Super-resolution study of PIAS SUMO E3-ligases in hippocampal and cortical neurons

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The SUMOylation machinery is a regulator of neuronal activity and synaptic plasticity. It is composed of SUMO isoforms and specialized enzymes named E1, E2 and E3 SUMO ligases. Recent studies have highlighted how SUMO isoforms and E2 enzymes localize with synaptic markers to support previous functional studies but less information is available on E3 ligases. PIAS proteins - belonging to the protein inhibitor of activated STAT (PIAS) SUMO E3-ligase family - are the best-characterized SUMO E3-ligases and have been linked to the formation of spatial memory in rodents. Whether however they exert their function co-localizing with synaptic markers is still unclear. In this study, we applied for the first time structured illumination microscopy (SIM) to PIAS ligases to investigate the co-localization of PIAS1 and PIAS3 with synaptic markers in hippocampal and cortical murine neurons. The results indicate partial co-localization of PIAS1 and PIAS3 with synaptic markers in hippocampal neurons and much rarer occurrence in cortical neurons. This is in line with previous super-resolution reports describing the co-localization with synaptic markers of other components of the SUMOylation machinery.

Key words: Neurons; SUMO; synapse; hippocampus; cortex.

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Introduction

In neurons post-translational modifications (PTMs) regulate the signaling cascade that guarantees an efficient, rapid and reversible response to extracellular stimuli.1,2 SUMOylation is one of the main regulators of neuronal activity3,4 and synaptic plasticity.5-7 It may also play a role in pathological processes that cause neurological disorders such as Parkinson’s and Alzheimer’s diseases (AD).4,8-10 SUMOylation involves the covalent conjugation of ubiquitin-like proteins, called small ubiquitin-like modifiers (or SUMO), to lysine residues of specific targets, by an enzymatic cascade.11 Five variants (SUMO1-5) have been found in mammals and brain cells very likely only express SUMO1-3.12,13 While their aminoacidic sequences differ, they share an almost identical three-dimensional structure.14,15

The proteins responsible for SUMO conjugations are the SAE1/SAE2 heterodimers, known as the SUMO-E1 enzyme, the SUMO-E2 ligase Ubc9, and various SUMO-E3 enzymes.12 SUMO proteins are initially activated by SAE1/SAE2 and are subsequently transferred to UbC9, which catalyzes the conjugation of SUMO proteins to lysine residues of the target substrates. UbC9 can be assisted during this conjugation by SUMO-E3 enzymes such as, for example, members of the PIAS family.16

Unlike the ubiquitin system, which comprises a multitude of E3 ligases,7 the SUMOylation pathway only can count on members of the PIAS family and a few other SUMO ligases (such as ZNF451 and RanBP2).16,17 SUMO can be removed from targets by specialized isopeptidases, such as members of the SENP family.18 All together, the SUMO isoforms, ligases and the SUMO proteases form the SUMOylation machinery.

Similarly to other cells, the SUMOylation machinery has nuclear and extranuclear functions in neurons. Among the extranuclear roles, it modulates synaptic activity - as first described in rat hippocampal neurons by Martin and colleagues.20 Since then, several reports have finely described how the SUMOylation machinery, in both hippocampal and cortical neurons, is required for proper neuronal functioning.21-23 Recently however, two reports have questioned the role of SUMOylation in synaptic plasticity. The authors failed to detect i) SUMOylation in synaptic fractions and ii) co-localization of SUMO1 with synaptic markers.24,25 Thus, they concluded that SUMOylation of synaptic proteins is, at best, very rare. Subsequent studies from our group employed structured illumination microscopy (SIM), a super-resolution technique that doubles the resolution of conventional microscopes, to systematically confirm the co-localization of several components of the SUMOylation machinery with both pre- and post-synaptic markers.26,27 Other groups have also pointed to new roles of SUMOylation in the physiology of synapses.28,29 Thus validating the likely role of SUMOylation in synaptic plasticity. Currently however, localization studies of members of the PIAS E3 SUMO ligase family in neurons are still lacking, limiting our understating of how E3 SUMO enzymes may affect synaptic function in health and disease. This group of proteins comprises five members: PIAS1, PIAS3, PIASXα, PIASXβ, and PIASγ (PIAS4).30,31 and PIAS1 and PIAS3 are also linked to central nervous system pathologies such as Huntington disease35,36 and schizophrenia4 and have other neuronal roles, too. For instance, PIAS1 can interact with necludin, a regulator of anti-apoptotic and pro-survival processes in neurons and neural stem cells,42 and act as a biomarker of stress susceptibility, as it modifies glucocorticoid receptors43 and transcription factors able to produce stress-induced changes44 that can lead to chronic social defeat stress.43

In view of the roles of PIAS1 and PIAS3 in physiological and pathological aspects of synaptic plasticity, we employed SIM to systematically examine their localization in hippocampal and cortical neurons, to complete the super-resolution characterization of the SUMOylation machinery in neurons.26,27

Materials and Methods

Animals

Procedures involving animals were conducted in conformity with the institutional guidelines at the Istituto di Ricerche Farmacologiche Mario Negri IRCCS, in compliance with national (Decree 26/2014; Authorization n. 19/2008-A issued March 6, 2008 by Ministry of Health) and international laws and policies (EEC Council Directive 2010/63/UE; the NIH Guide for the Care and Use of Laboratory Animals, 2011 edition). They were reviewed and approved by the Mario Negri Institute Animal Care and Use Committee, which includes ad hoc members for ethical issues, and by the Italian Ministry of Health (Decree n. 420/2017-PR). Animal facilities meet international standards and are regularly checked by a certified veterinarian who is responsible for health monitoring, animal welfare supervision, experimental protocols, and review of procedures.

Primary cultures

For the preparation of primary hippocampal and cortical neurons we employed the protocol previously described by Colnaghi et al.26 Dissected hippocampi and cortices from two-day-old CD1 mice were incubated at 37°C for 30 min in a solution of 5.8 mM MgCl2, 0.5 mM CaCl2, 3.2 mM HEPES, 0.2 mM NaOH (pH 7.4, 292 mOsm) and 20 U/mL papain (Sigma-Aldrich, St. Louis, MO, USA). The tissues were then dissociated and the single cells were plated in Ibidi micro-Slide 8-well plates to a concentration of 75,000 cells per well. Neurobasal Basal Medium (Gibco, Waltham, MA, USA) was used as medium, containing B27 (Gibco), penicillin/streptomycin and 2 mM glutamine.

Immuinofluorescence experiments

Immunofluorescence experiments followed the procedure previously described by Colnaghi et al.26 Primary neurons, after 12-14 days in vitro (DIV) were fixed in 4% paraformaldehyde (PFA) for 15 min. Neurons were then permeabilized with phosphate-buffered saline (PBS), pH 7.4, 0.2% Triton X-100 for 1 min, and incubated for 1 h with the PBS blocking solution containing 100 µg/mL BSA. To localize PIAS1 and PIAS3 proteins, neurons were incubated for 2 h at room temperature with primary antibodies against PIAS1 24.6 µg/mL (D33A7 XP; Cell Signaling, Danvers, MA, USA) and PIAS3 76 µg/mL (D59 XP, Cell Signaling). Co-local-
ization studies used antibodies against Map2 19 mg/mL (AB5392; Abcam, Cambridge, UK), synaptophysin 1 mg/mL (Sigma-Aldrich, S5768) and PSD-95 1 mg/mL (NeuroMabs, 75-028; Antibodies Incorporated, Davis, CA, USA). All primary antibodies were diluted in a PBS solution containing 100 μg/mL BSA and 0.2% Triton X-100.

Neurons were then treated with secondary antibodies (DyLight Fluor Antibody, Thermo Fisher Scientific) for 1 h at room temperature, followed by incubation with nuclei-staining solution Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA) at 2 μg/mL.

ProLong Glass Antifade Mountant (Thermo Fisher Scientific) was used to fix the neurons in the wells with the slides.

Confocal and structured illumination microscopy
Both the confocal images, acquired using a 40x objective, and the SIM images, acquired in 3D-SIM mode using a 100x objective, were obtained with a Nikon N-SIM microscope. Fiji software (ImageJ) was used to process the images.

Statistical analysis
We acquired forty five images from three different experiments for each condition, and analyzed the overlapping fluorescence signals with JACoP,45 a specialized toolbox for subcellular co-localization analysis under ImageJ. Pearson’s correlation coefficients were calculated to indicate the overlap between the signals. Mander’s M1 and M2 parameters were quantified to determine the co-localization between PIAS1 and PIAS3 with the synaptic markers synaptophysin or PSD-95.46 To avoid background noise, the threshold was set manually. Graphs were drawn using GraphPad Prism 7.

Results
To determine the localization of PIAS proteins in neurons, we employed SIM46 in cultured primary cells. Unlike conventional microscopy, SIM reaches a resolution of about 100 nm laterally and lends itself well suited to co-localization studies.48-51 We first determined the co-localization of PIAS1 and PIAS3 with synaptic markers in hippocampal primary neurons.52 We fixed 14-day cultured primary hippocampal neurons and incubated them with antibodies directed against PIAS3, MAP2 and the pre- and post-synaptic markers synaptophysin and PSD-95.49 To avoid background noise, the threshold was set manually. Graphs were drawn using GraphPad Prism 7.

Discussion
The SUMOylation machinery has been described in both hippocampal and cortical neurons in several reports.6,13 The data so far in primary hippocampal neurons are in line with previous SIM analysis, that showed partial co-localization between members of the SUMO machinery (such as SUMO1, SUMO2/3, Ubc9 and SENPs proteins) and synaptic markers.26-27 This all supports a role of the whole SUMOylation machinery in the formation of spatial memory and long-term potentiation (LTP).

In the last decade, several studies have investigated the implication of PIAS proteins in the formation of spatial memory. An increase in PIAS1 expression in hippocampal neurons of the CA1
area is linked to better spatial learning and memory performance than in rats whose PIAS1 expression was silenced. Although the mechanisms underlying PIAS1-mediated memory facilitation are not fully understood, PIAS1 has been shown: i) to increase SUMOylation of the spatial memory-related protein STAT1 (signal transducer and activator of transcription-1) and ii) to SUMOylate CREB. CREB is modified by several PTMs and its SUMOylation by PIAS1 is essential for spatial memory forma-

Figure 1. Co-localization of PIAS1 with synaptic markers in hippocampal neurons, by confocal microscopy and SIM. A) The images, obtained using a Nikon N-SIM confocal microscope with a 40x objective, represent neurons treated with antibodies directed against PIAS1 (cyan), synaptophysin (red) and Map2 (green), with a Hoechst stain for the nuclei (blue); scale bar: 50 μm. B) Analysis of 3D-SIM images acquired using a 100x objective, 2 μm scale; the colored squares indicate the details presented in (C). C) Merge images showing co-localization between PIAS1 (green) and synaptophysin (red); scale bar: 0.5 μm. D) Intensity profile, with the values normalized to 100 (arbitrary units), representing the co-localization between PIAS1 (green) synaptophysin (red) indicated by the blue arrow (C). E) Mander’s coefficients indicate the PIAS1 fraction that co-localizes with synaptophysin (M1) and the synaptophysin fraction that co-localizes with PIAS1 (M2). F) The Pearson correlation coefficients for PIAS1 and synaptophysin (SYN) data are the mean ± SD of 40 fields from four independent experiments. G) The images, obtained using Nikon N-SIM confocal microscope with a 40x objective, represent neurons treated with antibodies directed against PIAS1 (cyan), PSD-95 (red) and Map2 (green), with a Hoechst stain for the nuclei (blue); scale bar: 50 μm. H) Analysis of 3D-SIM images acquired using a 100x objective, 2 μm scale; the colored squares indicate the details presented in (I). I) Merge images showing co-localization between PIAS1 and (green) and PSD-95 (red); scale bar: 0.5 μm. J) Intensity profiles, normalized to 100 (arbitrary units), represent the co-localization between PIAS1 (green) PSD-95 (red) indicated by the light blue arrow (I). K) Mander’s coefficients indicate the PIAS1 fraction that co-localizes with PSD-95 (M1) and the PSD-95 fraction that co-localizes with PIAS1 (M2). L) The Pearson correlation coefficients of PIAS1 and PSD-95 data are the mean ± SD of 40 fields from four independent experiments.
PIAS3 too appears to be involved in learning processes, memory formation, and underlying long-lasting synaptic plasticity. nNOS, which has a key role in LTP, is SUMOylated by PIAS3 in the hippocampus and this modification is required for hippocampal LTP induction. That PIAS1 and PIAS3 are involved in the physiology of LTP could have implications for understanding how changes in the regulation of SUMOylation affect synaptic plasticity.

Figure 2. Co-localization of PIAS3 with synaptic markers in hippocampal neurons, by confocal microscopy and SIM. A) The images, obtained using a Nikon N-SIM confocal microscope with a 40x objective, represent neurons treated with antibodies directed against PIAS3 (cyan) synaptophysin (red) and Map2 (green) and a Hoechst stain for the nuclei (blue); scale bar: 50 μm. B) 3D-SIM images acquired using a 100x objective, 2 μm scale; the colored squares indicate the details presented in (C). C) Merge images showing co-localization between PIAS3 (cyan), Map2 (green) and synaptophysin (red); scale bar: 0.5 μm. D) Intensity profiles, normalized to 100 (arbitrary units), represent the co-localization between PIAS3 (green) and synaptophysin (red) indicated by the light blue arrow (C). E) Mander’s coefficients indicate the PIAS3 fraction that co-localizes with synaptophysin (M1) and synaptophysin fraction that co-localizes with PIAS3 (M2). F) Pearson correlation coefficients of PIAS3 and synaptophysin (SYN) are the mean ± SD of 40 fields from four independent experiments.

G) The images, obtained using a Nikon N-SIM confocal microscope with a 40x objective, represent neurons treated with antibodies directed against PIAS3 (cyan), PSD-95 (red) and Map2 (green) and with a Hoechst stain for the nuclei (blue); scale bar: 50 μm. H) Analysis of 3D-SIM images acquired using a 100x objective, 2 μm scale; the colored squares indicate the details presented in (I). I) Merge images showing co-localization between PIAS3 (green) and PSD-95 (red); scale bar: 0.5 μm. J) Intensity profiles normalized to 100 (arbitrary units), representing the co-localization between PIAS3 (green) and PSD-95 (red) indicated by the light blue arrow (I). K) Mander’s coefficients indicate the PIAS3 fraction that co-localizes with PSD-95 (M1) and the PSD-95 fraction that co-localizes with PIAS3 (M2). L) Pearson correlation coefficients of PIAS3 and PSD-95 are the mean ± SD of 40 fields from four independent experiments.
The modulation of synaptic plasticity in cortical neurons by the SUMOylation machinery has been less explored. While in cortical neurons SUMOylation can be induced by external stimuli —for of memory is further supported by evidence that their dysfunctions may cause spatial memory difficulties in different neurodegenerative diseases, such as Huntington’s disease or Rett syndrome.\textsuperscript{39,60,61} The modulation of synaptic plasticity in cortical neurons by the SUMOylation machinery has been less explored. While in cortical neurons SUMOylation can be induced by external stimuli —for

**Figure 3.** Co-localization of PIAS1 with synaptic markers in cortex neurons through Confocal and SIM microscopy. A) The images, obtained using Nikon N-SIM confocal microscope with a 40x objective, represent neurons treated with antibodies directed against PIAS1 (cyan), synaptophysin (red) and Map2 (green) and with a solution of Hoechst to stain the nuclei (blue); scale bar: 50 μm. B) Analysis of 3D-SIM images acquired using a 100x objective, 2 μm scale; the colored squares indicate the details represented in (C). C) Merge images showing co-localization between PIAS1 and (green) and synaptophysin (red); scale bar: 0.5 μm. (D) Intensity profile, whose values have been normalized to 100 (arbitrary unit), representing the co-localization between PIAS1 (green) synaptophysin (red) indicated by the light blue arrow (C). E) Mander’s coefficients indicate PIAS1 fraction that colocalizes with synaptophysin (M1) and synaptophysin fraction that colocalizes with PIAS1 (M2). F) Pearson Correlation Coefficient of PIAS1 and synaptophysin (SYN) data are the mean ± SD of 40 fields from four independent experiments. G) The images, obtained using Nikon N-SIM confocal microscope with a 40x objective, represent neurons treated with antibodies directed against PIAS1 (cyan), PSD-95 (red) and Map2 (green) and with a solution of Hoechst to stain the nuclei (blue); scale bar: 50 μm. H) Analysis of 3D-SIM images acquired using a 100x objective, 2 μm scale; the colored squares indicate the details represented in (I). I) Merge images showing co-localization between PIAS1 (green) and PSD-95 (red) indicated by the light blue arrow (I). K) Mander’s coefficients indicate PIAS1 fraction that colocalizes with PSD-95 (M1) and PSD-95 fraction that colocalizes with PIAS1 (M2). L) Pearson Correlation Coefficient PIAS1 and PSD-95 data are the mean ± SD of 40 fields from four independent experiments.
instance guanosine, a purine nucleoside linked to cell metabolism and stress, increases the conjugation of SUMO2/3⁶ - a direct role of the SUMOylation machinery in neuronal activity has not been described. This agrees with evidence that SUMO1-3 knock-down mice appear to have deficits in episodic memory processes and fear learning, phenotypes mediated by the hippocampus and amygdala and to a lesser extent cortical regions." In cortical neurons, in fact, we found that the co-localization of PIAS1 and PIAS3 with synaptic markers was rare, thus possibly suggesting different roles of the E3 ligases in the two neuronal populations.

Figure 4. Co-localization of PIAS3 with synaptic markers in cortex neurons, by confocal microscopy and SIM. A) The images, obtained using a Nikon N-SIM confocal microscope with a 40x objective, represent neurons treated with antibodies directed against PIAS3 (cyan), synaptophysin (red), Map2 (green) and a Hoechst stain for the nuclei (blue); scale bar: 50 μm. B) Analysis of 3D-SIM images acquired using a 100x objective, 2 μm scale; the colored squares indicate the details presented in (C). C) Merge images showing co-localization between PIAS3 (green) and synaptophysin (red); scale bar: 0.5 μm. D) Intensity profiles were normalized to 100 (arbitrary units), representing the co-localization between PIAS3 (green) synaptophysin (red) indicated by the light blue arrow (C). E) Mander’s coefficients indicate the PIAS3 fraction that co-localizes with synaptophysin (M1) and the synaptophysin fraction that co-localizes with PIAS3 (M2). F) Pearson’s correlation coefficients of PIAS3 and synaptophysin (SYN) are the mean ± SD of 40 fields from four independent experiments. G) The images, obtained using a Nikon N-SIM confocal microscope with a 40x objective, represent neurons treated with antibodies directed against PIAS3 (cyan), PSD-95 (red) and Map2 (green) and with Hoechst stain for the nuclei (blue); scale bar: 50 μm. H) Analysis of 3D-SIM images acquired using a 100x objective, 2 μm scale; the colored squares indicate the details presented in (I). I) Merge images showing co-localization between PIAS3 and (green) and PSD-95 (red); scale bar: 0.5 μm. J) Intensity profiles were normalized to 100 (arbitrary units), representing the co-localization between PIAS3 (green) PSD-95 (red) indicated by the light blue arrow (I). K) Mander’s coefficients indicate the PIAS3 fraction that co-localizes with PSD-95 (M1) and the PSD-95 fraction that co-localizes with PIAS3 (M2). L) Pearson correlation coefficient PIAS3 and PSD-95 are the mean ± SD of 40 fields from four independent experiments.

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In all these analyses, cultured hippocampal and cortical neurons were in the resting state, not chemically or electrically stimulated. It is therefore possible that neuronal activation might change the co-localization of PIAS1 or PIAS3 with synaptic markers.21 This would be in line with evidence that spatial training using a water maze not only increases PIAS1 expression in the rat hippocampus,32 but also significantly raises PIAS1 activity, resulting in inhibition of Stat1-mediated gene activation by PIAS1.33 In summary, the present data expand our understanding of the localization of components of the SUMO machinery in neurons. In previous studies, we observed the co-localization with synaptic markers of SUMO1, SUMO2/3, the E2 ligase Ubc9 and the SUMO isopeptidases SENP1, SENP6 and SENP7.26,27 The current analysis adds information on the E3 ligases PIAS1 and PIAS3 at super-resolution level. This study further supports the role of the SUMOylation machinery in the physiology of memory, suggesting our need to understand it better in the light of the connection with synaptopathies such as AD and Parkinson’s disease.

References

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