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Tropomyosin-Related Kinase B (TrkB) Regulates Neurite Outgrowth via a Novel Interaction with Suppressor of Cytokine Signalling 2 (SOCS2)

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Abstract

Brain-derived neurotrophic factor (BDNF) is highly expressed in the hippocampus, where it can initiate signalling pathways leading to neurite outgrowth, neuron survival, spine maturation and increased synapse strength. Although suppressor of cytokine signalling 2 (SOCS2) is primarily known to negatively regulate cytokine signalling, it is also highly expressed in the hippocampus and exerts neuron-specific functions in the brain, effecting the length and architecture of neurons. However, little is known about the role of SOCS2 in the hippocampus. In this study, we hypothesised that SOCS2 may have a regulatory role in BDNF-dependent neurite growth and hippocampal neuronal function. Here our data demonstrate that SOCS2 interacts with the kinase domain of the BDNF receptor TrkB. Germline overexpression of SOCS2 results in a BDNF-dependent increase in hippocampal neurite outgrowth, whereas deletion of SOCS2 results in shorter neurite outgrowth. Expression of SOCS2 also results in increased ubiquitination of the juxtamembrane region of TrkB, and alters the trafficking of TrkB into recycling endosomes. Collectively, our data suggest a novel role for SOCS2 in interacting with and regulating the trafficking of TrkB, leading to increased neurite outgrowth in hippocampus neurons.

Keywords TrkB · BDNF · SOCS2 · Ubiquitination · Endosome trafficking

Introduction

Neurite growth is an integral process required for normal neural development and maturity of the central nervous system. In its most fundamental form it begins with neurite extension, branching and continues through to synaptic refinement [1]. In the hippocampus, brain-derived neurotrophic factor (BDNF)/tropomyosin-related kinase receptor B (TrkB) signalling is a key factor regulating aspects of neuron development such as neuron survival, differentiation, dendritic and axonal growth and branching, maturation of dendritic spines and synaptic plasticity [2–6].

Recently, emerging data have identified that suppressor of cytokine signalling 2 (SOCS2) regulates the neurogenesis and dendritic spine morphology of adult hippocampal neurons. SOCS2 is most well characterised as an adaptor protein that negatively influences cytokine and growth hormone signalling [7]. However, it exhibits high neuron-specific expression in regions such as the fimbria, CA3 of the hippocampus and the thalamic neuroepithelium [7, 8]. Its expression profile suggests a vital role in neuronal development, as its highest expression coincides with embryonic neurogenesis and development. In SOCS2KO mice, the survival rate of newborn hippocampus neurons is reduced [9]. SOCS2 also plays an important role in neural repair. Its expression is increased in the CA1, SGZ and dentate gyrus regions of the hippocampus following forebrain ischemia. These cells were also labelled as neural stem cells, suggesting a role for SOCS2 in hippocampal neurogenesis after injury [10]. Transgenic overexpression of SOCS2 also reduces injury volume and enhances functional recovery after traumatic brain injury [9].

The question of how SOCS2 effects hippocampal neuron development remains to be elucidated. Given that TrkB exerts wide-ranging influences upon hippocampal neuron survival and neurite growth, suggests that SOCS2 could modulate TrkB signalling. SOCS2 is a multi-domain protein, containing a variable N-terminal region, a central Src

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homology domain (SH2) which recognises and binds to phosphotyrosine residues of cytokine receptors, and a conserved C-terminal SOCS BOX domain involved with the elongin B/C complex which targets proteins for ubiquitination and degradation [11, 12]. In this study, we show that expression of SOCS2 increases BDNF-dependent neurite growth in embryonic hippocampal neurons in vitro. Co-immunoprecipitation experiments revealed an interaction between the SH2 domain of SOCS2 and the kinase domain of TrkB, and also an association of the juxtamembrane region of TrkB with the BOX domain of SOCS2. Expression of SOCS2 increases the ubiquitination of TrkB, and alters its intracellular trafficking in vitro. Taken together, these results report a novel role for SOCS2 in regulating aspects of TrkB function, identifying a potential mechanism for SOCS2 in regulating neuronal development.

Material and Methods

Animals

All mice were back-crossed onto a C57Bl/6 background. Two strains of transgenic mice were used, SOCS2KO mice that lack the SOCS2 coding region and do not express SOCS2 [13] and SOCS2TG mice, that constitutively express FLAG-SOCS2 in all tissues, driven by the human ubiquitin C promotor [14]. Pregnant female mice were collected at day 18.5 of pregnancy and embryos were genotyped and used for further experiments. Postnatal day 2 rat pups were utilised for transfection experiments. The use of animals was approved by the animal experimental ethics committee of the Florey Institute of Neuroscience and Mental Health.

Hippocampal Culture and Transfection

Hippocampal cultures were prepared from individual embryonic mice previously described [15]. Cells were cultured in Neurobasal medium (Life technologies) supplemented with 2% B27 (50X, Life Technologies), 1% penicillin streptomycin (Life Technologies) and 1% Glutamax (Life Technologies). Cells were plated on 24 well or 6 well plastic plates (Falcon) coated with 50 μg/ml poly-DL-ornithine hydrobromide (Sigma) 20 μg/ml mouse laminin (Invitrogen). The same protocols were used to generate the rat hippocampal cultures. The Amaxa® Rat Neuron Nucleofector® Kit (Lonza) was used for nucleofection of rat hippocampal cells as per manufacturer's instructions. The neurons were seeded onto coated 24 well plates containing culture media ± 50 ng/mL BDNF (PeproTech). Cultures were fixed after 48 h in 4% *w*/*v* paraformaldehyde, permeabilized with methanol and immunostained for the neuronal marker βIII tubulin (#G7121, Promega,) and standard protocols, as previously described [15].

Neurite Measurement

Fluorescence images were captured with an Olympus 1X81 inverted microscope at × 20 magnification. All experiments were performed at least three independent times for cell lines or from at least three independent mice of each genotype. The length and number of neurites was measured by tracing from the cell body and along their extension. The Neuron J plugin for ImageJ was used for measurements [16]. For the mouse hippocampal cultures 300 neurons from three independent experiments were analysed, and for the rat hippocampal cultures, 100 cells from two separate experiments were analysed.

HEK293T Culture and Transfection

HEK293T cells were cultured in DMEM (Invitrogen) supplemented with 10% FCS, 4 mM L-glutamine and 50 μg/mL PenStrep at 37 °C with 5% CO_2. For protein interaction experiments, cDNA was transfected using polyethylenimine (PEI; Sigma). Constructs used were mouse SOCS2FL and mutants SOCS2ΔSH2, SOCS2ΔBOX, SOCS2ΔNT and (all sub-cloned into the pFLAG-CMV2 expression vector, as previously described [17]) and rat TrkA FL, TrkB FL and TrkB deletion mutants TrkBΔJM (amino acids 454–465 deleted), TrkBΔKD (amino acids 537–660 deleted) and TrkBΔDM (both regions deleted) (all sub-cloned into the pcDNA3 expression vector). For the analysis of TrkB phosphorylation, cells were transfected using PEI, then subsequently cultured in low serum (1%) conditions for 24 h then serum deprived for 4 h before BDNF (50 ng/ml, PeproTech) was added for either 5 or 15 min. Quantitative analysis of Western blots was undertaken using the ImageJ program (NIH). Briefly, ImageJ was used to determine uncalibrated optical density value for each band. The density value was subsequently corrected to TrkB, then normalised such that the level of TrkB phosphorylation following SOCS2 transfection in the absence of BDNF (0 min) represented an arbitrary value of 1. The data were subsequently graphed. For bimolecular fluorescence complementation (BiFc) analysis, lipofectamine 2000 (Invitrogen) was used for transfecting human Rab5, 7, 9, 11 and LAMP1 (in pDsRed-C1, Clontech), pBiFc-TrkB-VC155, pBiFc-VN173-UB (given by Dr. Jason Howitt) and pBiFc-VN173-SOCS2 (constructed by standard PCR techniques).

Immunoprecipitation

Cells were lysed with TNE buffer (20 nM Tris-HCL, PH 7.6, 135 mM NACL, 1.5 mM $MgCL_2$, 1 mM EDTA, 1%

Triton X-100) for the protein interaction experiments or with EDTA free buffer (1 M Tris PH:8, 5 M NaCl) for ubiquitination experiments. Complete Mini PI protease inhibitor cocktail (#11836153001, Roche) was added to lysis buffer. The protein concentration of each cell lysate was measured using the Pierce ™ BCA protein Assay Kit (Pierce, Thermo Fisher Scientific). Anti-Flag®Affinity gel (#A4596, Sigma) was used for immunoprecipitation of FLAG-tagged proteins. The beads were then added to the lysates and were rotated gently at 4 °C overnight then washed in TNE buffer and centrifuged. For the ubiquitination experiment, the supernatant was completely aspirated and Protein G Sepharose™ 4 fast flow (GE healthcare, Sigma). Ubiquitin P4D1 antibody (#3936, Cell Signalling Technology, 1:100,) was added to pre-cleared lysate. The lysates were rotated gently at 4 °C overnight. Protein G agarose beads (#20398, Thermo Scientific) were later added to the lysate and rotated for 2 h at 4 °C. Beads were precipitated, washed and aspirated. In both experiments 20 μl 2.5× Laemmli buffer was added to the beads. The beads were boiled at 95 °C for 5 min before separation by SDS-PAGE and transferred to PVDF and subjected to Western blotting. Antibodies employed were pan Trk (C-14) (sc-11 Santa Cruz Biotechnology), TrkB (H181) (sc-8316, Santa Cruz Biotechnology), Ubiquitin P4D1 (3936, Cell Signalling Technology) and Flag M2 (F1804, Sigma).

Bimolecular Fluorescence Complementation Analysis

Transfected 293T cells were incubated at 37 °C with 5% CO_2 for 48 h and fixed with 4%PFA for 20 min. Cells were washed with DAPI for 5 mins and Phalloidin 594 was used to stain the actin. Confocal imaging was performed with a × 60 magnification on a Zeiss Meta confocal microscope mounted on a Zeiss Axioplan upright microscope. A minimum of 20 cells per condition from three separate experiments was analysed and the average co-localisation calculated. The Velocity software (PerkinElmer 2009) was used to measure the co-localisation. Prior to measuring the co-localisation of the signals, positive and negative controls of each of the signals were analysed to set the minimum and maximum threshold of the signal intensities and remove background noise. To measure the co-localisation, Pearson correlation coefficient (PCC) generated a range from 0 to 1, 0 having no co-localisation and 1 having perfect co-localisation [18].

Statistical Analysis

Statistical tests were performed using GraphPad Prism (Prism-5, Version 5.02 2008) and R (Version 3.1.2–2014–10-31; The Foundation for Statistical Computing Platform). One way ANOVA followed by Bonferroni's post hoc test and Student's unpaired *t* test was used for statistical analysis.

Results

Altered Levels of SOCS2 Influences the Growth of Hippocampal Neurites in the Presence of Exogenous BDNF

To assess the influence that expression of SOCS2 exerts upon hippocampal neuron growth, hippocampi from SOCS2KO and wild-type littermate embryos were dissected; dissociated and 30,000 cells were plated in culture media ± 50 ng/mL BDNF. After 24 h, cultures were fixed, stained, and the total length of all individual neurites of a single neuron measured. Results show that the total length of all neurites in wild-type neurons was not affected by the addition of BDNF. However, neurons derived from SOCS2KO mice exhibited significantly decreased total neurite length compared to control neurons. Importantly, this decrease was normalised following addition of BDNF (Fig. 1a, b). As total neurite length is a function of both the length and number (branching) of neurites, to further evaluate the effect of SOCS2 on the growth and complexity of neurites, the number of neurite branches that each neuron exhibited was counted, and the results binned into neurons that contained 1–3 neurites, 4–6, 7–9, 10–12 and 13 or more neurites. Under control (-BDNF) conditions, there was no difference in the number of neurites per cell between the two genotypes (online resource Fig. 1). However, following treatment with BDNF, SOCS2KO neurons exhibited significantly greater branching, with more cells in the ≥ 13 category compared to wild-type neurons (Fig. 1c). Collectively, these data suggest that SOCS2 may exert a BDNF-dependent effect to limit overall neurite length and complexity/branching.

To complement this analysis, the same experiment was undertaken using hippocampal neurons derived from transgenic mice that over-express SOCS2 under the influence of the human ubiquitin C promotor (SOCS2TG). Hippocampal neurons from SOCS2TG and wild-type litter mates were dissected, plated and treated as above. As observed above, neurons derived from the wild-type control mice did not show any significant change in total neurite length following the addition of BDNF. The total length of neurites from neurons derived from SOCS2TG mice in the absence of BDNF was comparable to wild-type mice, but showed a significant increase in length following BDNF administration (Fig. 2a, b). In contrast to that observed in the SOCS2KO neurons, SOCS2TG neurons demonstrated no increase in neurite number, indicating that the increase in total neurite length was due to longer neurites rather than increased branching (Fig. 2c). Taken together, these data suggest that SOCS2 may modulate BDNF-dependent branching and growth of hippocampal neurons.

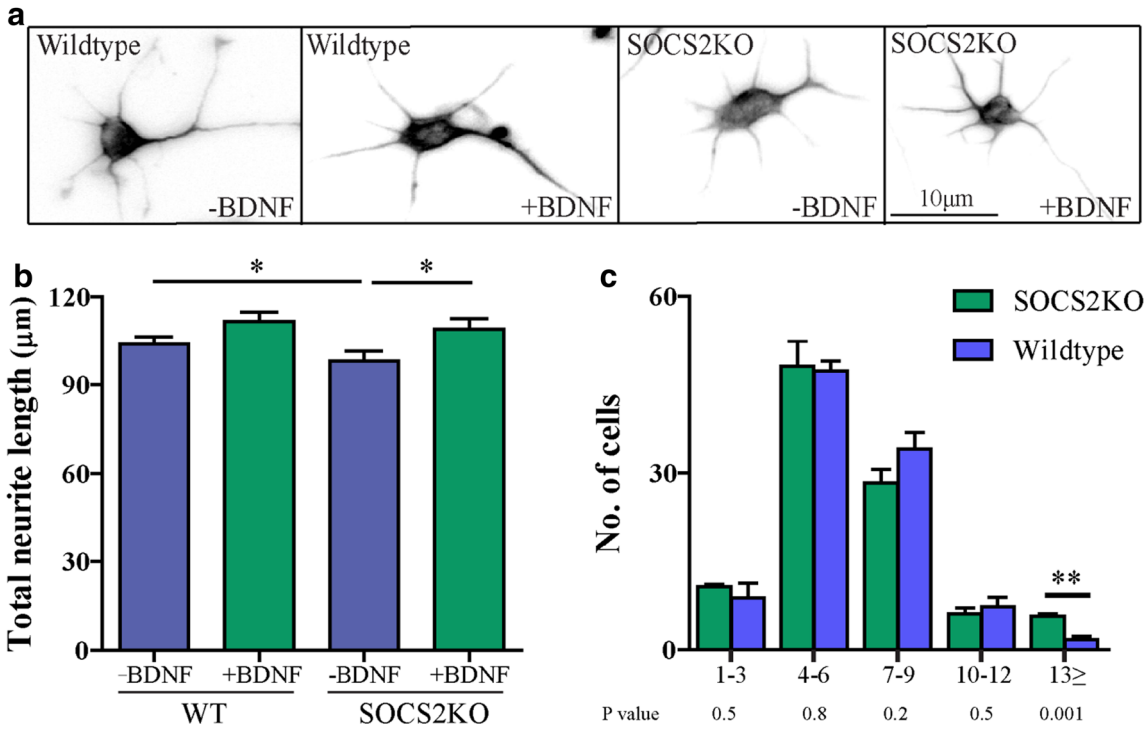

Fig. 1 SOCS2KO hippocampal neurons have more neurites. Embryonic (E18) hippocampal neurons derived from SOCS2KO and littermate wild-type mice were isolated, dissociated and cultured for 24 h ± BDNF (50 ng/ml), then fixed and immunostained for the neuronal marker βIII tubulin. Total neurite length from each genotype was assessed in the presence or absence of BDNF. **a** Representative images of neuron morphology of SOCS2KO and wild-type neurons (± BDNF). **b** SOCS2KO neurons had a significantly reduced total neurite length compared to wild-type neurons (WT) under basal (-BDNF) conditions. This normalised in response to BDNF. (**c**) Neurons were binned according to the number of neurites present, into five groups: 1–3 neurites, 4–6, 7–9, 10–12 and ≥ 13. In the presence of BDNF, the modal distribution between genotypes was the same; however, deletion of SOCS2 resulted in a significantly greater distribution of neurons with > 13 neurites. This suggests loss of SOCS2 results in increased neurite branching. (100 neurons per genotype were analysed and assessed in three independent experiments, data presented as mean ± SEM, statistics applied using the non-parametric Student's *t* test, $*P < 0.05$, $**P < 0.001$)

SOCS2 and TrkB Interact

As SOCS2 exerted a BDNF-dependent role in regulating neurite growth in vitro, this suggests that SOCS2 may regulate BDNF receptor signalling via TrkB. An interaction between both TrkA and TrkB receptors with SOCS2 has been previously demonstrated via co-immunoprecipitation analysis in transfected 293T cells [17]. This was confirmed in this study, showing that immunoprecipitation of Flag-tagged SOCS2 was clearly able to pull down both TrkA and TrkB (Fig. 3a). We next investigated whether co-expression of SOCS2 influenced TrkB phosphorylation. Analysis of transfected 293T cell lysates revealed that co-expression of SOCS2 robustly increased the level of TrkB phosphorylation (Fig. 3b, quantified in c). Negligible levels of TrkB phosphorylation were observed when TrkB was expressed alone in the absence of BDNF, with the addition of BDNF increasing levels of TrkB phosphorylation. Co-expression of SOCS2 induced a robust increase in TrkB phosphorylation both in the absence and presence of exogenous BDNF. Quantification of Western blot bands identified that SOCS2 significantly increased TkB phosphorylation both in the absence or presence of BDNF (Fig. 3c). This indicates that co-expression of SOCS2 increases the activation of TrkB, even in the absence of BDNF.

We investigated this interaction endogenously, immunoprecipitating TrkB from freshly isolated rat hippocampal lysates, and using an isotype IgG as a control. Probing the immunoprecipitates with SOCS2 antibodies generated a robust band in the lysates immunoprecipitated with the anti-TrkB antibody (Fig. 3d). The isotype control shows a weak band (~ 25 kD), suggesting either a weak background interaction or alternatively as SOCS2 is approximately the same size as the antibody light chain, a level of nonspecific background of the primary antibody. Nevertheless, the robust nature of the band observed in the TrkB immunoprecipitate is strongly suggestive of an endogenous interaction between SOCS2 and TrkB in hippocampal neurons.

SOCS2 Interacts with the Kinase Domain and Juxtamembrane Region of TrkB

We next sought to investigate the nature of the interaction between TrkB and SOCS2. To examine the role of the kinase domain in the interaction between TrkB and SOCS2, the

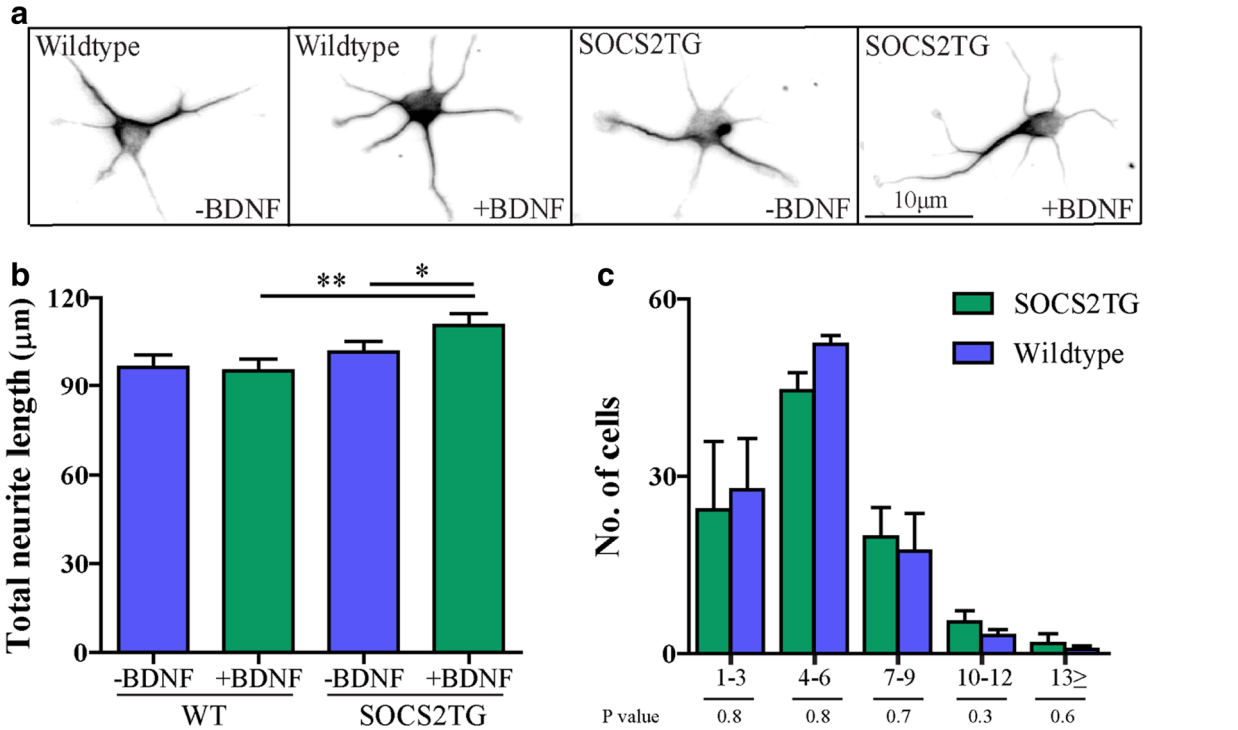

Fig. 2 Overexpression of SOCS2 increases hippocampal neurite length. Embryonic (E18) hippocampal neurons derived from SOCS2TG and littermate wild-type mice were isolated, dissociated and cultured for 24 h ± BDNF (50 ng/ml), fixed and immunostained for the neuronal marker βIII tubulin. Total neurite length from each genotype was assessed in the presence or absence of BDNF. **a** Representative images of neuron morphology of SOCS2TG and wild-type neurons (± BDNF). **b** SOCS2TG and wild-type (WT) neurons had similar total neurite length under basal (-BDNF) conditions. Following addition of BDNF, SOCS2TG neurons exhibited a significant increase in total neurite length. (**c**) Neurons were binned according to the number of neurites present, into five groups: 1–3 neurites, 4–6, 7–9, 10–12 and ≥ 13. In the presence of BDNF, the distribution of neurites was the same between genotypes. These data suggest the increase in total neurite length of the SOCS2TG neurons is due to increase in growth, not branching. (100 neurons per genotype were analysed and assessed in three independent experiments, data presented as mean ± SEM, statistics applied using the non-parametric Students *t* test, $*P < 0.05$ $**P < 0.001$)

truncated isoform of TrkB, TrkB T1, which contains the same extracellular, transmembrane and juxtamembrane regions of full-length TrkB (TrkB FL), but lacks the C-terminal kinase domain (schematically represented in Fig. 3e) was co-transfected with SOCS2 in 293T cells. Immunoprecipitation with Flag antibodies revealed a robust interaction with TrkB FL. Conversely, despite very high expression of TrkB T1, only a very weak band was detected, suggesting a weak interaction persists in the absence of the kinase domain (Fig. 3f). This suggests that the kinase domain of TrkB plays an important role in the interaction with SOCS2, but might not be solely responsible for the interaction and that the first 12 juxtamembrane amino acids in the intracellular domain might also be a region involved in interacting with SOCS2. To investigate this in more detail, mutant constructs of TrkB FL were generated, with deletions of the kinase domain (ΔKD), the juxtamembrane region (ΔJM), or both domains (ΔDM) (schematically depicted in Fig. 3e) to assess the roles that these regions play in the association with SOCS2.

HEK293T cells were co-transfected with FLAG-SOCS2 and either TrkB FL, TrkBΔJM, TrkBΔKD or TrkBΔDM. Analysis of cell lysates indicated differential expression levels of the deletion constructs, with the TrkBΔJM construct being expressed weakly and the TrkBΔKD construct being expressed very robustly compared to TrkB FL (middle panel, Fig. 3g). Lysates were immunoprecipitated with Flag antibodies and probed with an antibody directed against the extracellular domain of TrkB. Deletion of either the juxtamembrane region or kinase domain of TrkB resulted in a substantial reduction in binding with SOCS2, but neither completely abolished the interaction (Fig. 3g). The interaction was completely abolished following deletion of both domains (TrkBΔDM, Fig. 3g). These data suggest that both the juxtamembrane and kinase domains potentially interact with SOCS2, and that deletion of only one is not sufficient for disruption of the interaction.

Distinct Domains of SOCS2 Interact with TrkB

We next investigated the region of SOCS2 required for interaction with TrkB. SOCS2 contains 3 distinct protein domains; an amino (N) terminal domain (green), an SH2 domain (blue) that binds to phosphotyrosine residues, and the BOX domain (pink) involved in ubiquitination (schematically represented

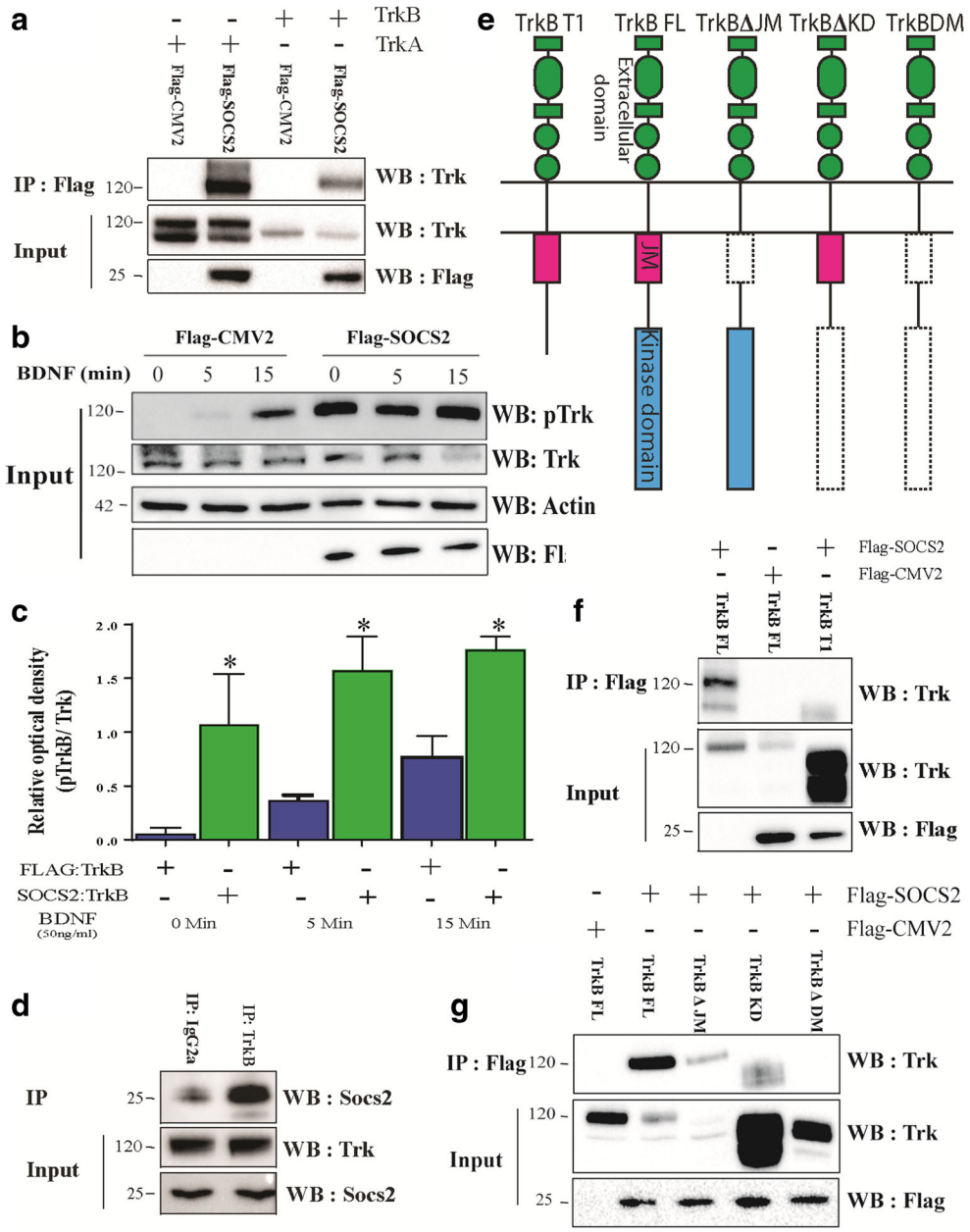

Fig. 3 SOCS2 interacts with TrkB. **a** FLAG-tagged SOCS2 (FLAG-SOCS2) and TrkA or TrkB were transfected in HEK293T cells, lysed and immunoprecipitated with FLAG-agarose beads. Immunoprecipitates and total lysates were subjected to SDS-PAGE and Western blots were probed with antibodies recognising FLAG and Trk. Both TrkA and TrkB showed an interaction with FLAG-SOCS2, whilst no interaction is shown with the empty vector FLAG-CMV2. **b** Representative Western blots showing TrkB phosphorylation (pTrk) levels in the absence of BDNF (0 min) or following either 5- and 15-min treatment with BDNF (50 ng/ml). **c** Densitometric quantification of Western blot bands ($n = 3$), normalised to total TrkB expression levels indicate that co-transfection of SOCS2 significantly increased TrkB phosphorylation at all time points. (Data = mean ± SEM; $^*P < 0.05$, paired Student's t test). **d** Hippocampal lysates from P1 rats were immunoprecipitated with antibodies directed against TrkB or an isotype control antibody (IgG2a), and Western blots probed for SOCS2. The result shows a robust association of TrkB with SOCS2, whilst the isotype control shows a weak band. **e** Schematic diagram of TrkB FL, TrkB T1 and deletion constructs. **f** FLAG-SOCS2 was co-transfected with either TrkB FL or the truncated isoform TrkB T1. Immunoprecipitation and Western blot analysis showed that in the absence of the kinase domain of TrkB, SOCS2 interacted weakly with TrkB T1. **g** Western blot analysis of TrkB mutant constructs co-transfected with SOCS2, immunoprecipitated with FLAG-agarose beads and probed with anti-Trk antibodies. Flag-SOCS2 FL associates strongly with TrkB FL, but only weakly with both TrkBΔJM and TrkBΔKD mutants. No interaction was observed with the TrkBΔDM mutant construct. Images are representative of at least three independent experiments

in Fig. 4a). Deletion constructs of each of these domains were generated: FLAG-SOCS2ΔNT, FLAG-SOCS2ΔSH2 and FLAG-SOCS2ΔBOX (Fig. 4a). These constructs were co-transfected with TrkB FL, and again differential levels of

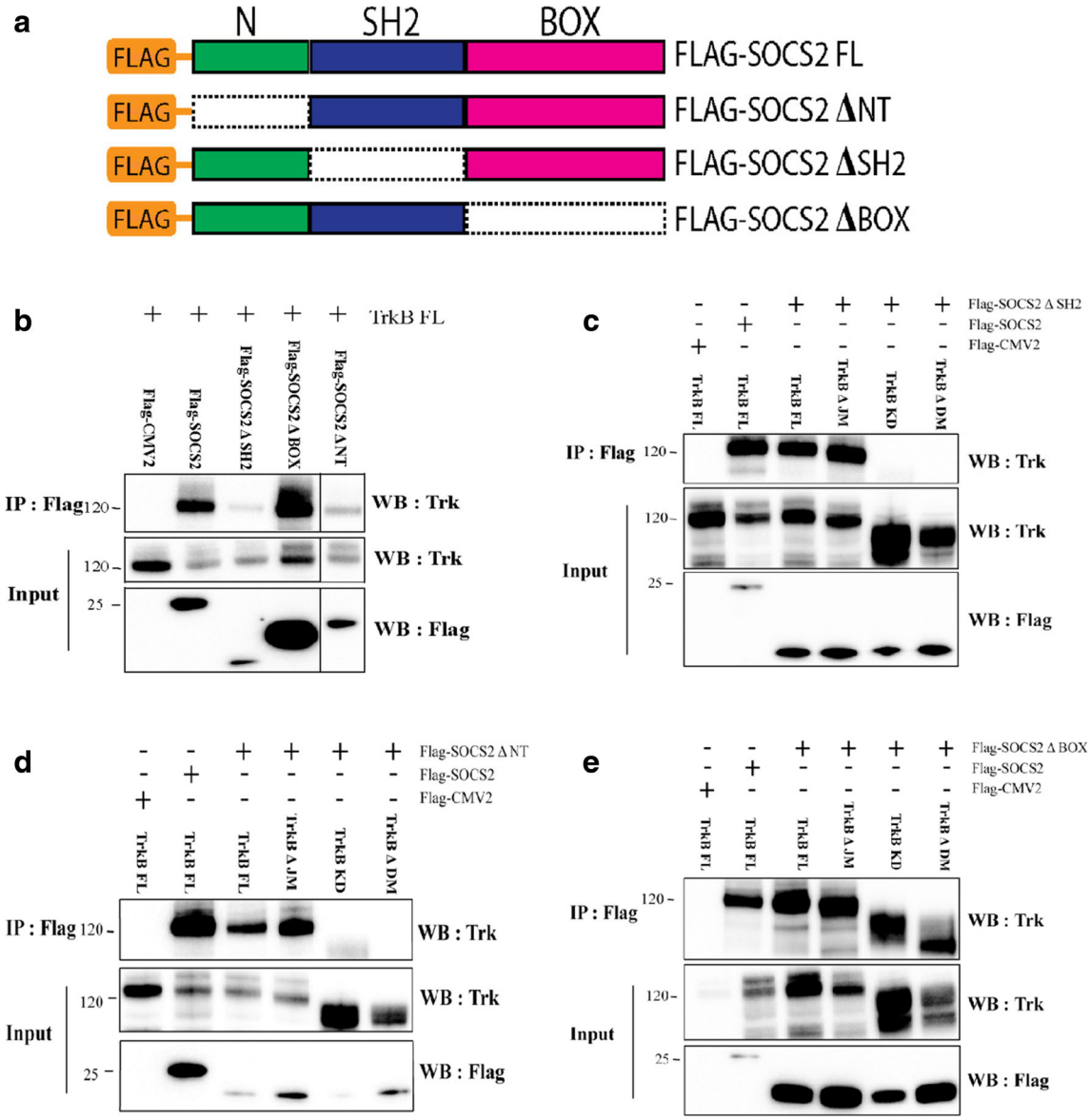

Fig. 4 The SH2 and N-Terminal domains of SOCS2 mediate the interaction with TrkB. **a** Schematic representation of the SOCS2 mutant constructs used to study the domains involved in the interaction with TrkB. **b** Immunoprecipitation analysis of TrkB FL with SOCS2 mutants. Both the SOCS2 FL and SOCS2ΔBOX constructs exhibited robust interaction with TrkB. In contrast, the SOCS2 ΔNT and SOCS2 ΔSH2 constructs showed a reduced level of interaction, suggesting both the N-terminal and SH2 domain of SOCS2 is important for the interaction with TrkB. **c** Immunoprecipitation analysis of Flag-SOCS2ΔSH2 with TrkB mutants. Deletion of the TrkB kinase domain disrupts the interaction of these two proteins, suggesting the SOCS2 N-terminal interacts with the kinase domain of TrkB. **d** Immunoprecipitation analysis of Flag-SOCS2ΔNT with TrkB mutants. Deletion of the TrkB kinase domain disrupts the interaction of these two proteins, suggesting the SOCS2 SH2 domain interacts with the kinase domain of TrkB. **e** Immunoprecipitation analysis of Flag-SOCS2ΔBOX with TrkB mutants. Flag-SOCS2ΔBOX interacts with all TrkB constructs, suggesting the SOCS2 SOCS BOX does not influence the interaction with TrkB. Images representative of at least three independent experiments

expression were observed, with in particular the FLAG-SOCS2ΔBOX construct being very robustly expressed (bottom panel, Fig. 4b). Interestingly, this also resulted in increased expression of TrkB FL (middle panel, Fig. 4b). Immunoprecipitation experiments revealed that all the deletion mutants interacted with TrkB to some degree (Fig. 4b). The full-length SOCS2 and ΔBOX mutant interacted with TrkB most robustly, strongly suggesting the BOX domain is not required for the interaction. Importantly, deletion of either the SH2 or N-terminal domain substantially reduced the interaction, suggesting these two domains exert independent effects upon the interaction with TrkB.

To further analyse the role of each SOCS2 domain in interacting with TrkB, each SOCS2 mutant construct was co-immunoprecipitated with the TrkB mutants. Our results illustrate that the SOCS2ΔSH2 construct interacted with the

TrkB FL and TrkBΔJM constructs, but did not show any interaction with TrkBΔKD or TrkBΔDM mutants (Fig. 4c), suggesting the SH2 domain of SOCS2 interacts with the kinase domain of TrkB. Similarly, the SOCS2ΔNT construct interacted with both TrkB FL and TrkBΔJM, but also did not interact with TrkBΔKD and TrkBΔDM (Fig. 4d), suggesting that the N-terminal domain also interacts with the kinase domain of TrkB. Finally, as expected, the SOCS2ΔBOX construct interacted with all the TrkB mutants (Fig. 4e) further suggesting the BOX domain plays no role no the interaction with TrkB. Again interestingly, the SOCS2ΔBOX construct was robustly expressed compared to SOCS2 FL (bottom panel, Fig. 4e), which also resulted in increased Trk FL expression (middle panel, Fig. 4e). Collectively this suggests a complex interaction, where both the N-terminal and SH2 domain of SOCS2 interact with the kinase domain of TrkB, and that deletion of either domain in SOCS2 results in a loss of interaction with TrkB. Interestingly, the expression levels of the TrkBΔKD construct were very high, suggesting the interaction with SOCS2 may regulate the expression level of TrkB. Given that co-expression with the SOCS2ΔBOX construct also resulted in high levels of TrkB expression suggests the BOX domain plays an important role in this regulation. The role that the juxtamembrane region of TrkB plays in the interaction with SOCS2 is unclear, and could not be further elucidated through immunoprecipitation with the SOCS2 mutants.

The SOCS2 BOX Domain Is Required for Neurite Growth

Having identified that the N-terminal and SH2 domains of SOCS2 are critical for interaction with TrkB, we next turned our attention to the influence that the SOCS2 BOX domain exerted upon TrkB. As our previous data had demonstrated that SOCS2 exerted a BDNF-dependent effect in increasing hippocampal neurite extension, we investigated the influence that the SOCS2ΔBOX mutant exerted upon neurite extension. To do this, we dissected and dissociated rat hippocampal neurons, Amaxa transfected them with GFP and either an empty vector, SOCS2 or SOCS2ΔBOX constructs, and plated 30,000 neurons in culture media ± BDNF (50 ng/ml). After 48 h, cultures were fixed, stained, and the total length, longest length and number of individual neurites of a single transfected GFP+ neuron measured. In the absence of BDNF, transfection of either SOCS2 or SOCS2ΔBOX resulted in a similar significant decrease in total neurite length compared to control (GFP) transfected neurons (Fig. 5a). Following the addition of BDNF, neither control (GFP) or SOCS2 overexpressing neurons increased total neurite length however, neurons overexpressing SOCS2ΔBOX exhibited a significant increase in total neurite length (Fig. 5a). Examining the basis of this change in mean neurite length,

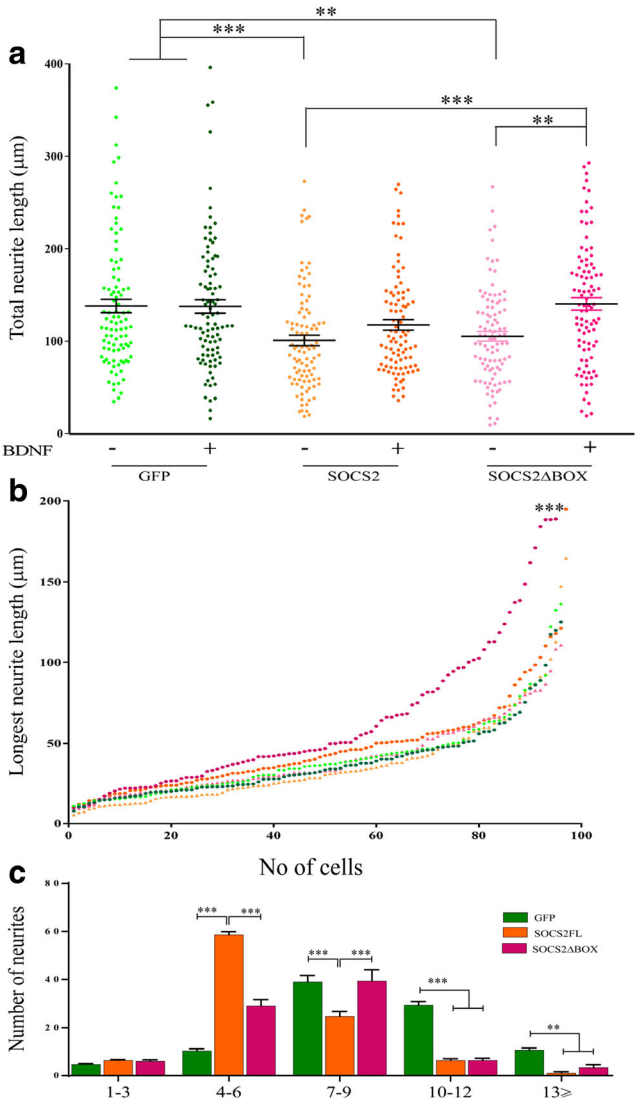

Fig. 5 Overexpression of SOCS2 increases neurite length of rat hippocampal neurons. Rat hippocampal cells were isolated, dissociated and transfected with GFP and either empty vector, SOCS2FL or SOCS2ΔBOX, plated down, treated ± BDNF (50 ng/ml), then after 48 h were fixed and immunostained for βIII tubulin and DAPI. Neurite length of GFP+ neurons was measured. **a** Expression of either SOCS2 or SOCS2ΔBOX resulted in a decrease in total neurite compared to control (GFP+) neurons. Total neurite length increased following BDNF treatment of SOCS2ΔBOX-transfected neurons, suggesting that the SH2/N-terminal-mediated interaction with TrkB is sufficient to promote neurite growth. **b** Comparison of the length of the longest neurite in rat hippocampal neurons. All groups showed a similar distribution, except for BDNF-treated SOCS2ΔBOX-transfected neurons which showed a significant increase in the length of the longest neurite. **c** Analysis of the number of neurites per cell following BDNF treatment. Neurons were binned according to the number of neurites present into five groups: 1–3 neurites, 4–6, 7–9, 10–12 and ≥ 13 neurites. Expression of SOCS2 or SOCS2ΔBOX reduced the branching of hippocampal neurons. Collectively, this suggests that SOCS2 reduces net neurite branching to favour increasing neurite length. (100 neurons per genotype were analysed and assessed in three independent experiments, data presented as mean ± SEM, statistics applied using two-way ANOVA and Tukey comparisons test, $F(8,24)$, $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***} = P < 0.0001$)

we assessed the length of the longest neurite of each neuron. In the absence of BDNF, the longest neurite of control (GFP), SOCS2 and SOCS2ΔBOX expressing neurons were all similar. The addition of BDNF had no effect upon the longest neurite of control (GFP) and SOCS2 transfected neurons, but SOCS2ΔBOX transfected neurons exhibited a significant increase in length (Fig. 5b). As total neurite length is a function of both the length and number (branching) of neurites, the number of neurite branches that each neuron exhibited was counted, and the results binned as above. Expression of SOCS2 and SOCS2ΔBOX exerted a similar effect on neurite number, independent of the provision of BDNF. Both in the absence of BDNF (online resource Fig. 1) and presence of BDNF (Fig. 5c), overexpression of either SOCS2 or SOCS2ΔBOX reduced the number of neurites, with an increasing number of cells with fewer neurites and decreasing number of cells with a large number of neurites. Collectively, these data are consistent in suggesting that SOCS2 exerts an instructive role in regulating neurite complexity by limiting branching and promoting length, and that the SOCS BOX exerts an important influence in modulating this effect.

SOCS2 Increases TrkB Ubiquitination

The C-terminal BOX domain of SOCS2 has been identified as an E3 ubiquitin ligase [19]. Given we have identified that the SH2 and the N-terminal domains interact with the kinase domain of TrkB, and that the BOX domain regulates neurite branching and extension in a BDNF-dependent manner, next we investigated whether SOCS2 increases TrkB ubiquitination. To address this, a bimolecular fluorescence complementation assay was used. pBiFc Venus vectors were transfected into HEK293T cells, with each vector containing one half of the GFP protein, which were independently non-fluorescent. To study ubiquitination of TrkB, pBiFc VN173-Ubiquitin (VN173UB) and pBiFc TrkB-VC155 (TrkBVC155) were co-transfected into 293T cells with either FLAG-SOCS2FL or empty vector (CMV-FLAG). The effect that SOCS2 exerted on TrkB ubiquitination was quantified by measuring the fluorescence intensity of the generated GFP signal. SOCS2 expressing cells exhibited significantly higher levels of GFP compared to empty vector expressing cells (qualitatively shown in Fig. 6a, quantitatively analysed in Fig. 6b), strongly suggesting that SOCS2 was increasing ubiquitination of TrkB.

The juxtamembrane region of TrkB contains a tri-peptide 'KFG' sequence which is conserved in all three Trk receptors [20]. The 'KFG' sequence of TrkA controls receptor protein levels through ubiquitination and regulating downstream signalling pathways [21]. As we found that TrkB ubiquitination was increased by SOCS2, ubiquitination of the juxtamembrane region of TrkB, in association with SOCS2 was investigated by immunoprecipitation. HEK293T cells were transfected with Ubiquitin, TrkB FL and empty vector CMV-FLAG, then lysates immunoprecipitated with Ubiquitin and probed with TrkB to show the basal level of TrkB ubiquitination. Modest levels of TrkB ubiquitination were observed (Fig. 6c, left lane). Next, the level of TrkB ubiquitination in the presence of SOCS2 was assessed, with an increase of TrkB ubiquitination readily apparent (Fig. 6c lane 2), suggesting that SOCS2 increases TrkB ubiquitination. To examine the role of the juxtamembrane region of TrkB on its ubiquitination, ubiquitin, TrkBΔJM and FLAG-SOCS2 were co-transfected, and resulted in a reduction in the levels of TrkB ubiquitination (Fig. 6c, right lane), approximately equivalent to the absence of SOCS2 (left lane). This suggests that SOCS2 was mediating ubiquitination of the juxtamembrane region of TrkB. Finally, to examine the role of the BOX domain on TrkB ubiquitination, Ubiquitin, TrkB FL and SOCS2ΔBOX were co-transfected and unexpectedly revealed a robust increase in TrkB ubiquitination (Fig. 6c, third lane), substantially greater than that observed when full-length SOCS2 was co-transfected (second lane). The reason behind this observation is unclear, but is consistent with this construct also causing a greater increase in neurite extension compared to SOCS2 (Fig. 5a, b).

Expression of SOCS2 Increases TrkB Receptor Recycling

Protein ubiquitination can exert multiple distinct outcomes, from internalisation to endosomal trafficking and proteasomal degradation. To address the influence that SOCS2 mediated ubiquitination exerted upon TrkB the bimolecular florescence (BiFC) assay was used. Venus vectors pBiFc TrkB-VC155 (TrkBVC155) and pBiFc VN173-SOCS2 (VN-SOCS2) were transfected into HEK293T cells, with the resultant GFP fluorescence an indication of the TrkB-SOCS2 interaction. As a control, GFP-tagged TrkB was transfected to enable tracking of TrkB trafficking in the absence of SOCS2. To investigate the specific subcellular localisation of TrkB-GFP and TrkB-SOCS2-GFP, tagged endosomal markers were co-transfected into 293T cells: Rab5 to mark early endosomes; Rab9 for late endosomes; Rab11 for recycling endosomes and Lamp1 for the lysosome. 16 h after transfection, cultures were starved for 4 h, treated ± BDNF (50 ng/ml for 4 h) before being fixed, visualised and the co-localisation of GFP with endosomal markers quantitated.

Comparing the co-localisation of GFP with the early endosome marker Rab5 identified that the expression of SOCS2 did not influence the initial stages of endocytosis, as co-localisation with GFP was similar between all groups (Fig. 7b). In contrast, expression of SOCS2 significantly increased TrkB co-localisation with late (Rab9+, Fig. 7c) and recycling (Rab11+, Fig. 7a, quantified in d) endosomes. Expression of SOCS2 also increased TrkB co-localisation

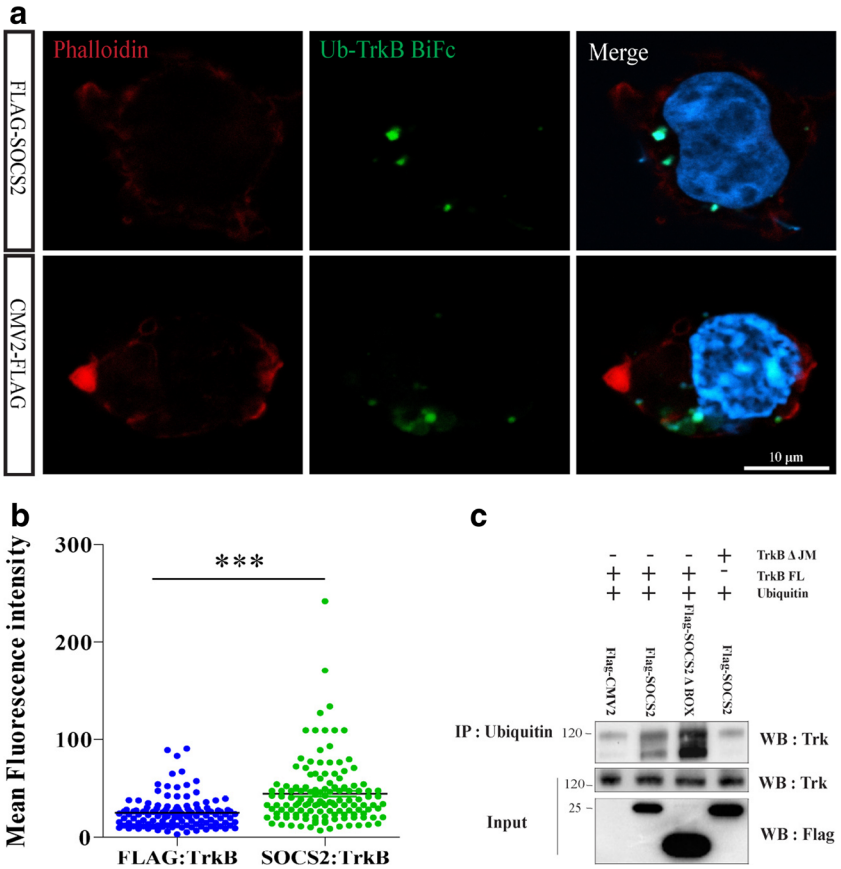

Fig. 6 SOCS2 increases TrkB ubiquitination. **a** GFP complementation following co-transfection of Ubiquitin (VN173-Ubi) and TrkB (TrkB-VC155) in 293T cells, in the presence or absence of SOCS2. GFP fluorescence indicates an interaction between TrkB and Ubiquitin. **b** The fluorescence intensity was measured and shows that co-expression of SOCS2 significantly increases ubiquination of TrkB (data is mean ± SEM, 125–130 cells from two separate experiments were analysed with unpaired Student's *t* test, ***$P < 0.0001$). **c** 293T cells were transfected with Ubiquitin, TrkB and SOCS2 constructs as indicated. Cells were lysed after 48 h and immunoprecipitated with an anti-Ubiquitin antibody. Western blot analyses show a basal level of TrkB ubiquitination in the absence of SOCS2. Co-expression of SOCS2 markedly increased TrkB ubiquitination, and SOCS2ΔBOX increased TrkB ubiquitination even more. Ubiquitination of TrkB reverts to basal levels if the juxtamembrane domain of TrkB is deleted (TrkBΔJM), suggesting this is the region ubiquitinated by SOCS2

with lysosomes (Lamp1+, Fig. 7e), but only in the presence to BDNF. These results suggests that SOCS2 does not affect the internalisation and initial phases of intracellular sorting of TrkB, rather it appears to selectively increase trafficking of TrkB within the cell. This implies that SOCS2 dependent ubiquitination of TrkB primarily results in increased trafficking of TrkB to late endosomes and recycling endosomes. Collectively, these data suggest that SOCS2 promotes the ubiquitination of the juxtamembrane region of TrkB to direct trafficking of the receptor to late and recycling endosomes, influencing the activation of downstream signalling by TrkB to promote an increase in neurite growth.

Discussion

Neurite growth, dendritic morphology and neuron survival are among the functions in the hippocampus strongly regulated by BDNF/TrkB signalling [4]. Recently it has been revealed that SOCS2 has high expression in different regions of the hippocampus [7], is involved in neurogenesis [22], newborn adult hippocampal neuron survival and maturation of spine morphology [23]. Here, we provide evidence that SOCS2 interacts with TrkB, promotes TrkB receptor ubiquitination, intracellular trafficking and expression, leading to a BDNF-dependent increase in hippocampal neurite growth. These data provide the first mechanistic insight into how SOCS2 could influence key aspects of hippocampal function, via interaction with TrkB and modulation of TrkB signalling.

Our data show that overexpression of SOCS2 results in a BDNF-dependent increase in hippocampal neurite length. This effect of SOCS2 is in general agreement with previous studies on dorsal root ganglion (DRG) neurons where high levels of SOCS2 resulted in longer neurites in the presence of the neurotrophin NGF [17]. Thus SOCS2 likely influences neuronal TrkA and TrkB receptor signalling in a similar manner to regulate neurotrophin-dependent neurite extension. Intriguingly though, overexpression of SOCS2 appeared to

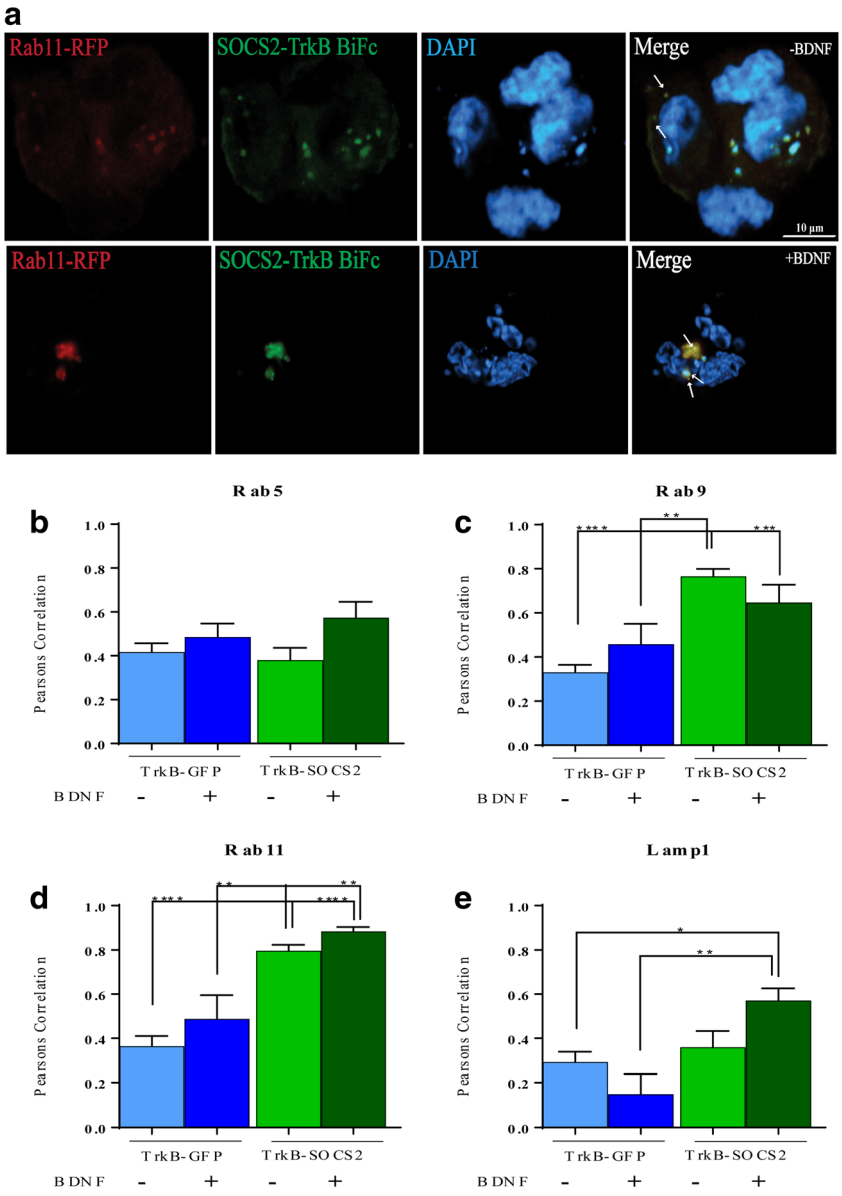

Fig. 7 SOCS2 increases TrkB recycling. Analysis of GFP complementation (TrkB-VC155 + VN173-SOCS2; SOCS2-TrkB BiFc) or TrkB-GFP co-localised with RFP-tagged endosome markers transfected in 293T cells. **a** Confocal microscopy images showing the co-localization of SOCS2:TrkB or TrkB-GFP with RFP-Rab11 in the presence and absence of BDNF. Arrows indicate co-localisation. **b**–**e** Co-localisation of red and green fluorescence was measured using Pearson's correlation (Manders et al. 1992). Results show that expression of SOCS2 directs TrkB trafficking into distinct subcellular locations. **b** SOCS2 does not affect the initial trafficking of TrkB; as TrkB:SOCS2 and TrkB both co-localise with the early endosome marker Rab5 with the same intensity. However, SOCS2 significantly increased the recycling of TrkB receptors, as indicated by increased co-localisation to late (Rab9+, **c**) and recycling (Rab11+, **d**) endosomes. SOCS2 increased co-localisation with the lysosome (Lamp1+, **e**) in the presence of BDNF. (Results represent mean ± SEM of $n = 10$–14 cells for TrkB: SOCS2 and 20–25 cells for GFP-TrkB from two separate experiments were analysed; one-way ANOVA followed by Bonferroni's post hoc test was used and $^{*}P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$)

exert contrasting effects on neuronal complexity between the DRG and hippocampal neurons. Whereas overexpression of SOCS2 resulted in increased branching of DRG neurites [17], we showed a general reduction in hippocampal neuron complexity. This suggests that there may either be some contextual differences in the influence that SOCS2 exerts on neuronal complexity between different neuronal subpopulations, or that there are differences in how SOCS2 regulates TrkA and TrkB signalling. Given the substantial phenotypic differences between DRG and hippocampal neurons, it is difficult to determine the cause of this difference. Regardless, the common finding between these two populations strongly suggests that SOCS2 plays an important role in neurotrophin-dependent neurite extension.

SOCS2 is a multi-domain protein, containing a centrally located SH2 domain, a structurally conserved domain well known for its ability to dock to phosphorylated tyrosine residues on other proteins [24–26]; and a C-terminal BOX domain, that complexes with elongin B and C to form a E3 ubiquitin ligase complex [19, 27]. SOCS2 is most well characterised as a negative regulator of cytokine signalling, utilising its SH2 domain to bind to phosphotyrosine residues on receptors such as the growth hormone and leptin receptors, and marking these activated receptors for proteasomal degradation via SOCS BOX dependent ubiquitination [6, 28–30]. These studies indicate explicit roles for the SH2 and BOX domains in how SOCS2 functions to down regulate receptor expression. Consistent with this, our data show that SOCS2 utilises its SH2 domain to interact with the kinase domain of TrkB. Indeed, the kinase domain of TrkB contains multiple phosphotyrosine sites: Y702, Y706 and Y707 in the kinase activation loop, and Y817 just distal to the kinase domain that

acts as docking site for PLC-γ [31, 32]. The fact that the kinase domain is highly conserved between the three different Trk receptors (TrkA, TrkB and TrkC) likely accounts for the fact that SOCS2 associates with all three Trk receptors [17]. Interestingly, at least in the case of TrkB, our data suggest the presence of a second site of potential interaction. We found that the naturally occurring truncated isoform of TrkB, TrkB-T1, which shares almost identical amino-acid identity in the juxtamembrane region but lacks the kinase domain, also interacted with SOCS2, albeit weakly. This region of the Trk receptor family contains a conserved 'SKFG' amino-acid motif, which in TrkA plays a major role in its ubiquitination [21]. Deletion of this domain results in a reduction of TrkA ubiquitination, leading to an increase in TrkA expression and activity, suggesting that ubiquitination at this site directly effects Trk receptor recycling [21]. It is interesting that SOCS2 may interact with TrkB in this region, and provides a potential mechanism whereby SOCS2 may play an important role in mediating the ubiquitination of TrkB, and potentially other Trk receptors, at this site. In addition, the juxtamembrane region of TrkB has also been established as a docking site for cyclin dependent kinase 5 (Cdk5), resulting in the phosphorylation of Ser478 of TrkB and an increase in hippocampal dendritic growth and neuronal survival [33]. As our data also show that SOCS2 influences hippocampal dendritic growth, SOCS2 may also regulate this interaction.

However, whilst we did observe an interaction between SOCS2 and TrkB, and consequent ubiquitination of TrkB, we did not observe the targeted proteasomal degradation of TrkB that might have otherwise been expected. Ubiquitin modification of proteins can regulate multiple intracellular functions, such as endosomal sorting [34], DNA repair [35], protein trafficking and proteasomal degradation [36, 37]. Whilst our data suggest that the interaction between SOCS2 and TrkB results in an increase in TrkB ubiquitination, consistent with the role of SOCS2 in ubiquitination, it did not lead to the proteasomal degradation of TrkB. The reason for this is not clear. However, ubiquitination is a complex post-translational modification that can result in an array of distinct topologies, such as mono-ubiquitination, multi-mono-ubiquitination and poly-ubiquitination, and the ubiquitin itself can be modified, acetylated, or phosphorylated [38]. These distinct types of protein ubiquitination result in distinct protein fates, and our data suggest that SOCS2-dependent ubiquitination of TrkB directs altered intracellular trafficking rather than proteasomal degradation. Our data indicating reduced TrkB ubiquitination when its juxtamembrane region (aa 454–465) is deleted suggests this region of TrkB, which contains the 'SKFG' amino-acid motif, could be involved in its ubiquitination. However, one confounding element of our results is that whilst the SOCS BOX is well characterised as bridging proteins to the ubiquitin protein complex, our data suggest that deletion of the SOCS BOX results in an increase in TrkB ubiquitination. This is surprising, as one would expect the opposite. However, the SOCS2ΔBOX construct actually exerts several effects that are unexpected: its expression is very much higher than SOCS2; it associates with TrkB when SOCS2 does not; it ubiquitinates TrkB more strongly; and exerts greater effects on neurite length than SOCS2. So deletion of the SOCS BOX completely changes the function of SOCS2 in a way that is difficult to explain, but is nevertheless absolutely consistent with a substantial gain of function. The mechanisms underpinning this are unclear, and worthy of further investigation.

Nevertheless, as a consequence of the SOCS2-mediated TrkB ubiquitination, our data suggest that TrkB trafficking is altered, with increased TrkB present in late (Rab9+) and recycling (Rab11+) endosomes, suggesting an increased proportion of TrkB being recycled. This is interesting, as the trafficking and localisation of TrkB is critical to its function. There is clear evidence that the internalisation and retrograde transport of the Trk receptors occurs via endosomal sorting, and as a consequence distinct signals, such as activation of either Erk1/2 or Erk5, and distinct functions, such as neurite survival or growth, can be separately modulated [39–46]. In the context of our data, we find that overexpression of SOCS2 results in a BDNF-dependent increase in neurite extension. It is established that the growth of neurites is dependent upon the neurotrophin being present at the growing axon tip [47], and that this leads to local activation of MEK1/ 2 and Erk1/ 2 [48, 49], as well as PI3K and Akt kinase activity [50]. Inhibition of either of these pathways results in a reduction in neurotrophin-induced axonal outgrowth. This suggests that SOCS2 regulated ubiquitination of TrkB could direct its trafficking to remain local to the growing axon tip, thus accounting for the significant increase in neurite extension observed. In support of this, BDNF increases the accumulation of recycling (Rab11+) endosomes in hippocampal dendrites, increasing dendritic localisation of TrkB and enhancing sensitisation to BDNF [51]. Collectively, SOCS2 could play an important role in directing TrkB to the recycling dendritic-localised endosomes, so that TrkB is retained in dendrites to increase local signalling and promote neurite extension.

Little is known about TrkB ubiquitination and the consequence of it. Here, we have shown that SOCS2 increases TrkB ubiquitination and recycling of the receptor, potentially accounting for how it increases hippocampal neurite extension. Whilst ubiquitination is certainly linked to protein recycling, further experiments are necessary to gain a greater understanding of the precise nature of TrkB ubiquitination which leads to its recycling. Understanding the molecular events that drive the survival, growth and development of hippocampal neurons will identify the underlying mechanisms of hippocampal neurogenesis and neuronal plasticity. This will allow us to better understand neural functions such as memory formation and ultimately develop better treatments for patients suffering

from injury or diseases resulting from loss of communication between neurons.

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Compliance with Ethical Standards

All experiments were conducted in compliance with the ARRIVE guidelines.

Conflict of Interest The authors declare that they have no conflict of interest.

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