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Phosphorylation and nitration of tyrosine residues affect functional properties of Synaptophysin and Dynamin I, two proteins involved in exo-endocytosis of synaptic vesicles

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ABSTRACT

Phosphorylation and nitration of protein tyrosine residues are thought to play a role in signaling pathways at the nerve terminal and to affect functional properties of proteins involved in the synaptic vesicle (SV) exo-endocytotic cycle. We previously demonstrated that the tyrosine residues in the C-terminal domain of the SV protein Synaptophysin (SYP) are targets of peroxynitrite (PN). Here, we have characterized the association between SYP and *c-src* tyrosine kinase demonstrating that phosphorylation of Tyr_{273} in the C-terminal domain of SYP is crucial in mediating SYP binding to and activation of *c-src*. SYP forms a complex with Dynamin I (DynI), a GTPase required for SV endocytosis, which may be regulated by tyrosine phosphorylation of SYP. We here report that, in rat brain synaptosomes treated with PN, the formation of SYP/DynI complex was impaired. Noteworthy, we found that DynI was also modified by PN. DynI tyrosine phosphorylation was down-regulated in a dose-dependent manner, while DynI tyrosine nitration increased. Using mass spectrometry analysis, we identified Tyr_{354} as one nitration site in DynI. In addition, we tested DynI self-assembly and GTPase activity, which are enhanced by *c-src*-dependent tyrosine phosphorylation of DynI, and found that both were inhibited by PN. Our results suggest that the site-specific tyrosine residue modifications may modulate the association properties of SV proteins and serve as a regulator of DynI function via control of self-assembly, thus influencing the physiology of the exo-endocytotic cycle.

1. Introduction

Neurotransmitter release is triggered by Ca^{2+}-dependent influx at the nerve terminal and relies on specific protein interactions between synaptic vesicles (SVs)[1] and presynaptic terminals resulting in the fusion process. After fusion, SV membrane proteins and lipids in turn are recycled at the periactive zone that surrounds the release site to restore functional SV pools [1]. To work appropriately, the synaptic proteins associate transiently in functional complexes with membrane promoting the correct steps from exo- to endocytosis.

Synaptophysin (SYP) is one of the most abundant integral proteins of SV membrane [2] involved in several steps of synaptic function including exocytosis, synapse formation, biogenesis, and endocytosis of SVs [3–5]. SYP interacts with other synaptic proteins, including Vesicle-Associated Membrane Protein 2 (VAMP2), also known as Synaptobrevin 2 [6], the vesicular proton pump V-ATPase [7], the adaptor protein AP1 [8] and Dynamin I (DynI) [5,9]. However, how the multiple associations of SYP are regulated is still to be clarified.

SYP shares with its homologous Synaptoporin and Pantophysin a common transmembrane topology, with four membrane-spanning domains and a short N- and a long C-terminal tail, both exposed on the cytoplasmic side [10]. SYP C-terminal cytoplasmic tail contains nine pentapeptide repeats, each starting with a tyrosine residue (YG(P/Q)QC) that may be the target of tyrosine kinases within the nerve terminal. Indeed, SYP is phosphorylated *in vitro* by the non-receptor tyrosine kinase *src* [11], but the functional role of its phosphorylation is still unknown [12]. It has been suggested that tyrosine phosphorylation of SYP may regulate its interaction with DynI, a GTPase required for SV endocytosis, since the tyrosine-based pentapeptide motifs are confined to DynI binding sites [5,9]. Actually, SYP forms a complex with DynI *in vivo* in a Ca^{2+}-dependent manner [9] and, recently, optical imaging and electrophysiological studies have provided evidence that the C-terminal cytoplasmic tail of SYP plays a role in facilitating rapid and efficient SV endocytosis [13].

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[1] Abbreviations used: PN, peroxynitrite; SYP, Synaptophysin; DynI, Dynamin I; SV, Synaptic Vesicles; VAMP2, Vesicle-Associated Membrane Protein 2; SNARE, Soluble N-ethylmaleimide sensitive fusion proteins Attachment Receptor; pTyr, Phosphotyrosine; NO_2Tyr, Nitrotyrosine; GST, Glutathione-S-Transferase; SH, *Src* Homology; PRD, Proline-Rich-Domain; AD, Alzheimer's Disease; SDS-PAGE, Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis; LC–MS/MS, Liquid Chromatography tandem Mass Spectrometry; PMSF, PhenylMethylSulfonylFluoride; WB, Western Blot.

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These findings indicate that SYP recruits DynI to the release site at nerve terminal and suggest that SYP/DynI complexes are involved in exo-endocytosis (for a review on DynI see [14]).

We previously demonstrated that in rat brain synaptosomes treated with peroxynitrite (PN), a potent oxidizing and nitrating species formed in a diffusion-limited radical–radical reaction between nitric oxide and superoxide anion [15], the tyrosine residues in the C-terminal cytoplasmic tail of SYP were either phosphorylated or nitrated [16]. The formation of 3-nitrotyrosine (NO_2Tyr) in proteins is an *in vivo* post-translational modification induced by nitric oxide-derived oxidants, such as PN [17]. PN-mediated nitration is a selective process where only a small amount of proteins is found nitrated and one or a few tyrosine residues are modified [17]. Because NO_2Tyr are poor substrates for tyrosine kinases, the nitration of a tyrosine residue may down-regulate phosphotyrosine (pTyr)-dependent signaling [18–20]. On the other hand, several *in vitro* studies have shown that PN may promote rather than inhibit tyrosine phosphorylation [21–26]. In the nervous system, protein tyrosine nitration is a major component of PN-dependent cytotoxicity in the course of neurodegenerative disorders and structural neuronal proteins have been found to be key targets for tyrosine nitration. For instance, α-synuclein undergoes oligomerization upon PN-dependent nitration, and oligomerized α-synuclein forms Lewis bodies, the hallmark of Parkinson's disease [27]. In addition, the presence of amyloid β nitrated at Tyr_{10} has been detected in Alzheimer's disease (AD) brain, as well as in brains from mouse models of AD [28]. Protein nitration may also contribute to amyotrophic lateral sclerosis pathogenesis by interfering with the function of specific proteins, protein degradation pathways and phosphorylation cascade [29].

In a previous work, we showed that in rat brain synaptosomes tyrosine-phosphorylated, but not tyrosine-nitrated SYP, binds to *c-src* tyrosine kinase and enhances its catalytic activity. Moreover, we identified by mass spectrometry analysis one nitration site at Tyr_{250} and two phosphorylation sites at Tyr_{263} and Tyr_{273} in the SYP C-terminal cytoplasmic tail [16]. To get additional insights into the role of PN-dependent tyrosine modifications of SYP C-terminal cytoplasmic tail, we have further characterized the functional effects of SYP tyrosine phosphorylation and nitration on *c-src* recruitment and activity. Moreover, we have analyzed the formation of SYP/DynI complex in synaptosomes treated with PN and we have identified a site-specific nitration in DynI at Tyr_{354} by mass spectrometry analysis. Since *c-src*-dependent tyrosine phosphorylation of DynI induces its self-assembly and increases its GTPase activity, we have also investigated the effect of PN on both GTPase activity and self-assembly and found that these functions were impaired. Collectively, our findings advance our understanding of the critical role that PN-induced post-translational modifications (phosphorylation and nitration) play at the nerve terminal in modulating mutual affinities of SYP with two binding partners, DynI and *c-src*, both involved in the exo-endocytotic cycle of SVs.

2. Materials and methods

2.1. Materials

Antibodies were obtained from the following sources: monoclonal anti-phosphotyrosine (pTyr) (clone 4G10) and anti-3-nitrotyrosine (NO_2Tyr) (monoclonal and polyclonal) from Millipore Bioscience Research Reagent (Billerica, MA, USA); anti-SYP (monoclonal and polyclonal) and monoclonal anti-Syntaxin 1 from Synaptic System (Göttingen, Germany); monoclonal anti-*v-src* (Ab-1, clone 327) from Calbiochem (EDM Chemical, Merck, Darmstadt, Germany); monoclonal anti-DynI from BD Biosciences (Franklin Lakes, NJ, USA); monoclonal anti-*lyn* (clone sc-15) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); peroxidase-conjugated goat anti-mouse and goat anti-rabbit from Bio-Rad (Hercules, CA, USA). The antibody against GST was in house-made [30]. Recombinant purified human *src* ($pp60^{src}$) was purchased from Millipore Bioscience Research Reagent. Immunopure-Protein G and Protein A immobilized to Trysacryl were purchased from Thermo Scientific (Rockford, IL, USA). Nitrocellulose paper from Shleicher and Schuell Bioscience Inc. (Dassel, Germany). [γ^{32}P] ATP (>3000 Ci/mmol) was obtained from DuPont NEN (Boston, MA, USA) and Chelex 100 from Bio-Rad. SYP C-terminal-derived peptides containing tyrosine 250 (aa sequence 274–260), tyrosine 263 (aa sequence 261–271) and tyrosine 273 (aa sequence 272–288) were synthesized by NeoMPS PolyPeptide Laboratories (Strasbourg, France). Complete™ protease inhibitor cocktail, EDTA-free, was obtained from Roche Diagnostics (Basel, Switzerland). Commercial PN was purchased from Alexis (Enzo Life Sciences Inc., Farmingdale, NY, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Methods

2.2.1. Synaptosomes preparation

Communities Council Directive (86/609/EEC) was formally guided by the Italian Ministry of Health. Synaptosomes were prepared as described by Carlin et al. [31] with minor modifications. Briefly, brain tissue was homogenized in 10 vol (w/v) of Buffer A (0.32 M sucrose, 0.5 mM EGTA, 4 mM Hepes-NaOH, pH 7.4, 10 mM NaF, 1 mM Na_3VO_4 and Complete™) using a Teflon-glass grinder. The homogenate was centrifuged at 1000 ×*g* for 5 min at 4 °C to remove nuclei and debris and the supernatant centrifuged at 13,800 ×*g* for 10 min at 4 °C. The resulting pellet was suspended in Buffer A, stratified on a discontinuous sucrose gradient (0.85, 1 and 1.2 M v/v in Hepes buffered sucrose) and centrifuged at 85,000 ×*g* for 2 h at 4 °C. The layer between 1.0 and 1.2 M sucrose (synaptosomal fraction) was collected and centrifuged at 140,000 ×*g* for 20 min. Protein content was determined by Bicinchoninic Acid Assay (BCA kit, Thermo Scientific, Waltham, MA, USA).

2.2.2. Peroxynitrite treatment

PN was synthesized as described by Radi et al. [32]. The concentration of PN was determined by absorbance at 302 nm in 1.5 M NaOH ($\varepsilon_{302} = 1700\ M^{-1}\ cm^{-1}$). Diluted stock solutions were freshly prepared in 0.1 M NaOH. To avoid metal-catalyzed oxidation of PN, the phosphate buffer was treated extensively with Chelex100 and all samples contained 0.1 mM diethylenetriaminepenta-acetic acid. PN was added as a single bolus directly to synaptosomes (1 mg/ml) in 150 mM phosphate buffer (pH 7.5). After 5 min at room temperature, synaptosomes were washed once with phosphate buffer (pH 7.5). Control experiments were performed with decomposed PN to exclude the participation of contamination products. Decomposed PN was obtained by adding PN to the phosphate buffer for 10 min at room temperature before the addition to synaptosomes. Treatment with 0.5 and 1 mM PN was performed in the presence of 25 mM sodium bicarbonate (at pH 7.5, CO_2 is 1.3 mM) to enable NO_2Tyr formation [33].

2.2.3. Immunoprecipitation and in vitro kinase assay

Immunoprecipitation and *in vitro* kinase assay were performed as previously described [34]. Synaptosomes were solubilized by incubation for 30 min at 0 °C with an equal volume of 4× RIPA buffer (100 mM Tris–HCl, pH 7.5, 0.6 M NaCl, 4% (w/v) Triton X-100, 4% (v/v) Na-deoxycholate, 0.4% (v/v) SDS, 0.4 mM Na_3VO_4, 20 μg/ml leupeptin, 20 μg/ml aprotinin, and 4 mM PMSF), diluted twice with TBS (50 mM Tris–HCl, pH 7.4, 150 mM NaCl) and then centrifuged at 12,000 ×*g* for 10 min at 4 °C. After preclearing with 20 μl of Protein A/G beads prepared as a 50% (v/v) suspension for 1 h at 4 °C, supernatants were incubated with the appropriate antibodies and the immunocomplexes were precipitated by addition of Protein A or Protein G bead suspensions (50% v/v). The immunoprecipitates were collected by centrifugation and washed twice with RIPA buffer and twice with TBS. Bound proteins were eluted with 4× SDS loading buffer, resolved on SDS-PAGE and

subjected to Western Blot (WB) analysis. For co-immunoprecipitation assays, synaptosomes were solubilized in 20 mM Hepes, pH 7.4, containing 140 mM KCl, 2 mM EDTA, 1% (v/v) Triton X-100, 0.1 mM PMSF, 0.1 mM Na_3VO_4 and Complete™ and incubated overnight with anti-SYP and anti-DynI antibodies. The kinase assay was performed by incubating the purified human pp60^{src} (0.5 U) with recombinant proteins or peptides in kinase buffer (20 mM Tris–HCl, pH 7.4, 10 mM $MnCl_2$, 0.1 mM Na_3VO_4) containing 1–2 μCi [γ^{32}P] ATP (>3000 Ci/mmol) for 10 min at room temperature. When indicated, an exogenous substrate, such as enolase, was added to the reaction mixture. The kinase reaction was stopped by adding 4× SDS loading buffer and the samples were subjected to SDS-PAGE. The dried gels were exposed to X-ray film for autoradiography.

2.2.4. Gel electrophoresis and Western Blot analysis

Proteins were separated on 10% SDS-PAGE or using precast 4–12% NuPAGE-Gels (Novex, precast gels from Invitrogen Life Science, Paisley, UK) and transferred to nitrocellulose paper. Blots were washed with TTBS (TBS containing 0.05% Tween-20) and blocked with 3% bovine serum albumin (w/v)-TTBS for 2 h. Washed nitrocellulose filters were incubated overnight at 4 °C with the appropriate antibodies. After extensive washes in TTBS, the immunoreactive bands were detected by chemiluminescence coupled to peroxidase activity according to the manufacturer's specifications (ECL kit, Thermo Scientific, Waltham, MA, USA). The specificity of the polyclonal and monoclonal anti-NO_2Tyr antibodies was established by competition experiments by a pre-incubation of the antibody with 5 mM NO_2Tyr for 10 min, which switched off the ECL signals obtained with both monoclonal and polyclonal antibodies.

2.2.5. Recombinant protein expression and purification

DNA coding for the C-terminal cytoplasmic region of SYP (aa 219–308) was amplified by RT-PCR of total RNA from mouse brain and subcloned into pCRIITOPO (Invitrogen Life Science, Paisley, UK). After EcoRI-restriction, SYP insert was subcloned into pGEX-4T-1 (GE healthcare Bio-Sciences Corp., Piscataway, NJ, USA) and the construct pGEX-SYP 219–308 was used to transform BL21 and TKB1 *E. coli* cells. To express glutathione S-transferase (GST)-fused recombinant protein, cells were incubated for 1 h at 37 °C in the presence of 0.5 mM isopropyl-β-D-thiogalactoside. Tyrosine-phosphorylated GST-SYP (GST-SYP$_{pY}$) was expressed in TKB1 *E. coli* cells (Agilent Technologies, Santa Clara, CA, USA), a strain that allows obtaining tyrosine-phosphorylated recombinant proteins. GST-Grb2-SH3 domain was expressed in *E. coli* BL21 strain [35]. GST-fusion proteins were purified by affinity chromatography on glutathione-Sepharose beads (GE healthcare Bio-Sciences Corp., Piscataway, NJ, USA) following the manufacturer's instructions. Nitrated GST-SYP (GST-SYP$_{NY}$) was prepared by treatment of GST-SYP with 1 mM PN, as described above, to obtain a site-specific nitration on Tyr_{250} previously identified by mass spectrometry analysis [16].

2.2.6. Pull-down experiments

GST-SYP, GST-SYP$_{pY}$ and GST-SYP$_{NY}$ conjugated with glutathione-Sepharose beads were used in binding assays of synaptosome extracts obtained by solubilization in binding buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 1% (v/v) Triton X-100, 0.1 mM PMSF and Complete™) for 1 h at 0 °C. After centrifugation at 15,000 ×*g* for 10 min, synaptosomal extracts (7.5 mg/ml) were incubated with GST fusion proteins (20 μl of beads suspension) overnight at 4 °C under gentle rotation. After binding, the beads were washed three times with 1 ml of binding buffer and boiled in 4× loading buffer, and eluted bound proteins resolved on SDS-PAGE and transferred to nitrocellulose. Sepharose-immobilized GST-Grb2-SH3 domain was used in pull-down experiments of untreated and PN-treated synaptosomes to purify endogenous DynI [36]. Five μl of Sepharose beads (25% suspension) was added to synaptosomal extracts (5 mg/ml) obtained after solubilization for 30 min at 0 °C in binding buffer and centrifugation at 10,000 ×*g* for 10 min. Then, the beads were washed seven times with binding buffer, solubilized in 4× loading buffer and subjected to SDS-PAGE and WB analysis. Biotynilated-peptides (2 mg/ml) used in pull-down assay were previously linked to Tetralink Tetrameric Avidin resin overnight at 4 °C.

2.2.7. In vitro DynI GTPase activity and self-assembly assay

DynI was immunoprecipitated, as described above, with specific antibodies from solubilized synaptosomes and the GTPase activity was measured in the immunocomplexes following the procedure described in the GTPase Assay System (Innova Biosciences, Cambridge, UK). DynI self-assembly was monitored by a sedimentation assay [37]. Endogenous DynI was purified from synaptosomes by GST-Grb2-SH3 as described above and eluted from beads in 20 mM PIPES, pH 6.8 and 2 M NaCl. After elution, the protein was dialyzed into high-ionic strength buffer (20 mM Hepes, pH 7.4, 150 mM NaCl, 2 mM EGTA, 2 mM $MgCl_2$, 0.1 mM PMSF) for 2 h with three buffer changes, followed by concentration in Microcon, cut-off 30 kDa (Millipore Bioscience Research Reagent, Billerica, MA, USA). Subsequently, the protein was diluted 10-fold into either high-ionic strength buffer or low-ionic strength buffer (20 mM PIPES, pH 7.4, 20 mM Hepes, 2 mM EGTA, 2 mM $MgCl_2$, 0.1 mM PMSF) on ice. Supernatant and pellet fractions were obtained after centrifugation at 150,000 rpm for 20 min in a TL100 (Beckman Instruments Inc., Fullerton, CA, USA). The pellet fractions were dissolved in 4× loading buffer, while DynI in the supernatant fractions was precipitated with TCA 20% (v/v). DynI in each fraction was resolved on 4–12% NuPAGE-Gels and revealed on gel by silver staining or on nitrocellulose by WB with anti-DynI antibody.

2.2.8. Identification of nitrated tyrosine residues in DynI protein by mass spectrometry

DynI obtained in the pull-down experiment with GST-Gbr2-SH3 from 0.5 mM PN-treated synaptosomes, after separation on 4–12% NuPAGE-Gels, was stained with Coomassie Colloidal Blue (Invitrogen Life Science, Paisley, UK). The detectable bands were cut away from the gel, cut again in small pieces, washed with water and destained with a solution containing 50 mM ammonium bicarbonate/acetonitrile (1:1 v/v) (CH_3CN, Merck, Darmstadt, Germany). The protein bands were subsequently treated to reduce and alkylate cysteine residues with DTT and iodoacetamide, respectively, and finally digested with 12.5 ng/ml trypsin (sequencing grade modified porcine trypsin, Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate at 37 °C as elsewhere described [38]. Peptide mixtures were analyzed by nanoflow reversed-phase liquid chromatography tandem mass spectrometry (RP-LC–MS/MS) using an HPLC Ultimate 3000 (DIONEX, Sunnyvale, CA, USA) connected on line with a linear Ion Trap (LTQ, ThermoElectron, San Jose, CA, USA). Peptides were desalted on a trap column (Acclaim PepMap 100 C18, LC Packings, DIONEX) and then separated in a reverse phase column – a 10 cm long fused silica capillary (Silica Tips FS 360-75-8, New Objective, Woburn, MA, USA) – slurry-packed in-house with 5 μm, 200 Å pore size C18 resin (Michrom BioResources, Auburn, CA, USA). Peptides were eluted with a linear gradient from 96% A (H_2O with 5% acetonitrile (ACN) and 0.1% formic acid) to 60% B (ACN with 5% H_2O and 0.1% formic acid) in 40 min, at 300 nl/min flow rate. Analyses were performed in positive ion mode and the HV Potential was set up around 1.7–1.8 kV. Full MS spectra ranging from m/z 400 to 2000 Da were acquired in a data-dependent mode: each full MS scan was followed by five MS/MS scans, where the five most abundant molecular ions were dynamically fragmented by collision-induced dissociation, using a normalized collision energy of 35%. Target ions already fragmented were dynamically excluded for 30 s. Tandem mass spectra were matched against SwissProt database containing rat proteins and through SEQUEST algorithm [39] incorporated in Bioworks software (version 3.3, Thermo Electron) using fully tryptic cleavage constraints with one miss cleavage permitted, static

carboamidomethylated cysteine residues (+57 Da), oxidated methionine (+16 Da) or nitrated tyrosine (+45 Da) as variable modification. Precursor and fragment ions tolerance were 1.5 Da and 1 Da. A peptide has been considered legitimately identified when it achieved cross correlation scores of 1.8 for $[M+H]^{1+}$, 2.5 for $[M+2\,H]^{2+}$, 3 for $[M+3\,H]^{3+}$, and a peptide probability cut-off for randomized identification of $p<0.001$.

3. Results

3.1. Phosphotyrosine 273 ($pTyr_{273}$) in SYP C-terminal domain is involved in the binding to and activation of c-src

We have previously demonstrated [16] that, following PN treatment, SYP can be both phosphorylated and nitrated in a dose-dependent manner. We also found that tyrosine-phosphorylated, but not tyrosine-nitrated, SYP bound to the *c-src* tyrosine kinase and enhanced its catalytic activity. Mass spectrometry analysis of the SYP C-terminal domain (aa 219–308), expressed as GST-fused recombinant protein, showed in GST-SYP_{pY} the presence of two phosphorylated tyrosine residues in the amino acids sequence 261–271 and 272–288, respectively. In GST-SYP_{NY} only one nitrated tyrosine was mapped in the amino acid sequence 247–260 [16]. MS/MS analysis allowed assigning the phosphorylation sites to Tyr_{263} and Tyr_{273}, and the nitration site to Tyr_{250} [16] (see peptide sequences in Fig. 1A and B). To further characterize the functional consequences of SYP tyrosine phosphorylation and nitration on *c-src* recruitment and activity, we performed pull-down and *in vitro* kinase assays using peptides corresponding to the identified sequences, containing Tyr_{263} (peptide 263) or Tyr_{273} (peptide 273), phosphorylated and non-phosphorylated, or Tyr_{250} (peptide 250) nitrated and non-nitrated. Each peptide was conjugated to biotin at the N-terminus to allow the binding to Tetralink-avidin beads. The conjugated peptides were used to pull-down *c-src* from rat brain synaptosomal extracts. As shown in Fig. 1C (left panel), WB analysis with an anti-*c-src* antibody revealed that the phospho-peptide containing $pTyr_{273}$ is able to precipitate *c-src* more efficiently, compared to the phospho-peptide containing $pTyr_{263}$. Notably, the nitro-peptide $NTyr_{250}$, as well as the non-modified analogous peptides (Tyr_{263}, Tyr_{273}, Tyr_{250}), did not bind to *c-src*. As expected, the pull-down with Sepharose-immobilized GST-SYP, GST-SYP_{pY} and GST-SYP_{NY} of synaptosomal extracts showed that GST-SYP_{pY} was able to bind to *c-src* more efficiently, compared to GST-SYP and GST-SYP_{NY} (Fig. 1C, central panel). Interestingly, GST-SYP_{pY} and phospho-peptide 273 also bind to *lyn* (Fig. 1C, right panel), another tyrosine kinase of the *src* family expressed in the nervous system, suggesting a common recognition mechanism between SYP and this class of kinases.

Since tyrosine-phosphorylated SYP enhances *c-src* kinase activity [16], we investigated which tyrosine residue in SYP C-terminal domain was responsible for the stimulation of the enzymatic activity in purified $pp60^{src}$. *In vitro* kinase assay was carried out in the presence of increasing concentrations (0.1–1 mM) of phosphorylated or non-phosphorylated peptides 273 and 263. The activity of $pp60^{src}$ was measured as autophosphorylation and phosphorylation of the exogenous substrate enolase (Fig. 2A). Both enolase phosphorylation and autophosphorylation of $pp60^{src}$ were found and significantly increased in a dose-dependent manner in samples containing the peptide $pTyr_{273}$ compared to the analogous non-phosphorylated peptide (Fig. 2A, upper panel). The peptide 263, either phosphorylated or non-phosphorylated, did not affect $pp60^{src}$ activity (Fig. 2A, lower panel). In the same experimental conditions, the nitro-peptide 250 failed to activate $pp60^{src}$ (data not shown). Again, activation of $pp60^{src}$ was also observed with GST-SYP_{pY} but not with GST-SYP_{NY} or

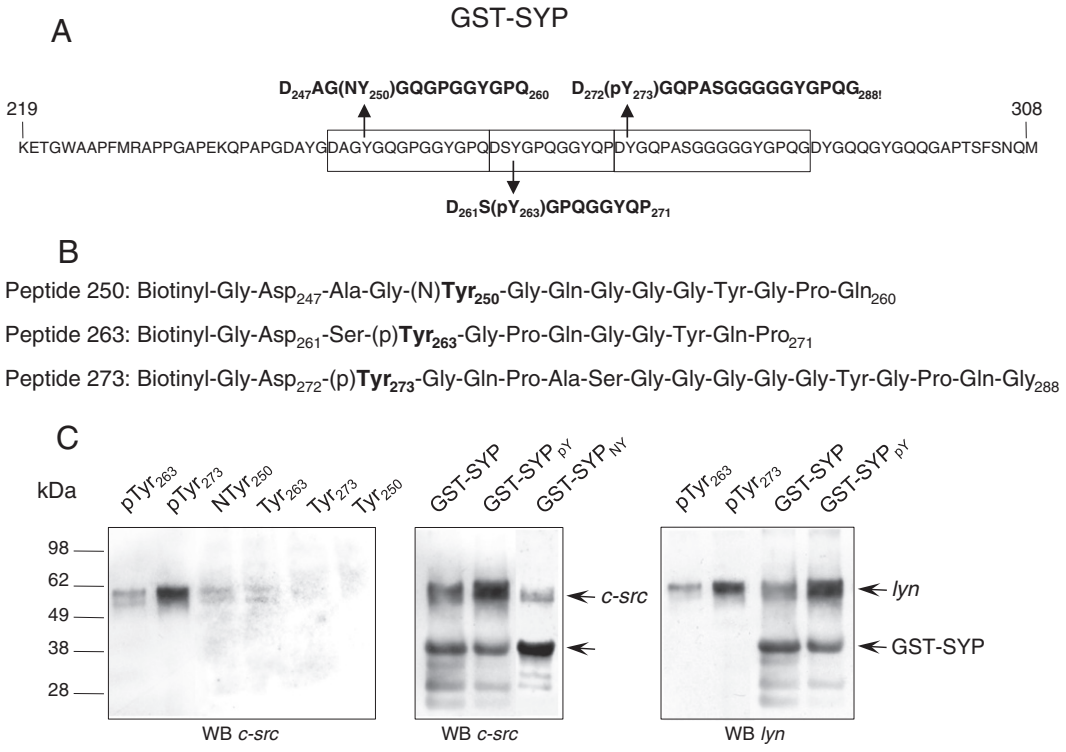

Fig. 1. SYP peptide containing phosphorylated Tyr_{273} binds to *c-src* and *lyn*. (A) Amino acid sequence of SYP C-terminal domain (219–308) expressed as protein fused to GST (GST–SYP). Boxes indicate the peptide sequences containing the nitrotyrosine residue ($NTyr_{250}$) and the two phosphotyrosine residues ($pTyr_{263}$ and $pTyr_{273}$), identified as targets of PN. (B) Amino acid sequence of the peptides conjugated with biotin at the N-terminus used in the experiments described below. (C) Pull-down assay of rat brain synaptosomal extract with peptides containing Tyr_{250}/$NTyr_{250}$, Tyr_{263}/$pTyr_{263}$ or Tyr_{273}/$pTyr_{273}$ linked to avidin-Tetralink beads and with GST–SYP, GST–SYP_{pY} and GST–SYP_{NY} immobilized to Sepharose beads. The presence of *c-src* and *lyn* in the pulled-down proteins was revealed by WB analysis with anti-*c-src* and anti-*lyn* antibodies, respectively. The immunoreactive bands were detected by chemiluminescence coupled to peroxidase activity (ECL). The molecular mass standards in kDa are indicated on the left. The results shown are representative of three independent experiments.

GST-SYP (Fig. 2B), in agreement with our previous report [16]. Moreover, the two tyrosine-phosphorylated residues (273 and 263) did not show additive effect, since in the sample containing both $pTyr_{273}$ and $pTyr_{263}$ (0.5 mM each) the autophosphorylation of $pp60^{src}$ was similar to that measured in the sample containing $pTyr_{273}$ (0.5 mM) only (Fig. 2B).

3.2. Peroxynitrite-dependent tyrosine modifications of SYP inhibit its binding to DynI

Although the functional role of SYP tyrosine phosphorylation as well as tyrosine nitration is still unknown, it is tempting to speculate that tyrosine phosphorylation of SYP may regulate the associations with its binding partners. In particular, it has been proposed to have a role in the interaction of SYP with DynI, since the tyrosine-based pentapeptide motifs in SYP C-terminal region contain DynI binding sites [9]. To test if PN-dependent modifications in SYP C-terminal tyrosine residues may modulate the interaction between SYP and DynI, two different approaches were conceived: pull-down assays from solubilized synaptosomes using Sepharose-immobilized GST-SYP and immunoprecipitation of SYP from untreated and PN-treated synaptosomes solubilized under conditions that preserve protein–protein interactions. The presence of DynI in the immunocomplexes was revealed by WB analysis using specific anti-DynI antibody. We found that the C-terminal tail of SYP (GST-SYP) very efficiently bound to and precipitated DynI. Noteworthy, $GST\text{-}SYP_{pY}$ and $GST\text{-}SYP_{NY}$ pulled-down DynI to a lesser extent than GST-SYP (~30% and ~50%, respectively, Fig. 3A, upper panel). Conversely, we attempted to pull-down DynI from solubilized synaptosomes using peptides (263, 273 and 250) immobilized on Tetralink-avidin beads, but we did not reveal the presence of DynI in any of the conditions tested (results not shown). The co-immunoprecipitation experiments using an anti-SYP antibody showed that the formation of SYP/DynI complex was impaired by modifications induced by PN (Co-IP SYP in Fig. 3B). Similarly, the co-immunoprecipitation with anti-DynI revealed that less SYP was associated to DynI in synaptosomal samples treated with PN (Co-IP DynI in Fig. 3B). As expected, neither anti-SYP nor anti-DynI immunocomplexes contained Syntaxin 1 (Fig. 3B) [9]. DynI typically migrates as one band in the SDS-PAGE, but in the Co-IP experiments, as in that described hereafter, WB analysis revealed a doublet (Fig. 3B, Input column, central panel), probably due to an alternative splicing of DynI, as described by Chan et al. [40].

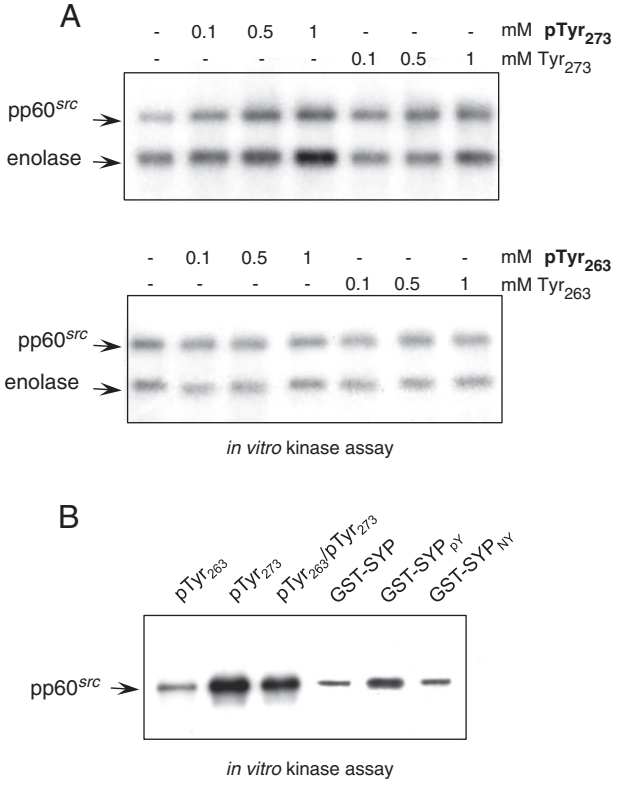

Fig. 2. SYP peptide containing phosphorylated Tyr_{273} activates $pp60^{src}$. (A) Autoradiographs of *in vitro* kinase assay of recombinant purified $pp60^{src}$ (0.5 U) performed in the presence of peptides containing Tyr_{273} and Tyr_{263} (upper and lower panels, respectively), phosphorylated (pTyr) and non-phosphorylated (Tyr), at concentrations of 0.1, 0.5 and 1 mM. Enolase (1 μg) was added to the reaction mixture as exogenous substrate. (B) Autoradiograph of *in vitro* kinase assay of $pp60^{src}$ (0.5 U) performed in the presence of peptides $pTyr_{263}$, $pTyr_{273}$ (0.5 mM) or a mixture of $pTyr_{263}/pTyr_{273}$ at a concentration of 0.5 mM each, and in the presence of GST–SYP, $GST\text{–}SYP_{pY}$ and $GST\text{–}SYP_{NY}$. The kinase assays were carried out in the presence of 1–2 μCi [γ^{32}P] ATP. [^{32}P]-labeled proteins were resolved on 10% SDS-PAGE and shown on dried gel by exposure to X-ray film. The results shown are representative of four independent experiments.

3.3. Peroxynitrite down-regulates tyrosine phosphorylation of DynI

Some components of the vesicular endocytic machinery have been shown to undergo regulated tyrosine phosphorylation mediated by the tyrosine kinase *c-src*. SYP is one of the major substrates of vesicle-associated *c-src*. Another target of *c-src* is DynI, which can directly interact with *src*-SH3 domain [41]. We investigated the tyrosine phosphorylation status of DynI following PN treatment of synaptosomes. DynI was immunoprecipitated with a specific antibody and tyrosine phosphorylation was revealed by WB analysis with an anti-pTyr antibody. As shown in Fig. 4(A), PN down-regulated DynI phosphorylation in a dose-dependent manner. Inhibition of DynI phosphorylation was also evident in synaptosomes treated with commercial PN (Alexis) at 0.3 mM concentration (Fig. 4A). As a control, the nitrocellulose was probed with an anti-DynI antibody to assess that equal amounts of DynI were immunoprecipitated in the different experimental conditions (Fig. 4A). We also performed an *in vitro* phosphorylation assay in the presence of [γ^{32}P] ATP by adding purified $pp60^{src}$ (0.5 U) to DynI-immunocomplex obtained from untreated and PN-treated synaptosomes. We found that the incorporation of [^{32}P] into DynI decreased dose-dependently after PN treatment (Fig. 4B). This experiment substantiates the results shown in Fig. 4(A), underlying PN-induced down-regulation of the DynI phosphorylation signal. Since DynI can directly interact with *src*-SH3 domain, we quantified both the amount and the enzymatic activity of *c-src* associated with DynI in co-immunoprecipitation assays from untreated and PN-treated synaptosomes. We found that PN dose-dependently inhibited both the interaction between DynI and *c-src*, as revealed by WB analysis with anti-*c-src* antibody (Fig. 4C), and the activity of *c-src* measured in the *in vitro* kinase assay as autophosphorylation and phosphorylation of enolase (Fig. 4D). The high molecular weight bands on the autoradiograph (Fig. 4D) corresponded to immunoprecipitated DynI, whose phosphorylation signal was reduced by PN treatment, as shown in Fig. 4B.

3.4. Identification of tyrosine 354 in DynI protein as target of peroxynitrite

One of the oxidative reactions induced by PN is the modification of tyrosine residues in proteins to yield NO_2Tyr, and it has been suggested that this modification impairs phosphotyrosine signaling. To investigate whether PN-dependent down-regulation of DynI tyrosine phosphorylation may be caused by NO_2Tyr formation, we immunoprecipitated DynI from PN-treated synaptosomes and subjected the immunocomplex to WB analysis using anti-NO_2Tyr antibodies. Unfortunately, the analysis failed to reveal any signal corresponding to the molecular weight of DynI (data not shown). To enrich for the protein, DynI was affinity purified from synaptosomal extract using recombinant SH3 domain, a technique that allows the isolation of high amounts of biologically active native DynI rapidly and reproducibly [36]. As affinity ligand, we used

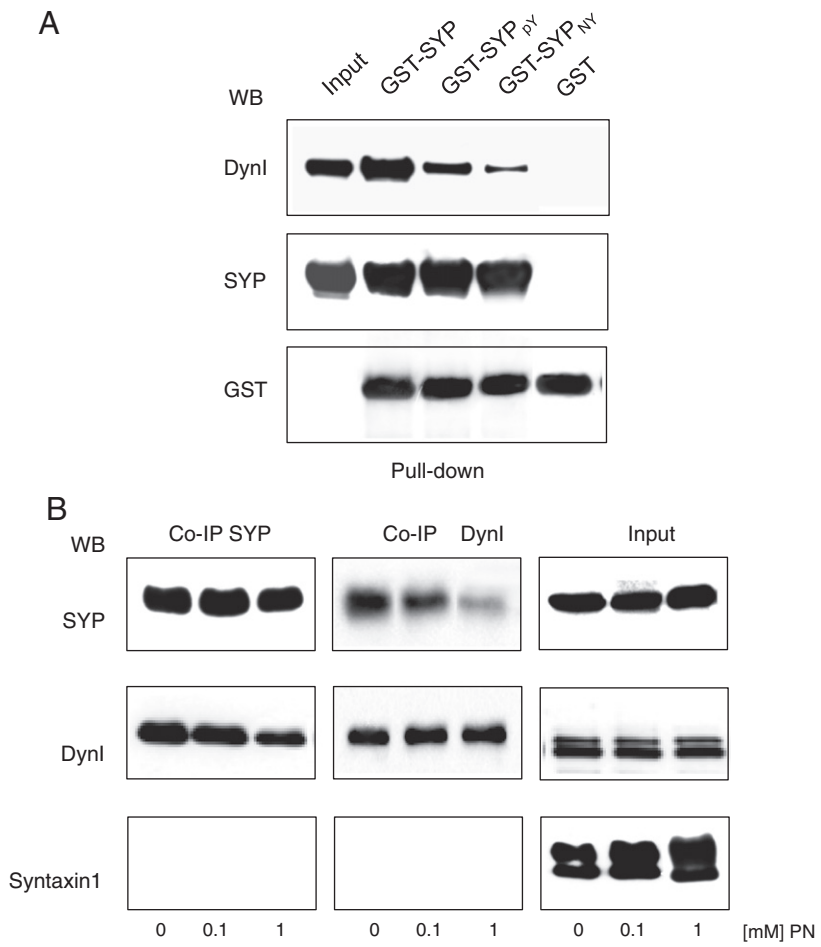

Fig. 3. PN-dependent modifications of SYP inhibit its binding to DynI. (A) Pull-down assay with Sepharose-immobilized GST–SYP, GST–SYP_{pY} and GST–SYP_{NY} from rat brain synaptosomal extract. The presence of bound DynI in pulled-down samples was revealed by WB analysis with anti-DynI antibody (upper panel). Input, starting material of synaptosomal extract. The pulled-down proteins were also probed with anti-SYP and anti-GST antibodies (central and lower panels, respectively), as control. (B) Co-immunoprecipitation (Co-IP) assays with anti-SYP and anti-DynI antibodies from untreated or 0.1–1 mM treated synaptosomes. After Co-IP, the presence of DynI and SYP was revealed using the respective specific antibodies. The nitrocelluloses were probed with anti-Syntaxin 1 as control. Input, starting material of synaptosomal extract. The results shown are representative of three independent experiments.

bacterially expressed GST fused to the Grb2-SH3, which recognizes DynI Prolin-Rich Domain (PRD). GST-Grb2-SH3 pulled-down a protein of about 100 kDa, revealed on gel by Coomassie Colloidal Blue staining (Fig. 5A) and identified as DynI by WB analysis with the anti-DynI antibody (Fig. 5B, left panel). Noteworthy, the same blot probed with an anti-NO_2Tyr antibody showed a strong NO_2Tyr signal associated with DynI band in the pull-down obtained from synaptosomes treated with 1 mM PN (Fig. 5B, right panel). To map the DynI tyrosine residues modified by PN, the native DynI, pulled-down with GST-Grb2-SH3 from synaptosomes treated with 0.5 mM PN, was separated on a 4–12% NuPAGE gel, the appropriate band was digested with trypsin and the peptide mixture analyzed by Liquid Chromatography tandem Mass Spectrometry (LC–MS/MS). The MS/MS analysis identified a nitrated Tyr_{354} located in the tryptic peptide containing amino acids 344–361 (Fig. 6). Not all the DynI molecules carried this modification as indicated by the presence of MS/MS spectrum of the same peptide without any modification. The extent of Tyr_{354} nitration was tricky to evaluate, although from the extract ion analysis, it appears to be less than 5% (supplementary Figure). In addition, LC–MS/MS analysis revealed that in untreated synaptosomes DynI showed a basal level of nitration. Indeed, we identified also nitrated Tyr_{125}, Tyr_{541} and Tyr_{669}, however, more interestingly, nitrated Tyr_{354} was detected only after PN treatment of synaptosomes.

3.5. Peroxynitrite inhibits GTPase activity and self-assembly of DynI

It is known that phosphorylation of DynI by *c-src* induces its self-assembly and increases its GTPase activity [42]. Upon recruitment via the C-terminal PRD, DynI oligomerizes into helical structures around the neck of budding vesicles and catalyzes vesicle scission in a GTP-hydrolysis-dependent manner. Based on the results described above, we investigated whether PN-dependent tyrosine modifications affected DynI GTPase activity. We measured the GTPase activity in the DynI immunocomplex obtained from PN-treated synaptosomes. The results clearly demonstrated that PN significantly down-regulated the DynI GTPase activity in a dose-dependent manner (Fig. 7). Commercial PN (Alexis) at 300 μM induced a decrease of DynI GTPase activity comparable to that measured in the 0.1 mM PN-treated sample (Fig. 7). Treatment of synaptosomes with decomposed PN did not affect DynI GTPase activity (Fig. 7).

Isolated DynI self-assembles *in vitro* in low-salt buffers into ordered formations of rings and helical arrays [37], similar morphologically to helical structures assembled at the necks of budding vesicles [43]. High-ionic strength conditions, conversely, do not permit DynI self-assembly. Because self-assembly of DynI is known to regulate its GTP hydrolysis rate [44], we hypothesized that the observed decrease in GTPase activity of DynI might depend on its diminished

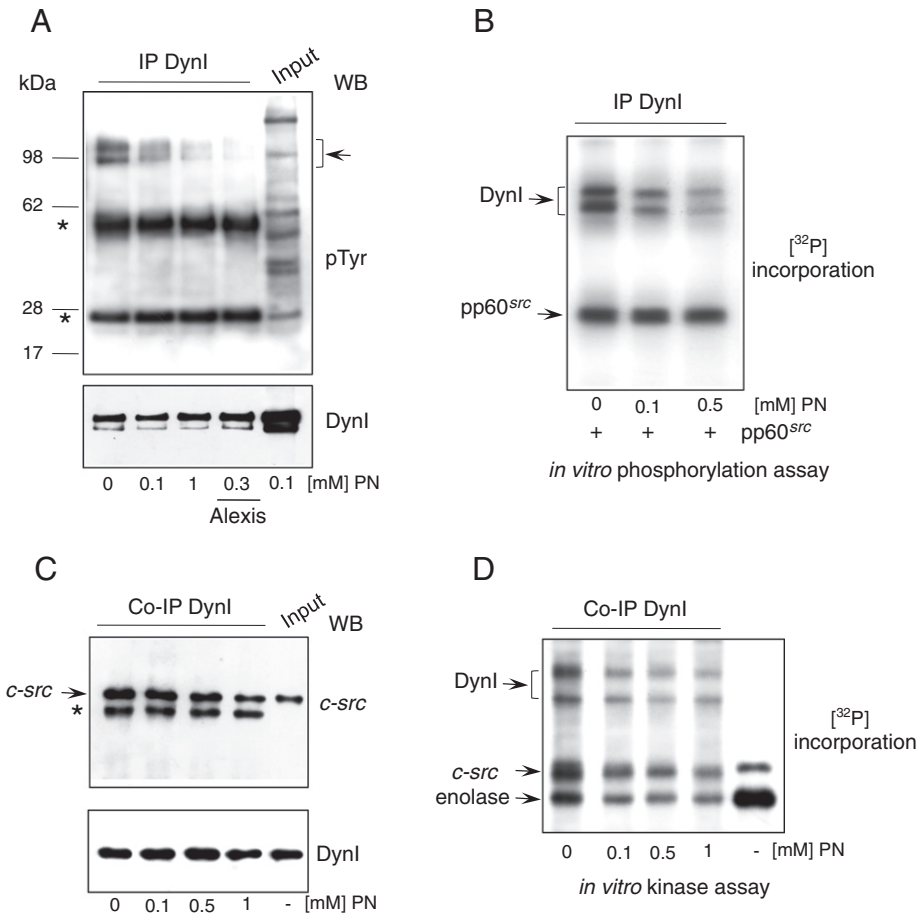

Fig. 4. PN down-regulates tyrosine phosphorylation of DynI. (A) WB analysis of DynI immunoprecipitated from synaptosomes (IP DynI), untreated or treated with 0.1 or 1 mM PN or with commercial 0.3 mM PN (Alexis), with anti-pTyr and anti-DynI antibodies. Input, starting material extract of synaptosomes treated with 0.1 mM PN. Equivalent DynI amounts were immunoprecipitated from the different samples as shown by probing the nitrocellulose with anti-DynI antibody (lower panel). The molecular mass standards in kDa are indicated on the left. The arrow shows the molecular weight of DynI and the asterisks indicate the migration of IgG high and low chains. (B) Autoradiograph of *in vitro* phosphorylation assay of DynI by exogenous kinase pp60^{src} (0.5 U). DynI was first immunoprecipitated from synaptosomes treated with PN at the concentrations indicated and the reaction was carried out in the presence of 1–2 μCi [γ^{32}P] ATP. [^{32}P]-labeled proteins were resolved on 10% SDS-PAGE and revealed on dried gel by exposure to radiographic film. (C) WB analysis of DynI immunoprecipitated (Co-IP DynI) from synaptosomes under conditions that preserve protein–protein interactions probed with anti-*c-src*. The asterisk indicates the IgG high chain. The lower panel shows that equivalent amounts of DynI were immunoprecipitated from the different samples. Input, total synaptosomal extract. (D) Autoradiograph of *in vitro* kinase assay of proteins co-immunoprecipitated with anti-DynI antibody (Co-IP DynI) from synaptosomes after PN treatment. DynI, *c-src* and enolase (1 μg), added to the reaction mixture as exogenous substrate, are indicated (arrows). The reaction was carried out in the presence of 1–2 μCi [γ^{32}P] ATP and the incorporation of [^{32}P] into the proteins revealed on dried gel by exposure to radiographic film.

self-assembly. Endogenous DynI protein was purified from untreated and 0.5 mM PN-treated synaptosomes using Sepharose-immobilized GST-Grb2-SH3 and eluted from beads in 2 M NaCl. WB analysis with anti-DynI of GST-Grb2-SH3-bound DynI was performed before and after elution to verify that the same amount of protein was recovered and subjected to self-assembly assay (Fig. 8A). After dilution into low-ionic or high-ionic strength buffers, DynI was collected by sedimentation at high-speed centrifugation. DynI in each fraction was revealed on gel by silver staining (Fig. 8B, upper panel) or on nitrocellulose with the anti-DynI antibody (Fig. 8B, lower panel). Interestingly, DynI coming from the PN-treated sample lost the ability to self-assembly, since at low salt it remained in its disassembled status in the supernatant compared to control sample. As expected, in high salt, DynI was present mainly in the supernatant in all samples tested (Fig. 8B).

4. Discussion

This study provides evidence of the role played by PN-induced protein tyrosine nitration and phosphorylation in modulating functional properties and association of proteins involved in exo-endocytosis of SVs, a process tightly linked to neurotransmitter release and synaptic transmission. Besides SV protein SYP [16], we found that DynI, a GTPase required for SV endocytosis, is also modified by PN. We observed that the oxidative changes induced by PN at the nerve terminal modulate the interactions of SYP with *c-src* and DynI, and affect DynI GTPase activity and self-assembly. We show for the first time that the functional properties of DynI are down-regulated in response to increasing concentration of PN, suggesting a new level of regulation of endocytotic machinery.

The reactive oxygen and nitrogen species can be critical for excitatory synaptic activity and, as we previously described, PN can significantly stimulate vesicle exocytosis and induce endogenous glutamate release from synaptosomes [45]. SVs fuse with the membrane of the nerve terminal during neurotransmitter release and are subsequently retrieved and recycled. This process relies on specific protein interactions to form transient functional complexes promoting the correct steps from exo- to endocytosis.

SYP is known to interact with other proteins of the SV membrane as VAMP2 [6] and the 38 kDa subunit of the vacuolar V-ATPase [7] to regulate exocytotic vesicle fusion at the level of SNARE complex or fusion pore formation [3]. Moreover, SYP is the major tyrosine

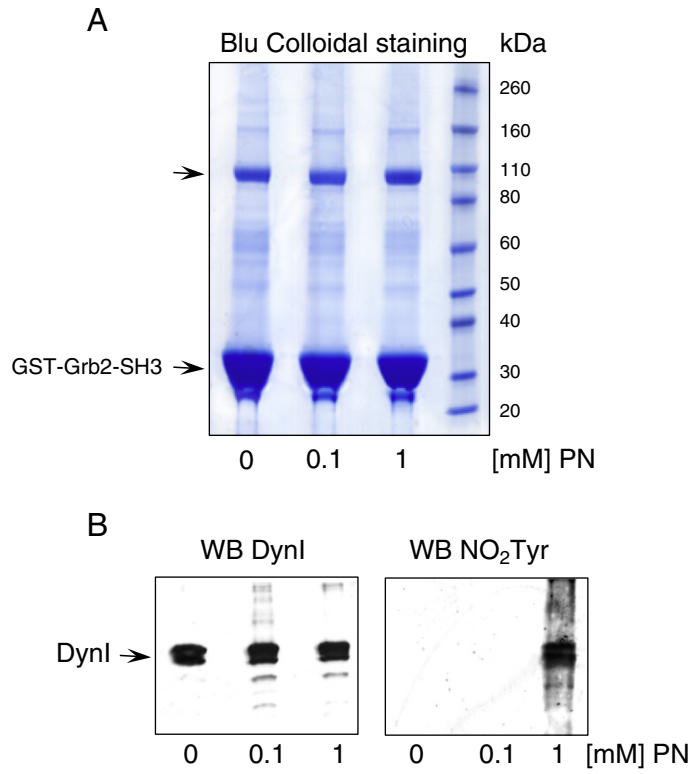

Fig. 5. DynI is nitrated by PN. Purification of native DynI from synaptosomal extracts using recombinat Grb2–SH3 domain. (A) Proteins pulled-down with Sepharose-immobilized GST–Grb2–SH3 were analyzed on 4–12% NuPAGE-Gel and stained with Coomassie Colloidal Blue. The arrow on the left indicates the migratory position of a ~100 kDa Grb2–SH3-bound protein. The migration of molecular mass markers in kDa is indicated on the right. (B) WB analysis with anti-DynI antibody showed that the ~100 kDa band in (A) corresponds to DynI (left panel). When the nitrocellulose was probed with anti-NO_2Tyr antibody, DynI resulted nitrated only in synaptosomes treated with 1 mM PN (right panel).

phosphorylated protein of SVs and its C-terminal tail is a substrate of the non-receptor tyrosine kinase *c-src in vitro* [11]. We previously showed that in rat brain synaptosomes treated with PN, depending on its concentrations, SYP can be tyrosine phosphorylated at Tyr_{263} and Tyr_{273} and tyrosine nitrated at Tyr_{250}. When phosphorylated, but not nitrated, SYP binds to *c-src*-SH2 domain and enhances *c-src* kinase activity [16]. In this study we have further characterized the association between SYP and *c-src* demonstrating that the peptide containing phosphotyrosine Tyr_{273}, but not phosphotyrosine Tyr_{263} or nitrotyrosine Tyr_{250}, specifically binds to and activates *c-src*. In addition, phosphotyrosine Tyr_{273} also recruits *lyn*, another kinase of the *src* family expressed in the nervous system, suggesting a common recognition mechanism between SYP and this class of kinases. It has been reported that *c-src* activity and *c-src*/SYP association are increased in the hippocampus of rats trained in a spatial memory task [46] and that tyrosine phosphorylation of SYP is enhanced upon synaptic stimulation, as it is known to occur during the long-term potentiation [47]. Since, in our experimental model, nitrated-SYP does not bind to nor activates *c-src*, we hypothesize that signaling processes relying on tyrosine phosphorylation may be potentially down-regulated. *In vivo*, site-specific nitration may serve as a pathogenic mechanism for inactivating catalytic proteins and promoting altered assembly of structural proteins in neurodegenerative diseases [48].

The C-terminal domain of SYP is also involved in the interaction with DynI. Indeed, a progressive removal of the nine YG(P/Q)QC repeats in SYP C-terminal results in a successive loss of DynI binding [9] and in reduction of endocytosis during neuronal activity [49]. Given the well-established role of DynI in endocytosis [14], SYP/DynI complex might take part in vesicle recycling by targeting DynI to SV membrane [49]. It has been suggested that SYP tyrosine phosphorylation may limit the pool of DynI competent to associate with SYP [9]. It is reasonable to hypothesize that tyrosine phosphorylation and/or nitration of SYP influences its interaction with DynI. In fact, we found that the association between SYP and DynI in rat brain synaptosomes was impaired by increasing concentrations of PN. Moreover, the formation of SYP/DynI complex was mediated by the full length C-terminal domain of SYP, in agreement with previous findings [9], since none of the peptides (250, 263 and 273) used in pull-down assays was able to precipitate DynI. The observation that SYP/DynI complex in brain extracts is promoted by dissociation of VAMP2 from SYP and by Ca^{2+} influx suggests that DynI only associates with SYP after the disassembly of SYP/VAMP2 complex [9]. It has been observed that, in rats infused with amyloid β1–40, SYP is both nitrated and phosphorylated and changes its ability to recruit VAMP2 [50]. Actually, we found that SYP associated with higher amounts of VAMP2 in synaptosomes treated with PN (C. Mallozzi, C. D'Amore and A.M.M. Di Stasi, unpublished observations), thus blocking the binding of DynI with SYP and affecting the correct targeting of DynI to the SV membrane.

At the nerve terminal, DynI is one of the substrates of *c-src* and directly interacts with *c-src*-SH3 through its PRD [41]. This association suggests a role for *src* in membrane traffic events [51]. The *c-src*-mediated tyrosine phosphorylation of DynI is required for agonist-induced endocytosis of β2-adrenergic [52] and M1 muscarinic acetylcholine [53] receptors, but the mechanisms whereby phosphorylation of DynI regulates SV endocytosis are still unclear. Here, we found that, in a dose-dependent manner, PN inhibits the interaction between DynI and *c-src* and down-regulates tyrosine phosphorylation of DynI. We also observed that DynI isolated from PN-treated synaptosomes is no longer recognized as a substrate by the exogenous tyrosine kinase $pp60^{src}$. In our experimental conditions, the kinase activity of DynI-associated *c-src* was not increased by PN. Onofri et al. [51] reported that another synaptic protein containing PRD, Synapsin I, recruits *c-src* through its

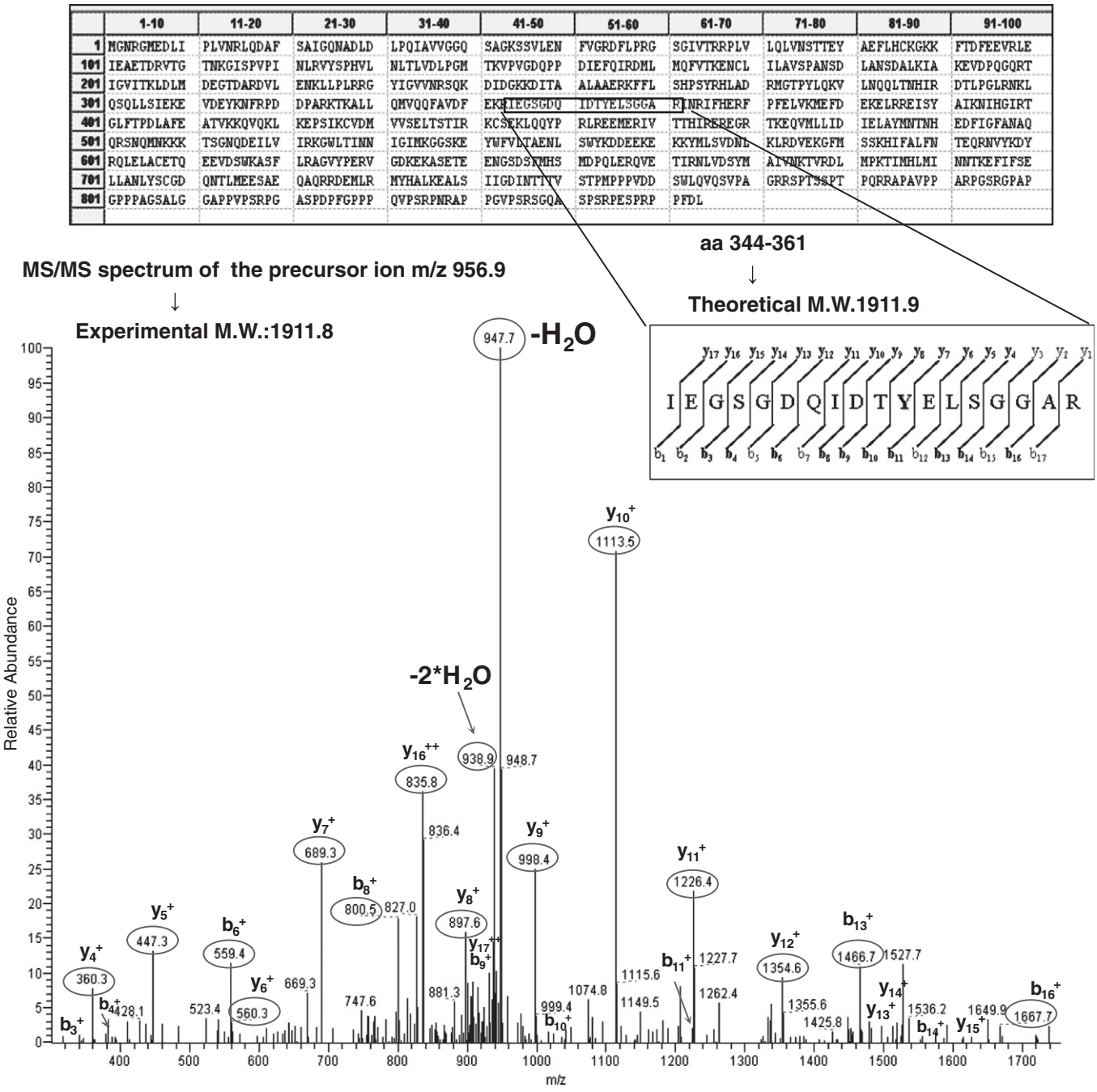

Fig. 6. Localization of nitrated tyrosine residue by MS/MS in DynI. Sequence of DynI is shown in the upper part of the figure with the sequence aa 344–361 enclosed in the square. In the lower part of the figure, the MS/MS spectrum of the precursor ion m/z 956.9 is displayed. The ion has a double charge, corresponding to a MW 1911.8 and is assigned to the tryptic peptide aa 344–361, carrying a nitrated tyrosine, as MW and pattern fragmentation demonstrate. Peaks matching the b and y series are indicated in the spectrum and in bold in the fragmentation scheme in the inset.

SH3 domain, inducing an initial activation of kinase activity, which in turn phosphorylates Synapsin I. Phosphorylated Synapsin I recognizes *c-src*-SH2, allowing further activation of *c-src*. We cannot exclude that DynI PRD interacts with *c-src*-SH3, but it is conceivable that DynI, after PN treatment, does not recruit *c-src*-SH2 and does not enhance *c-src* kinase activity.

Remarkably, we have identified by LC–MS/MS analysis one major nitration site at Tyr_{354} in DynI isolated from synaptosomes treated with PN. LC–MS/MS analysis revealed also that in untreated synaptosomes DynI showed a basal level of nitration on Tyr_{125}, Tyr_{541} and Tyr_{669}, however these sites of nitration were not modified by PN and did not affect DynI functional properties. Tyr_{354} was instead nitrated only after PN treatment of synaptosomes implying that this site-specific post-translational modification likely accounts for DynI dysfunction. It is interesting to note that Tyr_{354} has been previously identified as phosphorylated *in vitro* by *c-src* [42], while *in vivo* analysis showed that Tyr_{597}, but not Tyr_{354}, is phosphorylated in response to EGF stimulation in COS-7 cells [42]. Three tyrosine phosphorylation sites, at position 80, 125 and 354, have been identified in mouse brain DynI using large scale phosphoproteomic screens for tyrosine

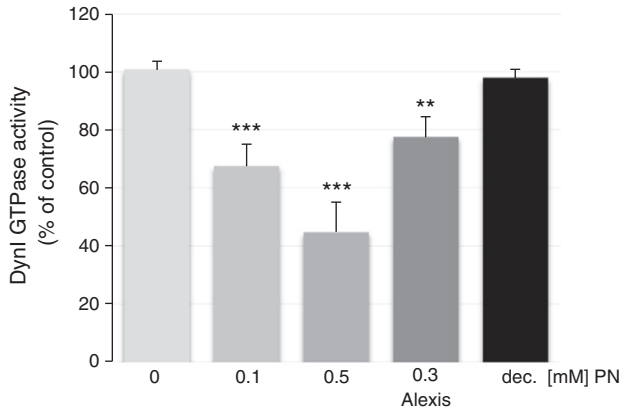

Fig. 7. PN treatment of synaptosomes down-regulates DynI GTPase activity. Representative histogram of the GTPase activity measured in DynI immunocomplex, obtained from synaptosomes treated with PN at concentrations indicated and with commercial PN (Alexis), following the procedure described in section 2. In the last column (dec.), GTPase activity was measured in samples treated with decomposed PN. GTPase activity is expressed as percentage of the value measured in control samples (100%). Values are represented as means ± SD from four separate experiments. Statistical analysis was performed according to Student's *t*-test. *** $p<0.001$; ** $p<0.01$ vs control.

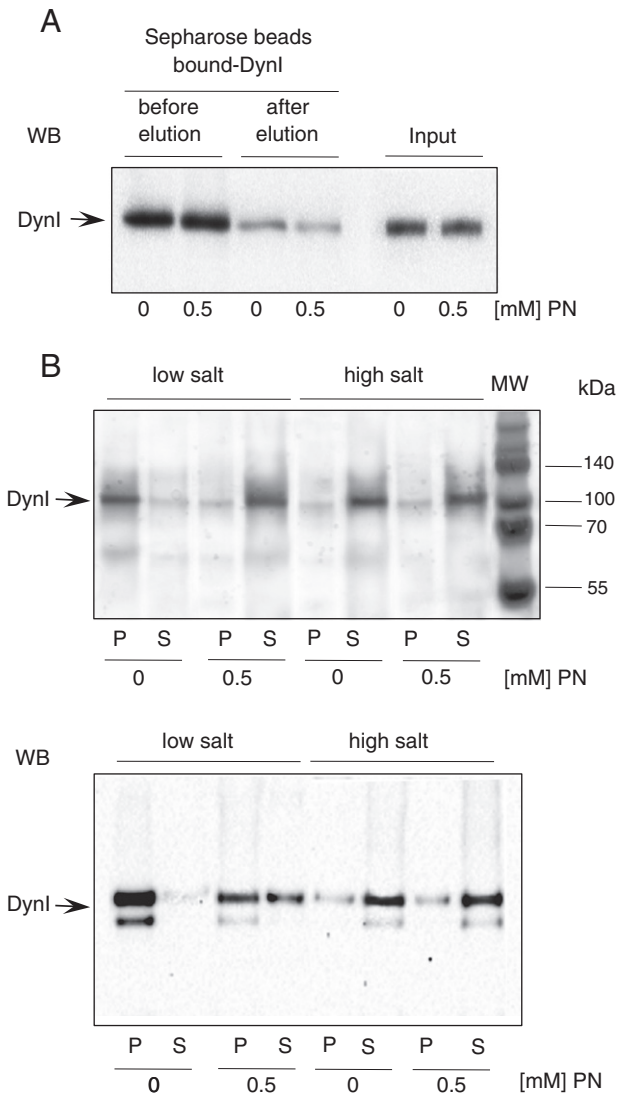

Fig. 8. PN impairs self-assembly of DynI. Endogenous DynI was purified from untreated or 0.5 mM PN-treated synaptosomes with Sepharose-immobilized GST–Grb2–SH3. (A) WB analysis with anti-DynI antibody of pulled-down DynI before and after elution from the Sepharose beads. Equal aliquots of solubilized synaptosomes were used (Input). (B) DynI self-assembly assay. Self-assembly was monitored by a sedimentation assay of DynI recovered after elution in 2 M NaCl and diluted in 10 vol of low or high salt buffers. DynI in each fraction was resolved on 4–12% NuPAGE-Gel and revealed on gel by silver staining (upper panel) or on nitrocellulose with anti-DynI antibody (lower panel). The migration of molecular mass markers in kDa is indicated on the right in the upper panel. The results are representative of four independent assays.

phosphorylation [54]. Nevertheless, the identification and the functional role of DynI tyrosine phosphorylation in controlling SV endocytosis remain open questions.

The presence of a nitro-group on a phosphorylable tyrosine residue may account for the impairment of phosphotyrosine signaling of DynI mediated by PN. Emerging data support a strong link between NO_2Tyr formation and both activation and interference with phosphotyrosine-dependent signaling pathways in a variety of cellular responses [17,18,55]. In this study we point out for the first time that DynI is the target of PN, which induces nitration of a specific tyrosine residue. The formation of NO_2Tyr in DynI well correlates with and may account for the failure of tyrosine phosphorylation due to PN treatment.

DynI is a mechanochemical GTPase that oligomerizes around the neck of clathrin-coated pits and catalyzes the "pinching off" of endocytosing vesicles in a GTP-hydrolysis-dependent manner. The molecular details of oligomerization and the mechanism of the mechanochemical coupling are currently unknown. It has been demonstrated that tyrosine phosphorylation of DynI by *c-src* induces its self-assembly and increases its GTPase activity. Isolated DynI self-assembles *in vitro* in low-salt buffers into ordered formations of rings and helical arrays [37], morphologically similar to helical structures assembled at the necks of budding vesicles [43]. DynI self-assembly itself is sufficient to stimulate GTPase activity several fold in the absence of any effector molecule. Noteworthy, we found that both the DynI GTPase activity and self-assembly were inhibited by PN. Because DynI self-assembly enhances its GTPase activity, these findings also suggest that PN-mediated dephosphorylation and/or nitration of DynI regulates the GTPase activity by controlling self-assembly. In addition to the well known mechanisms that regulate DynI activities, such as Serine phosphorylation [56] and the interaction with the adapter protein Amphiphysin I, a major DynI binding partner [57], the redox-dependent impairment of DynI properties suggest an additional regulation mechanism of the endocytotic process that may act during neurodegeneration.

One might argue that under neurodegenerative conditions the nitration level is relatively low, resulting in the modification of only 0.01%–0.05% of all tyrosine residues [58]. However, nitrated synaptic proteins may focally accumulate during lifetime, thereby contributing to a rate-limiting step for the SV cycle. While previously considered a late-stage epiphenomenon, nitration of tyrosine residues appears to be an early event that contributes to the onset and progression of several neurological diseases [48], as demonstrated for nitrated tau protein, which co-localizes with markers of early-stage of tauopathies [59]. In addition, endogenously nitrated proteins, including DynI at Tyr_{125}, have been identified in normal mouse brain and many of the nitrated proteins, localized in pre- and postsynaptic regions of the neuron, are more extensively nitrated during the progression of neurodegenerative disorders, suggesting a potential vulnerability of neurotransmission under oxidative stress and inflammation states [60].

In conclusion, our results suggest that post-translational modifications (phosphorylation and nitration) induced by PN at the nerve terminal play a critical role in modulating mutual affinities and functional properties of DynI and SYP, both involved in the exo-endocytosis cycle of SVs. Moreover, these findings provide a new insight into the regulation

of DynI function in endocytosis of SVs via control of self-assembly by the tyrosine residue modifications.

Acknowledgments

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