WIP modulates dendritic spine actin cytoskeleton by transcriptional control of lipid metabolic enzymes

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We identify Wiskott–Aldrich syndrome protein (WASP)-interacting protein (WIP) as a novel component of neuronal synapses whose absence increases dendritic spine size and filamentous actin levels in an N-WASP/Arp2/3-independent, RhoA/ROCK/profilinIIa-dependent manner. These effects depend on the reduction of membrane sphingomyelin (SM) due to transcriptional upregulation of neutral sphingomyelinase (NSM) through active RhoA; this enhances RhoA binding to the membrane, raft partitioning and activation in steady state but prevents RhoA changes in response to stimulus. Inhibition of NSM or SM addition reverses RhoA, filamentous actin and functional anomalies in synapses lacking WIP. Our findings characterize WIP as a link between membrane lipid composition and actin cytoskeleton at dendritic spines. They also contribute to explain cognitive deficits shared by individuals bearing mutations in the region assigned to the gene encoding for WIP.

INTRODUCTION

Synaptic function is among the most regulated cellular processes. Actin dynamics, which is driven by a network of actin-related molecules, is critical in this regulation (1). Knowledge of how this network is organized and responds to stimuli is crucial to understanding neuron function. The members of the Wiskott–Aldrich syndrome protein (WASP) family, together with the actin-related protein (Arp)2/3 complex, constitute a major nucleating center for filamentous actin (F-actin) assembly at the cell cortex (2). The role of these proteins was initially characterized in the immune system where they are central to T-cell activation (3). Neural (N)-WASP and the Arp2/3 complex also regulate actin in synapse development (4). The WASP-interacting protein (WIP) was first identified as a WASP partner in lymphocytes (5). Consistent with this role, a homozygous stop codon mutation in the WIPF1 gene (2q31.1), which encodes WIP, causes human immunodeficiency (6). However, several patients with overlapping deletions in a common region including only the WIPF1 gene showed variable clinical phenotype but lack of immune, Wiskott–Aldrich syndrome like, symptoms (7). Also supporting functions unrelated to WASP/N-WASP is the influence that WIP exerts on the actin cytoskeleton independently of its interaction with these proteins (8). WIP was recently reported in brain where it negatively regulates neuronal growth (9); its absence leads to accumulation of the post-synaptic density protein (PSD95) in dendritic spines, suggesting enhanced maturation of excitatory synapses. The underlying mechanism is at present unknown.

Stimulus-induced changes in synaptic morphology are due as much to actin cytoskeleton reorganization as to plasma membrane remodeling. Crosstalk between actin-related proteins and membrane lipids is thought to underlie synaptic activity-induced reshaping. The role of lipids is increasingly well recognized in this remodeling (10), which effects have been mainly evidenced in neurotransmitter receptor trafficking (11,12). The location of lipid metabolic enzymes at synapses supports an important role for the dynamics of lipid composition in synaptic plasticity. This is the case for the neutral sphingomyelinase (NSM) (13), which hydrolyzes the most abundant sphingolipid in neuronal membranes: sphingomyelin (SM). However, little is known of the molecular mechanisms triggered by lipid changes at synapses and of how these changes are achieved.

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Using mice lacking WIP (14), we here identify this protein as a new component of synapses with a WASP family independent ability to link actin cytoskeleton and membrane lipid composition.

RESULTS

Dendritic spine size and F-actin levels are increased in WIPko hippocampal neurons

Synapse strength is abnormally increased and PSD95 accumulation is greater in dendritic spines of hippocampal neurons from WIP knockout (WIPko) mice compared with controls (9). Since WIP is linked to actin regulation, a central element in dendritic spine physiology, we analyzed polymerized actin levels to study the molecular mechanisms underlying the WIP-deficient spine phenotype. Cultured hippocampal neurons from wild type (wt) and WIPko mice were phalloidin stained to label F-actin (15). To define dendritic spine areas, we colabeled cells with anti-MAP2 (microtubule-associated protein; to identify dendrites) and -PSD95 antibodies (to identify postsynaptic compartments). F-actin levels in these areas were 1.8-fold higher in WIPko dendritic spines compared with wt (Fig. 1A). This was accompanied by changes in dendritic spine size analyzed by electron microscopy in the hippocampal dentate gyrus from wt and WIPko mice. WIPko neurons showed a 32% increase in postsynaptic density length (Fig. 1B), which correlate directly with dendritic spine area. The results indicate WIP influence on dendritic spine size and F-actin levels.

Figure 1. Lack of WIP increases F-actin levels and size of dendritic spines. (A) Top: dendrites from wild type (wt) or WIPko hippocampal neurons stained for MAP2 (blue), PSD95 (green) and phalloidin (red); bottom: phalloidin staining only. Graph shows mean \( \pm \) SD of phalloidin fluorescence intensity per spine in arbitrary units \((n = 1200\) dendritic spines per mouse genotype, \(P = 0.0009\)). Bars: 5 \( \mu \)m. (B) Electron micrographs of synapses at the dentate gyrus of wt or WIPko hippocampus. Asterisks indicate postsynaptic compartments. Graph shows postsynaptic density length as the mean of 300 spines per mouse genotype (mean \( \pm \) SD; \(P = 0.0013\)). Bars: 0.35 \( \mu \)m.
N-WASP and Arp2/3 complex levels, membrane distribution and activity are unaltered in WIPko mouse synapses

To determine whether its influence is local, we tested for WIP in synaptosomal fractions. Western blots showed WIP expression in synapsonal of wt but not of WIPko mice (Fig. 2A), confirming antibody specificity, and the previously reported increase of PSD95 levels in WIPko synapses (9). WIP affects the actin cytoskeleton in non-neuronal cells through interaction with N-WASP and the Arp2/3 complex (8), which also participate in dendritic spine formation (4). To determine whether these proteins play a role on the spine phenotype in WIPko neurons, we evaluated N-WASP and Arp2/3 complex levels by western blot in total synaptosomal extracts (Fig. 2B), as well as in cytosolic (supernatant) and membrane (pellet) fractions obtained by high-speed centrifugation (Fig. 2C). We found no significant differences. In non-neuronal cells, the degree of N-WASP and Arp2/3 partitioning to membrane lipid raft domains regulates their actin-related activity in response to stimulus (16); the WASP–WIP complex is also recruited to membrane rafts in the stimulated immunological synapse (17). Hence, we tested whether lack of WIP in synaptosomes altered N-WASP and Arp2/3 complex levels in rafts, in steady-state conditions and after stimulation with 55 mM KCl for 15 min. Analysis of the flotation profile after cold Triton X-114 extraction of the raft marker flotillin 1 validated our raft isolation protocol and indicated no differences for this protein distribution in wt and WIPko synaptosomes in steady state or upon stimuli (Supplementary Material, Figure S1). The percentage in raft-containing insoluble (floating) fractions relative to total N-WASP (which displayed a broad, quite variable, distribution that was not dependent on the genotype) or Arp2/3 complex was similar in steady state for WIPko (40 ± 9 and 13 ± 2%, respectively) and wt synaptosomes (33 ± 15 and 9 ± 5%, respectively) (Fig. 2D). The distribution of these molecules did not differ after stimulation (32 ± 10 and 44 ± 13%, N-WASP in WIPko and wt; 6 ± 1 and 8 ± 5%, Arp2/3 in WIPko and wt) (Fig. 2D). The results suggest that N-WASP and Arp2/3 do not participate in the WIP deficiency effect on the spine actin cytoskeleton.

To further test this point, we monitored the activity of N-WASP in WIPko dendritic spines. Immunofluorescence analysis of cultured hippocampal neurons from wt and WIPko mice were performed using a conformation-sensitive antibody that recognizes the N-WASP in its open, active conformation but not in its closed, inactive conformation (18). Further supporting that the effect of WIP absence in dendritic spines is independent of N-WASP, we did not observe significant alterations in the levels of active N-WASP in WIPko spines (Fig. 2E).

Lack of WIP enhances the RhoA/ROCK/profilinIIa pathway in synaptosomes

The Rho GTPases RhoA, Cdc42 and Rac1 modulate actin polymerization in dendritic spines and have been associated with WIP; WIP-Cdc42 complex formation promotes actin nucleation in fibroblast motility (19) and filopodium generation (20). WIP also participates in Rac1-mediated membrane ruffle formation (21). We thus hypothesized that WIP deficiency could alter synaptic Rho GTPase levels. Whereas Cdc42 and Rac1 levels were normal (Supplementary Material, Figure S2), those of RhoA increased 3.1-fold in WIPko compared with wt synaptosomes (Fig. 3A). RhoA activity was 27% higher in WIPko than in wt synaptosomes, as determined by the amount of GTP-bound RhoA (Fig. 3A). Membrane attachment, a central event in RhoA activation, showed a 2.1-fold increase in WIPko relative to wt synaptosomes (Fig. 3B). There were also differences in RhoA membrane attachment poststimulation; membrane-bound RhoA increased 2.4-fold in KCl-stimulated compared with unstimulated wt synaptosomes, but not in stimulated WIPko synaptosomes, in which RhoA membrane binding decreased (Fig. 3B). To determine whether WIP deletion affected RhoA distribution in membrane domains, we analyzed RhoA partitioning in raft fractions in steady state and poststimulation. In flotation assays, there was a significantly larger proportion of RhoA in insoluble fractions of WIPko (82 ± 6%) than of wt synaptosomes (11 ± 5%) in steady state (Fig. 3C). After stimulation, the percentage of RhoA in insoluble fractions increased in wt (80 ± 6%). Instead, this percentage significantly decreased in WIPko synaptic membranes (47 ± 14%) (Fig. 3C). These findings indicate that, in the absence of WIP, synapses do not enhance RhoA membrane attachment and raft partitioning upon stimuli, in contrast to wt synapses. To analyze the RhoA–actin pathway triggered by lack of WIP, we measured levels of the RhoA-specific kinase ROCK and of profilinIIa. These RhoA downstream effectors increase dendritic spine F-actin levels after RhoA activation (15). ROCK and profilinIIa levels were higher (2.9- and 3.1-fold, respectively) in WIPko compared with wt synaptosomes (Fig. 3D).

Altogether these results suggest that RhoA/ROCK/profilinIIa pathway activation, through enhanced RhoA binding to membranes, contributes to F-actin increase in WIPko dendritic spines. To test this directly, phalloidin staining to label F-actin was performed in cultured hippocampal neurons from WIPko mice after incubation with the RhoA inhibitor Rh01. This inhibitor reduces efficiently the activity of the endogenous protein in different kind of cells including cultured neurons (22,23) through ADP-ribosylation in the effector-binding domain of this GTPase without affecting Cdc42 or Rac1 (24). Addition of Rh01 at 4 μg/ml for 5 h decreased 2.3-fold phalloidin intensity in dendritic spines (identified by colabeling with MAP2 and PSD95 antibodies) of WIPko neurons, which showed closer phalloidin intensity values to that of wt neurons (Fig. 3E). This result confirms the role of RhoA in F-actin alterations upon WIP deficiency.

Lack of WIP reduces membrane SM levels by transcriptional upregulation of NSM through active RhoA

The lipid composition of membranes, and specifically the amount of SM, influences RhoA binding to fibroblast membranes (25). SM is a major component of lipid rafts, in which we found increased RhoA in the absence of WIP (Fig. 3C). Interestingly, a microarray analysis we performed in WIPko fibroblasts showed a prominent 7.2-fold increase in the levels of the SM phosphodiesterase 3, neutral (Smpd3) and messenger RNA (mRNA) [NM_021491] [FDR (rank product < 0.05)] encoding NSM. NSM hydrolyzes SM at the plasma membrane (26). Consistently, staining with the SM-binding toxin lysenin (27) was 34% reduced on the WIPko fibroblast surface. This reduction was particularly evident in clusters of the lipid, for which lysenin shows special affinity (28) (Fig. 4A, Supplementary
Figure 2. WIP is found in synapses and its absence does not alter the levels, membrane distribution or activity of N-WASP or Arp2/3 complex. (A) Western blots for WIP, PSD95 and the non-synaptic membrane marker transferrin receptor (TfRc) in total brain (TOT) and synaptosomal (SYN) extracts from wt and WIPko mice. (B) Western blots for N-WASP, Arp2/3 complex and tubulin in wt and WIPko synaptosomal extracts. The graph shows mean ± SD normalized to tubulin \((n = 5)\). (C) N-WASP and Arp2/3 complex binding to membranes analyzed by western blot of supernatants (S) and pellets (P) after centrifugation \((100000 \text{g})\) of wt and WIPko synaptosomal extracts. Graphs show mean ± SD (expressed as pellet:supernatant ratio) \((n = 5)\). (D) Flotation assays of N-WASP and Arp2/3 complex in steady state and after 55 mM KCl stimulation (stimulated) analyzed by western blot of sucrose gradients after cold Triton X-114 extraction of wt or WIPko synaptosomes. Graphs show mean ± SD of the percentage of each protein in the floating fractions \((1–7)\) relative to total amount of the given protein \((n = 3)\). (E) Top: dendrites from wt or WIPko hippocampal neurons stained for phalloidin (red), PSD95 (blue) and active N-WASP (green); bottom: active N-WASP only. Graph shows mean ± SD of active N-WASP fluorescence intensity per spine in arbitrary units \((n = 600 \text{ spines per condition})\). Bars: 5 μm.
Figure 3. WIP absence activates the RhoA/ROCK/profilinIIa pathway in synapses. (A) Western blot for RhoA and tubulin in wt and WIPko synaptosomes. Graph shows RhoA mean ± SD normalized to tubulin ($n = 6$, $P = 0.002$). Right graph shows mean ± SD of RhoA activity/mg protein monitored by GTP-bound (active) RhoA in wt and WIPko synaptosomes ($n = 6$, $P = 0.007$). (B) RhoA binding to membranes in steady state and after 55 mM KCl stimulation, analyzed by western blot of supernatants (S) and pellets (P) after centrifugation (100 000 g) of wt and WIPko synaptosomal extracts. Graphs show mean ± SD (pellet:supernatant ratio) ($n = 3$, $P_{wt+KCl} = 0.004$, $P_{ko} = 0.003$). (C) RhoA membrane partitioning in steady state and after 55 mM KCl stimulation analyzed by western blot of sucrose gradients after cold TritonX-114 extraction of wt or WIPko synaptosomes. Graphs show mean ± SD of RhoA percentage in floating fractions (1–7) relative to RhoA total amount ($n = 3$, $P_{wt+KCl} = 0.0008$, $P_{ko} = 0.001$). (D) Western blots for ROCK, profilinIIa and tubulin in wt and WIPko synaptosomes. Graph shows mean ± SD normalized to tubulin ($n = 6$, $P_{ROCK} = 0.009$, $P_{profilinIIa} = 0.002$). (E) Top: dendrites from wt or WIPko hippocampal neurons (the latter alone or treated with RhoA inhibitor) were stained for MAP2 (blue), PSD95 (green) and phalloidin (red); bottom: phalloidin staining only. Graph shows mean ± SD phalloidin fluorescence intensity per spine ($n = 500$ dendritic spines per condition, $P_{wt} < 0.0001$, $P_{ko+RhoI} < 0.0001$). Bars: 5 μm.
Figure 4. Lack of WIP transcriptionally upregulates NSM and reduces SM levels. (A) Lysenin-stained unpermeabilized wt and WIPko fibroblasts. Graph shows mean ± SD of lysenin fluorescence intensity per area unit (n = 30 cells from each of three cultures, P = 0.00001). Bar = 100 μm. (B) Levels of NSM mRNA in WIPko fibroblasts treated or not with RhoA inhibitor. Graphs show mean ± SD referred to WIPko values considered as one (n = 3, P = 0.04). (C) Levels of WIP and tubulin in wt fibroblasts treated or not with Latrunculin A for 4 or 24 h. Graph shows mean ± SD normalized to tubulin (n = 3, P_{24h} = 0.03). (D) Levels of NSM mRNA in hippocampal extracts from wt and WIPko mice. Graph shows mean ± SD (n = 7, P = 0.004). (E) Western blot for NSM and tubulin in wt and WIPko synaptosomes. Graph shows mean ± SD normalized to tubulin (n = 6, P = 0.0008). (F) Levels of SM and cholesterol (nmol/mg protein) and GM1 (a.u.) in wt and WIPko synaptosomes. The graph shows mean ± SD (n = 4, P_{SM} = 0.03). (G) Lysenin-stained unpermeabilized wt and WIPko cultured hippocampal neurons. Graph shows mean ± SD of lysenin fluorescence intensity per area unit (n = 30 cells each from three cultures, P = 0.003). Bar = 100 μm.
NSM inhibition or SM addition restore RhoA binding to membranes, F-actin levels and synaptic function in WIPko synaptosomes and hippocampal neurons

Our results suggest a key role for NSM and SM on RhoA alterations induced by the absence of WIP by which RhoA increases its own binding to the synaptic membrane. This further enhances its activity resulting in the abnormally high F-actin levels in WIPko dendritic spines. To directly test the contribution of NSM and SM to these events, we modulated these molecules in synaptosomes from WIPko mice compared with wt (Fig. 4D). This was accompanied by a 2.6-fold increase in NSM protein levels (Fig. 4E) and a 32% SM reduction (Fig. 4F) in WIPko compared with wt synaptosomes. This change is SM specific as levels of other major lipid raft components (cholesterol, ganglioside GM1) are unaltered in the absence of WIP, as shown by enzymatic assays or by dot blot using cholera toxin, respectively (Fig. 4F).

Lysenin staining indicated the reduction of SM levels (22%) at the surface of WIPko compared with wt cultured hippocampal neurons (Fig. 4G, Supplementary Material, Figure S3).

These findings unveil a role for WIP in the regulation of membrane lipid composition in fibroblasts and neurons; more specifically, WIP modulates the levels of SM through the transcriptional control of NSM via active RhoA.

DISCUSSION

Our results identify WIP as a novel component of synapses with a key role in the modulation of F-actin levels in dendritic spines. WIP exerts this role by controlling the membrane attachment and thus activity of the small GTPase RhoA. Cooperative actions of Rho GTPases and the N-WASP/WIP complex to promote actin generation and/or recruitment and subsequent activation of the N-WASP/WIP complex contributing to F-actin increase in dendritic spines. An explanation for this discrepancy is that, in this neuronal compartment, WIP participates in a pathway mediated by SM, RhoA and its effectors ROCK and profilinIIa that is independent of Cdc42, N-WASP and the Arp2/3 complex. These findings highlight WIP versatility, which enables it to modulate SM breakdown products are mediating the effects of WIP deficiency, we added SM to WIPko synaptosomes in the presence of the NSM inhibitor GW4869 to prevent their formation. In these conditions, the reduction (3.3-fold) in RhoA membrane binding was still achieved (Fig. 5E) supporting the role for SM but not of its derivatives. SM addition to WIPko synaptosomes also reduced RhoA partitioning in rafts in steady state (39% of total RhoA) and recovered its capability to redistribute to these domains in response to stimuli (72%) (Fig. 5F), approaching the behavior observed in wt synaptosomes (see Fig. 3C). To determine whether SM addition restored F-actin levels in dendritic spines, we treated WIPko hippocampal neurons with 40 \( \mu \text{M} \) SM for 6 h. Lysenin staining showed a 19% SM increase on membranes of WIPko-treated neurons (Fig. 5G). F-actin levels, monitored by phalloidin staining, were 50% lower in dendritic spines (identified by PSD95 and MAP2 colabeling) of SM-treated WIPko neurons. These levels were even lower than those observed in wt neurons (Fig. 5H). SM addition to WIPko hippocampal neurons also restored PSD-95 levels in dendritic spines, which are abnormally high in WIPko neurons (Supplementary Material, Figure S4).

Finally, we aimed to determine whether SM addition had functional effects restoring the abnormally enhanced synaptic strength observed in WIPko neurons (9). Cultured WIPko neurons were treated or not with SM as in Figure 5G and H and electrophysiologically recorded to measure amplitude and frequency of spontaneous miniature excitatory postsynaptic currents (mEPSCs) (Fig. 6A). Addition of the lipid reduced mEPSC amplitude that is indicative of the postsynaptic strength of individual synapses (32) (Fig. 6B). We did not observe SM-induced changes in the mEPSC frequency that depends on synapse number and presynaptic properties (33) (Fig. 6C).

Altogether, these results confirm that increased NSM activity leading to reduced SM levels mediate the effects of WIP deficiency on dendritic spines and spontaneous synaptic function. They also show that modulation of these features is a potential strategy for reversion of WIP-dependent spine anomalies.
Figure 5. NSM inhibition or SM addition restores RhoA binding to membrane and F-actin levels in WIPko synaptosomes and hippocampal neurons. (A) SM levels (nmol/mg protein) in WIPko synaptosomes treated or not with the NSM inhibitor GW4869 (n = 3, P = 0.036). (B) RhoA binding to membrane in wt and in WIPko synaptosomes (the latter treated or not with GW4869) analyzed by western blot of supernatants (S) and pellets (P) after centrifugation (100 000g). Graph shows mean ± SD (pellet:supernatant ratio) (n = 3, Pwt = 0.003, Pko+GW = 0.03). (C) Top: dendrites from wt or WIPko hippocampal neurons (the latter treated or not with GW4869) stained for MAP2 (blue), PSD95 (green) and phalloidin (red); bottom: phalloidin staining only. Graph shows mean ± SD phalloidin fluorescence intensity per spine (n = 600 dendritic spines per condition, Pwt < 0.0001, Pko+GW < 0.0001). Bars: 5 μm. (D) SM levels (nmol/mg protein) in WIPko synaptosomes alone or SM-treated (n = 3, P = 0.038). (E) RhoA binding to membrane in WIPko synaptosomes treated or not with SM in the absence or presence of GW4869. Graph shows mean ± SD (RhoA pellet:supernatant ratio) (n = 3, Pko+SM = 0.03, Pko+SM+GW = 0.0033). (F) RhoA membrane partitioning in steady state and after 55 mM KCl stimulation analyzed by western blot of sucrose gradients after cold Triton X-114 extraction of WIPko synaptosomes treated or not with SM. Graph shows mean ± SD of RhoA percentage in floating fractions (1–7) relative to RhoA total amount (n = 3, Pko+SM = 0.003, Pko+SM+KCl = 0.002). (G) Graph shows mean ± SD of lysenin fluorescence intensity per area unit in WIPko hippocampal neurons treated or not with SM (n = 30 cells each from three cultures, P = 0.04). (H) Top: dendrites from wt hippocampal neurons or from alone or SM-treated WIPko hippocampal neurons stained for MAP2 (blue), PSD95 (green) and phalloidin (red); bottom: phalloidin staining only. Graph shows mean ± SD of phalloidin fluorescence intensity per spine (n = 1200 dendritic spines per condition, Pko = 0.0099, Pko+SM = 0.0009). Bars: 5 μm.
the actin cytoskeleton in several ways depending on cell type, cell site and the partners with which it associates.

Our results indicate an unanticipated role for WIP as a modulator of lipid metabolism that functionally links membrane lipids and the actin cytoskeleton at synapses. Upon T-cell activation the WIP-interacting protein WASP upregulates levels of the lipid raft marker GM1 at the plasma membrane, which promotes clustering of raft domains during the formation of the immunological synapse (36). Here we show that, in an apparently N-WASP-independent manner, WIP at synapses alters levels of another major raft lipid, SM, but not those of cholesterol or GM1. Lack of WIP has no effect on N-WASP activity or raft partitioning of N-WASP or Arp2/3 complex but enhances RhoA presence in raft domains due to SM decrease. This is not the first example of proteins that increase their presence in detergent resistant membranes despite the reduction of one of their lipid components. Neurons with reduced cholesterol levels showed enhanced TrkB amount in rafts (37). The possibility exists that upon the lipid change these membrane domains become looser thus facilitating the incorporation of certain proteins. Nevertheless, RhoA increase in raft domains might improve its stability, which would explain higher levels and activity of this protein in WIPko synaptosomes.

By providing mechanistic insight on how WIP influences lipid composition, we discover this protein as a transcriptional regulator of lipid metabolism. Lack of WIP increases mRNA levels of the SM catabolic enzyme NSM via active RhoA. Moreover, WIP levels are regulated by the amount of F-actin. Supporting the ability of WIP to also notice SM amount we found increased levels of the protein in synaptosomes from mice lacking acid sphingomyelinase (Supplementary Material, Figure S5), which have abnormally high SM levels (38).

METHODS

Mice

We compared 5-month-old male wt and WIPko SV129/BL6 littermate mice (14). Procedures followed European Union guidelines and were approved by the CBMSO Animal Welfare Committee.
Antibodies
We used anti-Rho A (Cell Signaling), -ROCK (BD Transduction Laboratories), -profilinIIa (donated by C.G. Dotti), -alpha tubulin (Abcam), -PSD95 (Neuromab), -MAP2 (donated by J. Avila), -N-WASP, -active-N-WASP (donated by J. Condellis), -NSM (Santa Cruz Biotechnology), -Arp2/3 complex, -GADPH (Abcam), -Cdc42, -Rac1 (BD Biosciences), -WIP (donated by R.S. Geha) and -transferrin receptor antibodies (Zymed). Horseradish peroxidase (PO)-conjugated secondary antibodies were from Dakocytomation.

Western blots
Western blots using different antibodies were carried out with the enhanced chemiluminescence detection system (ECL, Perkin Elmer Life Science, Waltham, MA, USA) and the digital camera ImageQuant LAS4000 mini (GE Healthcare). Quantification of the bands was performed by densitometry with the Quantity One software under conditions of non-saturated signal in order to ensure the linearity range.

Electron microscopy
Mice were perfused with phosphate-buffered saline (PBS) and fixative (4% paraformaldehyde, 2% glutaraldehyde in PBS). Brains were postfixed in 4% paraformaldehyde overnight and sectioned in 200-μm slices. Dentate gyrus sections were embedded in Epon, stained with uranyl acetate and lead citrate and viewed on a transmission electron microscope (JEM1010, Jeol). Synapses were sampled randomly and photographed at 10,000× magnification with a CMOS 4k TemCam-F416 camera (TVIPS, Gauting). Postsynaptic length was measured using ImageJ software (150 synapses/mouse; n = 3 mice/genotype).

Cell cultures
Primary hippocampal neuron cultures were prepared from wt and WIPko mouse embryos (42). Neurons were cultured (21 days; 21-DIV) on a supporting layer of astrocytes. Fibroblasts were derived from adult wt or WIPko mouse lungs and cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, 2 mm glutamine and antibiotics (21).
Immunofluorescence and imaging

Hippocampal neurons were immunostained and images acquired as in ref. (9). Phalloidin fluorescence intensity/spine was measured as integrated density using Image J.

Synaptosomes

Synaptosomes were obtained from mouse brains using Percoll gradients (43).

Addition of SM

SM from bovine brain (1.25 mM; Sigma) was added to freshly isolated synaptosomes and incubated (45 min, 37°C with gentle agitation). SM (40 μM) was added to 21-DIV wt or WIPko neurons for 6 h.

Lipid analysis

SM was quantified as in (44) in synaptosomal extracts containing 20% (w/v) Thesit (Sigma). Cells fixed with 4% paraformaldehyde and incubated with 0.2 μg/ml lysenin, anti-lysenin antibody (Peptanova) and donkey anti-rabbit antibody (Alexa 488). Images were obtained in an Axiovert 200 microscope and mean intensity per area unit determined in random areas along the dendrites using ImageJ. Cholesterol was quantified with the Amplex Red Cholesterol Assay Kit (Invitrogen) and GM1 by dot blot with PO-conjugated cholera toxin B subunit (Sigma) and ECL (GE Healthcare) in non-saturating conditions on a densitometer and Quantity One software.

Raft isolation

Synaptosomes were incubated in TNE buffer [100 mM Tris, 2 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4] containing 0.5% Triton X-114 and protease inhibitor cocktail (complete EDTA-free, Roche)(40 min, 4°C), then brought to 60% sucrose. A 35 and 5% sucrose step gradient in TNE was overlaid on samples and ultracentrifuged (19 h, 73 000 g). After centrifugation, 13 fractions of 1 ml each were collected from top to bottom of the tube. Detergent-insoluble material (rafts) was in the lighter fractions (1–7).

RhoA activity and inhibition

RhoA activity was measured in freshly isolated synaptosomal extracts using the G-LISA Rho A Activation Assay Biochem Kit (absorbance based). Rho inhibitor I (cytoskeleton), which inhibits RhoA but not Cdc42 or Rac1, was added to the medium of WIPko cultured hippocampal neurons or WIPko fibroblasts at 4 μg/ml for 5 or 24 h, respectively.

Latrunculin A treatment

WIPko fibroblasts were incubated with Latrunculin A (Sigma) at 0.5 μM for 4 or 24 h.

Measurement of NSM mRNA

Total RNA from hippocampal homogenates was obtained by Trizol Reagent (Ambion/RNA, Life Technologies) and chloroform extraction. Quantitative polymerase chain reaction (qPCR) was performed using GoTaq QPCR Master Mix (Promega) and ABI PRISM 7900HT SDS (Applied Biosystems). For the detection of NSM2 transcripts, we used as primers: Nsm2_fw: 5’-TGCTTGACACAAACGGTCT and Nsm2_re: 5’-GTGTTGCAGGGGTACACACAT. GAPDH, GUSB and HPRT1 were used as endogenous controls.

NSM inhibition

The specific inhibitor for NSM, GW4869 (Cayman), was added to freshly isolated WIPko synaptosomes at 15 μM for 1 h at 37°C or to the culture medium of WIPko hippocampal neurons at 10 μM for 1 h.

Microarray analysis

Total RNA was obtained from wt and WIPko fibroblasts and analyzed by Two-Color Microarray-Based Gene Expression Analysis (Agilent Technologies). Four biological replicates were hybridized independently for each transcriptomic comparison. Expression data were background corrected and normalized using LIMMA. Differentially expressed genes were evaluated by the RankProd non-parametric algorithm (Bioconductor).

Measurement of mEPSCs

mEPSCs were recorded from dissociated hippocampal neurons bathed in ACSF (containing 119 mM NaCl, 2.5 mM KCl, 1 mM NaH2PO4, 1 mM glucose, 26 mM NaHCO3, 2.5 mM CaCl2 and 1.3 mM MgCl2) in the presence of 1 μM tetrodotoxin and 100 μM picrotoxin at 29°C. Spontaneous activity was recorded during 3 min for each cell. mEPSCs were identified using pClamp software and corrected by eye on the basis of their kinetics.

Statistical analysis

Data are presented as mean ± standard deviation (SD) for at least three experimental groups. The P-values were determined by Student’s t-test; asterisks in figures indicate P values as *<0.05; **<0.005 and ***<0.001.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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