ion scores compared to lysine incorporation (Supplementary Figure 3). No information is at present available on differential functions of the different isoforms or the read-through amino acid because in functional rescue experiments of the null mutant the large cDNA was employed generating both small and large isoforms.^{37,42}

Vertebrate synapsins are substrates for several protein kinases like PKA, CaMKs, Src, cdk, and MAPK/Erk, which modulate their biochemical properties and functions.⁷ In vertebrates examined so far, the domain 'A' of synapsin contains the phosphorylation site 1 (RRxS) that has been identified as a target site for cAMP-dependent protein kinase (PKA) and calcium/calmodulin dependent protein kinase I/IV (CaMKI/IV). In *Drosophila*, this motif is conserved in the genome, but in the vast majority of late larval or adult transcripts, the enzyme adenosine deaminase acting on RNA (ADAR) edits the genomic site (RRFS) to RGFS.³⁸ *In vitro*, only undecapeptides containing the genomic but not the edited site are phosphorylated significantly by bovine PKA.³⁸ There is a second RRxS site at S533 in *Drosophila* synapsin. Whether this site can be phosphorylated by PKA or other kinases is not known and needs to be investigated. In the current study, we did not observe phosphorylation at either of these potential



1-26 A domain; 133-442 C domain; 538-581 E domain

Figure 5. Domains and identified phosphorylation sites in *Drosophila* synapsin. The black arrowhead refers to the amber stop codon which terminates the small isoform but can be read-through to generate the large isoform. The scale refers to amino acid residue number.

PKA sites. Vertebrate synapsin phosphorylation sites 2 and 3 (P2, P3) conform to the consensus RxS and can be phosphorylated by CaMK-II. The site S464 of *Drosophila* synapsin identified here conforms to this consensus and may, therefore, have a similar function as vertebrate P2/P3.

In a large-scale phosphoproteome analysis of *Drosophila* embryos, Zhai et al. reported synapsin phosphorylation at serines 509, 510, and 539.³⁹ None of these sites were detected here in synapsin from adult *Drosophila* heads with high ion score or significance, indicating that phosphorylation patterns may be highly specific for the developmental stage of the animal. Rather, we identified seven novel phosphorylation sites and verified them via phosphatase treatment: T86 and S87 near the domain A; S464, T466, and S538 near domain E; S961 and Y982 in the read-through proline rich region (Figure 5). MASCOT v2.2.06 predicted modification on T86 as sulfonation and Modiro v1.1 predicted T86 as either sulfonation or phosphorylation. As to the mass shift on T86, phosphorylation disappeared after phosphatase treatment and this represents evidence for phosphorylation rather than sulfonation. For additional evidence, T86 phosphorylation may be proven with a more precise instrumentation as, for example, electron capture dissociation (ECD) fragmentation and Fourier transform mass spectrometry.⁵⁴ Double phosphorylation at S961/Y982 was identified by Modiro. Modiro significance score is lower than 80 (76.6), but it was verified by phosphatase treatment (Supplementary Figure 2). Table 2 summarizes the present information on phosphorylation of *Drosophila* synapsins. In contrast to vertebrates,⁷ no functional implications of synapsin phosphorylation have been reported so far for *Drosophila*. Clearly, the present data on *Drosophila* synapsin phosphorylation are only a starting point because spatial and temporal differences in phosphorylation patterns have not been studied. However, based on the knowledge about novel verified phosphorylation sites, it should now be possible to generate phosphate-specific antibodies and study the tissue distribution of phospho-synapsin in *Drosophila* and analyze possible changes due to functional activity, in particular in relation to learning and memory.

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Supporting Information Available: Three figures and three tables as described in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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