Title: MEANS AND METHODS FOR IMPROVEMENT OF SYNAPTIC DYSFUNCTION DISORDERS

Abstract: The present invention relates to the diagnosis, prevention and/or treatment of neuronal disorders, more particularly to neurodegenerative disorders, even more particularly to synaptic dysfunction disorders, in particular Alzheimer's disease and Parkinson's disease. In particular, the present invention relates to TBC1D24, a GTPase-activating protein for synaptic Rab35, as a target for neurodegenerative disorders, and based thereon screening methods for compounds that ameliorate synaptic dysfunction in mammalian neuron cells. The present invention also relates to inhibiting agents targeting TBC1D24 and pharmacological compositions thereof, and their use in therapeutic applications of said disorders.
MEANS AND METHODS FOR IMPROVEMENT OF SYNAPTIC DYSFUNCTION DISORDERS

Field of the invention

The present invention relates to the field of neuronal disorders, more particularly to the field of neurodegeneration disorders, in particular Alzheimer's disease and Parkinson's disease, and the diagnosis, prevention and/or treatment thereof. In particular, the present invention relates to TBC1D24, a GTPase-activating protein for synaptic Rab35, as a target for neurodegeneration disorders, and based thereon screening methods for compounds that ameliorate synaptic dysfunction in mammalian neuron cells. The present invention also relates to inhibiting agents targeting TBC1D24 and pharmaceutical compositions thereof, and their use in therapeutic applications of said disorders.

Introduction to the invention

During long periods of stimulation, synaptic vesicles recycle locally to sustain neuronal communication. Although many proteins involved in vesicle fusion and reformation at the membrane have been characterized, the molecular mechanisms that regulate vesicle trafficking within nerve terminals are far less studied, and the transport of synaptic vesicles via organelles such as endosomes, remains debated. Following fusion, synaptic vesicles are reformed at the plasma membrane and are transported back to release sites to participate in a new round of release. In most cells, newly internalized transport vesicles are routed to recycling endosomes where proteins and lipids are sorted and packaged for transport to various locations in the cell or internalized into multivesicular bodies in an ESCRT-dependent manner. However, for synaptic vesicles the relevance of such an intermediate step remains unclear. At nerve terminals cisternae and membrane folds, reminiscent of endosomes, form upon intense neuronal stimulation, particularly in endocytic mutants, however electrophysiological evidence suggests these structures can be directly filled with neurotransmitter, as upon spontaneous fusion with the presynaptic membrane these cisternae elicit large-amplitude minis. Cisternae may thus not constitute bona fide endosomal sorting stations for synaptic vesicle proteins. In further support of this, most vesicles that underwent endocytosis and internalized a fluorescent membrane-bound dye in rat hippocampal neurons or in goldfish retinal bipolar neurons did not lose any of this labeling while traveling in the nerve terminal and intermixing of vesicle content is minimal. While the data suggest that under the conditions tested, the majority of synaptic vesicles do not traffic via an intermediate step, some endosomal trafficking may be vital to control the sorting of use-dependent, spoiled proteins and lipids resident on synaptic vesicles. It is known that numerous intracellular vesicle trafficking
events are regulated by a family of small Rab GTPases and their effectors (Zerial and McBride, 2001). However, only few Rabs, including Rab3, Rab5 and Rab27, have been implicated in the synaptic vesicle cycle (Fischer von mollard, 1994; Mahoney et al., 2006; Schluter et al., 2006; Schluter et al., 2004; Wucherpfennig et al., 2003; Yu et al., 2008).

Furthermore, the regulation of Rab GTPase activity, and therefore Rab function, remains enigmatic, particularly in synaptic vesicle traffic. Rab GTPases cycle between a GDP-bound inactive and a GTP-bound active state (Schimmoller et al., 1998; Zerial and McBride, 2001) and the active Rab GTPases bind effector molecules that control specific vesicle trafficking events (Corbeel and Freson, 2008; Stenmark, 2009). While Rabs harbor low endogenous GTPase activity, this activity, and thus the pool of active RabGTP, is directly controlled by specific GAPs (Pan et al., 2006; Takai et al., 2001). Despite their importance in regulating vesicle trafficking events in many cell types, a role for GAPs in the recycling of synaptic vesicles remains unexplored. In the present invention we identified Skywalker, a previously uncharacterized neuronal GAP protein that activates Rab35 GTPase activity to control endosomal traffic of synaptic vesicles. Using a combination of 10 kDa dextran labeling, FM 1-43 labeling, photoconversion of FM 1-43 for electron microscopy, multiple time-point electron microscopy, electron tomography and electrophysiology, we show that in sky loss-of-function mutants, an excessive amount of synaptic vesicles travel via sorting endosomes marked by 2xFYVE-GFP and Rab5-GFP, and to a lesser extend via recycling endosomes marked by Rab4-GFP. Both biochemical and genetic interaction data show that Sky-activity regulates Rab35 efficiently but not Rab5. Furthermore, animals expressing the constitutive active form of Rab35 as well as sky mutants show a dramatic increase in neurotransmitter release. While calcium influx upon stimulation is not affected in sky mutants, increased neurotransmitter release correlates with a larger size of the readily releasable synaptic vesicle pool. Consistent with our data we propose that facilitation of endosomal trafficking in sky mutants mediates the exchange of inactive synaptic vesicle proteins for functional ones. Indeed, lowering expression of ESCRT complex components, involved in endo-lysosomal traffic of ubiquitinated proteins destined for degradation, dramatically suppresses increased neurotransmitter release in sky mutants. Furthermore, the synaptic levels of a chimeric Synaptobrevin-Ubiquitin protein that we expressed in sky mutants are much lower compared to controls and this can be rescued by removing an ESCRT-gene copy, suggesting that increased endosomal traffic of this chimeric synaptic vesicle protein results in its degradation. Taken together, these and other data suggest that endosomes mediate synaptic vesicle rejuvenation, and this function is controlled by Sky and its main target Rab35, thus providing a novel mechanism by which neurons can regulate synaptic plasticity. It has recently been shown that presynaptic dysfunction is an early pathogenic event before neurodegeneration occurs in neurological diseases such as Alzheimer's disease and Parkinson's disease (Shen J. et al. 2010) Neurodegener. Dis. 7 (1-3):
80-3. Thus, the human homologue of skywalker can be used for screening to identify compounds which are able to increase the neurotransmitter release and induce vesicle cycling in neurons.

5 Figure legends

Figure 1: Characterization of sky mutants and localization of Sky protein

(A) Peculiar behavioral defects in third instar sky^200 mutants; other allelic combinations show a similar larval behavioral phenotype (data not shown).

(B) Electoretinograms of flies with homozygous FRT40A eyes (control) or homozygous sky^1 or sky^2 eyes (genotypes: see methods) and of sky^12 and of sky^R0 animals that carry a genomic sky^* rescue construct (sky^12; sky^* and sky^R0; sky^*). Light on and off positions are marked by arrowheads. Note the reduced or absence of on and off transients (coinciding with lights on and off) in sky mutants and rescue thereof upon introducing a sky^* rescue construct.

(C) SKY mRNA expression levels defined by quantitative real-time PCR in controls (FRT40A), in sky^12 mutants, in sky^200 mutants, in sky^100 mutants and in sky^260 mutants. Tukey test: **: p<0.0001. Error bars: SEM. Means are averages of 3 runs per genotype.

(D) Lethal phase of sky mutants and rescue experiments using neuronal nSyb-GAL4 (nSyb>) or muscular BG57- GAL4 (BG57>) expression in sky^20 or using nSyb-GAL4 and UAS-sky (nSyb>sky) as well as using the genomic rescue construct sky^*. E “”: animals do not reach the first instar larval stage; "L1”: first instar lethal; "P": Pupal lethal; "PA": pharate adult lethal; "Unc": few adult flies emerge but they are severely uncoordinated. "+": rescue of lethality; "-": no rescue of lethality; "ND": not defined.

(E) Schematic presentation of Sky protein structure (PA isoform; 587 aa). Locations of TBC and TLDc domains are shown and identity/similarity to human and nematode homologues.

(F) GFP-Sky (magenta) localization at the third instar larval GFP-sky^*/+ ventral nerve cord, double labeled with DLG (green), a synaptic marker.

(G-H) Immunolabeling of third instar larval ventral nerve cords with antibodies to Sky (magenta) and DLG (green) in controls (FRT40A) and sky^260 mutants. Note the reduced Sky labeling in the neuropile of the mutants.

(I-N) GFP-Sky localization at third instar larval GFP-sky^*/+ NMJs. Fillets are double labeled with anti-DLG, a pre- and postsynaptic marker (I, L); anti-Dynamin, a presynaptic marker (J, M) and anti-nSyb, a synaptic vesicle marker (K, N). Red outlines mark DLG, Dynamin and nSyb labeling respectively and encircle presynaptic GFP-Sky. Scale bar in F, for F-H and in I, for I-K is 20 µm; scale bar in L, for L-N is 5 µm.
Figure 2: Large endosomal-like structures at sky mutant NMJs do not form at the plasma membrane.

(A-C) FM 1-43 dye uptake and unloading in controls (FRT40A), sky^{ts2}, sky\textsuperscript{wD}. sky\textsuperscript{oD0}, sky\textsuperscript{yIO}, sky\textsuperscript{yIO} mutants and in nSyb>sky\textsuperscript{yIO} mutants that express Sky in their nervous system as well as in sky\textsuperscript{ts2} mutants that harbor a genomic sky\textsuperscript{*} rescue construct (sky\textsuperscript{ts2}; sky\textsuperscript{y/+}). For loading, dissected larvae were incubated in HL-3 with 90 mM KCl and 4 µM FM 1-43 for 1 min; for unloading, labeled larvae were incubated in HL-3 with 90 mM KCl for 5 min. (B) Mean boutonic labeling in controls and mutants following loading (black) and unloading (white) relative to loading in controls. (C) Number of accumulations observed per bouton area. Scale bar in A: 5 µm. Error bars: SEM. n is ≥ 10 per genotype. ANOVA and f-tests: p>0.05 in B, and **: p<0.01, ns: not significant.

(D, E) Transmission electron micrographs of control (D) and sky\textsuperscript{yIO} mutant (E) NMJ boutons stimulated for 1 min in 90 mM KCl and then fixed for TEM immediately following stimulation. Note the large cisternal-like structures in mutants (see also figure 3). Scale bar in F for D-F: 500 nm.

(F) TEM of sky\textsuperscript{yIO} mutant NMJ boutons loaded with FM 1-43 and photoconversion of DAB (see also Figure S2E-J). Vesicles (small arrowheads) and endosomal/cisternal-like structures (large arrowheads) are labeled by photoconverted DAB.

(G-L) Quantification of synaptic mitochondria per area (G), electron dense lysosomes per area (H), dense bodies per area (T-bars) (I), density of vesicles with a diameter smaller than 80 nm (J), density of vesicles with a diameter larger than 80 nm (K) and the mean vesicle diameter (L) in controls and in sky\textsuperscript{yIO} mutants. For quantification, at least 23 bouton profiles of 3-5 animals were analyzed. f-test: ns, not significant, *: p<0.05, **: p<0.001, ns: not significant. Error bars: SEM.

(M, N) FM 1-43 dye uptake during a 20 min 3 Hz stimulation paradigm in controls (FRT40A), sky\textsuperscript{ts2}, and in sky\textsuperscript{yIO} (M) and quantification of number of accumulations per bouton area (N). Scale bar in M: 5 µm. Error bars: SEM. n ≥ 5 per genotype. f-test: **: p<0.01.

(O, P) 10 kDa Dextran-Rhodamine uptake using 1 min of 90 mM KCl stimulation in controls (FRT40A), controls treated with 50 µM chlorpromazine and in sky\textsuperscript{yIO} mutants (O) and quantification of the number of the relative number of labeled synapses on muscle 12, 13 and 4 in abdominal segments compared to chlorpromazine treated samples. Scale bar in O: 5 µm. Error bars: SEM. n ≥ 4 per genotype or condition.

(Q, R) Quantification of membrane associated omega shapes (endocytic structures) in electron micrographs of stimulated control (FRT40A) and sky\textsuperscript{yIO} mutant. We quantified both omega structures with vesicle diameters of less than 80 nm (Q) and more than 80 nm (R). For quantification, at least 23 bouton profiles of 3-5 animals were analyzed. Error bars: SEM. t-test: ns, not significant.
(S, T) Surface rendered electron tomography models taken on 200-300 nm thick sections of control (FRT40A) and sky260 mutant boutons. Plasma membrane: blue; vesicular profiles: green; lysosomes: black; cisternal profiles with late endosomal features (e.g. MVB): yellow. Note that no obvious large diameter plasma membrane invaginations are observed in controls and mutants, furthermore, the endosomal-cisternal-like structures observed in sky mutants do not appear to be attached to the plasma membrane.

Figure 3: Stimulation-dependent formation of endosomal-cisternal-like structures that do not directly fuse with the membrane in sky mutants.

(A, B) Average mEJC amplitude and raw data traces of controls (FRT40A), sky^r2 and sky^260 mutant animals and cumulative probability histograms of mEJC amplitudes recorded prior to stimulation (A) and directly following 1 min 90 mM KCl stimulation (B). t-test: ns, not significant, error bars: SEM, n: number of tested animals indicated in the bars.

(C-H) TEM time-course of control (FRT40A) (C-E) and sky^260 mutant (F-H) boutons, prior to stimulation (C, F), fixed immediately after stimulation (1 min 90 mM KCl) (D,G) and fixed 1 h following stimulation (E,H). Scale bar in H, for C-H: 500 nm.

(I-N) Histograms of vesicle size binned per 10 nm of profiles found within boutons interiors (I-K) or of profiles located close (<200 nm) to active zones (T-bars) (L-N) of preparations that were not stimulated (I, L), fixed immediately following stimulation (1 min 90 mM KCl) (J, M) and fixed after resting for 1 h following stimulation (K, N). Frequencies of vesicle diameters are reported as percent of total vesicles analyzed (at least 799 vesicle profiles in I-K and at least 118 profiles in L-N). Insets in L-N show T-bars and associated vesicles in controls and sky^260 mutants prior to, immediately after and 1 h after stimulation, Scale bar in N for L-N: 100 nm. Note the vast accumulation of endosomal/cisternal structures in sky mutants fixed immediately after stimulation in bouton interiors (J), but not at active zones (M).

(O-P) Quantification of vesicle density with a diameter larger than 80 nm (O) and of profiles with a diameter smaller than 80 nm (P) prior to stimulation, immediately following stimulation and 1 h after stimulating control (FRT40A) (black) and sky^260 mutant (red) NMJs. For quantification, at least 17 bouton profiles of 3-5 animals were analyzed. Error bars: SEM. t-test: *: p<0.05, ***: p<0.001.

(Q-S) TEM (Q, R) and electron tomography surface rendering (S) of stimulated (1 min 90 mM KCl) sky^260 mutant boutons showing endosomal/cisternal-like structures with small -40 nm vesicles directly attached (arrowheads indicate some of these vesicles). Scale bar in R: 100 nm.
Figure 4: Sky facilitates the cycling of synaptic vesicles through an endosomal compartment.

(A-I) FM 1-43 loading (magenta) using 1 min of 90 mM KCl stimulation in controls expressing 2xFYVE-GFP (UAS-EGFP-myc-2xFYVE/+; nSyb-GAL4/+; sky^{v2} mutants expressing 2xFYVE-GFP (green) (sky^{l}sky^{2} UAS-EGFP-myc-2xFYVE; nSyb-GAL4/+ (A, B); and in controls expressing Rab5-GFP (UAS-GFP-Rab5/+; nSyb-GAL4/+) and sky^{v2} mutants expressing Rab5-GFP (green) (sky^{l}/sky^{2} UAS-GFP-Rab5; nSyb-GAL4/+) (C, D) as well as in controls expressing Rab4-GFP (UAS-EGFP-Rab4/nSyb-GAL4) and sky^{R2} mutants expressing Rab4-GFP (green) (sky^{v2}; UAS-EGFP-Rab4/nSyb-GAL4) (E, F). Arrows in A-F indicate the location of GFP spots. (G-I) Quantification of FM 1-43 that colocalizes with 2xFYVE-GFP (G), Rab5-GFP (H) and Rab4-GFP (I) indicated as [FM 1-43 area that colocalizes with GFP / FM 1-43 area]. f-test: **: p<0.01, error bars: SEM. Scale bar in A for A-F: 5 \mu m. n ≥ 4 per genotype.

(J-Z) Immunolabeling of control and sky mutant NMJs with synaptic vesicle markers of samples fixed prior to 1 min 90 mM KCl stimulation, immediately following stimulation and after a rest period at various time points following stimulation. (J-L) Quantification of the number of boutonic accumulations observed with anti-CSP labeling prior to stimulation (‘US’ in J) and immediately following stimulation (J, KCI) or with anti-Sytl labeling prior to stimulation ("US" in K, L), immediately following stimulation (K, L; KCI or 0') or after a rest period at various time points following stimulation (5', 10', 30' and 60') (L) in controls (UAS-EGFP-myc-2xFYVE/+; nSyb-GAL4/+) (J-L), in sky^{R2} mutants (sky^{l}/sky^{2} UAS-EGFP-myc-2xFYVE; nSyb-GAL4/+) (J-L) and in sky^{R2} mutants that harbor a genomic sky^{+} rescue construct (sky^{l}/sky^{2} UAS-EGFP-myc-2xFYVE; nSyb-GAL4/sky^{+}) (J, K). (M, N) Quantification of CSP (M) or Sytl (N) colocalization with 2xFYVE-GFP in controls and sky^{R2} mutants indicated as [synaptic vesicle marker area that colocalizes with GFP / synaptic vesicle marker area]; see also Figure S3. f-test: ns: not significant, *: p<0.05 and **: p<0.01, error bars: SEM. n ≥ 5 per genotype. (O-Z) anti-CSP (O-R) and anti-Sytl (S-Z) labeling in unstimulated 2xFYVE-GFP expressing sky^{R2} mutants and in 2xFYVE-GFP controls (not shown, data not different from sky^{R2} (O, S "US") and in 2xFYVE-GFP expressing controls (P, T, W, Y) and sky^{R2} mutants (Q, U, X and Z) that were fixed immediately following stimulation (0'), that rested for 5 min following stimulation (5') or that rested for 1 h following stimulation (60') and in sky^{R2} mutants with a sky^{+} rescue construct that were fixed immediately following stimulation (0') (R, V), Scale bar in O for O-Z: 5 \mu m.

Figure 5: Sky is a Rab35 GAP.

(A) FM 1-43 loading using 1 min of 90 mM KCl stimulation at control third instar NMJ boutons (nSyb-GAL4/) and at boutons of larvae expressing each of a collection of 31 CA Rab GTPases in the nervous system (nSyb>rab^{CA}). Arrows indicate sub-synaptic accumulations of FM 1-43 upon expression of Rab5^{CA}, Rab23^{CA} and Rab35^{CA}. 
(B) Time-course measurements of GTP hydrolysis for GST-Rab35 in absence and presence of different concentrations of HIS-Sky (indicated in µM). Solid lines are fitted curves of the pseudo-first-order-Michaelis-Menten model function. Inset: calculated k_{cat}/Km values ± SEM for GTP hydrolysis of GST-Rab35, GST-Rab23 and GST-Rab5 tested at identical HIS-Sky concentrations as those used for Rab35.

(C) Mean survival of sky^{2D} animals that express Rab35^{DN} (sky^{2n}; rab35^{DN/nSyb-GAL4}) or Rab5^{DN} (sky^{2D}; rab5^{DN/nSyb-GAL4}) in their nervous system compared to the third instar larval stage relative to the survival of sky^{2n} animals. Note that compared to sky mutants, more than double the amount of sky mutants expressing Rab35^{DN} survive, and some animals survive until the adult stage (not shown), while all sky mutants that express Rab5^{DN} succumb much earlier in development and die as first instar larvae.

(D-N) FM 1-43 dye loading and quantification of accumulations per bouton area in controls (FRT40A) (D, E) in sky^{2n} (D, F), in animals that express Rab35^{DN} in their nervous system (rab35^{DN/nSyb-GAL4}) (D, G) in sky^{2n} mutants that express Rab35^{DN} in their nervous system (D, H), in heterozygous rab35 mutants {rab35^{D/+} and rab35^{D/+}} (D, I, K), in sky^{2n} mutants heterozygous for either rab35 allele (D, J, L), in animals heterozygous for rab5 (rab5^{D/+}) (D, M) and in sky^{2n} mutant animals heterozygous for rab5 (D, N). Note that loss of Rab35 function, but not loss of Rab5 function, rescues sky related vesicle trafficking defects. Scale in E for E-N: 5 µm. f-test: ns: not significant, **: p<0.01, error bars: SEM. n ≥ 5 per genotype.

(O) Quantification of SytI accumulations per bouton area in control (nSyb-GAL4/) in Rab35^{DN} expressing animals (rab35^{DN/nSyb-GAL4}), in sky^{2n} and in sky^{2n} animals that are expressing Rab35^{DN} in their nervous system (sky^{2n}; rab35^{DN/nSyb-GAL4}). f-test: ns not significant, *: p<0.05, **: p<0.01, n ≥ 5 (number of animals tested). Error bars: SEM.

(P-Q) Immunolabeling of third instar larval control (FRT40A) NMJs with a-HRP, a presynaptic marker (green), and anti-Rab35 (magenta) (P) and of GFP-sky^{+} animals with anti-GFP (green) and anti-Rab35 (magenta). Rab35 is enriched synaptically similar to Sky. Scale bar in P, for P, Q: 20 µm.

Figure 6: Neurotransmitter release and the readily releasable pool are increased in sky mutants.

(A-B) Sample EJP traces from controls (FRT40A) (black), sky^{lop}, sky^{12}, sky^{2n}, sky^{160} and sky^{2mo} mutants (green) (A) recorded in 0.5 mM external calcium and quantification of the average EJP amplitudes recorded in various external Ca^{2+} concentrations (0.3-1 mM) from controls and sky^{12} mutants (n>5 animals per condition) (B). Quantification of average EJP amplitudes recorded in 0.5 mM calcium of other sky genotypes also shows significantly
increased amplitudes compared to controls (not shown). Error bars: SEM; f-test: *p < 0.05, **p < 0.01.

(C-E) Sample EJC traces recorded in 0.5 mM external Ca\(^{2+}\) in controls (black) and sky\(^{12}\) mutants (green) (C) and average EJC amplitude in control, sky\(^{12}\), sky\(^{2D/1}\), in sky\(^{12}\) mutants harboring a sky\(^{+}\) genomic rescue construct (sky\(^{12}\); sky\(^{+/+}\)), in sky\(^{2D/1}\) mutants with a sky\(^{+}\) rescue construct (sky\(^{1/3}\); sky\(^{+/+}\)), in nSyb-GAL4/+ animals (nSyb>), in sky\(^{2D/1}\) mutants that express Sky in their nervous system using nSyb-GAL4 (nSyb>sky\(^{2D/1}\)) and in sky\(^{2D/1}\) animals that express Sky in their nervous system using UAS-sky (sky\(^{2D/1}\); nSyb>sky) (60 EJCs recorded at 1 Hz per animal) as well as junctional quantal content calculated as the average EJC amplitude (D) divided by the average mEJC amplitude (not shown) for each genotype (average mEJC amplitude for each of the genotypes shown is not statistically different, ANOVA and f-test) (E). Error bars: SEM; n: number of animals tested; f-test: ns: not significant; **p < 0.01.

(F-G) Average EJC amplitude recorded in 0.5 mM calcium relative to controls (nSyb-GAL4/+ in sky\(^{2E/1}\) (green) and in animals with neuronal expression of Rab5\(^{CA}\), Rab23\(^{CA}\) and Rab35\(^{CA}\) (nSyb>rab\(^{CA}\)) (blue) as well as in nSyb-GAL4/+, in animals neuronally expressing Rab35\(^{DN}\) (nSyb>rab\(^{35DN}\)) (red) and in sky\(^{2D/1}\) mutants that express Rab35\(^{DN}\) in neurons (green) (sky\(^{2E/1}\); nSyb>rab\(^{35DN}\) (F) and junctional quantal content (average mEJC amplitude for each of the genotypes shown is not statistically different, ANOVA and f-test) (G). Error bars: SEM; n: number of animals tested; f-test: ns: not significant; *p<0.05, **p < 0.01.

(H-I) Sample EJC traces recorded at 100 Hz in 5 mM external Ca\(^{2+}\) from controls (FRT40A) (black) and sky\(^{12}\) mutants (green) (H) and the cumulative released quantal content versus stimulus number (I). The slope of the trend line at steady state (points 20-40) is a measure of the readily releasable pool refilling rate (dotted lines) and its y-intercept provides a measure of the average RRP size.

(J) Calcium imaging using G-CaMP1.6 neuronally expressed in controls (UAS-G-CaMP1.6/Y; nSyb-GAL4/+ (grey and black) and in sky\(^{12}\) mutants (UAS-G-CaMP1.6/Y; sky\(^{12}\); nSyb-GAL4/+ (green) during a 200 ms (light), 400 ms (medium colored) and a 1 s (dark) 100 Hz stimulation train. Fluorescence changes (delta F) are reported relative to G-CaMP1.6 fluorescence intensity prior to stimulation (Fo). n > 4 animals, differences between genotypes not statistically different (ANOVA).

(K-M) Average charge transfer induced by application of 0.5 M sucrose measured using TEVC in control (FRT40A) and in sky\(^{2D/1}\) mutants during the first 60 s of sucrose application (K) and during the 70\(^{th}\) and the 90\(^{th}\) s of application (L) and sample traces for control (black) and sky\(^{2D/1}\) (green) (M). Error bars: SEM; n: number of animals tested; f-test: *p < 0.05.
Figure 7: Synaptic vesicle proteins are sorted at endosomes in sky mutants.

(A) Average EJC amplitudes recorded in 0.5 mM calcium of control (FRT40A), of sky\(^{12}\) mutants, of animals heterozygous for ESCRT complex components (hrs/+, vps23/+, vps25/+ and vps32/) and of sky\(^{12}\) mutants heterozygous for the ESCRT complex components. Error bars: SEM; n: number of animals tested. f-test: ns: not significant, **p < 0.01.

(B-D) FM 1-43 labeling (C, D) and quantification of accumulations per bouton area (B) for the genotypes indicated in (A). Scale bar in C and D: 5 µm. Error bars: SEM; n: number of animals tested. f-test: *p < 0.05, **p< 0.01. n ≥ 5 per genotype.

(E-L) Anti-HA labeling (magenta) and anti-HRP labeling (green) (G-L) and quantification of boutonic HA labeling intensity relative to the intensity measured in controls expressing nSybHA (E) or to controls expressing Ub-nSybHA (F) in controls neuronally expressing nSybHA (nSyb-GAL4/UAS-nSybHA) (E, G) or Ub-nSybHA (nSyb-GAL4/UAS-Ub-nSybHA) (E, F, J), in sky\(^{12}\) mutants neuronally expressing nSybHA (sky\(^{12}\); nSyb-GAL4/UAS-nSybHA) (E, H) or Ub-nSybHA (sky\(^{12}\); nSyb-GAL4/UAS-Ub-nSybHA) (E, F, K) as well as in heterozygous hrs animals expressing nSybHA (hrs/+; nSyb-GAL4/UAS-nSybHA) (not shown), in heterozygous hrs mutants neuronally expressing Ub-nSybHA (hrs/+; nSyb-GAL4/UAS-Ub-nSybHA) (F) and in sky\(^{12}\) mutants that are heterozygous for hrs and that neuronally express nSybHA (sky\(^{1}\) hrs/sky\(^{2}\); nSyb-GAL4/UAS-nSybHA) (I) or in sky\(^{12}\) mutants that are heterozygous for hrs and that neuronally express Ub-nSybHA (sky\(^{1}\) hrs/sky\(^{2}\); nSyb-GAL4/UAS-Ub-nSybHA) (F, L). Note the reduced Ub-nSybHA levels compared to nSybHA levels in controls that are reduced even further in sky mutants and are partially rescued by removing a copy of hrs. Also note that HA immunoreactivity in sky\(^{12}\), hrs/+ animals expressing nSybHA is not statistically significantly different from that in hrs/+ animals expressing nSybHA (not shown). Error bars: SEM. n ≥ 7 (number of animals tested). f-test: **p <0.01.

(M) Model. Under normal conditions (wild type), Skywalker limits cycling of synaptic vesicles via endosomes (End) by accelerating Rab35 GTPase activity. In sky mutants, Rab35-mediated endosomal trafficking via endosomes is stimulated, allowing efficient ESCRT dependent sorting into multivesicular bodies (MVB) and exchange of synaptic vesicle proteins resulting in degradation of spoiled, ubiquitinated proteins (red) at the lysosome (Lys), thus resulting in a rejuvenated synaptic vesicle protein population (green).

Detailed description of the invention

Synaptic vesicles recycle locally at the synapse and in the present invention we show for the first time that the synapse holds the capacity to regulate the sorting of synaptic vesicle proteins at endosomes. In a Drosophila screen, Skywalker (Sky) was identified as an inhibitor for the trafficking of synaptic vesicles, via endosomes, during neuronal stimulation. Skywalker is a neuronally expressed GTPase activating protein (GAP) which restricts endosomal trafficking of
synaptic vesicles at Drosophila neuromuscular junction boutons, chiefly by controlling Rab35 GTPase activity, in other words skywalker is a synaptic Rab35 GAP. Skywalker mutants harbor a larger readily releasable pool of synaptic vesicles and show a dramatic increase in basal neurotransmitter release.

Accordingly, in one embodiment the present invention relates to a method for enhancing the endosomal trafficking at the synapse and/or for enhancing the release of neurotransmitters comprising the inhibition of SEQ ID NO: 1 or SEQ ID NO: 2. Accordingly, the invention relates to the use of SEQ ID NO: 1 or SEQ ID NO: 2 to enhance the endosomal trafficking at the synapse and/or to enhance the release of neurotransmitters.

SEQ ID NO: 1 depicts the nucleotide sequence of the human TBC1D24 gene. TBC1D24 is the homologue of the *Drosophila* skywalker gene. SEQ ID NO: 2 depicts the amino acid sequence of the human TBC1D24 protein.

The terms "enhancing", "activating", "stimulating" are used interchangeably and mean an up-regulation of endosomal trafficking at the synapse and/or an up-regulation of the release of neurotransmitters. The terms "inhibition", "lowering the activity", "lowering the expression", "decreasing" are used herein interchangeably and mean a down-regulation of the expression of SEQ ID NO: 1 or SEQ ID NO: 2. More specifically, down-regulation in the context of SEQ ID NO: 1 relates the a down-regulation of the gene, the mRNA or any other step in the synthesis of its protein product (i.e. SEQ ID NO: 2).

The terms "protein", "polypeptide", "peptide" are interchangeably used further herein.

The term "compound" is used herein in the context of a "test compound" or a "drug candidate compound" described in connection with the methods of the present invention. As such, these compounds comprise organic or inorganic compounds, derived synthetically or from natural resources. The compounds include polynucleotides, lipids or hormone analogs that are characterized by low molecular weights. Other biopolymeric organic test compounds include small peptides or peptide-like molecules (peptidomimetics) comprising from about 2 to about 40 amino acids and larger polypeptides comprising from about 40 to about 500 amino acids, such as antibodies or antibody conjugates.

Examples of assay methods for identifying compounds in the context of the present invention are described in the Example section, without the purpose of being limitative. It should be clear to the skilled artisan that the present screening methods might be based on a combination or a series of measurements, particularly when establishing the link with neurotransmitter release and/or endosomal trafficking changes. Also, it should be clear that there is no specific order in performing these measurements while practicing the present invention.
For high-throughput purposes, compound libraries may be used. Examples include, but are not limited to, natural compound libraries, allosteric compound libraries, peptide libraries, antibody fragment libraries, synthetic compound libraries, etc.

Determining the enhanced level of neurotransmitters produced can be done by using specific ELISAs using antibodies specifically recognizing the neurotransmitters. An increase in the level of neurotransmitters is preferably by at least 5%, more preferably by at least 10%, and most preferably by at least 25%, 50% or more.

Assays can be performed in eukaryotic cells, advantageously in mammalian cells, such as human cells, preferably of neuronal cells, more preferably in neuron cells. In a particular embodiment appropriate assays can also be performed in prokaryotic cells, reconstituted membranes, and using purified proteins *in vitro*.

Non-limiting examples of neuronal cells which can be used are neuroblastoma cells and PC-12 cells.

Non-limiting examples of neuron cells are primary cortical neurons, primary basal forebrain cholinergic neurons, primary neural stem cells, sensory neurons (e.g. retinal cells, olfactory epithelium cells), motoneurons (e.g. spinal motor neurons, pyramidal neurons, Purkinje cells) and interneurons (e.g. dorsal root ganglia cells).

Polypeptide therapeutics and in particular antibody-based therapeutics have significant potential as drugs because they have exquisite specificity to their target and a low inherent toxicity. In particular, the features of monoclonal antibodies such as high affinity, high selectivity, and distinct structure and function domains amenable to protein engineering for therapeutic delivery, make them potential drug candidates. Given the growing potential for the utilization of monoclonal antibodies as therapeutics, SEQ ID NO: 2-specific monoclonal antibodies can be generated using techniques well-known by the skilled person as these form part of the current state of the art and the effectiveness of these antibodies as enhancers of neurotransmitter release and/or enhancers of synaptic endosomal trafficking can also be determined in the context of the present invention.

Active fragments of the above described antibodies form also part of the invention. The term "active fragment" refers to a portion of an antibody that by itself has high affinity for an antigenic determinant, or epitope, and contains one or more CDRs accounting for such specificity. Non-limiting examples include Fab, F(ab')2, scFv, heavy-light chain dinners, camelid antibodies (also designated as nanobodies), domain antibodies, and single chain structures, such as a complete light chain or complete heavy chain.
The antibodies of the invention, or their active fragments, can be labeled by an appropriate label, said label can for instance be of the enzymatic, colorimetric, chemiluminescent, fluorescent, or radioactive type.

It is known by the skilled person that an antibody which has been obtained for a therapeutically useful target requires additional modification in order to prepare it for human therapy, so as to avoid an unwanted immunological reaction in a human individual upon administration. The modification process is commonly termed "humanization". It is known by the skilled artisan that antibodies raised in species, other than in humans, require humanization to render the antibody therapeutically useful in humans (1) CDR grafting: Protein Design Labs: US61 80370, US5693761; Genentech US6054297; Celltech: EP626390, US5859205; (2) Veneering: Xoma: US5869619, US5766886, US5821 123). Humanisation of antibodies entails recombinant DNA technology, and is departing from parts of rodent and/or human genomic DNA sequences coding for H and L chains or from cDNA clones coding for H and L chains. Techniques for humanization of non-human antibodies are known to the skilled person as these form part of the current state of the art. Non-human mammalian antibodies or animal antibodies can be humanized (see for instance Winter and Harris 1993). The antibodies or monoclonal antibodies according to the invention may be humanized versions of for instance rodent antibodies or rodent monoclonal antibodies.

In a further aspect, the invention also relates to the use of SEQ ID NO: 1 or SEQ ID NO: 2 to diagnose or prognose neurological diseases. The use as meant here is any use of the nucleic acid or protein, and may be, as a non-limiting example, the genomic DNA, for the detection of mutation, the mRNA or derived cDNA for the analysis of the expression, or the protein, for the analysis of translated protein. Methods for mutation and SNP analysis, expression analysis and detection and quantification of protein (e.g. via antibodies recognizing the protein) are known to the person skilled in the art.

Examples of neurological diseases in the context of the present invention are Alzheimer's disease, Parkinson's disease, Frontal temporal lobe dementia and schizophrenia. In a particular embodiment the agents of the invention characterized in that they enhance synaptic endosomal trafficking and/or enhanced neurotransmitter release, are selected from the list of a small interfering RNA (siRNA) or an artificial microRNA or an antisense polynucleotide or a ribozyme, wherein the latter agents comprises a nucleic acid sequence engineered from SEQ ID NO: 1, and an antibody with a specificity for SEQ ID NO: 2, are used for the treatment of synaptic dysfunction diseases. Particularly preferred examples of synaptic dysfunction diseases are Alzheimer's disease and Parkinson's disease.
In still another embodiment, the present invention relates to an agent such as an antisense polynucleotide, a ribozyme, or a small interfering RNA (siRNA) or an artificial microRNA, characterized in that it enhances synaptic endosomal trafficking and/or enhancer neurotransmitter release, and wherein said agent comprises a nucleic acid sequence complementary to, or engineered from SEQ ID NO: 1.

The terms "polynucleotide", "polynucleic acid", "nucleic acid" are interchangeably used further herein.

The term "siRNA" refers to a small interfering RNA(s), which also has been referred to in the art as short interfering RNA and silencing RNA, among others. siRNAs generally are described as relatively short, often 20-25 nucleotide-long, double-stranded RNA molecules that are involved in RNA interference (RNAi) pathway(s). Generally, siRNAs are, in part, complementary to specific mRNAs and mediate their down regulation (hence, "interfering"). siRNAs thus can be used for down regulating the expression of specific genes and gene function in cells and organisms. siRNAs also play a role in related pathways. The general structure of most naturally occurring siRNAs is well established. Generally, siRNAs are short double-stranded RNAs, usually 21 nucleotides long, with two nucleotides single stranded "overhangs" on the 3′ of each strand. Each strand has a 5′ phosphate group and a 3′ hydroxyl (-OH) group. In vivo, the structure results from processing by the enzyme "dicer," which enzymatically converts relatively long dsRNAs and relatively small hairpin RNAs into siRNAs.

The term siNA refers to a nucleic acid that acts like a siRNA, as described herein, but may be other than an RNA, such as a DNA, a hybrid RNA:DNA or the like. siNAs function like siRNAs to down regulate expression of gene products.

The term "RNA interference" which also has been called "RNA mediated interference" refers to the cellular processes by which RNA (such as siRNAs) down regulate expression of genes; i.e., down regulate or extinguish the expression of gene functions, such as the synthesis of a protein encoded by a gene. Typically, double-stranded ribonucleic acid inhibits the expression of genes with complementary nucleotide sequences. RNA interference pathways are conserved in most eukaryotic organisms. It is initiated by the enzyme dicer, which cleaves RNA, particularly double-stranded RNA, into short double-stranded fragments 20-25 base pairs long. One strand of the double-stranded RNA (called the "guide strand") is part of a complex of proteins called the RNA-induced silencing complex (RISC). The thus incorporated guide strand serves as a recognition sequence for binding of the RISC to nucleic acids with complementary sequences. Binding by RISC to complementary nucleic acids results in their being "silenced." The best studied silencing is the binding of RISCs to RNAs resulting in post-transcriptional gene silencing. Regardless of mechanism, interfering nucleic acids and RNA
interference result in down regulation of the target gene or genes that are complementary (in pertinent part) to the guide strand.

A polynucleotide can be delivered to a cell to express an exogenous nucleotide sequence, to inhibit, eliminate, augment, or alter expression of an endogenous nucleotide sequence, or to affect a specific physiological characteristic not naturally associated with the cell. The polynucleotide can be a sequence whose presence or expression in a cell alters the expression or function of cellular genes or RNA. A delivered polynucleotide can stay within the cytoplasm or nucleus apart from the endogenous genetic material. Alternatively, DNA can recombine with (become a part of) the endogenous genetic material. Recombination can cause DNA to be inserted into chromosomal DNA by either homologous or non-homologous recombination.

A polynucleotide-based gene expression inhibitor comprises any polynucleotide containing a sequence whose presence or expression in a cell causes the degradation of or inhibits the function, transcription, or translation of a gene in a sequence-specific manner. Polynucleotide-based expression inhibitors may be selected from the group comprising: siRNA, microRNA, interfering RNA or RNAi, dsRNA, ribozymes, antisense polynucleotides, and DNA expression cassettes encoding siRNA, microRNA, dsRNA, ribozymes or antisense nucleic acids. SiRNA comprises a double stranded structure typically containing 15 to 50 base pairs and preferably 19 to 25 base pairs and having a nucleotide sequence identical or nearly identical to an expressed target gene or RNA within the cell. An siRNA may be composed of two annealed polynucleotides or a single polynucleotide that forms a hairpin structure. MicroRNAs (miRNAs) are small noncoding polynucleotides, about 22 nucleotides long, that direct destruction or translational repression of their mRNA targets. Antisense polynucleotides comprise a sequence that is complimentary to a gene or mRNA. Antisense polynucleotides include, but are not limited to: morpholinos, 2'-O-methyl polynucleotides, DNA, RNA and the like. The polynucleotide-based expression inhibitor may be polymerized in vitro, recombinant, contain chimeric sequences, or derivatives of these groups. The polynucleotide-based expression inhibitor may contain ribonucleotides, deoxyribonucleotides, synthetic nucleotides, or any suitable combination such that the target RNA and/or gene is inhibited.

Polynucleotides may contain an expression cassette coded to express a whole or partial protein, or RNA. An expression cassette refers to a natural or recombinantly produced polynucleotide that is capable of expressing a sequence. The cassette contains the coding region of the gene of interest along with any other sequences that affect expression of the sequence of interest. An expression cassette typically includes a promoter (allowing transcription initiation), and a transcribed sequence. Optionally, the expression cassette may include, but is not limited to, transcriptional enhancers, non-coding sequences, splicing signals,
transcription termination signals, and polyadenylation signals. An RNA expression cassette typically includes a translation initiation codon (allowing translation initiation), and a sequence encoding one or more proteins. Optionally, the expression cassette may include, but is not limited to, translation termination signals, a polyadenosine sequence, internal ribosome entry sites (IRES), and non-coding sequences. The polynucleotide may contain sequences that do not serve a specific function in the target cell but are used in the generation of the polynucleotide. Such sequences include, but are not limited to, sequences required for replication or selection of the polynucleotide in a host organism.

In a particular embodiment, the agents of the present invention encompass short interfering RNA (siRNA) molecules that down regulate expression of SEQ ID NO: 1 mRNA by RNA interference. The siRNA molecules of the present invention encompass SEQ ID NO: 1 siRNAs, which are useful for research to analyse the function of SEQ ID NO: 1, and which may be used for therapy in humans, e.g. in the prevention and/or treatment of a neurological disease, in particular Alzheimer’s disease and Parkinson’s disease.

Based on the RNA sequence of SEQ ID NO: 1, siRNA molecules with the ability to knock-down TBC1D24 activity, can be obtained by chemical synthesis or by hairpin siRNA expression vectors. There are numerous companies that provide the supply of costumer-designed siRNAs on a given RNA sequence, e.g. Ambion, Imgenex, Dharmacon.

The TBC1D24 siRNAs of the invention may be chemically modified, e.g. as described in US20030143732, by phosphorothioate internucleotide linkages, 2'-0-methyl ribonucleotides, 2'-deoxy-2'fluoro ribonucleotides, "universal base" nucleotides, 5-C-methyl nucleotides, and inverted deoxyabasic residue incorporation. The sense strand of TBC1D24 siRNAs may also be conjugated to small molecules or peptides, such as membrane-permeant peptides or polyethylene glycol (PEG). Other siRNA conjugates which form part of the present invention include cholesterol and alternative lipid-like molecules, such as fatty acids or bile-salt derivatives.

In a further embodiment, the present invention relates to an expression vector comprising any of the above described polynucleotide sequences encoding at least one TBC1D24 siRNA molecule in a manner that allows expression of the nucleic acid molecule, and cells containing such vector. The polynucleic acid sequence is operably linked to regulatory signals (promoters, enhancers, suppressors etc.) enabling expression of the polynucleic acid sequence and is introduced into a cell utilizing, preferably, recombinant vector constructs. A variety of viral-based systems are available, including adenoviral, retroviral, adeno-associated viral, lentiviral, herpes simplex viral vector systems. Selection of the appropriate viral vector system,
regulatory regions and host cell is common knowledge within the level of ordinary skill in the art.

As gene delivery and gene silencing techniques improve, the selective deletion of TBC1 D24 in particular tissues or cellular populations may prove useful in order to limit the impact of protein deletion to a particular system under study. The TBC1 D24 siRNA molecules of the invention may be delivered by known gene delivery methods, e.g. as described in US20030143732, including the use of naked siRNA, synthetic nanoparticles composed of cationic lipid formulations, liposome formulations including pH sensitive liposomes and immunoliposomes, or bioconjugates including siRNAs conjugated to fusogenic peptides. Delivery of siRNA expressing vectors can be systemic, such as by intravenous or intramuscular administration or by any other means that would allow for introduction into the desired target cell (see US 20030143732).

In still another aspect, the invention relates to a pharmaceutical composition comprising a therapeutically effective amount of any of the above described agents and at least one of a pharmaceutically acceptable carrier, adjuvant or diluents. Any of the above agents or said pharmaceutical composition can be used for the manufacture of a medicament to prevent and/or treat neurological disorders described herein. One of ordinary skill in the art will recognize that the potency and, therefore, an "effective amount" can vary for the inhibitory agents of the present invention. One skilled in the art can readily assess the potency of the inhibitory agent.

A medicament to prevent and/or to treat a neurological disorder, in particular Alzheimer's disease or Parkinson's disease, relates to a composition comprising agents as described above and a pharmaceutically acceptable carrier or excipient (both terms can be used interchangeably) to treat or to prevent neurological diseases as described herein.

The administration of pharmaceutical compositions may be by way of oral, inhaled or parenteral administration. In particular, pharmaceutical compositions can be delivered through intrathecal or intracerebroventricular administration. The active ingredient may be administered alone or preferably formulated as a pharmaceutical composition. An amount effective to treat a neurological disease depends on the usual factors such as the nature and severity of the disorder being treated and the weight of the mammal. It is greatly preferred that the pharmaceutical composition is administered in the form of a unit-dose composition, such as a unit dose oral, parenteral, or inhaled composition. Such compositions are prepared by admixture and are suitably adapted for oral, inhaled or parenteral administration, and as such may be in the form of tablets, capsules, oral liquid preparations, powders, granules, lozenges, reconstitutable powders, injectable and infusible solutions or suspensions or suppositories or
aerosols. Tablets and capsules for oral administration are usually presented in a unit dose, and contain conventional excipients such as binding agents, fillers, diluents, tableting agents, lubricants, disintegrants, colourants, flavourings, and wetting agents. The tablets may be coated according to well-known methods in the art. Suitable fillers for use include cellulose, mannitol, lactose and other similar agents. Suitable disintegrants include starch, polyvinylpyrrolidone and starch derivatives such as sodium starch glycollate. Suitable lubricants include, for example, magnesium stearate. Suitable pharmaceutically acceptable wetting agents include sodium lauryl sulphate. These solid oral compositions may be prepared by conventional methods of blending, filling, tableting or the like. Repeated blending operations may be used to distribute the active agent throughout those compositions employing large quantities of fillers. Such operations are, of course, conventional in the art. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups, or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, for example sorbitol, syrup, methyl cellulose, gelatin, hydroxyethylcellulose, carboxymethyl cellulose, aluminium stearate gel or hydrogenated edible fats, emulsifying agents, for example lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (which may include edible oils), for example, almond oil, fractionated coconut oil, oily esters such as esters of glycerine, propylene glycol, or ethyl alcohol; preservatives, for example methyl or propyl p-hydroxybenzoate or sorbic acid, and if desired conventional flavouring or colouring agents. Oral formulations also include conventional sustained release formulations, such as tablets or granules having an enteric coating. Preferably, compositions for inhalation are presented for administration to the respiratory tract as a snuff or an aerosol or solution for a nebulizer, or as a microfine powder for insufflation, alone or in combination with an inert carrier such as lactose. In such a case the particles of active compound suitably have diameters of less than 50 microns, preferably less than 10 microns, for example between 1 and 5 microns, such as between 2 and 5 microns. For parenteral administration, fluid unit dose forms are prepared containing a compound of the present invention and a sterile vehicle. The active compound, depending on the vehicle and the concentration, can be either suspended or dissolved. Parenteral solutions are normally prepared by dissolving the compound in a vehicle and filter sterilising before filling into a suitable vial or ampoule and sealing. Advantageously, adjuvants such as a local anaesthetic, preservatives and buffering agents are also dissolved in the vehicle. To enhance the stability, the composition can be frozen after filling into the vial and the water removed under vacuum. Parenteral suspensions are prepared in substantially the same manner except that the compound is suspended in the vehicle instead of being dissolved and sterilised by exposure to ethylene oxide before suspending in the sterile vehicle. Advantageously, a surfactant or wetting agent is included in
the composition to facilitate uniform distribution of the active compound. Where appropriate, small amounts of bronchodilators for example sympathomimetic amines such as isoprenaline, isoetharine, salbutamol, phenylephrine and ephedrine; xanthine derivatives such as theophylline and aminophylline and corticosteroids such as prednisolone and adrenal stimulants such as ACTH may be included. As is common practice, the compositions will usually be accompanied by written or printed directions for use in the medical treatment concerned.

The terms "therapeutically effective amount", "therapeutically effective dose" and "effective amount" mean the amount needed to achieve the desired result or results (modulating TBC1D24 activity; treating or preventing a neurological disease).

"Pharmaceutically acceptable" means a material that is not biologically or otherwise undesirable, i.e., the material may be administered to an individual along with the compound without causing any undesirable biological effects or interacting in a deleterious manner with any of the other components of the pharmaceutical composition in which it is contained.

A "carrier", or "adjuvant", in particular a "pharmaceutically acceptable carrier" or "pharmaceutically acceptable adjuvant" is any suitable excipient, diluent, carrier and/or adjuvant which, by themselves, do not induce the production of antibodies harmful to the individual receiving the composition nor do they elicit protection. Preferably, a pharmaceutically acceptable carrier or adjuvant enhances the immune response elicited by an antigen. Suitable carriers or adjuvants typically comprise one or more of the compounds included in the following non-exhaustive list: large slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers and inactive virus particles.

A "diluent", in particular a "pharmaceutically acceptable vehicle", includes vehicles such as water, saline, physiological salt solutions, glycerol, ethanol, etc. Auxiliary substances such as wetting or emulsifying agents, pH buffering substances, preservatives may be included in such vehicles.

It should be clear that the inhibitory agents of the present invention for a particular neurological disease (e.g. Alzheimer's disease) can also be used in combination with any other neurological disease (e.g. AD disease) therapy known in the art.

In yet another particular embodiment the invention provides for method for identifying compounds that enhance synaptic endosomal trafficking and/or neurotransmitter release in a neuron or a neuronal cell comprising the steps of:

a) providing a neuron or a neuronal cell culture expressing a polypeptide encoded by SEQ ID NO: 1;
b) administering a test compound and a dye with a specificity for endosomal vessels (e.g. a lipophilic dye) to said cell culture;

c) stimulating endosomal vessel trafficking;

d) imaging the labeled endosomal vessels of the cells in said cell culture;

wherein, under the same test conditions, a deviation in said imaging compared to the same imaging of cells of a corresponding cell culture without said test compound, identifies said test compound as a compound that enhances synaptic endosomal trafficking and/or neurotransmitter release in a neuronal cell.

In other embodiments the assay for the selection of compounds which are able to reduce the activity of skywalker (or the human TBC1D24 orthologue) hinges on the possibility to inhibit the enzymatic function of skywalker, i.e. the GAP-activity (i.e. GTPase Activating Protein) of skywalker (or TBC1D24). The present invention shows that Rab35 is a natural substrate of skywalker. During our experimental work we also showed that skywalker efficiently activates the GTPase activity of the ARF6 protein, in particular the GTP-loaded form of ARF6. ARF6 is a member of the human ARF gene family (which is part of the RAS super-family). The ARF genes encode small guanine nucleotide-binding proteins that stimulate the ADP-ribosyltransferase activity of cholera toxin and play a role in vesicular trafficking and as activators of phospholipase D. The protein sequence of the human ARF6 protein is depicted in SEQ ID NO: 4.

In a particular embodiment said stimulation of the endosomal trafficking is an electrical stimulation. In another particular embodiment said stimulation is carried out by the addition of a salt (e.g. potassium chloride) to the neuronal or neuron cell culture.

In yet another embodiment the invention provides for a method for identifying compounds that enhance synaptic endosomal trafficking and/or neurotransmitter release in a neuronal cell or a neuron comprising the steps of:

e) providing a purified SEQ ID NO: 2 polypeptide and a GTP-loaded Rab35 protein (SEQ ID NO: 3) and/or a purified SEQ ID NO: 2 polypeptide and a GTP-loaded ARF6 protein (SEQ ID NO: 4);

f) administering a test compound;

10) measuring the inorganic phosphate production in a coupled enzymatic assay;

h) wherein, a decrease in said inorganic phosphate production compared to a condition wherein no test compound was administered, identifies said test compound as a compound that enhances synaptic endosomal trafficking and/or enhanced neurotransmitter release when administered to a neuron or a neuronal cell.

In a particular embodiment the methods for identifying compounds provide a compound which is a therapeutic candidate for the prevention and/or treatment of neurological diseases.
The amino acid sequence of the Rab35 protein is depicted in SEQ ID NO: 3.

The monitoring of the enhancement of synaptic endosomal trafficking is a morphological parameter and can be monitored as described herein further in the examples section. Differences in morphological phenotype between neuronal or neuron cell cultures in the presence of test compounds can be screened in a high-throughput imaging setup (e.g. InCell 2000, GE Healthcare; see also Example 4).

In a preferred embodiment, a compound will "enhance" or "stimulate" the activity of the endocytic pathway. Said stimulation is preferably by at least 5%, more preferably by at least 10%, and most preferably by at least 25%, 50% or more. Assays and methods for measuring the activity of the endocytic trafficking pathway are known in the art.

The following examples are intended to promote a further understanding of the present invention. While the present invention is described herein with reference to illustrated embodiments, it should be understood that the invention is not limited hereto. Those having ordinary skill in the art and access to the teachings herein will recognize additional modifications and embodiments within the scope thereof. Therefore, the present invention is limited only by the claims attached herein.

Examples

1. Skywalker encodes a putative Rab GTPase Activating Protein

In a forward genetic screen for defects in synaptic transmission in Drosophila melanogaster we isolated two EMS-induced mutants on the left arm of the second chromosome that fail to complement each other (Verstreken et al., 2005; Verstreken et al., 2009). Homozygous sky^1 mutants are embryonic lethal, while homozygous sky^2 or heteroallelic sk^+/sky^0 (sky^0^d^0^) mutant third instar larvae survive, but in contrast to control larvae, they show a particular phenotype: they paralyze and erect themselves in the medium (Figure 1A). Based on this observation we name this complementation group skywalker (sky). When homozygous only in the eye and heterozygous in the body (Newsome et al., 2000), flies carrying the sky mutations survive to adulthood, but they show defects in electroretinogram recordings (ERGs) (Figure 1B). ERGs measure differences in extracellular potential between the photoreceptors and the thorax during a short light flash. While the main depolarization in an ERG is caused by activation of the phototransduction cascade in the photoreceptors, the 'on' and 'off' transients at the start and completion of the light pulse (arrowheads in Figure 1B) arise in the postsynaptic neurons (Heisenberg, 1971). Given that depolarization in response to light is normal in sky mutant eyes, yet 'on' and 'off' transients are dramatically reduced compared to
controls, our data suggest that sky mutations disrupt the normal neuronal communication in the fly eye. To map the gene encoding sky, we used meiotic recombination mapping with P elements (Zhai et al., 2003) as well as complementation tests with large deficiencies (Sinclair, 1983; Wright et al., 1976). These assays allowed us to map the mutants independently to the cytological interval, 38F. Next, we used complementation tests with small deficiencies whose breakpoints are molecularly defined (Parks et al., 2004; Ryder et al., 2004). Sky mutants fail to complement two small deletions Df(2L)Exel7079 and Df(2L)Exel7080 that overlap and together uncover only 5 genes. Using additional complementation tests with lethal alleles in this region we identified 7 P elements that fail to complement one another as well as both sky alleles (Figure 1D). All P elements are inserted in CG9339, suggesting sky is CG9339. We therefore sequenced CG9339 in the sky EMS alleles and used quantitative real-time PCR to determine the RNA expression levels of CG9339 in the EMS and P element alleles. Sequencing revealed a C to T nucleotide transition in sky\textsuperscript{1} that would result in the incorporation of a bulky arginine instead of a glycine at position 543 in the Sky protein (Figure F). We did not find lesions in the open reading frame of sky\textsuperscript{2}. However, we also performed quantitative real-time PCR on third instar larvae of different sky allelic combinations. As shown in Figure 1C, we observed a dramatic loss of SKY RNA expression in all sky mutants tested as well as a concomitant loss of Sky protein expression (see below). Given that SKY RNA levels in sky\textsuperscript{1} and sky\textsuperscript{2O} are very similar, the data show that sky\textsuperscript{1} leads to a near complete loss of sky expression, representing a severe loss of function or null allele. In addition, the data also show that expression levels in sky\textsuperscript{003436} (sky\textsuperscript{d0}), a P-element insertion in the locus, are severely reduced and similar to those measured in the sky\textsuperscript{2} allele. Hence, sky mutants affect the expression of the CG9339 gene. To determine if the phenotypes we observe in sky mutants are caused solely by loss of CG9339 expression we used the UAS/GAL4 system as well as a genomic fragment that we generated using recombineering (Venken et al., 2006). First, we took advantage of the UAS sites present in the P element in sky\textsuperscript{d0} that allows expressing sky using GAL4 (Actin-Gal4). Unlike sky\textsuperscript{2O} animals, sky\textsuperscript{d0} animals that express GAL4 easily survive, all inflate their wings and almost all (90 %) can fly (data not shown). We also generated flies with a UAS-sky cDNA expression construct. Expression of wild type Sky in sky mutants (using nSyb-Gal4) circumvents lethality (nSyb>sky, Figure 1D) and these flies can fly. Similarly, addition of a genomic fragment, that allows to express CG9339 (sky\textsuperscript{+}) endogenously, to sky mutants rescues their lethality (Figure 1D), ERG defects (Figure 1B) as well as the cellular phenotypes we recorded in these mutants (see below). Thus, the sky mutants we isolated are loss-of-function alleles that specifically affect CG9339. Our analyses led to the identification of 9 sky alleles that fail to complement. To determine severity of the mutants, we measured the lethal phase of different allelic sky combinations grown under optimal conditions (Figure 1D). Based on the lethal phase of homozygous animals and that of the alleles in trans over a deficiency, our data indicate that
the P elements f06707 and e01084 as well as sky\(^1\) are null alleles or severe hypomorphic alleles, while sky\(^2\) and the other P element alleles are less severe hypomorphic alleles (Figure 1D). These data are consistent with the expression levels of SKY RNA we measured using quantitative real-time PCR (Figure 1C) and we therefore suggest the following allelic series: 

\[
sky^1 = sky^{06707} = sky^{e01084} > sky^2 = sky^{v0} = sky^{h0} = sky^{62146} = sky^{l2487} = sky^{10707}.\]

Sky encodes a protein with a predicted size of 65-68 kDa, depending on the isoform (www.flybase.org). All isoforms harbor two conserved domains: a TBC domain and a TLDc domain, commonly found in GAPs (Bernards and Settleman, 2004; Doerks et al., 2002). BLAST searches reveal a well conserved Sky homologue in human: KIAA1171 (TBC1D24) that is 45% similar at the amino acid level compared to Sky (Figure 1E). Although Sky orthologues have not been studied in vivo or in vitro, the C. elegans homologue C31H2.1 was identified in a systematic RNAi screen for genes implicated in synaptic function (Sieburth et al., 2005). These observations underscore a possible function for this putative GAP in vesicle trafficking and neuronal communication.

2. Sky is enriched synaptically and required in the nervous system

To study the protein expression pattern and subcellular localization of Sky we created a genomic EGFP-tagged construct allowing us to express GFP-Sky under endogenous control (Venken et al., 2008). Determining the expression pattern of GFP-Sky, our data indicate the protein is enriched at synaptic areas in the ventral nerve cord (VNC) of third instar larvae and strongly overlaps with the neuropile marker DLG (Figure 1F) (Mendoza et al., 2003). We also find abundant labeling at neuromuscular junction (NMJ) boutons (Figure 1I-N) and we scanned single confocal sections of double labeled boutons with pre- and postsynaptic markers (anti-DLG, a pre- and postsynaptic marker, anti-Dynamin and anti-nSyb, both pre-synaptic markers). As shown in Figure 1I-N, our data indicate Sky is present both pre- and postsynaptically at the NMJ. To further scrutinize these results we also raised a polyclonal antibody against bacterially expressed full length HIS-Sky protein. Consistent with GFP-Sky localization, labeling with Sky antibodies shows an enrichment of immunoreactivity in the neuropile of the larval brain (Figure 1G) and this staining is strongly reduced in sky hypomorphic mutants (more than 2.1-fold lower than in controls), indicating the labeling is specific (Figure 1G-H). Furthermore, similar to GFP-Sky, we also observe an enrichment of Sky immunoreactivity at the pre- and postsynaptic sides of NMJ boutons. In conclusion, our data indicate Sky is widely expressed and is abundantly present in the nervous system at synapses, including at presynaptic boutons, in line with a role in neuronal communication. To determine if Sky is required in the nervous system, we expressed Sky in neurons of sky mutants. Interestingly, while sky\(^{l2487}\) hypomorphic mutant animals die, sky\(^{h0}\) animals that express GAL4 under control of a neuron specific promoter (nSyb-GAL4) survive and all inflate
their wings. In contrast, when Sky is expressed only in muscles of sky<sup>10</sup> animals using BG57-GAL4, the lethality associated with sky mutations is not rescued. Also more severe sky mutants (sky<sup>10</sup>) that express Sky in the nervous system using UAS-sky under control of nSyb-GAL4, survive to adulthood (Figure 1D). Such animals can fly similar to control flies and do not show the cellular phenotypes we recorded in sky mutants (see below). These data corroborate our labelling experiments and indicate a critical role for Sky in the nervous system and at the presynaptic side of the NMJ.

3. Sky mediates vesicle recycling in neurons

To test for functional defects in sky motor neurons we used the lipophilic dye FM 1-43 at third instar larval NMJs (Betz and Bewick, 1992; Ramaswami et al., 1994; Verstreken et al., 2008). FM 1-43 binds the membrane, and upon nerve stimulation of control synapses, newly formed vesicles are fluorescently labeled by the dye (Figure 2A). Similar to controls, we also find abundant fluorescent labeling of synapses following stimulation in the different sky mutants tested, indicating membrane internalization per se is not impaired (Figure 2A, B). However, while in controls the vesicle pool marked by FM 1-43 organizes in a typical doughnut-like pattern, the dye internalized in sky mutants consistently concentrates in sub-boutonic structures (Figure 2A, C). Interestingly, FM 1-43 dye can be released during a second stimulation round, indicating that in sky mutants, synaptic membrane can cycle to and from these sub-boutonic structures (Figure 2B). The dye mislocalization we observe in mutants is specific to sky mutations, as expression of Sky in neurons of sky mutants, or addition of a genomic sky<sup>+</sup> rescue construct to sky mutants results in an FM 1-43 labeling pattern indistinguishable from controls (Figure 2A, C). Furthermore, the trafficking defects are not caused by major morphological changes at the NMJ synapse of sky mutants, as evaluation of NMJ size, bouton number and active zone density visualized using immunohistochemistry does not reveal differences from controls. Hence, our data show that Sky regulates vesicle traffic, preventing the internalization of newly internalized membrane to sub-boutonic structures in a cell autonomous manner. To further explore the presynaptic organellar composition in sky mutants we performed transmission electron microscopy (TEM) at third instar NMJ boutons.

We stimulated control and sky mutant preparations for 1 min using 90 mM KCl and prepared the samples for TEM (Figure 2D, E). Several ultrastructural features, including the number of mitochondria, lysosomes and active zone (T-bar) number per synaptic area are not different between controls and sky mutants (Figure 2G-I). However, our data indicate a dramatic accumulation of large cisternal-like structures and a concomitant decrease in small synaptic vesicles in mutants when compared to controls (Figure 2D, E and J-L), in line with a defect in membrane transport. To directly determine if these large cisternal-like structures take part in the synaptic vesicle cycle, we tested if FM 1-43 accumulates in the cisternal-like structures...
observed by TEM using diaminobenzidine (DAB) photoconversion. We labeled sky mutant synapses using 1 min of 90 mM KCl stimulation in the presence of FM 1-43 and then used the fluorescence of this dye to convert DAB into an electron dense precipitate visible by TEM (Akbergenova and Bykhovskaia, 2009; Denker et al., 2009; Harata et al., 2001; Schikorski and Stevens, 2001; Vijayakrishnan et al., 2009). As shown in Figure 2F, in sky mutants several small synaptic vesicles contain DAB precipitates (small arrowheads). In addition, numerous cisternal membranes are also labeled, indicating they participate in the vesicle cycle (large arrowheads). These data further suggest that in sky mutants synaptic membranes cycle via cisternal-endosomal-like structures.

4. sky mutations do not induce bulk endocytosis.

Next, we tested if these cisternal/endosomal-like structures in stimulated sky mutants form by bulk membrane retrieval. First, we assessed FM 1-43 labeling in sky mutants and controls upon 20 min of 3 Hz stimulation. Although such a low frequency stimulation protocol does not induce bulk membrane retrieval in controls (Clayton and Cousin, 2008; Wu and Wu, 2007), FM 1-43 dye internalized in sky mutants still distributes in sub-boutonic structures, suggesting they do not form as a result of bulk retrieval (Figure 2M, N). Second, we also used 10 kDa Dextran-Rhodamine that, due to its large size, specifically labels membranes internalized by bulk endocytosis (Araki et al., 1996; Berthiaume et al., 1995; Clayton and Cousin, 2009; Holt et al., 2003; Teng et al., 2007). We used control and sky mutant preparations and also prepared samples treated with chlorpromazine that induces the internalization of large membrane patches (Kasprowicz et al., 2008). Prior to stimulation we incubated larval preparations in 1 mM collagenase for 30 s, a treatment commonly used to remove a protective sheet around the muscles when preparing *Drosophila* embryos for electrophysiology (Broadie et al., 1994; Chen et al., 2009). We used it here to allow the 10 kDa Dextran-Rhodamine to gain access to the synapses. We then stimulated the animals in 90 mM KCl in the presence of 10 kDa Dextran-Rhodamine and following washing in calcium free HL-3, we monitored uptake of the probe. Compared to chlorpromazine treated animals, we found much less labeling in controls as well as in sky mutants (Figure 20, P), arguing against increased bulk retrieval in sky mutants. If bulk retrieval is the major pathway of synaptic membrane endocytosis in sky mutants, we expect to observe an increased number of large diameter (> 80 nm) membrane-attached cisternae in the process of internalization; so-called ‘omega structures’. We quantified the number of small (<80 nm) and large diameter (>80 nm) omega structures in sky mutant samples fixed immediately following stimulation but did not find a difference in the number of these structures per boutonic perimeter in comparison to stimulated controls (Figure 2Q, R). We also further analyzed the synaptic membrane of stimulated sky mutants and controls in three dimensions using electron tomography on 200-300 nm thick sections. Taken together,
our results indicate that cisternal-endosomal structures in sky mutants are not created by bulk membrane endocytosis but form following endocytosis of regularly sized small synaptic vesicles.

5. Synaptic membrane trafficking in sky mutants

In several endocytic mutants in Drosophila, cisternae filled with neurotransmitter accumulate at synapses, and their spontaneous fusion with the membrane elicits larger than normal miniature excitatory junctional current (mEJC) amplitudes (Fergestad et al., 1999; Koh et al., 2004; Marie et al., 2004; Verstreken et al., 2009; Zhang et al., 1998). A difference in mEJC amplitudes may be caused by the fusion of larger vesicles that contain more neurotransmitter or by a larger postsynaptic glutamate receptor field. To determine if the cisternal-like structures in sky mutants can fuse directly with the synaptic membrane, we performed two-electrode voltage clamp (TEVC) and recorded mEJCs both from synapses at rest as well as from synapses that were stimulated for 1 min with 90 mM KCl. As shown in Figure 3A, mEJC amplitudes recorded from synapses at rest are similar in sky mutants and controls. While KCl stimulation is known to result in larger mEJC amplitudes likely as a result of some cisternae that may form (Akbergenova and Bykhovskaia, 2009), the distribution of mEJC amplitudes between KCl stimulated sky and controls does not show a difference (Figure 3B). In addition, labeling of glutamate receptor clusters using anti-GluRIIC/III antibodies (Marrus et al., 2004), shows very similarly sized postsynaptic glutamate receptor fields (Figure S2C, D). Given that neurotransmitters are thought to be efficiently loaded into synaptic vesicles (Daniels et al., 2006), the data suggest that transmitters in sky mutants are only released from vesicles comparable in size to those found in control boutons; however a defect in transmitter loading cannot be formally excluded. We reasoned that if synaptic vesicles travel via sub-boutonic endosomal-like structures in sky mutants, such organelles may form transiently upon stimulation. We therefore performed TEM on unstimulated control and sky mutant boutons as well as on boutons left to recover for 1 h following a 1 min 90 mM KCl stimulation protocol (Figure 3C-H). We quantified several features in these synapses as well as in synapses that were fixed immediately after 1 min 90 mM KCl stimulation, including frequency of vesicle size, frequency of vesicle size at the active zones, density of vesicles with a diameter larger than 80 nm and density of vesicles with a diameter smaller than 80 nm (Figure 3I-3P). While unstimulated control and sky mutant synapses show very similar vesicle size distributions (Figure 3I, L) and number (Figure 30, P), the most obvious feature we observe in stimulated sky mutants is the accumulation of endosomal-like membranes (Figure 3J, M, O) at the expense of small synaptic vesicles (Figure 3P) and these endosomal-like profiles dissipate upon rest (Figure 3K, N, O). While we find endosomal-like structures throughout sky mutant boutons (Figure 3G and Figure 2E, F and T), they do not accrue to a larger extent at active
zones compared to controls (Figure 3L-N). These data suggest that unlike synaptic vesicles, endosomal-like structures in sky mutants may not directly participate in neurotransmitter release, in line with our observation that mEJC amplitudes are not increased in sky mutants (Figure 3A, B). The transient nature of the endosomal-like structures in sky mutants suggests synaptic vesicles may form or fuse at these sites. We therefore carefully examined cisternae in stimulated sky mutant boutons using both regular TEM as well as using electron tomography and we find numerous endosomal-like membranes with small synaptic vesicle profiles attached that appear to be either fusing or leaving (arrowheads, Figure 3Q-S). Taken together, our data suggest that endosomal-like structures in sky mutants serve as intermediate stations that form as a result of neuronal stimulation.

6. Synaptic vesicles cycle through an endosomal-like compartment in sky mutants
To characterize the molecular nature of the sub-boutonic structures in sky mutants, we labeled mutant and control boutons with FM 1-43 while simultaneously expressing endosomal markers in neurons. We used 2xFYVE-GFP or Rab5-GFP, both markers of sorting endosomes, as well as Rab4-GFP, a marker of recycling endosomes (Bokel et al., 2006; Dyer et al., 2007; Gillooly et al., 2000; Simonsen et al., 1998; Stenmark et al., 1996; Wucherpfennig et al., 2003). While the excitation maxima of GFP and FM 1-43 overlap, emitted light can be well separated using spectral unmixing such that both fluorescent probes can be detected independently. Our data indicate extensive colocalization between 2xFYVE-GFP and FM 1-43 or between Rab5-GFP and FM 1-43 internalized in sky mutant boutons. In contrast, in controls, FM 1-43 does not concentrate in 2xFYVE-GFP or Rab5-GFP labeled areas (Figure 4A-D, G, H) (FM 1-43/2xFYVE: control 10±1%; sky 64±2 %. FM 1-43/Rab5: control 7±1 %; sky 59±5 %). Furthermore, we also determined co-localization between FM 1-43 internalized in sky mutants and Rab4-GFP. While compared to controls, we also find increased colocalization between Rab4-GFP and FM 1-43 internalized in sky mutants, the overlap between FM 1-43 and Rab4-GFP is less pronounced than with Rab5-GFP or 2xFYVE-GFP in sky mutants (Figure 4E, F and I). The colocalization between FM 1-43 labeled structures in sky mutants and the endosomal markers is specific to sky induced endosomal structures, because when we induce the internalization of large FM 1-43-labeled cisternae following chlorpromazine administration (Kasprowicz et al., 2008), we cannot find appreciable overlap between FM 1-43 and 2xFYVE-GFP or Rab5-GFP (FM 1-43/2xFYVE: without chlorpr 12±2 %; with chlorpr 17±2 %. FM 1-43/Rab5 without chlorpr 9±2%; with chlorpr 5±1 %). Taken together, our data provide evidence that the sub-boutonic structures that form in sky mutants harbor features of sorting endosomes. Next, we tested if the endosomal-like structures in KCl stimulated sky mutants also harbor synaptic vesicle markers by labeling mutant and control synapses with anti-CSP, anti-Sytl, anti-VGlut and anti-nSyb (Daniels et al., 2004; Littleton et al., 1999; Wu et al., 1999;
Zinsmaier et al., 1994). While in controls the boutonic vesicle pool labeled by these markers distributes in a doughnut-like pattern, in stimulated sky mutants clear sub-boutonic accumulation of synaptic vesicle protein labeling is visible and these accumulations often colocalize with the endosomal marker 2xFYVE-GFP (Figure 4J-V). Furthermore, the localization of synaptic vesicle markers to endosomal 2xFYVE-GFP labeled compartments in stimulated sky mutants is specific as these phenotypes can be rescued by adding a genomic rescue sky* construct to sky mutants (Figure 4J, K, R, V). Thus, our data suggest that in stimulated sky mutants, synaptic vesicles travel via endosomal compartments. To further scrutinize these data we performed time-lapse experiments and quantified the localization of the synaptic vesicle marker Sytl in 2xFYVE-GFP expressing sky mutants and controls. Sytl distribution in sky mutants and controls prior to stimulation is very similar and does not concentrate at endosomes. However, as indicated above, 1 min of KCl stimulation in sky mutants results in Sytl accumulation in sub-boutonic structures that often colocalize with 2xFYVE-GFP, a phenotype not observed in controls (Figure 4L, N, S-U). Finally, imaging Sytl and 2xFYVE-GFP in sky mutants at various time points following stimulation shows a gradual decline in accumulations of Sytl, and Sytl labeling returns to the doughnut-like pattern seen in controls (Figure 4L, W-Z). Together with the EM data, our results show that upon stimulation of sky mutant synapses, excessive amounts of newly internalized synaptic vesicle membrane is 'pushed' via an endosomal-like compartment.

7. Sky is a Rab35 GAP
Skywalker harbors a TBC and a TLDc domain commonly found in GAPs (Bernards and Settleman, 2004; Doerks et al., 2002). GAPs facilitate the conversion of RabGTP to RabGDP and thus mediate the inactivation of their substrates (Stenmark, 2009). Hence, in sky mutants we expect the pool of RabGTP to be increased. To identify potential Sky substrates relevant to vesicle trafficking we first used an in vivo approach and screened constitutive active Rabs that phenocopy FM 1-43 dye uptake defects observed in sky mutants. We expressed a collection of 31 constitutive active (CA) Rab GTPases in neurons (Zhang et al., 2007), stimulated vesicle trafficking using 90 mM KCl and labeled newly endocytosed membrane with FM 1-43 (Figure 5A). While expression of most CA Rabs does not lead to altered distribution of labeled membrane in synaptic boutons, expression of CA Rab5-YFP, Rab23-YFP and Rab35-YFP leads to an accumulation of dye in sub-boutonic structures that can be unloaded upon a next round of stimulation (Figure 5A). Visualization of YFP fluorescence indicates that CA Rab23-YFP and CA Rab35-YFP, and to a lesser extend also CA Rab5-YFP, concentrate at the neuropile of third instar larval brains. Thus, the data show that expression of Rab5CA, 23CA or 35CA results in cell autonomous synaptic membrane trafficking defects that are reminiscent of the deficit we observed in sky mutants. Therefore, these data point to Rab5, Rab23 and Rab35
as candidate-substrates for Sky. To test Sky substrate specificity in vitro we determined the
GTP hydrolysis efficiency of purified Rab5, Rab23 and Rab35 with and without adding full
length purified Sky. First, bacterially expressed *Drosophila* GST-Rab5, GST-Rab23 and GST-
Rab35 were loaded with GTP and intrinsic GTP hydrolysis activity of these Rabs was followed
by measuring inorganic phosphate production in real time in a coupled enzymatic assay
(EnzChek, Invitrogen) (Pan et al., 2006; Webb, 1992). As shown in Figure 5B (orange trace),
Rab35, but also Rab5 and Rab23 (not shown) hydrolyze GTP, indicating the enzymes are
functional. Next, we added different concentrations of full length bacterially expressed HIS-Sky
to the GTP-loaded Rabs and measured inorganic phosphate production. Addition of Sky to
GTP-loaded Rab35 facilitates inorganic phosphate production more effectively than for Rab5
and Rab23 (Figure 5B and data not shown). Fitting the data to a Michaelis-Menten kinetic
model (black curves in Figure 5B for Rab35) yields an average $k_{cat}/K_m$ for Rab35-Sky of
121.3±12.9 M$^{-1}$s$^{-1}$, while the $k_{cat}/K_m$ for Rab5-Sky is 53.8±5.7 M$^{-1}$s$^{-1}$ and for Rab23-Sky is
88.7±3.1 M$^{-1}$s$^{-1}$. Thus, without excluding other possible substrates as well, our data show that
Sky most efficiently facilitates Rab35 mediated GTP hydrolysis in vitro. Next, to test Sky
substrate specificity in vivo, we performed genetic interactions analyses. We did not pursue
experiments with Rab23 as, in contrast to Rab5$^{CA}$ and Rab35$^{CA}$, expression of Rab23$^{CA}$ does
not phenocopy *sky* neurotransmitter release defects (see below). First, in an attempt to restore
the RabGTP/RabGDP balance in *sky* mutants we expressed constitutive GDP-bound Rab5
(Rab5$^{DN}$) and Rab35 (Rab35$^{DN}$) in neurons and determined if these conditions can rescue the
*sky* phenotypes. While *nSyb-Gal4* mediated neuronal expression of Rab35$^{DN}$ or Rab5$^{DN}$ in
controls does not affect lethality, expression of Rab35$^{DN}$ in *sky* mutants significantly improves
their survival, as measured by the number of animals that survive to the third instar stage, and
some *sky* mutant animals that normally die as third instar larvae or pupae, now eclose as
uncoordinated adults (Figure 5C). Interestingly, expression of Rab5$^{DN}$ in *sky* mutants results in
an opposite effect and strongly exacerbates lethality of *sky* mutants that now die very early as
first instar larvae (Figure 5C). Furthermore, expression of Rab35$^{DN}$ in *sky* mutants rescues the
cellular phenotypes observed in the mutants as we now find a normal distribution of FM 1-43
dye and a significant rescue of the abnormal Syt1 distribution that we observe in stimulated *sky*
mutants (Figure 5D-H, O). In addition, also functional defects in *sky* mutants at the level of
neurotransmitter release are rescued by expression of Rab35$^{DN}$ (see below). Second, we also
created *sky* mutant animals heterozygous for *rab35* or *rab5* and assessed FM 1-43 dye
distribution following 1 min of 90 mM KCl stimulation (Figure 5D, I-N). Heterozygous *rab35* or
*rab5* show normal FM 1-43 dye labeling under the conditions tested and removing a single
copy of *rab5* in *sky* mutants does not appreciably alter FM 1-43 dye distribution seen in *sky*
mutants (Figure 5D, M, N). However, removing a single copy of *rab35* in *sky* mutants
significantly rescues s/cy-associated defects in FM 1-43 distribution that now shows in a
doughnut-like vesicle pool similar to controls (Figure 5D, I, J). Also heterozygosity for an independent rab35 allele in sky mutants results in normal FM 1-43 distribution upon stimulation, indicating specificity (Figure 5D, K, L). Taken together these results suggest that in vivo, Sky mainly activates Rab35, but not Rab5, to regulate endosomal traffic of synaptic vesicles. To find further evidence for a Rab35-Sky partnership in vivo we performed Rab35 localization studies. We labeled third instar larval fillets with anti-Rab35 (Zhang et al., 2009) and anti-HRP and find Rab35 enriched in the larval brain neuropile (data not shown). We also find abundant Rab35 synthetically at NMJ boutons in a similar pattern to anti-Sky labeling (Figure 5P, data not shown). The labeling observed is specific as anti-Rab35 immunoreactivity is reduced in hypomorphic homozygous rab35 mutants (data not shown). Furthermore, double labeling of Sky and Rab35 using Sky-GFP and Rab35 antibodies also indicates that the subcellular distribution of Rab35 at synapses is very similar to that of Sky (Figure 5Q), consistent with Sky activating Rab35 GTPase activity.

8. Sky mutants show increased neurotransmitter release
To determine if sky mutations affect neurotransmitter release, we electrically stimulated motor neurons at 1 Hz while recording the muscle membrane potential. As shown in Figure 6A, the amplitude of excitatory junctional potentials (EJPs) recorded in bath solution with 0.5 mM calcium are increased in mutants when compared to controls. EJP amplitudes are also increased at lower or higher calcium concentrations (0.3 mM - 1 mM), but saturate at concentrations above 2 mM CaCl₂ (Figure 6B, data not shown), suggesting that sky mutations result in increased transmitter release but harbor a normal calcium dependence of release. We also find that this increased neurotransmission in sky mutants does not result in quicker synaptic depression during trains of repetitive stimulation. While 10 min of 10 Hz stimulation in 2 mM calcium results in synaptic depression in most endocytic mutants (Delgado et al., 2000; Marie et al., 2004; Verstreken et al., 2002; Verstreken et al., 2009), sky mutants, similar to controls, maintain release well (Figure S5). These results suggest efficient recycling of vesicles in sky mutants despite exuberant trafficking of vesicles via an endosomal-like compartment. Next, to estimate junctional quantal content we measured evoked release using TEVC. Similar to EJPs, EJCs recorded in 0.5 mM calcium are dramatically increased in sky mutants and EJCs are rescued in larvae expressing wild type Sky (Figure 6C, D). Given that the quantal amplitude (mini amplitude) is not significantly different between sky mutants and controls (Figure 3A), we calculate the quantal content to be nearly doubled in the mutants compared controls (-180% of control) (Figure 6E). Our data suggest that increased endosomal trafficking facilitates neurotransmission. This notion is further supported by two electrode voltage clamp recordings made from animals that express CA Rabs that result in increased endosomal traffic. Although EJC amplitudes are not increased upon expression of Rab23CA, expression of
Rab5CA and Rab35CA results in a more than 2-fold EJC amplitude increase compared to controls (Figure 6F). Given that the quantal amplitude at the NMJs of Rab5CA or Rab35CA expressing animals is not significantly different from controls (data not shown), quantal content is dramatically increased, similar to our observations with sky mutants (Figure 6G). Hence, membrane trafficking via synaptic endosomal-like compartments facilitates vesicle fusion and neurotransmitter release.

To test if Rab35 mediated traffic of synaptic vesicles via endosomes is limiting for neurotransmitter release we quantified quantal content upon expression of Rab35DN. EJC amplitudes in Rab35DN expressing animals are significantly reduced to 74% of control amplitudes (Figure 6F). Given that mini amplitudes are not different, quantal content upon expression of Rab35DN is reduced (Figure 6G). Similarly, expression of Rab35DN in sky restores the increased quantal content measured in sky mutants. Thus, Rab35 activity is a limiting factor and a critical regulator of neurotransmitter release (Figure 6F, G).

9. The Readily Releasable Pool of vesicles is enlarged in sky mutants

Given that sky mutants show a normal density of release sites at their synaptic boutons, (Figure 21 and Figure S2B, D), increased neurotransmission may arise from a larger functional vesicle pool that participates in release. To determine the total number of releasable vesicles (quanta) in sky mutant and control synapses we blocked new vesicle formation using the temperature sensitive shhet mutation, and counted the total number of quanta that can be released by dividing the summed EJC amplitudes by the mEJC amplitude. Stimulation of shhet; sky mutants or shhet controls at the restrictive temperature in 2 mM calcium depletes the vesicle pool and exhausts release after about 5 min at 10 Hz (Delgado et al., 2000; Verstreken et al., 2002). Calculation of the total amount of released quanta indicates no statistical significant difference between the two genotypes (shhet; sky^rd0: 109253±15515 quanta; shhet: 90967±6325 quanta; f-test: p>0.3). These data suggest the size of the functional vesicle pool in sky mutants is comparable to controls. While performing the experiments with shhet; sky animals we noticed that the time course of depletion was different compared to controls. The half maximum EJC amplitude during depletion recurrently occurred later in sky mutants when compared to controls (shhet; sky^rd0: 70.7±20.9 s vs shhet: 47.6±8.1 s). These observations suggest that in sky mutants a larger proportion of the vesicle pool is readily available for immediate participation in release during a 10 Hz stimulation paradigm. We therefore more directly determined the size of the readily releasable pool (RRP) in sky mutants and controls. We first used a short high frequency (100 Hz) stimulation train leading to a near complete depression of EJCs already after 150 ms (15 stimuli), representing depletion of the RRP (Figure 6H) (Habets and Borst, 2007; Lee et al., 2008; Millar et al., 2002). Remaining release following this initial depression is caused by refilling of the releasable vesicle pool. Both RRP
size and refilling rate can be determined from plots of the cumulative released number of quanta over time (Figure 6I). As shown in Figure 6H and 6I, stimulation of sky mutants and controls at 100 Hz in 5 mM Ca^{2+} shows consistent facilitation of EJC amplitudes not observed in controls. In addition, following initial depletion, EJCs in sky mutants remain larger (within the 400 ms stimulation paradigm) when compared to controls (Figure 6H, I). These data are consistent with an increased RRP size in sky mutants as well as an increased refilling rate (RRP size control: 2108±188 quanta; sky 3142±238 quanta, f-test: p<0.05; refilling rate control: 3679±1302 quanta/s; sky 8239±1269 quanta/s. f-test: p<0.05).

Facilitation during a 100 Hz stimulation train could also be explained by an increased calcium influx in sky mutants during intense stimulation. We therefore directly measured calcium influx under such stimulation conditions using G-CaMP imaging. However, fluorescence intensity changes in controls and in sky mutants during a 200 ms, 400 ms or a 1 s 100 Hz stimulation train are very similar, indicating that calcium influx in the mutants is not affected under the conditions tested (Figure 6J, supplemental movie 3, 4). Next, we tested RRP size independently of external calcium and puffed hypertonic sucrose in the absence of calcium over the NMJ while recording the ensuing charge transfer in voltage clamp. Sky mutants show a 2.4 fold increase in charge transfer within the first minute of sucrose application (Figure 6K-M). Continued hypertonic sucrose application following the initial triggering of RRP vesicle fusion results in a steady state of release (Rosenmund and Stevens, 1997). Also during this phase (70-90 s) the charge transfer in sky mutants is increased compared to controls, suggesting efficient refilling and recycling of RRP vesicles (Figure 6L, M). Hence, these data are in further support of a larger RRP in sky and may provide a functional explanation for the increased neurotransmission seen in the mutants.

Increased ESCRT-mediated protein sorting in sky mutants

Membrane trafficking via endosomal-like compartments may facilitate the exchange of synaptic vesicle proteins and/or lipids, thus promoting vesicle function and neurotransmitter release. A major determinant controlling endosome-to-lysosome traffic of ubiquitinated proteins destined for degradation are the ESCRT complexes, consisting of ESCRT 0 that includes Hrs, a protein involved in recognizing ubiquitinated proteins (Katzmann et al., 2001; Raiborg et al., 2002) and three other complexes (ESCRTI-III) (Raiborg and Stenmark, 2009; Wollert et al., 2009). The ESCRT proteins mediate endosomal membrane invagination to create multivesicular bodies (Bache et al., 2003; Doyotte et al., 2005; Katzmann et al., 2003); however, a role in clearing dysfunctional synaptic vesicle components remains elusive. To determine if increased transmitter release in sky mutants is facilitated by ESCRT-mediated sorting, we removed a single copy of hrs in sky mutants and monitored neurotransmitter release and FM 1-43 dye distribution following stimulation. Compared to controls, heterozygosity for hrs does not affect
neurotransmitter release recorded in 0.5 mM external calcium, nor FM 1-43 dye uptake or distribution following a 1 min 90 mM KCl application (Figure 7A-C). However, while FM 1-43 dye accumulates in endosomal structures in sky mutants as well as in sky mutants heterozygous for hrs (Figure 7B-D), removing a copy of hrs in sky mutants significantly rescues the increased EJC amplitude (Figure 7A). To further scrutinize these results, we also created sky mutants heterozygous for additional ESCRT components, including vps23, a member of ESCRTI, vps25, a member of ESCRTII and vps32, a member of ESCRTIII. Heterozygous ESCRT mutants do not affect synaptic function as we find normal neurotransmitter release as well as normal FM 1-43 loading and distribution in these animals (Figure 7A-C). However, while FM 1-43 dye travels via endosomal-like structures in sky mutants heterozygous for the ESCRT genes (Figure 7B, D), this manipulation results in a significant suppression of the increased neurotransmitter release seen in sky mutants (Figure 7A). Thus, ESCRT mediated endo-lysosomal sorting facilitates the increased synaptic transmission we observe in sky mutants. To further test if endosomal protein sorting is a prominent feature in sky mutants, we determined if proteins destined for degradation are more effectively cleared upon loss of sky function. We therefore generated an artificial ESCRT cargo by constructing a chimeric synaptic vesicle protein, Synaptobrevin (nSyb), fused at its cytoplasmatic tail to Ubiquitin and at its luminal side to HA (Ub-nSybHA). We also created "control nSyb" (nSybHA) and used Phi-C-31 mediated integration to insert both constructs at the same genomic location, ensuring identical expression levels of both constructs. Neuronal expression of Ub-nSybHA or of nSybHA does not affect NMJ morphology nor FM 1-43 dye uptake, indicating the constructs do not cause deleterious effects to the synapse. Furthermore, nSybHA behaves like a synaptic vesicle protein as it shows a very similar boutonic labeling pattern to nSyb. In addition, similar to other synaptic vesicle proteins, nSybHA expressed in sky mutants accumulates in sub-boutonic structures upon stimulation, and when expressed in shi mutants that are stimulated at the restrictive temperature, nSybHA redistributes to the presynaptic membrane as a result of endocytic blockade and synaptic vesicle pool depletion (Estes et al., 1996; Yao et al., 2009). These results indicate the chimeric protein is synaptic vesicle associated and we subsequently expressed Ub-nSybHA or nSybHA in neurons of control and sky mutant animals and labeled them with anti-HA antibodies. As expected, synaptic bouton levels of Ub-nSybHA in controls are much lower compared to nSybHA levels, indicating clearance of the ubiquitinated protein (Figure 7E, G, J). Interestingly, in sky mutants, boutonic levels of Ub-nSybHA are reduced even further compared to controls (Figure 7E, H, K). This result is in line with the idea that increased endosomal traffic facilitates synaptic vesicle protein sorting in sky mutants. To determine if the improved clearance of Ub-nSybHA in sky mutants is dependent on the ESCRT machinery we created sky mutant animals that are heterozygous for hrs and also express Ub-nSybHA or nSybHA. Interestingly, while expression
of Ub-nSybHA is similar in controls and in controls that lack a copy of hrs (Figure 7F). Ub-
nSybHA levels in sky mutants that lack a copy of hrs are significantly higher than Ub-nSybHA levels in sky mutants (Figure 7F, K, L). Thus, our data suggest a model where in sky mutants, ubiquitinated proteins are more efficiently shuttled to lysosomes for degradation leaving a more performant pool of synaptic vesicle proteins to populate the vesicle cycle (Figure 7M).

Materials and methods

Genetics

All fly stocks were kept on standard corn meal and molasses medium at room temperature. For experiments, mutants and controls were grown in optimal conditions on grape juice plates with fresh yeast paste. Deficiencies, mutant and transgenic stocks (Ahmad et al., 2009; Bellen et al., 2004; Brand and Perrimon, 1993; Dockendorff et al., 2000; Hendel et al., 2008; Littleton and Bellen, 1994; Parks et al., 2004; Roseman et al., 1995; Ryder et al., 2004; Spradling et al., 1999; Sweeney et al., 2006; Zhang et al., 2007) were obtained from the Bloomington stock center (Indiana) and from Harvard University or were gifts. Controls are y w P(ry+ ey-FLP.N)2 P(GMR-LacZ.C(38.1))TPN1; P(ry+ neoFRT)40A unless otherwise indicated; sky1 is y w P(ry+ ey-FLP.N)2 P(GMR-LacZ.C(38.1))TPN1; sky1 P(ry+ neoFRT)40A, sky2 is y w P(ry+ ey-FLP.N)2 P(GMR-LacZ.C(38.1))TPN1; sky2 P(ry+ neoFRT)40A, sky10 is y; P(XP)CG9339; sky0 is PBac[WH]CG9339; sky601084 is PBac(RB)CG9339; PBac(PB)CG9339; PBac[WH]CG9339; PBac[WH]CG9339; PBac[WH]CG9339. The stock deficiency (sky0) used in experiments is Df(2L)Exel7080, rab35Δ is w1118 P(w+)ED7635, rab35NP is y w P(GawB)rab35NP3216, rab5Δ is Df(2L)BSC455, hrs is hrs028, vps23 is PBac[WH]TSG101; vps25 is vps251 and vps32 is P(LacW)shrbK12101. UASrabaDN and UASrabaΔ constructs are described in (Zhang et al., 2007). UAS-EGFP-myc-2xFYVE, UAS-GFP-rab5 and UAS-GFP-rabA are gifts from M. Gonzalez-Gaitan (Universite de Geneve) and we received w UAS-G-CaMP1.6 from H. Bellen (Baylor College of Medicine). Transgenes or insertion constructs where combined using classical genetic techniques (see figure legends for genotypes). For ERGs from sky mutants, flies with homozygous sky eyes were y w P(ry+ ey-FLP.N)2 P(GMR-LacZ.C(38.1))TPN1; sky1 or sky2 P(ry+ ey-FLP.N)2 P(neoFRT)40A / (l2)cl-2LP(w+) P(ry+, neoFRT)40A, controls were y w P(ry+ ey-FLP.N)2 P(GMR-LacZ.C(38.1))TPN1; P(ry+, ry+ 25F P(neoFRT)40A / (l2)cl-2LP[w+], P(ry+, neoFRT)40A controls were y w P(ry+ ey-FLP.N)2 P(GMR-LacZ.C(38.1))TPN1; P(ry+ 25F P(neoFRT)40A / (l2)cl-2LP[w+], P(ry+, neoFRT)40A (Newsome et al., 2000). To determine survival of sky mutants (Figure 1) and sky mutants that express rabaDN (Figure 5) a fixed number of sky2tm, sky2tm; nSyb-Gal4/UASraba35DN (sky2tm, nSyb-Gal4/rab35DN) and sky2tm; nSyb-Gal4/rab5DN (sky2tm, nSyb-Gal4/rab5DN) embryos were followed during development and either the latest developmental stage or the percentage of animals that at least survive until the third instar larval stage normalized to the number of
sky^{2D}' animals that survive is shown. nSyb>rab35^{DN} and nSyb>rab5^{DN} show identical survival rates compared to nSyb-Gal4 ("nSyb>" in the figures) control animals.

Molecular Biology and biochemistry

UAS-sky was generated by PCR amplification of BDGP cDNA clone LD101 17 (Sky-RA) and UAS-nSybHA by PCR amplification of genomic DNA from flies with a UAS-nSybEGFP construct (Zhang et al., 2002). The HA tag was added at the nSyb C-terminal by adding the HA sequence to the primer. UAS-Ub-nSybHA consists of residues 1-74 from fly ubiquitin Rpl40 fused N-terminally to nSybHA with the spacer Gln-Gln-Ser-Arg separating ubiquitin and nSyb. The carboxy-terminal glycine residues of ubiquitin were omitted to prevent its removal by Ubiquitin Isopeptidase2, as previously described (Raiborg et al., 2002). All constructs were cloned into the EcoRI and NotI restriction sites of pUAST-attB and sequenced (Bischof et al., 2007), and primers used are listed in Table S1. The genomic sky* and GFP-sky* constructs were generated using recombineering mediated gap repair as previously described (Venken et al., 2006; Venken et al., 2008). Sky fragments were retrieved from BACR06G10 and primers used to generate either construct are listed in Table S1. UAS-sky, UAS-nSybHA, UAS-Ub-nSybHA as well GFP-sky* and sky* were inserted into the genome using phi-C-31 mediated transgenesis in following docking sites on the third chromosome: VK33 for UAS-sky, attP2 for GFP-sky* and sky* and VK27 for UAS-nSybHA and UAS-Ub-nSybHA (Genetic Services, Sudbury, MA, USA).

For quantitative real-time PCR, total RNA from whole larvae was isolated using TRI Reagent (Sigma-Aldrich) according to the manufacturer's protocol. Subsequently, the RNA samples were cleaned using the RNeasy Mini Kit with on-column DNase treatment (Qiagen). RT-PCRs were performed using the SuperScriptIII First-Strand Synthesis System (Invitrogen). 10-30 ng cDNA of each sample was used. SYBR Green PCR Master mix (Applied Biosystems) and the following primers were used: CG9339-F (5' GGGACTGAGTACCCGTATA 3') and CG9339-R: (5' TTAGATGCCACGAATCGTAG 3'). All experiments were performed in triplicate and run on an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The data were normalized to RP49 using the following primers: RP-49-F (5' ATCGGTTCAGGATCGAACAA 3') and RP-49-R (5' GACAATCTCCTTTGCGCTTCT 3').

RabGTPases were PCR amplified from the following BDGP cDNA clones: GH24702 (Rab5); RH23273 (Rab23) and LD21953 (Rab35) (DGRC) and cloned into the EcoRI and Notl sites of pGEX4T-1 for expression as an N-terminal GST fusion. Sky was PCR amplified from the BDGP cDNA clone LD101 17 and cloned into the EcoRI and Notl sites of pET28a to express Sky as an N-terminal 6xHis fusion protein. All constructs were verified by sequencing. Primers are described in Table S1. Constructs were transformed in OneShot BL21 Star (DE3) E. coli cells (Invitrogen). 250 ml cultures at \(\sim\)OD\(_{600}\) 0.8 were overnight induced by addition of 0.2 % the inducer (MolecuLAb) to GST cultures and 1 % the inducer to His cultures and grown at
room temperature. Soluble GST fusion proteins, extracted with BugBuster Master Mix (Novagen) with complete Protease inhibitor cocktail (Roche), were purified on glutathione Sepharose 4B (GE Healthcare) and eluted with 20 mM reduced glutathione in 50 mM Tris-HCl pH 8.0. His fusion proteins were purified on Nickel Beads (Affiland) and eluted with 500 mM Imidazole in 100 mM NaCl, 0.1 % Triton X-100, 20 mM Tris-HCl pH 7.5. Proteins were concentrated using Amicon Ultra centrifugal filters (Millipore) and washed 6 times with 0.5 M Tris pH 7.5.

Rat Sky antibodies against bacterially expressed full length HIS-Sky were produced and immuno-purified at Eurogentec.

The single-turnover kinetics of intrinsic and GAP-accelerated GTP hydrolysis of Rab5, Rab23 and Rab35 were performed as previously described (Pan et al., 2006). GST-Rab proteins were loaded with GTP (25 molar excess) as follows: in 20 mM HEPES pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol for 1 h incubation at 25°C and free nucleotide was removed using Zeba desalt spin columns (Pierce Biotechnology). For real time measurements of GTP hydrolysis, 10 μM Rab5-GTP, 5 μM Rab23-GTP and 20 μM Rab35-GTP and various concentrations of Sky (GAP) (indicated in the figure legend) were used in the following reaction mix: 20 mM HEPES pH 7.5, 150 mM NaCl, 0.15 mM 2-amino-6-mercaptop-7-methylpurine ribonucleoside, 0.75 U/ml purine nucleoside phosphorylase and 10 mM MgCl₂ (EnzChek phosphatase kit, Invitrogen). The absorbance at 360 nm was monitored every 16 s with an ultrospec 2100 pro UV visible spectrophotometer (Biochrom LTD). Absorbance data were analyzed by fitting them to a pseudo-first-order Michaelis-Menten model: A(t) = (A∞ - A₀)(1-exp(-k₀t)) + A₀, where k₀ = kₘᵣ + (k₉t/K₉) [GAP] using GraphPad Prism 5.03. The catalytic efficiency (k₉t/K₉) and intrinsic rate constant for GAP hydrolysis of each Rab without Sky (k₉t) were treated as global parameters and k₉t/K₉ for each RabGTP-Sky reaction was determined from at least 2 sets of independent experiments using 3 different Sky concentrations.

Immunohistochemistry

NMJs were dissected in HL-3 (see below), fixed in 3.7 % formaldehyde for 20 min and processed for immunohistochemistry as previously described (Kasprowicz et al., 2008). For stimulated samples, preparations were incubated in HL-3 with 90 mM KCl for 1 min and fixed in 3.7 % formaldehyde immediately following this treatment. For stimulated samples that were left to rest following stimulation, preparations were also incubated in HL-3 with 90 mM KCl for 1 min and then washed in HL-3 and left to rest for 5, 10, 30 or 60 min (HL-3 was replaced every 10-15 min) prior to fixation. For VGlut staining, larvae were fixed in Bouin's fixative for 5 min (Daniels et al., 2004). Primary antibodies used: Rat anti-Sky pAb 1:200, Rabbit anti-GFP pAb 1:1000 (Invitrogen), mouse anti-Dyn mAb (41) 1:50 (BD Transduction Laboratories), mouse anti-DLG mAb (4F3) 1:50, mouse anti-Bruchpilot mAb (NC82) 1:50, (both from Developmental studies Hybridoma bank), Rat anti-nSyb pAb (R29) 1:250, Rabbit anti-Sytl pAb (DSYT-2),
1:10000 (Littleton et al., 1999) (both gifts from H. Bellen, Baylor College of Medicine, Houston, TX), Rabbit anti-HRP pAb 1:1000 (Jackson ImmunoResearch laboratories), mouse anti-CSP mAb (49/92), Rabbit anti-GluRIII/IIC pAb 1:2500 (Marrus et al., 2004), Rabbit anti-VGlut pAb 1:10000 (Daniels et al., 2004) (both gifts from A. Di Antonio, Wash U, St Louis, MO), Rat anti-

Rab35 pAb 1:100 (a gift from Matthew Scott and Kaye Suyama, Stanford U, CA), mouse anti-

HA mAb (HA.11 clone 16B12) 1:1000 (Covance). Secondary Alexa-488 or -555 conjugated antibodies (Invitrogen) were used at 1:1000. Rab5-GFP, Rab4-GFP and 2xFYVE-GFP were imaged without further enhancement and Sky-GFP and 2xFYVE-GFP when double labeled with VGlut were also labeled with anti-GFP antibodies. Fluorescent images were captured using a Zeiss 510 Meta confocal microscope through a 63x 1.4NA oil lens. Labeling intensities were determined in ImageJ by measuring mean boutonic pixel intensity, subtracting background levels and normalizing data to anti-HRP labeling intensity measured in the same boutons. To determine the number of accumulations per synaptic area, inclusions per NMJ were manually counted and normalized over NMJ surface area measured in ImageJ using anti-HRP labeling. To determine the percent overlap between vesicle markers and endosomal markers, both data sets were manually thresholded and thresholded images were multiplied. The resulting area of colocalization was normalized to the vesicle marker area.

FM 1-43 and dextran labeling and FM 1-43 photoconversion

Third instar larvae were dissected in HL-3: (mM) NaCl (110), KCl (5), NaHCO₃ (10), HEPES (5), sucrose (30), trehalose (5), MgCl₂ (10) pH 7.2 and motor neurons were cut. Larvae were then stimulated in the presence of FM 1-43 (4 μM) (Invitrogen), either at 3 Hz for 20 min in HL-3 with 1.5 mM CaCl₂ or for 1 min in HL-3 with 90 mM KCl: (mM) NaCl (25), KCl (90), NaHCO₃ (10), HEPES (5), sucrose (30), trehalose (5), MgCl₂ (10), CaCl₂ (1.5), pH 7.2. Larvae were washed with HL-3 to remove non-internalized dye and imaged. For unloading, loaded terminals were stimulated in HL-3 with 90 mM KCl and 1.5 mM CaCl₂ for 5 min. Images were captured with a Zeiss 510Meta confocal microscope and 63x 1.0NA water immersion lens. For imaging of FM 1-43 and GFP together, we used 488 nm excitation and a 510/20 nm band pass filter to detect GFP emission as well 570-620 nm emission using the 510Meta detector to capture FM 1-43 fluorescence. We performed control experiments using FM 1-43 labeled samples excited at 488 nm while detecting emission using the 510/20 nm band pass filter to ensure we did not detect FM 1-43 fluorescence in the GFP channel. Quantification of number of FM 1-43 accumulations per synaptic area and colocalization between FM1-43 inclusions and GFP markers was performed as described above in the immunohistochemistry section. Quantification of labeling intensity was performed as described (Verstreken et al., 2008).

For photoconversion, dissected larvae were incubated in HL-3 with 90 mM KCl, 1.5 mM CaCl₂ and FM 1-43FX (4 μM) (Invitrogen) for 1 min and washed 3x in HL-3. Samples were fixed in 1 % glutaraldehyde and 4 % paraformaldehyde in 0.1 M Na-Cacodylate buffer for 15
min at room temperature and washed in 0.1 M phosphate buffered saline (PBS) pH 7.4 (Vijayakrishnan et al., 2009). Subsequently, fixed samples were incubated 2x5 min in 2 mg/ml 3,3 diaminobenzidine (DAB, Sigma) in PBS. For conversion of DAB into electron dense precipitates, samples were illuminated through a 40x 0.8NA water immersion objective on a Nikon FN1 microscope for 20 min using an Intensilight (C-HGFI, Nikon) light source filtered through a standard FITC filter. Samples were finally washed 3x10 min in PBS pH 7.4. The presence of brown precipitates as a sign of efficient photoconversion was verified using a standard light microscope. Samples were then further fixed for TEM (see below).

For 10 kDa tetramethylrhodamine-dextran (dextran) (invitrogen) labeling, larvae were dissected in HL-3 and were treated with collagenase (1 mg/ml) for 30 s to make the NMJ more accessible for dextran. Preparations were then stimulated in HL-3 with 90 mM KCl, 1.5 mM CaCl$_2$ and 100 μM dextran. Labeled preparations were washed in HL-3 and preparations were imaged on a Nikon FN1 microscope through a 60x 1.0NA water immersion lens with a cooled CCD camera (Andor DR-328G-C01-SIL). For control experiments with chlorpromazine, dissected animals were first incubated for 18 min in 50 μM chlorpromazine (Sigma) in Schneider’s medium prior to collagenase treatment. For quantification of labeling, the number of labeled NMJs on muscles 12, 13 and 4 were counted per animal and data was normalized to controls treated with chlorpromazine.

Transmission electron microscopy

For TEM, larvae were dissected in HL-3. They were either directly fixed (unstimulated), stimulated for 1 min in HL-3 with 90 mM KCl and 1.5 mM CaCl$_2$ and then fixed (stimulated) or washed for 1 h in HL-3 after stimulation in 15 min intervals and then fixed (stimulation + 1 h rest). Fixation solution contained 1 % glutaraldehyde, 4 % paraformaldehyde and 1 mM MgCl$_2$ in 0.1 M Na-cacodylate buffer pH 7.4 as previously described (Kasprowicz et al., 2008). Subsequently, specimens were osmicated in Os04/Na-cacodylate buffer for 2 h and stained in 2 % aqueous uranyl acetate (1.5 h). After dehydration using a graded series of ethanol, specimens were embedded in Agar 100 (Laborimpex, Agar Scientific). Ultrathin sections (60-70 nm) were collected at butvar-coated grids (Laborimpex, Agar Scientific) and contraststained with lead citrate (according Reynolds) and 4 % uranyl acetate. Sections were imaged using a JEM2100 [JEOL] transmission electron microscope at 200 kV. We used ImageJ for quantification of profile diameters, areas and grey values. Ultrastructural features were quantified from boutonic profiles whose surface area is at least 1 μm$^2$. Diameters of organelles were measured from profiles with clearly visible membranes. Means and histograms in figure 2 and 3 were calculated from at least 17 boutons collected from 3-5 animals per genotype and per experimental condition.

Serial-tilt EM for tomography was performed on 200-300 nm thick sections collected on formvar-carbon coated grids, covered with colloidal gold particles (15 nm-fiducial marker) at
200 kV. Micrographs were recorded at tilt from -60° to 60° in 2° intervals in X-axis, and digitized using Jeol TEM-recorder software. 3D-reconstructions were obtained using R-weighted back-projection in IMOD (Kremer et al., 1996). Tomograms were manually segmented and visualized in IMOD.

Electrophysiology

ERGs were recorded as described in Venken et al. 2008. Flies were immobilized with 'liquid Pritt glue'. We used digitally controlled LED green light to deliver 1 s light pulses. Data were stored in Clampex 10 and processed with Clampfit. Voltage clamp and current clamp in third instar larvae were performed on muscle 6 in HL-3 using <15 MΩ intracellular electrodes (<30 MΩ for current clamp), as described (Verstreken et al., 2002; Verstreken et al., 2009). The membrane holding potential for two electrode voltage clamp was -70 mV, voltage errors were <1.5 mV for 100 nA EJCs and input resistances were ≥ 5 MΩ. All data were filtered at 1 kHz (minis at 600 Hz). Synaptic currents or membrane potentials were monitored with an Axoclamp 900A amplifier and digitized using a Digidata 1440A (Molecular Devices). Excitatory junctional currents (EJCs) or excitatory junctional potentials (EJPs) in external calcium concentrations as indicated in the figures were evoked by stimulation of the cut segmental nerve at 2x threshold. Miniature EJCs (mEJCs) were recorded in the presence of 0.5 μM TTX (Alomone labs) and 0.5 mM CaCl₂. We also recorded minis in HL-3 with 0.5 mM CaCl₂ and 0.5 μM TTX immediately following stimulation using 1 min of 90 mM KCl in HL-3 with 1.5 mM CaCl₂.

Basal EJC or EJP amplitudes were determined in Clampfit 10.2 by calculating the average of 60 traces. mEJC amplitudes and frequencies were quantified using the event detection module in Clampfit 10.2 and calculated from 5 min recordings. Quantal content was determined by dividing the average EJC amplitude by the average mEJC amplitude. For voltage clamp recordings with shi₃₀⁶, animals were kept at 33 °C and stimulated at 10 Hz. Total quantal content was determined by summing all EJC amplitudes and dividing this by the mEJC amplitude. To determine readily releasable pool (RRP) size using the cumulative quantal content method, motor neurons were stimulated at 100 Hz for 400 ms. EJC amplitudes are relative to the initial baseline (corrected for drift during the 400 ms stimulation paradigm) and are plotted as cumulative quantal content. The trendline slope through points 200-400 ms is a measure for RRP refilling rate and the y-intercept corresponds to the RRP size (Habets and Borst, 2007; Lee et al., 2008; Millar et al., 2002) To independently determine RRP size, we also measured the charge transfer during application of hypertonic sucrose in 3rd instar control and mutant larval fillets by puffing 0.5 M sucrose for several seconds over third instar NMJs bathed in HL-3 without CaCl₂. The puff pipette had a tip diameter of 2 mm, and the tip was placed close to the NMJ on muscle 6/7 in segment A2. Junctional currents were recorded in TEVC using a holding potential of -70 mV and the charge transfer during the first 60 s of
sucrose application as well as during the 70-90th s of application were quantified using Clampfit 10.

**Calcium measurements**

G-CaMP1.6 was expressed in neurons of controls and sky mutants using nSyb-Gal4 and calcium influx at 3rd instar larval NMJ boutons was measured while bathing animals in HL-3 with 5 mM CaCl₂ and 100 mM 1-Naphthylacetyl spermine trihydrochloride (an AMPA receptor blocker that suppresses muscle contractions in Drosophila larvae) (Sigma). Larvae were stimulated at 100 Hz for 200 ms, 400 ms, 1 s and 2 s (not shown) and images were captured every 385 ms on a Nikon Eclipse F1 fluorescence microscope using a cooled CCD camera (Andor DR-328G-C01-SIL) and a 60x 1.0NA water immersion objective. Data evaluation and signal processing were performed as described (Hendel et al., 2008). Briefly, background intensity was subtracted from boutonic fluorescence. Then, three frame points before and at least sixteen frames after the stimulus train were taken to fit an exponential decay that represents bleaching and this function was subtracted from all data points. To calculate AF/Fo of the resulting time-series, fluorescence intensities were divided by average boutonic intensity in the three frames before stimulation (=Fo).
References


1. A method for identifying compounds that enhance synaptic endosomal trafficking and/or neurotransmitter release in a neuronal cell or a neuron comprising the steps of:
   a) providing a purified SEQ ID NO: 2 and a GTP-loaded Rab35 protein or a purified SEQ ID NO: 2 and a GTP-loaded ARF6 protein;
   b) administering a test compound;
   c) measuring the inorganic phosphate production in a coupled enzymatic assay;
   d) wherein, a reduction in said inorganic phosphate production compared to a condition wherein no test compound was administered, identifies said test compound as a compound that enhances synaptic endosomal trafficking and/or enhanced neurotransmitter release when administered to a neuron of a neuronal cell.
2. A method for identifying compounds that enhance synaptic endosomal trafficking and/or neurotransmitter release in a neuron or a neuronal cell comprising the steps of:
   a) providing a neuronal cell or neuron culture expressing a polypeptide encoded by SEQ ID NO: 1;
   b) administering a test compound and a dye with a specificity for endosomal vessels to said cell culture;
   c) stimulating endosomal vessel trafficking;
   d) imaging the labeled endosomal vessels of the cells in said cell culture; wherein, under the same test conditions, a deviation in said imaging compared to the same imaging of cells of a corresponding cell culture without said test compound, identifies said test compound as a compound that enhances synaptic endosomal trafficking and/or neurotransmitter release in a neuronal cell or a neuron.
3. Use of SEQ ID NO: 1 or 2 to identify compounds that enhance synaptic endosomal trafficking and/or enhanced neurotransmitter release.
4. Use of SEQ ID NO: 1 or 2 to enhance synaptic endosomal trafficking and/or enhanced neurotransmitter release in an in vitro neuron or neuronal cell culture.
5. SEQ ID NO: 1 or 2 for diagnosing and/or prognosing neurological diseases.
6. An agent characterized in that it enhances synaptic endosomal trafficking and/or enhanced neurotransmitter release, wherein said agent is selected from the list of: a small interfering RNA (siRNA), an artificial microRNA, an antisense polynucleotide, a ribozyme, wherein the latter agents comprises a nucleic acid sequence engineered from SEQ ID NO: 1, or an antibody with a specificity for SEQ ID NO: 2.
7. A pharmaceutical composition comprising a therapeutically effective amount of the agent according to claim 6 and at least one of a pharmaceutically acceptable carrier, adjuvant or diluent.

8. The agent according to claim 6 or the pharmaceutical composition according to claim 7 for use in the prevention and/or treatment of neurological diseases.

9. The use according to claim 8 in which the neurological disease is selected from Alzheimer's disease or Parkinson's disease.
Figure 7

A. ESCRT amplitude (pA)

B. Accumulated button area (μm²)

C. Control: hrs+/ vps23+/ vps25+/ vps32+/
   sky²/ hrs+/ vps23+/ vps25+/ vps32+/
   sky²/ hrs+, vps23+/ vps25+/ vps32+/

D. Relative labeling intensity

E. Relative labeling intensity

F. Ub-nSybHA

G. Control: hrs+/ vps23+/ vps25+/ vps32+/
   sky²/ hrs+, vps23+/ vps25+/ vps32+/

H. sky²/

I. sky²/, hrs+

J. Ub-nSybHA

K. HA HRP

L. HA HRP

M. Wild type: Lys MVB End
   Skywalker: Lys MVB End

- used protein
- fresh protein

Rab-GDP
Rab-GTP
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/50 A61K39/00 C07K14/00 C12N15/00

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category* Citation of document, with indication, where appropriate, of the relevant passages


the whole document, in particular abstract; page 367, column 1, second full paragraph; paragraph spanning pages 368 and 369; page 369, column 1, first full paragraph; Figure 2

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X Further documents are listed in the continuation of Box C.

See patent family annex.

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Date of the actual completion of the international search

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Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2

NL - 2280 HV Rijswijk

Tel. (+31-70) 340-2040,

Fax: (+31-70) 340-3016

Authorized officer

Weber, Peter

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<td>VALERIE UYTTERH0EVEN ET AL: &quot;Abstract 71: Skywalker is a novel GTPase activating protein that restrains synaptic endosomal trafficking and neurotransmitter release&quot;, 2010 ASBMB SPECIAL SYMPOSIA: BIOCHEMISTRY OF MEMBRANE TRAFFIC: SECRETORY AND ENDOCYTIC PATHWAYS, 28 October 2010 (2010-10-28), page 1,82, XP55024586, Granlibakken Resort, Tahoe City, CA abstract</td>
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