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Flotillin-1 Promotes Formation of Glutamatergic Synapses in Hippocampal Neurons

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Abstract

Synapse malformation underlies numerous neurodevelopmental illnesses, including autism spectrum disorders. Here we identify the lipid raft protein flotillin-1 as a promoter of glutamatergic synapse formation. We cultured neurons from the hippocampus, a brain region important for learning and memory, and examined them at two weeks *in vitro*, a time period rich with synapse formation. Double-label immunocytochemistry of native flot-1 with glutamatergic and GABAergic synapse markers showed that flot-1 was preferentially colocalized with the glutamatergic presynaptic marker vesicular glutamate transporter 1 (VGLUT1), compared to the GABAergic presynaptic marker glutamic acid decarboxylase-65 (GAD-65). Triple-label immunocytochemistry of native flot-1, VGLUT1, and NR1, the obligatory subunit of NMDA receptors, indicates that Flot-1 was preferentially localized to synaptic rather than extrasynaptic NR1. Furthermore, electrophysiological results using whole-cell patch clamp showed that Flot-1 increased the frequency of miniature excitatory postsynaptic currents (mEPSCs) but not miniature inhibitory postsynaptic currents (mIPSCs), whereas amplitude and decay kinetics of either type of synaptic current was not affected. Corresponding immunocytochemical data confirmed that the number of glutamatergic synapses increased with flot-1 overexpression. Overall, our anatomical and physiological results show that flot-1 enhances the formation of glutamatergic synapses but not GABAergic synapses, suggesting that the role of flot-1 in neurodevelopmental disorders should be explored.

Keywords

rat; lipid raft; development; GABA; synaptogenesis

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Dedication: This manuscript is dedicated to the memory of Robert J. Wenthold, Ph.D.

†deceased

INTRODUCTION

Neuronal development of the hippocampus is important for learning and memory. Neurons undergo various stages of growth, starting with differentiation of their processes into axons and dendrites (for review, Craig and Banker, 1994). These processes then elongate and branch into complex arborization patterns in a phase known as neurite outgrowth. As neurites grow, they form synapses with other neurons in order to establish neuronal circuitry. Dynamic addition and removal of synapses persists throughout adulthood and is necessary for interacting with environmental stimuli. Therefore, proteins that regulate synapse formation could have an important function in modulating the synaptic plasticity needed for cognition.

One factor that influences synapse formation is lipid rafts. Lipid rafts are microdomains of the plasma membrane rich in cholesterol and sphingolipids that act as signaling platforms (Pike, 2006). Glia-derived cholesterol induces the formation of synapses (Mauch et al., 2001). However, cholesterol synthesis within the neuron can also enhance synaptogenesis by increasing the number of lipid rafts (Suzuki et al., 2007). In addition, intact lipid rafts (Hering et al., 2003) and cholesterol homeostasis (Goritz et al., 2005; Karasinksa et al., 2009) are necessary for synapse stability.

The lipid raft-associated protein flotillin-1 (flot-1) has previously been shown to be important for early stages of neuronal development. Flot-1 is a 428 amino acid protein with a prohibitin homology domain and a flotillin domain. It associates with the cytoplasmic side of lipid raft membranes and oligomerizes with flot-2 to form homomeric and heteromeric tetramers (Solis et al., 2007). It is ubiquitously expressed in virtually all cell types (for review, Morrow and Parton, 2005), but its expression is enriched during development (Volonte et al., 1999), as well as in regenerating axons of goldfish (Schulte et al., 1997) and zebrafish (Munderloh et al., 2009). Flot-1 induces filopodia formation in mammalian cell lines (Hazarika et al., 1999; Neumann-Giesen et al., 2004). Furthermore, flot-1 promotes hippocampal neuronal differentiation (Langhorst et al., 1998; Munderloh et al., 2009) and neurite outgrowth (Swanwick et al., in press). We investigated whether flot-1 could also mediate synapse development. Using cultured hippocampal neurons, we demonstrate that flot-1 enhances the formation of glutamatergic synapses without affecting GABAergic synapses.

METHODS

Cell culture

All experiments involving animals were performed according to National Institutes of Health (NIH) guidelines. Hippocampal neurons were cultured as previously described (Sans et al., 2003). Briefly, hippocampi were dissected from embryonic day 18 Sprague-Dawley rats, dissociated in 0.25% trypsin and incubated with 0.01% DNAse. Neurons were triturated and plated onto poly-lysine/fibronectin coated glass coverslips at a density of 50,000 cells per well of a 6-well dish in Neurobasal medium containing 2% fetal bovine serum and 2% B27 supplement. The culture media was changed to neurobasal medium plus B27 after 3 days *in vitro* (DIV).

cDNA Constructs

Flot-1-GFP was a gift from Dr. Benjamin Nichols (MRC Laboratory of Molecular Biology, Cambridge, UK) and was created with the pEGFP-N1 vector (Clontech). The company's updated version of this vector, pAcGFP1-N1, was used to express GFP alone. Based on WB experiments performed on HEK293 cells transfected with flot-1, we estimate that the level of flot-1 increased approximately 4-fold with overexpression.

Antibodies

Primary antibodies included a monoclonal flot-1 antibody (1:50, BD Biosciences # 610820), guinea pig polyclonal VGLUT1 antibody (1:5000, Millipore #AB5905), monoclonal GAD-65 antibody (1:1000, Millipore #MAB351), rabbit polyclonal GluR1 antibody raised in this laboratory (1:300, available at Millipore #AB1504) and rabbit monoclonal antibody raised against the C2 cassette of NR1 (1:2000, Millipore #AB9864). Specificity of the flot-1 antibody has been demonstrated by siRNA knockdown experiments previously performed by this laboratory (Swanwick et al., in press). Secondary antibodies included Alexa 488, 555, or 647 fluorochromes (Invitrogen) at a concentration of 1:500.

Immunocytochemistry

To visualize flot-1, neurons were fixed at 13-16 DIV with 4% paraformaldehyde/4% sucrose for 10 min at room temperature (RT), washed with PBS, and treated with methanol for 5 min at 20°C. Neurons were then permeabilized with 0.2% saponin for 10 min at RT and blocked with 10% normal goat serum for 15 min at RT before primary antibodies were applied. Primary antibody against flot-1 was exposed to neurons overnight at 4°C; all other primary antibodies were applied to neurons for 1 hr at RT. For double- and triple-labeling, primary antibodies were applied in succession. Afterwards, neurons were treated with fluorescent secondary antibodies for 45 min at RT before their coverslips were mounted on slides using ProLong® Gold Anti-fade reagent (Invitrogen).

Imaging and Analysis

For double-label immunocytochemistry, images were captured on an E1000 Nikon microscope mounted with a CCD camera using a 60X PlanApo (1.4 N.A.) objective. For triple-label immunocytochemistry, images were taken using a PE Ultraview confocal microscope with a 100X PlanApo (1.4 NA) objective.

Colocalization was quantified using the Multi-Wave Cell Sorting function in Metamorph software (Molecular Devices). For images taken at 60X, flot-1 puncta were defined by 1-6 pixels wide (0.1-0.6 μm) with fluorescent intensity >2 times stronger than background. Postsynaptic NR1 and GluR1 puncta were defined as 2-11 pixels wide (0.2-1.2 μm), and presynaptic VGLUT1 and GAD-65 puncta were defined as 2-13 pixels wide (0.2-1.4 μm). The area of a puncta that is 1 pixel in diameter (the minimum definition of flot-1) is equal to 0.785 pixels^2. Therefore, for flot-1 to be scored as colocalized in 60X, the minimum area of overlap was set at 1 pixel. (MWCS software accepts only round numbers). To maintain the width measurements for images captured at 100X, flot-1 puncta were defined as 2-9 pixels wide, postsynaptic NR1 and GluR1 puncta were defined as 3-19 pixels wide, and

presynaptic VGLUT1 and GAD-65 puncta were defined as 3-22 pixels wide. Therefore, for flot-1 to be scored as colocalized in 100X, the minimum area of overlap was set at 3 pixels.

Numbers of synaptic puncta were quantified in 40 μm dendrite segments created using the Region Measurement tool of Metamorph. All dendrites chosen for analysis were secondary dendrites. We defined primary dendrites as those protruding from the cell body and secondary dendrites as those branching immediately from primary dendrites. Three dendrite regions were averaged per neuron. When selecting cells for analysis, we consistently chose flot-1-GFP-transfected neurons with the brightest intensity, at similar levels of plasmid expression.

Statistical comparisons between two groups were analyzed by unpaired student's t-tests. Adobe Photoshop was used to adjust brightness and contrast of images for publication.

Electrophysiological Recording and Analysis

Neurons were transiently transfected from 13-15 DIV using CalPhos Mammalian Transfection Kit (BD Biosciences). When selecting cells for recording, we consistently chose flot-1-GFP-transfected neurons with pyramidal morphology and exhibiting similar levels of plasmid expression, at the brightest intensity. Whole-cell patch clamp recordings were conducted with an Axopatch 200B amplifier and pCLAMP software (Molecular Devices). Thick-walled (1.5 mm outer diameter, 0.86 mm inner diameter) borosilicate patch electrodes (World Precision Instruments) were pulled on a P-97 Flaming-Brown horizontal puller (Sutter Instruments) to a final resistance of 2-5 MΩ. Series resistance and capacitance were compensated for each neuron.

mEPSCs with both NMDA and AMPA receptor components were recorded at a holding potential of −70 mV according to the method of Myme et al. (2003). Briefly, due to the difficulty of recording NMDAR-mediated mEPSCs in hippocampal neurons, neurons were recorded in 30 second intervals for up to 10 minutes. These intervals alternated between and a Mg^{2+}-free extracellular solution containing 10 μM D-serine, 1 μM tetrodotoxin, and 50 μM bicuculline and a "baseline" extracelluar solution containing 2 mM Mg^{2+} in which the neuron could rest. All the intervals recorded in the Mg^{2+}-free solutions were compiled for analysis. A potassium gluconate-based internal solution was used for both types of recording intervals.

mIPSCs were recorded in extracellular solution containing 1 μM TTX, 50 μM APV, and 5 μM NBQX at a holding potential of −60 mV. Internal and external solutions contained equimolar concentrations of chloride ions. Neurons were recorded for up to 30 minutes. To measure alterations in mIPSCs over time, each 5-minute epoch of the overall recording length was recorded as a separate file for individual analysis.

MiniAnalysis (Synaptosoft) and Clampfit 9.2 software (Molecular Devices) was used for analysis. For mEPSCs, AMPAR amplitudes were determined by the peak current, and NMDAR ampltiudes were measured from 18-23 ms after the peak current, a time when the contribution of AMPAR current had mostly declined. The NMDA:AMPA ratio is reported as a ratio of these two currents. Decays of average mEPSCs were fit with a biexponential

curve and calculated with a weighted mean decay time constant, where $T_w = [I_f/(I_f + I_s)] * t_f + [I_s/(I_f + I_s)] * t_s$. For mIPSCs, amplitude was measured by the median peak current and average decays were fit with biexponential curves, where T_1 describes the fast component and T_2 evaluates the fast component.

RESULTS

Synaptic Localization of Flot-1

To investigate the synaptic distribution of flot-1, we performed double-label immunocytochemistry in hippocampal neurons cultured for 2 weeks *in vitro* with antibodies against flot-1 and either vesicular glutamate transporter 1 (VGLUT1) as a marker of glutamatergic synapses or the 65 kDa isoform of glutamic acid decarboxylase (GAD-65) as a marker of GABAergic synapses (Fig. 1). VGLUT1 (left, red) and GAD-65 (right, red) were expressed as puncta of relatively equal size. Flot-1 (green) was often colocalized with VGLUT1 (left, yellow) but rarely with GAD-65 (right, yellow). When quantified, the percentage of flot-1 puncta colocalized with VGLUT1 puncta (24.4 ± 0.9%) was much higher than with GAD-65 puncta (1.2 ± 0.2%, $p < 0.0001$, n = 18 neurons each). Similarly, 63.5 ± 2.1% of VGLUT1 puncta overlapped with flot-1 puncta, but only 31.8 ± 2.3% of GAD-65 puncta were colocalized with flot-1 puncta ($p < 0.0001$).

To further explore the synaptic distribution of flot-1, we determined the localization of flot-1 to synaptic vs. extrasynaptic glutamate receptors. NMDA receptors (NMDARs) exist as tetramers containing two obligatory NR1 subunits, so we performed triple-label immunocytochemistry of native NR1 subunits of NMDARs (red), with flot-1 (green) and VGLUT1 (blue) in hippocampal neurons cultured for 2 weeks *in vitro* (Fig. 2). Synaptic NMDARs were identified by colocalized sites of NR1 and VGLUT1, whereas extrasynaptic NMDARs were defined as NR1 clusters that were localized outside of VGLUT1 puncta. Many NR1 clusters were localized to extrasynaptic sites, but few of these extrasynaptic clusters were colocalized with flot-1. In contrast, most synaptic NR1 clusters were colocalized with flot-1. When quantified, the percentage of synaptic NR1 clusters overlapped with flot-1 puncta was more than double that of extrasynaptic NR1 clusters, with approximately 58.1 ± 2.6% of synaptic NR1 clusters colocalized compared to 22.0 ± 1.5% of extrasynaptic NR1 clusters colocalized ($p < 0.0001$, n = 24 neurons each).

Effect of Flot-1 on Synapse Development

To determine the effect of flot-1 on synaptic function, we used whole-cell patch clamp electrophysiology to measure miniature postsynaptic currents (mPSCs), the smallest unit of synaptic transmission whose kinetics reflect properties of individual synapses (Fig. 3). We recorded both miniature excitatory postsynaptic currents (mEPSCs) and miniature inhibitory postsynaptic currents (mIPSCs) in cultured hippocampal neurons transfected from 13-15 DIV with GFP or flot-1-GFP. The mEPSCs contained both NMDAR and AMPAR components and were recorded based on a protocol from Myme et al. (2003). We found that neurons overexpressing flot-1 produced more mEPSCs, represented by downward spikes in the trace (Fig. 3A). When quantified, the frequency of mEPSCs approximately doubled with flot-1 overexpression ($p < 0.05$, n = 13-14 neurons each), and typical cumulative probability

density functions show that neurons with flot-1 overexpression exhibited synaptic events with shorter inter-event intervals than neurons with GFP alone. In contrast, the frequencies of mIPSCs from neurons overexpressing flot-1 were similar to those with GFP alone (Fig. 3C, $p = 0.85$, $n = 11$-13 neurons each). However, amplitude and decay kinetics of mEPSCs and mIPSCs were unchanged by flot-1 overexpression (Supp. Fig. 1).

To determine whether increased mEPSC frequency corresponded with increased synapse formation, we performed double-label immunocytochemistry in cultured hippocampal neurons transfected from 13-15 DIV with GFP or flot-1-GFP (Fig. 4). We defined synapses as colocalized puncta of presynaptic and postsynaptic markers, using VGLUT1 (blue) as a presynaptic glutamatergic marker and the AMPA receptors subunit GluR1 (red) as a postsynaptic marker. When these images were merged in control neurons expressing GFP alone, a fraction of GluR1 clusters were colocalized with VGLUT1 (pink), but many GluR1 clusters remained noncolocalized, in extrasynaptic locations (Fig. 4A). However, when flot-1 was overexpressed, colocalization of GluR1 and VGLUT1 increased (Fig. 4B). When quantified (Fig. 4C), neurons expressing GFP alone contained 23.9 ± 2.1 colocalized VGLUT1 + GluR1 puncta per 40 μm segment of dendrite, with $32.7 \pm 2.4\%$ of GluR1 clusters colocalized with VGLUT1. Neurons expressing flot-1-GFP showed a higher number of synapses, with 31.8 ± 2.9 colocalized VGLUT1 + GluR1 puncta per 40 μm segment of dendrite and $40.7 \pm 3.1\%$ of GluR1 clusters colocalized with VGLUT1 ($p < 0.05$ each, $n = 29$-30 neurons each). Interestingly, the total number of GluR1 clusters remained unchanged with flot-1 overexpression, from 78.1 ± 5.5 clusters per 40 μm segment of dendrite in GFP-expressing neurons to 82.5 ± 6.2 clusters per 40 μm dendrite segment in flot-1-overexpressing neurons ($p = 0.60$). Likewise, the total number of VGLUT1 puncta remained constant, from 46.0 ± 4.6 to 53.0 ± 4.9 puncta per 40 μm dendrite segment ($p = 0.30$).

DISCUSSION

In summary, we identify the lipid raft protein flot-1 as a novel enhancer of glutamatergic synapse formation. It is preferentially localized to glutamatergic synapses compared to GABAergic synapses. It is also preferentally localized to synaptic glutamate receptors compared to extrasynaptic glutamate receptors. Most importantly, it increases the frequency of mEPSCs without affecting mIPSCs or the amplitude or decay kinetics of either type of current. In support, it increases the corresponding number of glutamatergic synaptic puncta. Our results show that flot-1 induces the formation of glutamatergic synapses and suggest that flot-1 may be used as a molecular target to regulate glutamatergic synaptogenesis.

It should be noted that flot-1 puncta are relatively small and abundant, increasing the probability that flot-1 may overlap with synaptic puncta simply by chance. Several lines of evidence indicate otherwise. First, the puncta of the synaptic markers VGLUT1 and GAD-65 are similar in size, yet flot-1 is preferentially associated with VGLUT1. Second, the number of extrasynaptic NR1 clusters is much higher than synaptic NR1 clusters, but the probability of flot-1 puncta associating with synaptic NR1 clusters is higher. Therefore, we conclude that flot-1 is specifically targeted to synaptic glutamate receptors.

Flot-1 has been reported as a marker of a clathrin-independent endocytic pathway (Glebov et al., 2006). Oligomerization of flotillins induces membrane invagination and vesicle budding characteristic of internalization (Frick et al., 2007), and this process is regulated by activation of Fyn kinase (Riento et al., 2009). However, overexpression of flot-1 did not affect the amplitude of glutamatergic synaptic currents, indicating that the number of synaptic glutamate receptors remained constant. The lack of detectable change in synaptic receptor number is consistent with the fact that glutamate receptor trafficking is thought to be mainly mediated by clathrin-dependent pathways (for review, Nong et al., 2004). The flot-1 internalization pathway might only be utilized under conditions of high neuronal activity, as has been suggested for other clathrin-independent pathways (for review, Mayor and Pagano, 2007). Therefore, alterations in mEPSC amplitude might only be detected if clathrin is blocked.

Our results identify flot-1 as a molecular link between lipid rafts and glutamatergic synapse formation. Flot-1 is well-established as a lipid-raft associated protein (Bickel et al., 1997; for review, Browman et al., 2007), and is present in a subpopulation of lipid rafts distinct from those associated with the lipid raft marker caveolin (Glebov et al., 2006). The presence of lipid rafts in the plasma membrane is necessary to maintain synapses (Hering et al., 2003), and lipid rafts and their associated proteins have been purified from synaptic membrane fractions (for review, Suzuki, 2002). In addition, cholesterol, a major component of lipid rafts, can induce synapse formation (Mauch et al., 2001). This cholesterol can be produced from either glia (Mauch et al., 2001) or the neuron itself (Goritz et al., 2005; Karasinska et al., 2009). Extensive evidence supports the idea that glia release proteins responsible for enhancing the formation of both glutamatergic (Christopherson et al., 2005; Eroglu et al., 2009) and GABAergic synapses (Hughes et al., 2010). It is unclear whether neuronal expression of flot-1 can be modulated by glia, but future experiments should explore factors which influence flot-1 expression.

Potential regulators of flot-1 induced synapse formation include cell adhesion molecules. These are contact-forming molecules that bind together across cell junctions and include superfamilies of proteins such as cadherins and integrins (for review, Arikkath and Reichardt, 2008). Flot-1 is often localized at sites of cell contact (Lang et al., 1998). Moreover, we found that Synaptic Adhesion-Like Molecule (SALM) 4, a cell adhesion molecule family initially discovered and characterized by our laboratory (Wang et al., 2006), requires flot-1 for its enhancement of neurite branching (Swanwick et al., in press). Whether flot-1 also mediates the abililty of SALM4 to promote synapse formation remains to be tested (Wang et al., 2006; Ko et al., 2006).

Finally, it is likely that flot-1 promotes synapse formation by accelerating dendritic spine formation and maturation. Flot-1 can induce filopodia formation in mammalian cell lines (Hazarika et al., 1999; Neumann-Giesen et al., 2004). It also enhances neurite outgrowth in hippocampal neurons by increasing the number of neuronal processes and complexity of neurite branching (Swanwick et al., in press). Glutamatergic synapses are housed in the head of dendritic spines. Mature dendritic spines are mushroom-shaped, with glutamatergic synapses housed at the tip. Immature dendritic spines are long, thin, and filopodia-like. We previously found that flot-1 interacts with synaptic proteins such as NMDARs (Swanwick et

al., 2009). Due to the central role of lipid rafts as membrane platforms that organize neuronal signaling and trafficking (for review, Lingwood and Simons, 2010), it is highly likely that flot-1 interacts with additional postsynaptic proteins involved in synaptic plasticity. (Notably, we found that the percentages of presynaptic and postsynapitc puncta colocalized with flot-1 were approximately equal (~60%), suggesting that flot-1 is equally distributed to both sides of the synapse.) To enhance synapse formation, flot-1 may induce the formation of filopodia, advance the transition of dendritic spines from immature filopodia into mature mushroom-shaped spines, or both. Whereas our evidence showed that flot-1 increased the colocalization of presynaptic and postsynaptic markers, the total number of presynaptic and postsynaptic markers remained unchanged, suggesting that flot-1brings together pre-existing presynaptic and postsynaptic terminals during synapse maturation. Future studies should further explore how flot-1 affects the development and maturation of dendritic spines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

Arikkath J, Reichardt LF. Cadherins and catenins at synapses: roles in synaptogenesis and synaptic plasticity. Trends Neurosci. 2008; 31:487–494. [PubMed: 18684518]

Bickel PE, Scherer PE, Schnitzer JE, Oh P, Lisanti MP, Lodish HF. Flotillin and epidermal surface antigen define a new family of caveolae-associated integral membrane proteins. J Biol Chem. 1997; 272:13793–13802. [PubMed: 9153235]

Browman DT, Hoegg MB, Robbins SM. The SPFH domain-containing proteins: more than lipid raft markers. Trends Cell Biol. 2007; 17:394–402. [PubMed: 17766116]

Christopherson KS, Ullian EM, Stokes CC, Mullowney CE, Hell JW, Agah A, Lawler J, Mosher DF, Bornstein P, Barres BA. Thrombospondins are astrocyte-secreted proteins that promote CNS synaptogenesis. Cell. 2005; 120:421–433. [PubMed: 15707899]

Craig AM, Banker G. Neuronal polarity. Annu Rev Neurosci. 1994; 17:267–310. [PubMed: 8210176]

Eroglu C, Allen NJ, Susman MW, O'Rourke NA, Park CY, Ozkan E, Chakraborty C, Mulinyawe SB, Annis DS, Huberman AD, Green EM, Lawler J, Dolmetsch R, Garcia KC, Smith SJ, Luo ZD, Rosenthal A, Mosher DF, Barres BA. Gabapentin receptor alpha2delta-1 is a neuronal thrombospondin receptor responsible for excitatory CNS synaptogenesis. Cell. 2009; 139:380–392. [PubMed: 19818485]

Frick M, Bright NA, Riento K, Bray A, Merrified C, Nichols BJ. Coassembly of flotillins induces formation of membrane microdomains, membrane curvature, and vesicle budding. Curr Biol. 2007; 17:1151–1156. [PubMed: 17600709]

Glebov OO, Bright NA, Nichols BJ. Flotillin-1 defines a clathrin-independent endocytic pathway in mammalian cells. Nat Cell Biol. 2006; 8:46–54. [PubMed: 16341206]

Goritz C, Mauch DH, Pfrieger FW. Multiple mechanisms mediate cholesterol-induced synaptogenesis in a CNS neuron. Mol Cell Neurosci. 2005; 29:190–201. [PubMed: 15911344]

Hazarika P, Dham N, Patel P, Cho M, Weidner D, Goldsmith L, Duvic M. Flotillin 2 is distinct from epidermal surface antigen (ESA) and is associated with filopodia formation. J Cell Biochem. 1999; 75:147–159. [PubMed: 10462713]

Hering H, Lin CC, Sheng M. Lipid rafts in the maintenance of synapses, dendritic spines, and surface AMPA receptor stability. J Neurosci. 2003; 23:3262–3271. [PubMed: 12716933]

Hughes EG, Elmariah SB, Balice-Gordon RJ. Astrocyte secreted proteins selectively increase hippocampal GABAergic axon length, branching, and synaptogenesis. Mol Cell Neurosci. 2010; 43:136–145. [PubMed: 19850128]

Karasinska JM, Rinninger F, Lutjohann D, Ruddle P, Franciosi S, Kruit JK, Singaraja RR, Hirsch-Reinshagen V, Fan J, Brunham LR, Bissada N, Ramakrishnan R, Wellington CL, Parks JS, Hayden MR. Specific loss of brain ABCA1 increases brain cholesterol uptake and influences neuronal structure and function. J Neurosci. 2009; 29:3579–3589. [PubMed: 19295162]

Ko J, Kim S, Chung HS, Kim K, Han K, Kim H, Jun H, Kaang BK, Kim E. SALM synaptic cell adhesion-like molecules regulate the differentiation of excitatory synapses. Neuron. 2006; 50:233–245. [PubMed: 16630835]

Lang DM, Lommel S, Jung M, Ankerhold R, Petrausch B, Laessing U, Wiechers MF, Plattner H, Stuermer CA. Identification of reggie-1 and reggie-2 as plasmamembrane-associated proteins which cocluster with activated GPI-anchored cell adhesion molecules in non-caveolar micropatches in neurons. J Neurobiol. 1998; 37:502–523. [PubMed: 9858255]

Langhorst MF, Jaeger FA, Mueller S, Sven HL, Luxenhofer G, Stuermer CA. Reggies/flotillins regulate cytoskeletal remodeling during neuronal differentiation via CAP/ponsin and Rho GTPases. Eur J Cell Biol. 2008; 87:921–931. [PubMed: 18722032]

Lingwood D, Simons K. Lipid rafts as a membrane-organizing principle. Science. 2010; 327:46–50. [PubMed: 20044567]

Mauch DH, Nagler K, Schumacher S, Goritz C, Muller EC, Otto A, Pfrieger FW. CNS synaptogenesis promoted by glia-derived cholesterol. Science. 2001; 294:1354–1357. [PubMed: 11701931]

Mayor S, Pagano RE. Pathways of clathrin-independent endocytosis. Nat Rev Mol Cell Biol. 2007; 8:603–612. [PubMed: 17609668]

Morrow IC, Parton RG. Flotillins and the PHB domain protein family: rafts, worms and anaesthetics. Traffic. 2005; 6:725–740. [PubMed: 16101677]

Munderloh C, Solis GP, Bodrikov V, Jaeger FA, Wiechers M, Malaga-Trillo E, Stuermer CA. Reggies/flotillins regulate retinal axon regeneration in the zebrafish optic nerve and differentiation of hippocampal and N2a neurons. J Neurosci. 2009; 29:6607–6615. [PubMed: 19458231]

Myme CI, Sugino K, Turrigiano GG, Nelson SB. The NMDA-to-AMPA ratio at synapses onto layer 2/3 pyramidal neurons is conserved across prefrontal and visual cortices. J Neurophysiol. 2003; 90:771–779. [PubMed: 12672778]

Neumann-Giesen C, Falkenbach B, Beicht P, Claasen S, Luers G, Stuermer CA, Herzog V, Tikkanen R. Membrane and raft association of reggie-1/flotillin-2: role of myristoylation, palmitoylation and oligomerization and induction of filopodia by overexpression. Biochem J. 2004; 378:509–518. [PubMed: 14599293]

Nong Y, Huang YQ, Salter MW. NMDA receptors are movin' in. Curr Opin Neurobiol. 2004; 14:353–361. [PubMed: 15194116]

Pike LJ. Rafts defined: a report on the Keystone Symposium on Lipid Rafts and Cell Function. J Lipid Res. 2006; 47:1597–1598. [PubMed: 16645198]

Riento K, Frick M, Schafer I, Nichols BJ. Endocytosis of flotillin-1 and flotillin-2 is regulated by Fyn kinase. J Cell Sci. 2009; 122:912–918. [PubMed: 19258392]

Sans N, Prybylowski K, Petralia RS, Chang K, Wang YX, Racca C, Vicini S, Wenthold RJ. NMDA receptor trafficking through an interaction between PDZ proteins and the exocyst complex. Nat Cell Biol. 2003; 5:520–530. [PubMed: 12738960]

Schulte T, Paschke KA, Laessing U, Lottspeich F, Stuermer CA. Reggie-1 and reggie-2, two cell surface proteins expressed by retinal ganglion cells during axon regeneration. Development. 1997; 124:577–587. [PubMed: 9053333]

Solis GP, Hoegg M, Munderloh C, Schrock Y, Malaga-Trillo E, Rivera-Milla E, Stuermer CA. Reggie/flotillin proteins are organized into stable tetramers in membrane microdomains. Biochem J. 2007; 403:313–322. [PubMed: 17206938]

Suzuki S, Kiyosue K, Hazama S, Ogura A, Kashihara M, Hara T, Koshimizu H, Kojima M. Brain-derived neurotrophic factor regulates cholesterol metabolism for synapse development. J Neurosci. 2007; 27:6417–6427. [PubMed: 17567802]

Suzuki T. Lipid rafts at postsynaptic sites: distribution, function, and linkage to postsynaptic density. Neurosci Res. 2002; 44:1–9. [PubMed: 12204288]

Swanwick CC, Shapiro ME, Vicini S, Wenthold RJ. Flotillin-1 mediates neurite branching induced by synaptic adhesion-like molecule 4 in hippocampal neurons. Mol Cell Neurosci. 2010 in press, doi: 10.1016/j.mcn.2010.06.012.

Swanwick CC, Shapiro ME, Yi Z, Chang K, Wenthold RJ. NMDA receptors interact with flotillin-1 and -2, lipid raft associated proteins. FEBS Lett. 2009; 583:1226–1230. [PubMed: 19298817]

Volonte D, Galbiati F, Li S, Nishiyama K, Okamoto T, Lisanti MP. Flotillins/cavatellins are differentially expressed in cells and tissues and form a hetero-oligomeric complex with caveolins in vivo. Characterization and epitope-mapping of a novel flotillin-1 monoclonal antibody probe. J Biol Chem. 1999; 274:12702–12709. [PubMed: 10212252]

Wang CY, Chang K, Petralia RS, Wang YX, Seabold GK, Wenthold RJ. A novel family of adhesion-like molecules that interacts with the NMDA receptor. J Neurosci. 2006; 26:2174–2183. [PubMed: 16495444]

Wang PY, Seabold GK, Wenthold RJ. Synaptic adhesion-like molecules (SALMs) promote neurite outgrowth. Mol Cell Neurosci. 2008; 39:83–94. [PubMed: 18585462]

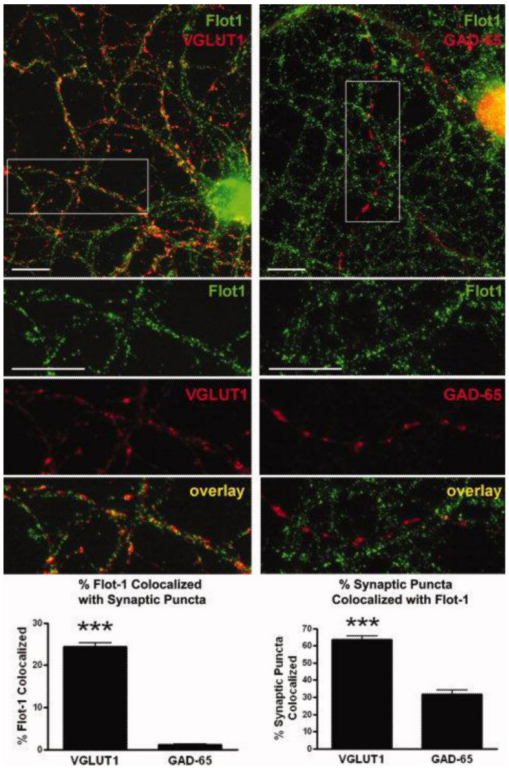

Figure 1. Flot-1 is preferentially localized at glutamatergic synapses

The synaptic localization of flot-1 was examined using vesicular glutamate transporter (VGLUT1) as a glutamatergic synaptic marker and the 65 kDa isoform of glutamic acid decarboxylase (GAD-65) as a GABAergic synaptic marker. Double-label immunocytochemistry of flot-1 (left, green) and VGLUT1 (left, red) shows numerous puncta colocalized (left, yellow). In contrast, double-labeling for flot-1 (right, green) and GAD-65 (right, red) reveals few puncta colocalized (right, yellow). Quantification of either the percentage of flot-1 (left) or synaptic marker (right) colocalized confirms that flot-1 is preferentially localized to glutamatergic synapses (*** = $p < 0.0001$, student's t-test, n = 18 neurons each). All images captured at 60X. Scale bar = 10 mm. Insets illustrated below images.

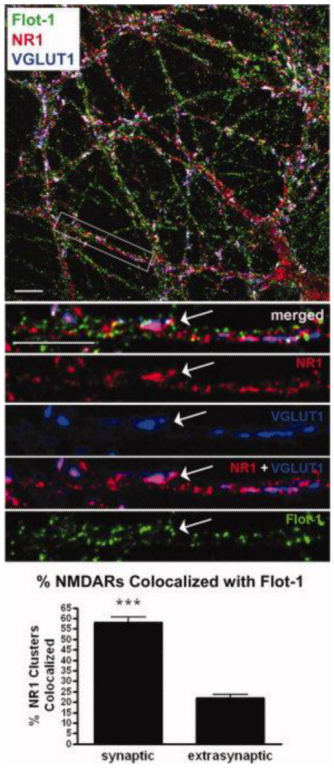

Figure 2. Flot-1 is preferentially localized with synaptic NMDARs compared to extrasynaptic NMDARs

The synaptic localization of the flot-1/NMDAR interaction was explored using triple-label immunocytochemistry of native flot-1 (green), NR1 (red), and vesicular glutamate transporter 1 (VGLUT1, blue) in cultured hippocampal neurons. Synaptic NMDARs were defined by NR1 clusters that overlapped with VGLUT1 puncta. Although most flot-1 puncta were extrasynaptic, most of these puncta did not overlap with extrasynaptic NR1 clusters. In contrast, a majority of synaptic NR1 clusters were colocalized with flot-1 puncta. When quantified, a larger percentage of synaptic NR1 clusters compared to extrasynaptic NR1 clusters were colocalized with flot-1 puncta (*** = $p < 0.0001$, student's t-test, n = 24 neurons each). Images captured at 100X. Arrow marks examples of colocalization of flot-1 with synaptic NMDAR clusters. Scale bar = 5 μm. Insets illustrated below images.

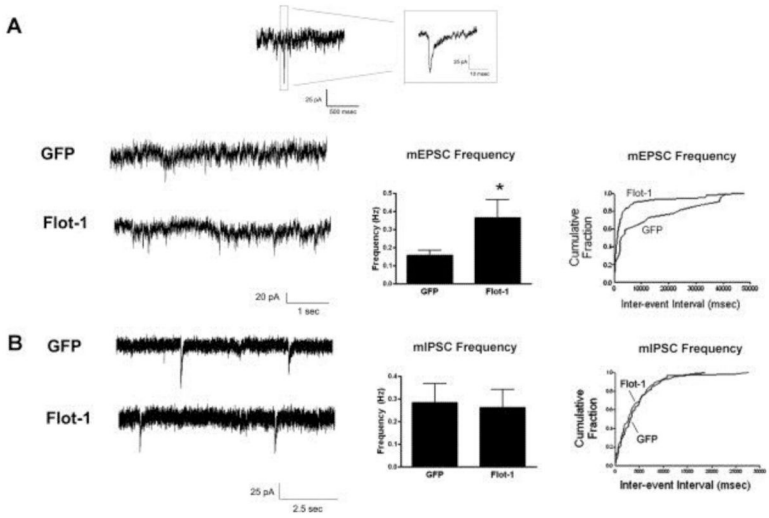

Figure 3. Flot-1 increases the frequency of mEPSCs but not mIPSCs

Whole-cell patch clamp electrophysiology was used to record miniature excitatory postsynaptic currents (mEPSCs) or miniature inhibitory postsynaptic currents (mIPSCs) in cultured hippocampal neurons transfected with either GFP or flot-1-GFP from 13-15 DIV. A) Neurons expressing flot-1-GFP exhibited more mEPSCs, represented by downward spikes in the trace (left). When quantified, the frequency of mEPSCs approximately doubled with flot-1 overexpression (middle, $p < 0.05$, student's t-test, $n = 13$-14 neurons each), and typical cumulative probability density functions show that a higher number of mEPSCs had shorter inter-event intervals with flot-1 overexpression than with GFP alone (right). B) Frequencies of mIPSCs from neurons overexpressing flot-1 were similar to those with GFP alone.

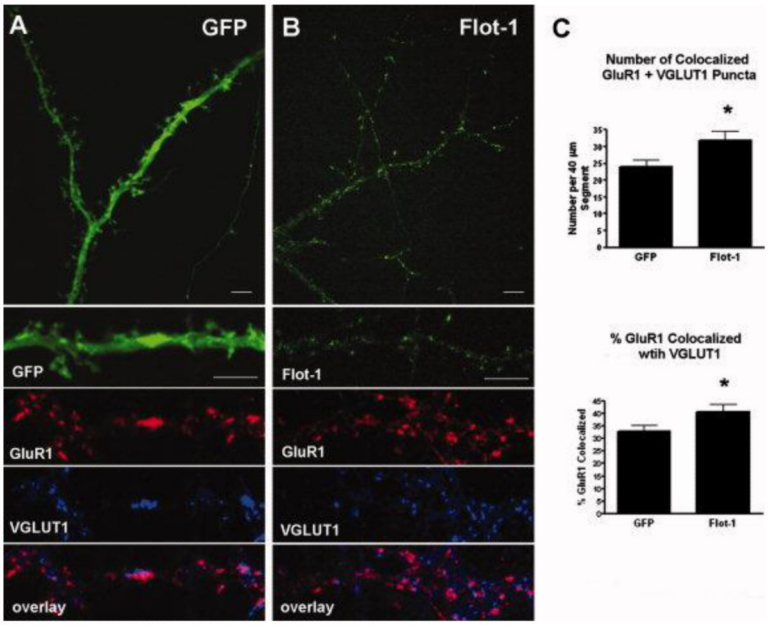

Figure 4. Flot-1 increases the number of glutamatergic synapses

Cultured hippocampal neurons were transfected with GFP alone or flot-1-GFP and double-labeled for presynaptic and postsynaptic glutamatergic markers. Vesicular glutamate transporter 1 (VGLUT1) was used as a presynaptic glutamatergic marker and the AMPA receptor subunit GluR1 was used as a postsynaptic marker. A) Neurons expressing GFP (green) had many clusters of GluR1 (red) and VGLUT1 (blue). When these images were merged, a fraction of GluR1 clusters were colocalized with VGLUT1 (pink), but many GluR1 clusters remained noncolocalized, in extrasynaptic locations. B) Neurons expressing flot-1-GFP (green) also contained many GluR1 clusters (red) and VGLUT1 puncta (blue), but when merged, a higher percentage of GluR1 clusters were colocalized with VGLUT1 puncta (pink) compared to neurons expressing GFP alone. C) When quantified, both the number of colocalized GluR1 and VGLUT1 puncta per 40 µm segment (top) and the percentage of GluR1 clusters colocalized with VGLUT1 puncta increased with flot-1 overexpression ($p < 0.05$ each, student's t-test, n = 29-30 neurons each). Images captured at 100X. Scale bar = 10 mm.