Activity-Dependent Netrin-1 Secretion Drives Synaptic Insertion of GluA1-Containing AMPA Receptors in the Hippocampus

Highlights

- Activity-dependent secretion of netrin-1 occurs at dendrites of excitatory neurons
- Neuronal netrin-1 is required for long-term potentiation of synaptic transmission
- Netrin-1 is sufficient to trigger NMDAR-independent accumulation of GluA1 at synapses
- Netrin-1 promotes maturation of immature or nascent synapses

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In Brief

Glasgow et al. demonstrate depolarization- and NMDAR-dependent secretion of netrin-1 from the dendrites of hippocampal neurons. They report that genetic deletion of netrin-1 from excitatory neurons impairs hippocampal synaptic plasticity and that netrin-1 application is sufficient to trigger the potentiation of excitatory synaptic transmission via the insertion of GluA1 AMPARs.
Activity-Dependent Netrin-1 Secretion Drives Synaptic Insertion of GluA1-Containing AMPA Receptors in the Hippocampus

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SUMMARY

Dynamic trafficking of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptors (AMPARs) to synapses is critical for activity-dependent synaptic plasticity underlying learning and memory, but the identity of key molecular effectors remains elusive. Here, we demonstrate that membrane depolarization and N-methyl-D-aspartate receptor (NMDAR) activation triggers secretion of the chemotropic guidance cue netrin-1 from dendrites. Using selective genetic deletion, we show that netrin-1 expression by excitatory neurons is required for NMDAR-dependent long-term potentiation (LTP) in the adult hippocampus. Furthermore, we demonstrate that application of exogenous netrin-1 is sufficient to trigger the potentiation of excitatory glutamatergic transmission at hippocampal Schaffer collateral synapses via Ca²⁺-dependent recruitment of GluA1-containing AMPARs, promoting the maturation of immature or nascent synapses. These findings identify a central role for activity-dependent release of netrin-1 as a critical effector of synaptic plasticity in the adult hippocampus.

INTRODUCTION

Glutamatergic synaptic transmission in the adult brain is primarily mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid glutamate receptors (AMPARs), with changes in synaptic strength due to alterations in both synaptic structure and receptor composition (Bosch and Hayashi, 2012; Kessels and Malinow, 2009). Long-term potentiation (LTP), an intensively studied experimental model of synaptic plasticity and memory, modifies postsynaptic function through N-methyl-D-aspartate receptor (NMDAR)-dependent recruitment of AMPARs, cytoskeletal reorganization, and modification of synaptic adhesion (Bosch et al., 2014; Kasai et al., 2010). Netrin-1 is a secreted protein that directs axon outgrowth and synaptogenesis during development (Goldman et al., 2013; Kennedy et al., 1994; Serafini et al., 1994). The netrin receptor deleted in colorectal cancer (DCC) directs cell motility by regulating intracellular calcium, RhoGTPases, Src family kinases, focal adhesion kinase, phospholipase C, phosphoinositol 3-kinase, p21-activated kinase, and local protein synthesis, all of which influence adhesion, cytoskeletal organization, and synaptic function (Lai Wing Sun et al., 2011). During neural development, restricted sources of netrin-1 direct the rapid local recruitment of synaptic proteins (Colón-Ramos et al., 2007; Goldman et al., 2013; Poon et al., 2008). Furthermore, a form of synaptic consolidation in Aplysia neurons, studied in primary cell culture, requires netrin-1-dependent local protein synthesis, suggesting a critical role for netrin-1 in the modification of synaptic function (Kim and Martin, 2015). In the mature mammalian brain, netrin-1 and DCC are enriched at synapses, and DCC co-fractionates with detergent-resistant components of the postsynaptic density (PSD). Selective deletion of DCC from excitatory forebrain neurons in the mature brain reduces dendritic spine volume, severely attenuates LTP at Schaffer collateral hippocampal synapses, and impairs hippocampal-dependent learning and memory (Horn et al., 2013). Several families of ligands have been identified for DCC, including draxins, cerebellins, and netrins (Ahmed et al., 2011; Wei et al., 2012); however, the enrichment of netrin-1 at synapses suggests that it may be a key ligand for DCC to regulate synaptic function.

Here, we show that activity-dependent secretion of netrin-1 from neurons regulates synaptic transmission in the adult hippocampus. Selective genetic deletion of netrin-1 from principal...
excitatory neurons severely attenuates NMDAR-dependent LTP in the adult mammalian hippocampus. Furthermore, application of exogenous netrin-1 to Schaffer collateral synapses is sufficient to induce a lasting potentiation of evoked synaptic responses through the recruitment of GluA1-containing, GluA2-lacking AMPARs. Taken together, our study identifies the activity-dependent secretion of netrin-1 as a critical component of LTP expression downstream of NMDAR activation.

RESULTS

Netrin-1 Is Secreted in Response to Neuronal Depolarization and NMDAR Activation

Netrin-1 is highly enriched in intracellular vesicles in synaptosomes (Horn et al., 2013), suggesting that action potential generation and neuronal depolarization might regulate netrin-1 release at synapses. To initially examine if netrin-1 protein may be secreted from neurons in an activity-dependent manner, we collected and concentrated cell-culture supernatants from embryonic rat neurons (14 days in vitro [DIV]) that had been depolarized for 20 min with 20 mM KCl. Immunoblots of the concentrated supernatants revealed significantly increased levels of netrin-1 compared with supernatants from cultures treated with vehicle alone (Figure S1), indicating that netrin-1 is secreted in response to strong depolarization.

Sustained neuronal activity can mobilize vesicular release (Ryan and Smith, 1995). To determine if increased neuronal spiking induces secretion of netrin-1, we used the designer receptors exclusively activated by designer drugs (DREADD) approach to excite primary hippocampal neurons infected with AAV8-Syn-hM3D(Gq)-mCitrine. Bath application of the synthetic hM3D(Gq) agonist clozapine-N-oxide (CNO; 10 μM) triggered rapid depolarization and spiking of mCitrine-expressing neurons (Figures S1C and S1D). We detected a large increase in netrin-1 protein in supernatants from cultures expressing hM3D compared with untransfected control cultures following bath application of CNO (10 μM) for 1 hr (Figure 1A), demonstrating that prolonged spiking and depolarization of hippocampal neurons promotes secretion of netrin-1.

The neuronal specific promoter (synapsin) used in the DREADD experiments indicates that depolarization causes netrin-1 to be released by neurons. However, whether netrin-1 is released perisynaptically by axons or dendrites remained unresolved. To reveal the subcellular localization of netrin-1 secretion, we developed a full-length functional netrin-1 fused to super-ecliptic pHluorin (NTN1-SEP) that increases fluorescence intensity upon shifting from acidic to neutral pH following vesicle fusion with the plasma membrane (Figures S2A and S2B). Co-expression of NTN1-SEP and the synaptic marker Homer1C-DsRed in cultured rat hippocampal neurons (14 DIV) revealed NTN1-SEP positive puncta along dendrites at synaptic (Homer 1C-DsRed positive) and extrasynaptic sites (Figure 1B), in a distribution similar to endogenous netrin-1 (Figures S2C–S2E). Fluorescence was quenched by a brief pulse of acidic solution (pH 5.5), confirming that it corresponds to extrasynaptic secreted NTN1-SEP. Netrin-1 secreted from dendrites should be initially present in an intra-dendritic endosomal pool. Intracellular distributions of NTN1-SEP were detected by applying an extracellular NH4Cl solution, alkalinizing the intracellular compartment and revealing large clusters of NTN1-SEP throughout dendritic shafts and spines (Figures 1C and 1D).

To address whether netrin-1 secretion is constitutive or mobilized in response to synaptic activity, we performed wide-field fluorescence video imaging of NTN1-SEP in neurons co-transfected with Homer1C-DsRed. To detect exocytotic events, pre-existing cell-surface fluorescence was first photobleached (Yudowski et al., 2007). We then monitored the spontaneous appearance of new bright puncta that reached full fluorescence amplitude, within one or two imaging time points, consistent with rapid exocytosis of NTN1-SEP (Figures 1E and 1F). We observed that NTN1-SEP fluorescence decay frequently lasted for minutes, consistent with NTN1-SEP remaining extracellularly at synapses following secretion (Figures 1G and 1H).

Figure 1. Netrin-1 Is Secreted by Neurons in Response to Depolarization and NMDAR Activation

(A) Western blots (left) and group data (right) of concentrated media supernatants and cell lysates of cultured hippocampal neurons (14 DIV) expressing hM3D(Gq) and treated with vehicle (black) or depolarized with CNO (10 μM, blue) for 1 hr (control supernatant, 1.00 ± 0.11; CNO supernatant, 1.51 ± 0.17; n = 4, t0 = 2.60, p = 0.04, independent-samples t test, n = 4 independent cultures per condition). Note that an Alexa 633-conjugated secondary antibody (40 ng/tube) was added to the medium as a loading control.

(B) Low (left) and high (right) magnification of NTN1-SEP (green) appears as puncta at synaptic sites (Homer1C-DsRed-positive; red) in cultured hippocampal neurons (14 DIV).

(C) Representative segment of a hippocampal dendrite expressing NTN1-SEP (green) and Homer1C-DsRed (red) during baseline (0 min), following exposure to extracellular solution at pH 5.5 (14 min), recovery (18 min), and bath application of NH4Cl (32 min).

(D) Group data show decrease in NTN1-SEP fluorescence intensity following bath application of extracellular solution at pH 5.5 (orange line) and an increase at intra-dendritic sites following bath application of NH4Cl (blue line; n = 7 cells from two independent cultures).

(E) High magnification of Homer1C-DsRed (red) positive puncta showing NTN1-SEP (green).

(F) Time lapse of a Homer1C-DsRed puncta (red; in E) prior to and following a NTN1-SEP exocytotic event.

(G) Kymograph of NTN1-SEP fluorescence of a Homer1C-DsRed puncta (dashed line in F).

(H) Raw group data (average in black, SEM in gray) and fitted curve (red) show amplitude and duration of NTN1-SEP exocytotic events.

(I) Time-lapse images of dendritic segment shown in (E) during baseline (top) and following cLTP induction (bottom). Note the NTN1-SEP exocytotic events (white arrows).

(J) Group data show frequency of exocytotic NTN1-SEP events in during baseline and following cLTP induction (baseline, 0.020 ± 0.005; cLTP, 0.055 ± 0.011; t1 = 2.56, p = 0.02, paired-samples t test, n = 14 dendritic segments from five independent cultures).

(K) Group data show frequency of NTN1-SEP exocytosis during baseline and following cLTP induction in the presence of NMDAR antagonist APV (50 μM) (APV, 0.019 ± 0.005; cLTP, 0.025 ± 0.007; t8 = 1.70, p = 0.14, n = 7 dendritic segments from two independent cultures, paired-samples t test).

Data are shown as mean ± SEM.
NMDAR activation is critical for several major forms of activity-dependent synaptic plasticity (Bliss and Collingridge, 2013). To determine if NMDAR activation drives netrin-1 secretion, we monitored the dynamic appearance of NTN1-SEP fluorescent puncta in hippocampal neurons following chemical LTP (cLTP), induced by applying an extracellular solution of 0 mM Mg$^{2+}$, 200 μM glycine, and 30 μM bicuculline that promotes NMDAR conductance and potentiates AMPAR-mediated currents (Lemieux et al., 2012; Lu et al., 2001). Inducing cLTP increased the frequency of NTN1-SEP exocytosis and promoted rapid secretion of long-lasting NTN1-SEP puncta (Figures 1I and 1J; Video S1). To examine whether the increase in frequency of NTN1-SEP exocytosis requires NMDAR activation, we repeated these experiments in the presence of 2-amino-5-phosphonovaleric acid (APV; 50 μM), a potent and specific NMDAR antagonist. cLTP stimulation in the presence of APV failed to increase the frequency of NTN1-SEP exocytotic events (Figure 1K), indicating that the increase in secretion requires NMDAR activation. Together, these findings demonstrate that NMDAR activation promotes netrin-1 secretion from dendrites.
CA1 Neurons in the Adult Hippocampus Express Netrin-1

RNA sequencing (RNA-seq) analysis previously detected netrin-1 mRNA in adult mouse hippocampal CA1 neurons (Cembrowski et al., 2016). Using a validated netrin-1-specific monoclonal antibody (Bin et al., 2015), immunohistochemical analyses of hippocampal sections from Thy1-GFP L15 mice revealed a punctate distribution of endogenous netrin-1 protein across all layers of the CA1 region, including strata oriens, pyramidale, and radiatum (Figure S3A). These findings indicate that ntn1 is expressed by hippocampal neurons and that endogenous netrin-1 protein can be detected in all layers of the adult hippocampus.

The subcellular distribution of netrin-1 was then assessed throughout stratum radiatum in adult Thy1-GFP L15 mice using super-resolution confocal microscopy (Figures 2A, S3A, and S3B). The apical dendrites of CA1 pyramidal neurons, visualized by membrane-targeted GFP expression, show punctate endogenous netrin-1 immunoreactivity throughout the neuropil (Figures S3A and S3B). Immunostaining for netrin-1 shows clusters of endogenous protein throughout the dendritic shafts and spines. Many of these netrin-1 clusters are contained within dendritic spines, as revealed by opaque surface renderings of the membrane-targeted GFP (Figure 2A). Consistent with NTN1-SEP live imaging (Figure 1), these results localize endogenous netrin-1 protein within dendrites and spine heads of CA1 pyramidal neurons.

Netrin-1 Is Necessary for LTP at Schaffer Collateral Synapses

High-frequency stimulation of the Schaffer collaterals induces NMDAR-dependent LTP in the adult hippocampus (Malenka and Bear, 2004). Because NMDAR activation by cLTP stimulation increases NTN1-SEP exocytosis at synaptic sites, we hypothesized that the endogenous release of netrin-1 may contribute to LTP induced by high-frequency stimulation of the Schaffer collateral hippocampal pathway. To test this, we selectively deleted a floxed ntn1 allele from excitatory neurons by Cre expression regulated by the CaMKIIα promoter (T29-CaMKIIα-Cre/NTN1fl/fl). Cre recombinase is first expressed ~2.5 weeks postnatally in these mice and is detected throughout all subfields of the hippocampus, exclusively in glutamatergic neurons, by 1 month of age. Critically, expression occurs after developmental axon guidance is complete (Tsien et al., 1996). Hippocampal homogenates from 3- to 6-month-old T29-CaMKIIα-Cre-NTN1fl/fl (NTN1 cKO) mice showed reduced levels of netrin-1 protein compared with control littermates (Cre-negative NTN1fl/fl), consistent with deletion of ntn1 expression from excitatory forebrain neurons (Figure 2B). Furthermore, immunohistochemical analyses demonstrated that netrin-1 immunoreactivity is substantially reduced in stratum radiatum of NTN1 cKO mice compared with control littermates (Figure S3B).

Previous work has suggested that DCC may function as a dependence receptor, and therefore reducing the level of netrin-1 might increase apoptosis (Forcet et al., 2001). However, levels of cleaved caspase-3 were not found to be significantly different between 3- and 6-month-old NTN1 cKO mice and control littermates (Figure S3C).

To determine if netrin-1 contributes to basal synaptic transmission, we measured evoked excitatory postsynaptic currents (eEPSCs) from CA1 pyramidal neurons following Schaffer collateral stimulation of acute hippocampal slices from 3- to 6-month-old NTN1 cKO mice and control littermates in the presence of picrotoxin (PTX; 100 μM) to block GABAA-mediated synaptic transmission. We detected no significant differences in AMPAR or NMDAR eEPSC amplitudes, paired-pulse ratios, rectification, and AMPAR-to-NMDAR ratios, indicating that basal evoked synaptic transmission was not affected by deletion of ntn1 (Figures S3D–S3J). To assess whether conditional deletion of ntn1 impaired the relative strength of individual synapses, we recorded AMPAR-mediated miniature EPSCs (mEPSCs) in CA1 pyramidal neurons from control and NTN1 cKO brain slices in the presence of PTX (100 μM) and tetrodotoxin (TTX; 1 μM) (Figure S4A). The amplitude of mEPSCs was slightly but significantly reduced in CA1 pyramidal neurons from NTN1 cKO compared with littermate controls, whereas no changes were detected in mEPSC event or instantaneous frequency (Figures S4B–S4G).

These results provide evidence that conditional deletion of ntn1 from principal excitatory neurons leads to a modest weakening of synapses onto CA1 pyramidal neurons.

To assess whether netrin-1 may contribute to NMDAR-dependent LTP, we recorded eEPSCs in CA1 pyramidal neurons from adult NTN1 cKO mice and control littermates in the presence of the GABA A receptor antagonist PTX (100 μM). Brief high-frequency stimulation (HFS; 1 s at 100 Hz) resulted in significant short-term facilitation of eEPSCs in CA1 pyramidal neurons from both NTN1 cKO and control littermates. The amplitude of eEPSCs remained significantly potentiated in slices from control littermates after 20–25 min compared with baseline values. However, the amplitude of synaptic potentiation was significantly attenuated in slices from NTN1 cKO mice (Figures 2D and 2E). Moreover, we observed significant increases in AMPAR-mediated current and AMPAR-to-NMDAR ratio in slices from control littermates following tetanic stimulation, but not in slices from NTN1 cKO mice (Figures 2F–2H). These findings demonstrate that deletion of ntn1 from principal excitatory neurons significantly attenuates NMDAR-dependent LTP and indicate that netrin-1 plays a central role in synaptic plasticity in the adult hippocampus.

Netrin-1 Potentiates CA3-CA1 Schaffer Collateral Synapses via an NMDAR-Independent Postsynaptic Mechanism

Genetic studies in C. elegans provided initial evidence that netrin directs the local enrichment of synaptic proteins during synaptogenesis (Colón-Ramos et al., 2007; Poon et al., 2008; Stavee and Colón-Ramos, 2012). In mammalian neurons, synaptic proteins, such as PSD95, are rapidly recruited to synaptic sites in response to a local source of netrin-1 (Goldman et al., 2013). The impaired LTP detected in T-29-CaMKIIα-Cre/NTN1fl/fl mice suggested that netrin-1 secreted by neurons may be a critical effector of activity-dependent LTP in the adult hippocampus that functions in parallel to or downstream of NMDAR activation. To determine if netrin-1 might be sufficient by itself to elicit changes in synaptic strength, we assessed netrin-1 gain of function by perfusing hippocampal slices from adult wild-type...
C57/B6 mice (2–3 months old) with exogenous recombinant ne-trin-1. Schaffer collateral-evoked AMPAR-mediated eEPSCs were measured using whole-cell patch-clamp recordings from CA1 pyramidal neurons held at $-70\text{ mV}$ in the presence of PTX (100 nM). Bath perfusion of netrin-1 (100–200 ng/mL, 5 min) without tetanic stimulation elicited a dose-dependent potentiation of synaptic responses that persisted for at least 40 min (Figure 3A).

To determine if netrin-1 potentiation of eEPSCs was associated with facilitation of presynaptic release, we assessed the paired-pulse ratio across a range of inter-stimulus intervals (10–200 ms ISI) prior to and 20 min after netrin-1 application. We detected no change, consistent with the lack of an effect on presynaptic release properties (Figure 3B). Furthermore, individual paired comparisons failed to detect any change in paired-pulse ratio. These findings suggest that exogenous netrin-1 facilitates AMPAR-mediated synaptic currents in CA1 pyramidal neurons independently of changes in presynaptic glutamate release.

Glutamatergic synapses can be potentiated by the postsynaptic recruitment of AMPARs (Kessels and Malinow, 2009). We found that AMPAR-mediated current (measured at $-70\text{ mV}$) was significantly increased following perfusion of netrin-1, whereas NMDAR-mediated current (measured at +40 mV, 50 ms post-tetanus) was unchanged, resulting in an increased AMPAR-to-NMDAR current ratio (Figures 3C–3F and S5). These findings provide evidence that the addition of netrin-1 is sufficient to rapidly and selectively facilitate glutamatergic synaptic transmission.
transmission in the hippocampus via the postsynaptic insertion of AMPARs into hippocampal synapses.

AMPAR insertion into immature spines can lead to synaptic maturation and contribute to potentiation of synaptic responses in the hippocampus (Lisman and Raghavachari, 2006; Morita et al., 2013). To determine if bath application of netrin-1 increases the number of functional synapses by recruiting immature synapses, we recorded AMPAR-mediated mEPSCs in CA1 pyramidal neurons from adult mouse acute hippocampal slices in the presence of tetrodotoxin (1 µM) and picrotoxin (100 µM) before and after 5 min bath application of netrin-1 (200 ng/ml). (B and C) Cumulative distribution plots (left) and group data (right) of mEPSC amplitude (B) (16.5 ± 1.1 pA in baseline versus 16.4 ± 1.3 pA following netrin-1, t2 = 0.35, p = 0.73, paired-samples t test) and frequency (C) (4.2 ± 1.8 Hz in baseline versus 7.1 ± 1.7 Hz following netrin-1, t8 = 3.37, p < 0.01, paired-samples t test). n = 9 cells from five mice. Kolmogorov-Smirnov test for cumulative distribution data.

(D–F) Synaptic responses evoked by minimal stimulation (raw traces in gray, averaged in black, D) and individual response amplitudes (E) during baseline and 20 min following netrin-1 (blue in E). Group data show a significant decrease in failure rate following bath application of netrin-1 (200 ng/mL) (F) (64 ± 11% in baseline versus 24 ± 11% following netrin-1, t5 = 3.46, p = 0.018, paired-samples t test; n = 6 cells from six mice).

Data are shown as mean ± SEM.

Figure 4. Netrin-1 Recruits New Synapses in CA1 Pyramidal Neurons during Activity-Dependent Long-Term Potentiation

(A) Representative voltage-clamp recordings of mEPSCs from CA1 pyramidal neurons held at −70 mV in an acute adult mouse hippocampal slice in the presence of tetrodotoxin (1 µM) and picrotoxin (100 µM) before and after 5 min bath application of netrin-1 (200 ng/ml).
Exogenous Netrin-1 Occludes Activity-Induced LTP

The rapid and long-lasting potentiation of synaptic responses after bath application of netrin-1, as well as the attenuation of potentiation induced by HFS in hippocampal slices from in T-29-CaMKIIα-Cre/NTN1<sup>fl/fl</sup> mice, suggests that activity-dependent secretion of netrin-1 may contribute to the expression of LTP induced by HFS. To assess whether netrin-1 is linked mechanistically with HFS-induced LTP, we first examined the effect of netrin-1 on previously potentiated synapses. Following a 5 min baseline period, LTP was induced by brief HFS (1 s at 100 Hz). Synaptic responses were significantly potentiated compared with baseline values and resulted in a significant increase in the AMPAR-to-NMDAR ratio, consistent with insertion of AMPARs at Schaffer collateral synapses in the adult mouse brain following HFS. Bath application of netrin-1 failed to elicit additional enhancement of AMPAR-mediated evoked synaptic responses, nor alter AMPAR-to-NMDAR ratios compared with post-HFS levels (Figures 5A–5E). These data indicate that activity-dependent HFS LTP can successfully occlude netrin-1-mediated potentiation.

We then assessed whether netrin-1-induced potentiation can occlude the expression of LTP induced by HFS. Bath application of exogenous netrin-1 resulted in rapid potentiation of synaptic responses. Twenty minutes following the application of netrin-1, we attempted to induce LTP using HFS. A single burst

(8E) Group data show that HFS results in potentiation of AMPAR-mediated synaptic responses (B) but does not occlude further potentiation by 5 min netrin-1 application (C) (218 ± 34% of baseline following HFS versus 290 ± 51% following netrin-1, F<sub>2,23</sub> = 21.84, p < 0.001, one-way repeated-measures [RM]-ANOVA; pairwise comparisons, baseline versus HFS: p = 0.001, baseline versus netrin-1: p = 0.002, HFS versus netrin-1: p = 0.106). NMDAR-mediated EPSCs were not significantly changed between all conditions (D) (105 ± 12% of baseline following HFS versus 120 ± 15% following netrin-1, F<sub>2,23</sub> = 1.05, p = 0.374, one-way RM-ANOVA), whereas AMPAR-to-NMDAR ratios were significantly elevated following both HFS and netrin-1 (E) (baseline: 2.8 ± 0.7; netrin-1: 6.6 ± 1.5; F<sub>2,23</sub> = 11.01, p = 0.006, one-way RM-ANOVA; pairwise comparisons, baseline versus HFS: p = 0.018, baseline versus netrin-1: p = 0.029, HFS versus netrin-1: p = 0.311, n = 8 cells from five mice).

(F) Representative traces of evoked synaptic responses during baseline (1, black), 25 min following bath application of netrin-1 (2, blue, 5 min at 200 ng/mL), and following HFS (3, red, 1 s at 100 Hz, arrow in (G)).

(G) Bath application of netrin-1 (blue) leads to significant potentiation of AMPAR-mediated synaptic responses but that HFS (1 s at 100 Hz, arrow) does not further increase evoked amplitude.

(H) Group data show AMPAR-mediated synaptic responses following application of netrin-1 and following HFS (201 ± 26% of baseline following netrin-1 versus 237 ± 31% of baseline following HFS, F<sub>2,13</sub> = 11.33, p < 0.001, one-way RM-ANOVA; pairwise comparisons, baseline versus netrin-1: p = 0.001, baseline versus HFS: p < 0.001, netrin-1 versus HFS: p = 0.92, n = 6).

(I) NMDAR-mediated synaptic responses were not significantly affected by netrin-1 (99 ± 8% of baseline following netrin-1, 98 ± 8% following HFS, F<sub>2,13</sub> = 1.57, p = 0.233).

(J) AMPAR-to-NMDAR ratio was significantly elevated following netrin-1 but was not further increased following HFS (baseline: 3.9 ± 0.5; netrin-1: 7.5 ± 1.4; HFS: 8.5 ± 1.2; F<sub>2,13</sub> = 17.28, p < 0.001; baseline versus netrin-1: p = 0.004, baseline versus HFS: p < 0.001, netrin-1 versus HFS: p = 0.39, pairwise comparisons); n = 14 cells from nine mice. All pairwise comparisons using Tukey’s multiple comparison test.

Data are shown as mean ± SEM.

Figure 5. Netrin-1 Occludes HFS LTP in the Adult Hippocampus

(A) Representative traces of EPSCs during baseline (1, black), 20 min following HFS (2, red, 1 s at 100 Hz arrow in B), and following 5 min bath application of netrin-1 (200 ng/mL) (3, blue).
Figure 6. Netrin-1 Potentiation of AMPA Current Requires Increased Intracellular Ca\(^{2+}\) and CaMKII Activation Independent of NMDAR Function

(A–C) Evoked synaptic responses in CA1 pyramidal neurons from acute mouse hippocampal slices after bath application of netrin-1 (200 ng/mL, blue) in the presence of APV (A) (50 \(\mu\)M, purple; \(n = 9\) cells from five mice). Representative traces (B) and group data (C) prior to (1 in A) and following bath application of netrin-1 (2 in A) in the presence of APV (259 ± 53% of baseline; \(t_8 = 4.66, p = 0.001\), paired-samples t test).

(D) Effect of netrin-1 (D, blue bar) on synaptic responses in CA1 pyramidal neurons from acute mouse hippocampal slices from aged DCC cKO (>6 months old CaMKII\(^{a-Cre/DCC^{fl/fl}}\), red, \(n = 7\) cells from five mice), young DCC cKO (4–6 weeks old CaMKII\(^{a-Cre/DCC^{fl/fl}}\), green, \(n = 5\) cells from three mice), and control aged littermates mice (>6 months old CaMKII\(^{a-Cre/neg/DCC^{fl/fl}}\), black, \(n = 7\) cells from five mice).

(E) Group data show that netrin-1 increases amplitude of AMPAR-mediated current in aged littermates and young DCC cKO mice but not in aged DCC cKO (aged control littermate: 200 ± 33% of baseline, \(p = 0.002\); young DCC cKO: 153 ± 15% of baseline, \(p = 0.04\); aged DCC cKO: 89 ± 8% of baseline, \(p = 0.44\); two-way RM-ANOVA: interaction between genotype \(\times\) LTP: \(F_{2,17} = 5.54, p = 0.014\)).

(F) AMPAR-to-NMDAR ratio was significantly increased in aged littermates and young DCC cKO following bath application of netrin-1 but not in aged DCC cKO (aged control littermate: 3.0 ± 0.6 in baseline versus 5.5 ± 0.9 following netrin-1, \(p = 0.001\); young DCC cKO: 2.1 ± 0.2 in baseline versus 3.8 ± 0.4 following netrin-1, \(p = 0.001\)).

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of HFS resulted in a brief increase in eEPSC amplitude, consistent with a post-tetanic short-term facilitation of Schaffer collateral inputs. However, by 15 min after HFS, we observed no significant potentiation compared with pre-tetanus eEPSC amplitudes (Figures 5F–5J). This occlusion suggests that the application of exogenous netrin-1 saturates a mechanism required for HFS-induced LTP. Failure to induce LTP may also have been due to whole-cell dialysis at the induction time point. We therefore applied HFS 30 min after whole-cell break-in and found that it was sufficient to induce LTP (Figure S6). Together, these findings support the conclusion that HFS promotes netrin-1 secretion, which in turn activates downstream effectors that recruit AMPARs to hippocampal synapses.

Netrin-1 Facilitates Synaptic Responses via NMDAR-Independent Increases in Intracellular Ca\(^{2+}\) Levels and Activation of CaMKII

NMDAR activation triggers activity-dependent LTP of AMPAR currents (Bliss and Collingridge, 2013). To assess the role of NMDARs in netrin-1 potentiation of synaptic responses, we bath-applied exogenous netrin-1 in the presence of the NMDAR antagonist APV (50 μM). Netrin-1 resulted in strong potentiation of evoked AMPAR-mediated current (Figures 6A–6C), indicating that NMDAR activation is not required for netrin-1 potentiation of synaptic responses. Taken together with the demonstration that NMDAR-dependent cLTP stimulation increases netrin-1 secretion, we conclude that NMDAR activation promotes the release of netrin-1 and that netrin-1 triggers downstream mechanisms that are sufficient to potentiate synaptic responses independent of NMDAR activation.

Netrin-1 binding to DCC mediates axonal chemoattraction during development (Keino-Masukata et al., 1996; Lai Wing Sun et al., 2011). To determine if netrin-1 potentiation requires DCC, we deleted a floxed dcc allele from glutamatergic neurons in the adult forebrain by generating T-29-CaMKIIα-Cre/DCCfl/fl mice (DCC cKO) (Horn et al., 2013). Bath perfusion of netrin-1 potentiated eEPSCs in acute slices from control littersmates and young 4- to 6-week-old DCC cKO mice that still express high levels of residual DCC but failed to potentiate eEPSCs in CA1 pyramidal neurons from 6-month-old adult DCC cKO mice (Figures 6D and 6E). Additionally, although bath application of netrin-1 increased AMPAR-mediated current and AMPAR-to-NMDAR ratios in control littersmates and 4- to 6-week-old DCC cKO mice, no increase was detected in aged DCC cKO (Figure 6F). We conclude that netrin-1 potentiation of Schaffer collateral inputs requires DCC.

Both NMDAR and DCC activation result in Ca\(^{2+}\) influx and increased intracellular Ca\(^{2+}\) concentration, which in turn can promote changes in AMPAR trafficking via Ca\(^{2+}\)/calmodulin kinase II (CaMKII) activation (Hayashi et al., 2000; Opazo et al., 2010). To assess the contribution of increased intracellular Ca\(^{2+}\) to netrin-1 potentiation, we included the Ca\(^{2+}\) chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA; 20 mM) in the intracellular recording pipette. BAPTA completely blocked netrin-1 potentiation of AMPAR-mediated synaptic responses and the increase in AMPAR-to-NMDAR ratio (Figures 6G–6J), indicating that intracellular Ca\(^{2+}\) is critical for netrin-1 potentiation.

CaMKII activation and stargazin phosphorylation are required to trap and accumulate GluA1-containing AMPARs at synapses (Opazo et al., 2010). Bath application of netrin-1 resulted in increased CaMKII phosphorylation in hippocampal slice homogenates (Figure 6K), suggesting that netrin-1-mediated activation of CaMKII may potentiate synaptic responses. Consistent with this, bath application of the CaMKII inhibitor KN62 (5 μM) completely blocked netrin-1-induced increases in the amplitude of evoked AMPAR-mediated currents and AMPAR-to-NMDAR ratio in acute hippocampal slices (Figures 6L–6O). These results support the conclusion that netrin-1 activates CaMKII and that CaMKII activation is required for netrin-1 potentiation of evoked responses.

Netrin-1 Potentiation Is Mediated via Postsynaptic Accumulation of GluA1-Containing Receptors

Increases in intracellular Ca\(^{2+}\) can trigger AMPAR recruitment to synapses (Hayashi et al., 2000). AMPARs are composed of homo- or heteromeric configurations of four different subunits (GluA1–4), with Q/R-edited GluA2-containing receptors lacking Ca\(^{2+}\) permeability (Burnashev et al., 1992; Holllmann et al., 1991). Early stages of LTP require interactions mediated by the “TGL” PDZ domain binding motif at the C terminus of the GluA1 AMPAR subunit (Hayashi et al., 2000). However, recent studies suggest that multiple forms of AMPARs contribute to this type of synaptic plasticity (Granger et al., 2013). We assessed the subunit composition of AMPARs inserted into the plasma membrane 20 min following application of netrin-1 (200 ng/mL, 5 min) using cell-surface biotinylation of isolated

\[
\text{netrin-1}, p = 0.005; \text{aged DCC cKO: } 2.8 \pm 0.5 \text{ in baseline versus } 2.7 \pm 0.3 \text{ following netrin-1, } p = 0.91; \text{two-way RM-ANOVA: interaction between genotype } \times \text{ LTP: } F_{2,17} = 6.22, p = 0.009. \\
\text{G–J): Effect of netrin-1 (blue) on average synaptic responses (G) and representative evoked EPSCs (H) recorded in CA1 pyramidal neurons in acute mouse hippocampal slices with intracellular BAPTA (20 mM, dark blue, } n = 6 \text{ cells from four mice). Group data show that netrin-1 did not significantly potentiate AMPAR-mediated synaptic responses (J) (118 \pm 22\% of baseline, } t_6 = 0.66, p = 0.54, \text{paired-samples } t \text{ test) following netrin-1 (200 ng/mL, 5 min, blue bar). Group data show no significant increase in AMPAR-mediated current (N) (95 \pm 7\% of baseline, } t_6 = 0.73, p = 0.49, \text{paired-samples } t \text{ test) or AMPAR-to-NMDAR ratio (O) (3.2 \pm 0.6 in baseline versus } 3.5 \pm 0.6 \text{ following netrin-1, } t_6 = 0.56, p = 0.59, \text{paired-samples } t \text{ test) following netrin-1 (200 ng/mL, 5 min, blue bar). Data are shown as mean } \pm \text{ SEM.}
\]
Figure 7. Synaptic Recruitment of Ca\(^{2+}\)-Permeable GluA1-Containing AMPA Receptors by Netrin-1

(A and B) Western blots (left) and group data (right) of GluA1 (A) (306 ± 73% of control, \(t_2 = 2.81, p = 0.04\), independent-samples t test) and GluA2 (B) (145 ± 49% of control, \(t_2 = 0.45, p = 0.44\), independent-samples t test) surface membrane distribution in acute hippocampal slices from adult mice with (blue) or without (black) 5 min bath application of netrin-1 (200 ng/mL; \(n = 4\) slices per mouse, \(n = 3\) mice per condition).

(C) Representative traces of synaptic rectification experiments during baseline (black) and following netrin-1 (200 ng/mL; blue).

(D) Effect of netrin-1 on I-V curve of AMPAR-mediated synaptic responses.

(E) Group data show rectification index during baseline and following netrin-1 (2.7 ± 0.7 in baseline versus 7.7 ± 1.9 following netrin-1, \(t_4 = 3.13, p = 0.03\), paired-samples t test). \(n = 5\) cells from three mice.

(F–I) Effect of netrin-1 (200 ng/mL; blue) on Schaffer collateral-evoked synaptic response in CA1 pyramidal neurons from acute adult hippocampal slices during continuous bath application of the CP-AMPAR antagonist IEM1460 (F) (50 \(\mu\)M, purple bar; \(n = 7\) cells from four mice). Representative evoked EPSCs (G) and group data show AMPAR-mediated current (H) (112 ± 22% of baseline, \(t_6 = 0.01, p = 0.99\), paired-samples t test) and AMPAR-to-NMDAR ratio (I) (3.5 ± 0.9 in baseline versus 3.3 ± 0.9 following netrin-1, \(t_6 = 0.16, p = 0.87\), paired-samples t test) in the presence of IEM1460 following bath application of netrin-1.

(legend continued on next page)
acute adult hippocampal slices. Bath application of netrin-1 significantly increased the amount of cell surface GluA1 compared with control slices from the same animal that were not exposed to netrin-1 (Figure 7A). In contrast, levels of biotinylated GluA2 were not different between conditions (Figure 7B). These findings indicate that netrin-1 selectively increases the amount of GluA1-containing, GluA2-lacking AMPARs at the neuronal surface.

GluA2-lacking AMPARs composed of hetero- or homomeric combinations of GluA1, GluA3, and GluA4 subunits are permeable to Ca\(^{2+}\) and characterized by pronounced inward rectification due to blockade by endogenous polyamines (Jonas and Burnashev, 1995). Bath application of netrin-1 in the presence of GABA\(_A\) and NMDAR antagonists significantly enhanced rectification of AMPAR-mediated currents at depolarized voltages (Figures 7C–7E). To determine if netrin-1-induced potentiation is mediated by Ca\(^{2+}\)-permeable AMPARs (CP-AMPARs), netrin-1 was co-applied with the CP-AMPAR blocker IEM1460 (50 μM) (Burnashev et al., 1992). Blockade of CP-AMPARs eliminated the netrin-1-induced potentiation of synaptic responses and blocked the increases in AMPAR-mediated current and AMPAR-to-NMDAR ratio (Figures 7F–7I). These findings are consistent with GluA2-lacking CP-AMPARs underlying the netrin-1-induced synaptic potentiation, likely engaging the incorporation of hetero- or homotetramers of GluA1. To test this, we then blocked the incorporation of GluA1-containing AMPARs by including the competitive peptide Pep1-TGL (100 μM) in the intracellular solution (Edelmann et al., 2015; Hayashi et al., 2000). Pep1-TGL, which prevents SAP97 binding to the “TGL” PDZ-binding motif at the GluA1 C terminus, completely blocked netrin-1 potentiation of AMPAR-mediated current (Figures 7J–7L). To determine if netrin-1 promotes GluA1 synaptic localization, we co-expressed super-ecliptic pHluorin-tagged GluA1 (SEP-GluA1) and the postsynaptic marker Homer1C-DsRed in cultured rat hippocampal neurons (14 DIV). Bath application of netrin-1 (200 ng/mL) triggered a rapid increase in SEP-GluA1 fluorescence at a subset of synapses compared with cultures not treated with netrin-1 (Figures 7M and 7N; Videos S2 and S3). Moreover, bath application of netrin-1 in the presence of peptide Pep1-TGL caused an increase in the number of SEP-GluA1 positive synapses (“up” puncta) while decreasing the number of SEP-GluA1 negative synapses (“down” puncta) compared with control cultures without netrin-1 application (Figure 7O). Together, these findings indicate that netrin-1 promotes the insertion of GluA1-containing AMPARs at a subset of synaptic sites in hippocampal pyramidal neurons.

### DISCUSSION

Netrin-1 is essential for normal embryonic neural development, directing cell migration, axon guidance, and synaptogenesis. Neuronal expression and enrichment at synapses in the adult CNS suggested that netrin-1 might also influence the plasticity of mature neural circuits. Here, we demonstrate that netrin-1 expressed by neurons plays a critical role in the modification of glutamatergic transmission at Schaffer collateral synapses between CA3 and CA1 pyramidal neurons in the adult mammalian hippocampus. Our findings reveal that netrin-1 is secreted by neurons in response to depolarization and NMDAR activation. We demonstrate that neuronal expression of netrin-1 in principal forebrain neurons is critical for activity-dependent LTP, that brief application of exogenous netrin-1 triggers long-lasting synaptic potentiation, and that netrin-1-induced synaptic potentiation occludes LTP induced by HFS. Furthermore, we show that netrin-1-induced potentiation enhances glutamatergic synaptic transmission through DCC-mediated recruitment of GluA1-containing AMPARs to hippocampal synapses in a CaMKII-dependent manner. These findings reveal an essential role for neuronal secretion of netrin-1 in glutamatergic synaptic plasticity in the adult hippocampus that is critical for classic activity-dependent LTP.

### Netrin-1 Contributes to Activity-Dependent LTP

Long-lasting changes in synaptic strength in the adult hippocampus depend on activation of postsynaptic NMDARs (Herron et al., 1986). Here, we report that netrin-1 is enriched in dendrites (Figure 2) and that NMDAR activation can result in netrin-1 exocytosis (Figure 1), suggesting a role in activity-dependent synaptic plasticity in the hippocampus. Consistent with this, conditional loss of netrin-1 from excitatory hippocampal neurons results in attenuated expression of LTP with no change in short-term plasticity following HFS (Figure 2). Short-term facilitation of synaptic responses following HFS is mediated through changes in presynaptic Ca\(^{2+}\) that enhance transmitter exocytosis (Regehr, 2012). In contrast, changes in the distribution of postsynaptic receptors can be detected within 5–10 min after HFS, consistent with the time course observed following exogenous application of netrin-1. Moreover, our findings indicate that following LTP-inducing stimulation, the NMDAR-mediated Ca\(^{2+}\) influx mobilizes the secretion of netrin-1 from dendrites at or near synaptic sites (Figure 1). We propose that netrin-1 then activates postsynaptic signaling mechanisms such as CaMKII, independent of NMDAR activation, that locally recruit GluA1-containing CP-AMPARs to excitatory synapses, resulting in postsynaptic potentiation of synaptic transmission.

**(A–L)** Inhibition of GluA1 insertion with intracellular Pep1-TGL (100 μM, yellow bar, n = 8 cells from four mice) blocks netrin-1 potentiation of synaptic responses (J). Representative evoked EPSCs (K) and group data (L) show mean AMPAR-mediated current during baseline and following netrin-1 in neurons loaded with Pep1-TGL (I) (93 ± 9% of baseline, t = 0.13, p = 0.89, paired-samples t test).

**(M)** Sample images of dendritic segments expressing both SEP-GluA1 (left, green on right) and Homer1C-DsRed (middle, red on right) during baseline (top) and after netrin-1 (200 ng/mL, bottom) in dissociated rat hippocampal neurons. Overlay (right) shows SEP-GluA1 insertion at Homer1C-DsRed sites (white arrows). Scale bar, 5 μm.

**(N)** Average SEP-GluA1 fluorescence variation at Homer1C-DsRed puncta for each neuron (control ΔF\(_{\text{median}}\) = −0.0364 versus netrin-1 ΔF\(_{\text{median}}\) = 0.0226, n = 12 dendritic segments from five independent cultures). Wilcoxon test for paired observations: p < 0.05.

**(O)** Circular plots show proportion of synaptic states (blue, “down”; black, “stay”; red, “up”; see STAR Methods) after netrin-1 compared with control sister cultures. Data are shown as mean ± SEM.

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Activity-Dependent Secretion of Netrin-1 Enhances Synaptic Transmission through Maturation of Immature Synapses

Intracellular vesicles isolated from adult rat brain synaptosomes contain readily detectable netrin-1 and DCC. Patches of DCC immunoreactivity decorate dendritic spines, and DCC is associated with a detergent-resistant component of PSD (Horn et al., 2013). Postsynaptic expression of LTP requires soluble N-ethylmaleimide-sensitive factor-attachment proteins (SNAPs), which promote vesicle exocytosis in a t-SNARE-dependent manner (Gu et al., 2016; Liedo et al., 1998). DCC interacts with the t-SNARE synaptin-1 and v-SNARE Ti-VAMP. However, it remains to be demonstrated that netrin-1 activation of DCC in dendritic spines is required for SNARE function during activity-dependent AMPAR recruitment.

Netrin-1 is secreted from Homer1C-positive sites along dendrites in response to plasticity-inducing stimulation (Figure 1). We also observed exocytotic events at sites lacking Homer1C-DsRed. Homer1C is an adaptor for PSD-enriched proteins, where it regulates glutamatergic synaptic transmission through interactions with Group I metabotropic glutamate receptors (mGluR1/5) (Brakeman et al., 1997). Recent evidence indicates that ~20% of excitatory synapses in the hippocampus lack the PSD proteins necessary for synaptic stabilization (Berry and Nedivi, 2017) and that these non-PSD-containing synapses exhibit relatively low AMPAR-to-NMDAR ratios (Lambert et al., 2017; Zito et al., 2009). Bath application of netrin-1 potently increases the AMPAR-to-NMDAR ratio (Figure 3), indicating that netrin-1 secretion promotes insertion of GluA1-containing AMPARs, recruiting these receptors to immature synapses, possibly including those that initially lack PSD proteins. This conclusion is further supported by the demonstration that challenging cultured rat pyramidal neurons with an immobilized netrin-1 coated micro-bead is sufficient to rapidly recruit PSD-95 (Goldman et al., 2016; Lledo et al., 1998) and promotes synaptic maturation (El-Husseini et al., 2000).

Previous work suggests that HFS LTP selectively recruits GluA1-containing CP-AMPARs and increases AMPAR single channel conductance (Benke et al., 1998; Zhou et al., 2018). Consistent with increases in immature synapses in the absence of netrin-1, conditional deletion resulted in significantly decreased amplitude of mEPSCs compared with control littermates, suggesting that lack of netrin-1 results in reduced AMPAR density at synapses (Figure S4). In contrast, following bath application of exogenous netrin-1 to acute hippocampal slices from wild-type mice, we observed an ~2-fold increase in mEPSC frequency, with no appreciable change in mEPSC amplitude. These findings suggest that netrin-1 may recruit CP-AMPARs to immature synapses to serve as “placeholders” for subsequent synaptic stabilization. Indeed, previous work has suggested that LTP can lead to increases in mEPSC frequency (Oliet et al., 1996) by increasing the density of mature synapses (Kerchner and Nicoll, 2008). Therefore, netrin-1 may facilitate the maturation of immature synapses by promoting the incorporation of transient GluA1-containing AMPARs, which may be replaced with GluA2-containing AMPARs by synaptic local protein synthesis (Kim and Martin, 2015; Plant et al., 2006; Sutton et al., 2006).

AMPA trafficking is a key process for LTP expression in the hippocampus and is regulated in part through NMDAR-mediated increases in intracellular Ca\(^{2+}\) and CaMKII activation (Lee et al., 2009). We report that netrin-1 increases phosphorylation of CaMKII and that pharmacological inhibition of CaMKII impairs netrin-1-induced potentiation of evoked synaptic responses (Figure 6), indicating that CaMKII activation is necessary for netrin-1 potentiation. However, we cannot rule out the possibility that non-CaMKII-dependent processes may also contribute to AMPAR subunit reorganization (Yasuda et al., 2003). Interestingly, LTP results in enlargement of thin-type dendritic spines, which are largely devoid of AMPARs under control conditions (Bélique et al., 2006; Matsuoka et al., 2001). Increases in thin-type spine volume are dependent, in part, on activation of CaMKII and RhoGTPases including RhoA and Cdc42 (Murakoshi et al., 2011). Netrin-1 binding to DCC, which is critical for netrin-1-mediated potentiation of AMPAR currents (Figure 6), directs cytoskeletal reorganization in developing neuronal growth cones by regulating Cdc42, Rac1, and RhoA (DeGeer et al., 2013; Moore et al., 2008; Shekarabi et al., 2005). However, it remains to be determined how netrin-1 may contribute to AMPAR trafficking and structural modification of dendritic spines associated with LTP in the adult hippocampus.

We have demonstrated that netrin-1 is enriched in the stratum radiatum of adult mice and is detected intracellularly within dendritic shafts and spines of CA1 pyramidal neurons (Figure 2). Our findings suggest that NMDAR-dependent exocytosis of netrin-1 during activity-dependent LTP promotes recruitment of Ca\(^{2+}\)-permeable GluA1-containing AMPARs at immature or nascent synapses. Although certain forms of activity-dependent plasticity do not require GluA1 receptor insertion (Granger et al., 2013), trafficking of GluA1-containing AMPARs is dependent on increases in intracellular Ca\(^{2+}\) and is critical for LTP in the adult hippocampus (Zhou et al., 2018). Application of netrin-1 is sufficient to trigger a local increase in the concentration of intracellular Ca\(^{2+}\) in developing neurons (Hong et al., 2000; Wang and Poo, 2005). We propose that NMDAR-mediated synaptic Ca\(^{2+}\) transients in dendritic spines facilitate netrin-1 release, which in turn locally increases intracellular Ca\(^{2+}\) from internal stores to activate CaMKII, and promote the focal insertion and synaptic trapping of GluA1-containing AMPARs. Together, our findings indicate that NMDAR-mediated increases in intracellular Ca\(^{2+}\) promote the activity-dependent secretion of netrin-1, which functions as a critical effector of long-term synaptic plasticity.

**STAR METHODS**

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○ Cell surface biotinylation and phosphorylation assays in acute brain slice
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SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and three videos and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.09.028.

ACKNOWLEDGMENTS

We thank Wayne Sossin and the members of the Kennedy, Ruthazer, Seguela, and De Koninck labs for comments on drafts of the manuscript. We also thank Francine Nault, Charleen Salesse, Nathalie Marcal, Yi Jiang, and Hanan Eilam for technical assistance. S.D.G. was supported by a graduate scholarship from the Natural Sciences and Engineering Research Council (NSERC) of Canada. J.G. was supported by a postdoctoral fellowship from FRQS. The project was supported by grants from NSERC (P.S., RGPIN-2015-04876; P.D.K., RGPIN-2017-06171; and E.S.R., EQPEQ-458696-2014), CIHR (R.A.M., MOP-86724; P.S., PJT-153098; P.D.K., PJT-153107; E.S.R., FDN-143238; and T.E.K., PJT-366649 and PJT-114965), and the Alzheimer Society of Canada (T.E.K.). E.S.R. holds a FRQs Research Chair (FRQs-31038).

AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: April 26, 2017
Revised: May 30, 2018
Accepted: September 6, 2018
Published: October 2, 2018

REFERENCES


## STAR METHODS

### KEY RESOURCES TABLE

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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Timothy E. Kennedy (timothy.kennedy@mcgill.ca).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The study was conducted in accordance with the guidelines of the Canadian Council for Animal Care and approved by the McGill University and Université Laval Animal Care Committees. All animals were housed in group housing, and provided ad libitum access to food and water. For experiments using wild-type animals, C57/B6 mice (8-12 weeks) were used (Charles River, St Constant, Canada). Thy1-GFP (line 15) mice were used in immunohistochemical experiments, and express low levels of membrane-targeted EGFP (mGFP)-positive cells within the CA1 region of the hippocampus under the control of Thy1.2 promoter (De Paola et al., 2003). Floxed alleles of ntn1 (NTN1fl/fl, RRID: MGI:5755388) and dcc (DCCfl/fl) were generated in mice and maintained on a C57BL/6 genetic background as described (Bin et al., 2015; Horn et al., 2013). T29-1 CaMKIIa-Cre mice (JAX 005359, RRID:IMSR_JAX:005359) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and maintained on a C57BL/6 background. Both male and female T-29-CaMKIIa-Cre/netrin-1fl/fl (T-29-CaMKIIa-Cre/NTN1fl/fl > 3 months old) and T-29-CaMKIIa-Cre/DCCfl/fl (T-29-CaMKIIa-Cre/DCCfl/fl > 6 months old) mice were used for molecular biological and electrophysiology experiments. We observed no statistically significant differences between sexes, and therefore all data were pooled for analysis. Control experiments were performed using littermates that were negative for Cre and homozygous for floxed alleles of ntn1 and dcc.

METHOD DETAILS

Hippocampal neuronal culture
For cell biological assays, hippocampi from E18 or P1 Sprague Dawley rats were isolated, dissociated, plated and cultured as described (Goldman et al., 2013; Horn et al., 2013; Lavoie-Cardinal et al., 2016).

Immunohistochemistry
Immunohistochemistry was performed on free-floating sections derived from Thy1-GFP (line 15). Briefly, animals were deeply anesthetized by intraperitoneal injection of a mixture of 2,2,2 – tribromoethyl alcohol and tert-amyl alcohol diluted at 2.5% in PBS, and transcardially perfused with cold PBS (pH: 7.4) followed by 4% paraformaldehyde (PFA). Brains were removed and postfixed for 24 h in 4% PFA, followed by 24 h cryoprotection in 30% sucrose in PBS. Thirty μm sections were cut using a cryostat (Leica CM1850), and stored in PBS at 4°C.

Antigen retrieval was performed by boiling sections in 0.1M citrate buffer for 10 min, and allowing to cool to RT. Sections were then washed three times in PBS-T (0.3% Triton X-100), and blocked for 1.5 h in PBS-T containing 3% BSA. Brain sections were incubated sequentially overnight with rabbit monoclonal anti-netrin-1 (1:500, Abcam, RRID:AB_11131145) and chicken polyclonal anti-GFP.
For protein media concentration experiments, rat hippocampal neurons from E18 Sprague Dawley rats were plated at 2.5 × 10^5 cells and used at 14 DIV. For hM3D/CNO experiments, cells were infected at 6 DIV with pAAV8-hSyn-HA-hM3D(Gq)-IRES-mCitrine (MOI: 1:1000; Neurophotonics Molecular Tools Platform, Université Laval, Québec, QC, Canada). At 14 DIV, cells were washed 3x with warm (37°C) control ACSF containing (in mM): 135 NaCl, 3.5 KCl, 1.2 MgCl₂, 2 CaCl₂, 10 HEPES, and 20 D-Glucose (pH: 7.4, 300 mOsm). Cells were then incubated at 37°C in ACSF with or without clozapine-N-oxide (CNO, 10 μM) for 1 h. For KCl experiments (Figure S1), cells were washed 3x with serum-free warmed NeuroBasal media (NBM, ThermoFisher Scientific). Cells were then incubated in NBM supplemented with high KCl (20 mM) for 30 min at 37°C. Protease inhibitors (in 1 mg/ml aprotonin, 1 mg/ml leupeptin, 100 mM PMSF, and 0.5 M EDTA) were then added to the media, and cells and media were rapidly cooled to 4°C. Equal volumes of supernatant were collected into a 4 ml Amicon Ultra Centrifugal tube with Ultracel 10-membrane (EMD Millipore), and goat anti-chicken Alexa 633-conjugated secondary antibody (40 ng per tube) was added to ensure equal concentration. Control and experimental media tubes were then centrifuged at 5,000 × g for 15 min, and washed twice with cold PBS. Proteins were then eluted from beads using Laemmli sample buffer, and boiled for 5 min.

Protein media concentration

For protein media concentration experiments, rat hippocampal neurons from E18 Sprague Dawley rats were plated at 2.5 × 10^5 cells and used at 14 DIV. For hM3D/CNO experiments, cells were infected at 6 DIV with pAAV8-hSyn-HA-hM3D(Gq)-IRES-mCitrine (MOI: 1:1000; Neurophotonics Molecular Tools Platform, Université Laval, Québec, QC, Canada). At 14 DIV, cells were washed 3x with warm (37°C) control ACSF containing (in mM): 135 NaCl, 3.5 KCl, 1.2 MgCl₂, 2 CaCl₂, 10 HEPES, and 20 D-Glucose (pH: 7.4, 300 mOsm). Cells were then incubated at 37°C in ACSF with or without clozapine-N-oxide (CNO, 10 μM) for 1 h. For KCl experiments (Figure S1), cells were washed 3x with serum-free warmed NeuroBasal media (NBM, ThermoFisher Scientific). Cells were then incubated in NBM supplemented with high KCl (20 mM) for 30 min at 37°C. Protease inhibitors (in 1 mg/ml aprotonin, 1 mg/ml leupeptin, 100 mM PMSF, and 0.5 M EDTA) were then added to the media, and cells and media were rapidly cooled to 4°C. Equal volumes of supernatant were collected into a 4 ml Amicon Ultra Centrifugal tube with Ultracel 10-membrane (EMD Millipore), and goat anti-chicken Alexa 633-conjugated secondary antibody (40 ng per tube) was added to ensure equal concentration. Control and experimental media tubes were then centrifuged at 5,000 x g for 60 min. The resultant supernatant was concentrated at 200x, lysed in Laemmli sample buffer, and boiled for 5 min. Cells were harvested and processed as described above.

Cell surface biotinylation and phosphorylation assays in acute brain slice

Cell surface biotinylation and phosphorylation assays were performed as described (Gabriel et al., 2014). For surface biotinylation in brain slices, acute hippocampal slices from 2-3 month old C57/B6 mice were made using the same protocol as for electrophysiological experiments. Slices were then transferred to a recording chamber and continuously perfused with control ACSF. Exogenous netrin-1 (200 ng/ml) or chicken anti-HA (1:1000, Abcam, RRID:AB_1131145) was diluted in blocking solution and incubated for 2 h at room temperature. After washes in PBS, secondary antibodies (goat anti-rabbit STAR488, Abberior, 1:1000) was diluted in blocking solution and incubated for 45 min at room temperature. Coverslips were washed 2 × 5 min with PBS-T followed by 2 × 5 min in PBS and mounted on glass microscope slides.

Immunofluorescence images were obtained using either a Zeiss Axiovert (S100TV) with epifluorescence illumination at 40x (0.75 N.A.), or a Zeiss LSM 880 laser-scanning confocal microscope equipped with an Airyscan detection unit using a high NA oil immersion alpha Plan-Apochromat 100X/1.46 Oil DIC M27 (Zeiss) with a zoom adjustment to 1.8. All imaging was performed using Immerol 518 F immersion media (n = 1.518 (23°C); Carl Zeiss). Detector gain set to 700 and pixel dwell times were adjusted for each dataset keeping them at their lowest values in order to avoid saturation and bleaching effect. The pinhole was adjusted to have an AU of ~1.25 considering the axial position in tissue. The different fluorophores were acquired sequentially with appropriate filter conditions. The information is listed in the following as “fluorophore – excitation wavelength – dichroic mirror – detection filter.” Alexa Fluor 488 – 488 nm (Argon) – MBS488 – BP 495-550 + LP 570; CF 568 – 561 nm (DPSS 561-10) - MBS 458/561 – BP 495-550 + LP 570; Alexa Fluor 647 – 633 nm (HeNe633) – MBS 488/561/633 – BP 570-620 + LP 645. For processing Airyscan images, Zen Black 2.3 software was used to enhance the spatial resolution 1.7-fold of the diffraction limit by processing each of the 32 Airy detector channels separately with filtering, deconvolution and pixel reassignment. The visualization and rendering was processed with Imaris 8.1.2 (Bitplane AG, Zurich CH).

For images in Figure S2C-E, cultured hippocampal neurons were fixed for 10 min in 4% PFA solution (0.1 M phosphate buffer, 4% sucrose, and 2 mM EGTA, pH 7.4). Neurons were then washed once with PBS and twice with 0.1 M PBS/glycine and were permeabilized for 30 min in blocking solution (PBS, 2% normal goat serum, and 0.1% Triton X-100). Primary antibody against netrin-1 (1:500, Abcam, RRID:AB_1131145) was diluted in blocking solution and incubated for 2 h at room temperature. After washes in PBS, secondary antibodies (goat anti-rabbit STAR488, Abberior, 1:1000) was diluted in blocking solution and incubated for 45 min at room temperature. Coverslips were mounted in Prolong Gold Antifade mounting media (Thermofisher). Images were acquired on a confocal system (LSM700; Carl Zeiss) using a 63x 1.4 NA oil immersion objective.

Figure S1

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For phosphorylation assays, hippocampi were dissected in ACSF and harvested in RIPA buffer supplemented with protease and phosphatase inhibitors (1mM Na3VO4 and 1mM NaF), homogenized, and centrifuged for 10 min at 13,800 g. Supernatant was then collected, lysed in Laemmli sample buffer, and boiled for 5 min.

### Western blot analysis

Netrin-1 expression was assessed from hippocampal and cerebellar homogenates derived from 2-3 month old C57/B6 or 3 month old T-29-CaMKIix-Cre/NTN1<sup>Cre</sup> mice. Briefly, mice were deeply anaesthetized, and the brain was quickly extracted in ice-cold Ringer solution. Cerebellum, cortex, and hippocampi were dissected and isolated in cold DMEM (Life Technologies) supplemented with protease and phosphatase inhibitors, and homogenized in cold RIPA buffer containing (10 mM phosphate buffer [pH 7.2], 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS) with protease and phosphatase inhibitors (aprotinin 2 mg/ml, leupeptin 5 mg/ml, EDTA 2 mM, sodium orthovanadate 1 mM, sodium fluoride 1 mM, and PMSF 1 mM).

Extracts from protein media concentration supernatant, cell lysates, whole hippocampal homogenates, or hippocampal slice homogenates were assessed using Western protein assay (Pierce BCA kit, ThermoFisher Scientific). Equal protein levels were loaded and subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes, as described (Horn et al., 2013). Western blots were analyzed using mouse beta-III tubulin (1:1000; Sigma Aldrich, RRID:AB_477590), rabbit anti-active caspase 3 (1:1000; Abcam, RRID:AB_288785), rabbit anti-phospho-Thr185 (1:1000; PhosphoSolutions, RRID: AB_2492051). Blots were developed using Immunoblot Western Chemiluminescent HRP Substrate (Millipore). Densitometry and quantification of relative protein levels were performed on scanned images of immunoblots using Fiji image software (Schindelin et al., 2012).

### Optical imaging of NTN1-SEP

NTN1-SEP was generated by subcloning the SEP coding sequence (GenBank: AY533296.1) (Ng et al., 2002) lacking the initiating methionine in frame on the sequence encoding netrin-1 (Serafini et al., 1994) in plasmid eGFP-N3 (Clontech). For optical imaging of NTN1-SEP, rat hippocampal neurons were plated at a density of 75 cells / mm<sup>2</sup> on glass coverslips coated with poly-D-lysine and maintained in Neurobasal/B27 (Thermo Fisher Scientific). Neurons were transfected with plasmids encoding NTN1-SEP and Homer-DsRed using Lipofectamine 2000 (Thermo Fisher Scientific) at 12 DIV for NTN1-SEP imaging, as described (Hudmon et al., 2005). Neurons (13-14 DIV) were imaged at 32-35 °C in an open perfusion chamber (0.2-0.5 ml/min) (Warner Instruments) mounted on an Olympus IX-71 inverted microscope equipped with a 100X objective (N.A. = 1.49) with Topctica Chrome MLE laser sources, and a backlit thinned CCD (Princeton ProEm 512B-FT). Signals for NTN1-SEP and Homer-DsRed were discriminated using the following laser lines and Semrock filters: SEP, excitation 488nm, emission Brightline 520/35; Homer1C-DsRed, excitation 543nm, emission Brightline 617/73.

To image netrin-1 exocytosis, a segment of dendrite expressing NTN1-SEP and Homer1C-DsRed was selected, and subsequently photobleached to 30%–40% of the initial fluorescence by exposing the dendrite to approximately 40 s of high power laser (488nm) light. Movies of 1000 images, at 100 ms per image, were then acquired at lower laser power, with neurons in baseline extracellular solution (in mM: 102 NaCl, 5 KCl, 10 NaH<sub>2</sub>PO<sub>4</sub>, 1 MgCl<sub>2</sub>, 1.2 CaCl<sub>2</sub>, 10 Glucose, pH 7.3-7.4, 225-230 mOsm) followed by cLTP solution (same as baseline, except lacking Mg<sup>2+</sup>, and with 200 μM glycine and 30 μM bicuculline).

Image analysis was performed using a custom-made MATLAB (Mathworks Inc., Natick, USA) script allowing semi-automatic detection of exocytotic events. Based on any single exocytotic event occurring faster than the image integration time, we computed maps of the events by subtracting each image from the previous frame. Each map was then segmented to identify regions exhibiting a fluorescence increase, which were extracted to calculate the ΔF/F on 500 ms preceding the event and 1500 ms following. We then computed the area under the ΔF/F curve, using a detection threshold of 2.

### Brain slice in vitro electrophysiology

Acute horizontal brain slices containing the hippocampus were obtained from adult C57/B6 mice (8 to 12 weeks), and CaMKIix-Cre/NTN1<sup>Cre</sup> (3-6 months old) and age-matched control littermates. Mice were deeply anaesthetized by intraperitoneal injection of a mixture of 2,2,2-tribromoethanol and tert-amyl alcohol diluted at 2.5% in PBS, and transcardially perfused with ice-cold choline chloride-based solution containing (in mM): 110 choline-Cl, 1.25 NaH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 7 MgCl<sub>2</sub>, 0.5 CaCl<sub>2</sub>, 2.5 KCl, 7 glucose, 3 pyruvic acid, and 1.3 ascorbic acid, bubbled with carbogen (O<sub>2</sub> 95%, CO<sub>2</sub> 5%). The brain was rapidly removed, thick horizontal brain slices (300 μm) containing the hippocampus were cut using a vibrating microtome (VT1000s, Leica), and allowed to recover for 1h in artificial cerebrospinal fluid (ACSF) containing, in mM: 124 NaCl, 5 KCl, 1.25 NaH2PO<sub>4</sub>, 2 MgSO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 2 CaCl<sub>2</sub>, and 10 Glucose saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH 7.3-7.4, 300 mOsm) at room temperature (22–24 °C).

Individual brain slices were placed in a custom-built recording chamber, and continuously perfused with warmed (30 ± 2 °C) ACSF (TC324B, Warner Instruments). Voltage- recordings of CA1 hippocampal pyramidal neurons were performed on an upright microscope (Nikon Eclipse or Scientifica Slicescope 2000) equipped with a micromanipulator (Sutter MP-225 or Scientifica Patchstar),
a 40x or 60x water immersion objective (0.8 or 1.0 N.A., respectively), differential interference contrast optics, and coupled to a near-infrared charge-coupled device camera (NC70, MTI or SciCam, Scientifica). Borosilicate glass pipettes (Sutter Instruments) were pulled with resistances of 4–8 MΩ. The intracellular solution for voltage-clamp recordings was (in mM): 120 CsMeSO₄, 20 CsCl, 10 HEPES, 7 di-tris phosphocreatine, 2 MgCl₂, 0.2 EGTA, 4 Na₂ATP, 0.3 Tris-GTP, and in some experiments, 5 N-ethylidooxycaine chloride (QX-314) (pH 7.2-7.26, 280-290 mOsm). AMPAR-mediated and NMDAR-mediated synaptic responses were evoked using a bipolar platinum/iridium electrode (FHC, CE2C275) placed in the Schaffer collaterals, ~200 μm from the recorded cell. For synaptic experiments, a pair of 0.1 ms biphasic current pulses (50 ms ISI) was delivered via a stimulus isolation unit (Isolflex, A-MPI), and stimulus intensity was adjusted to evoke a response 65%–75% of the maximal response at 200 μA. After 10-20 min of stable baseline recording in the presence of PTX (100 μM, PTX), netrin-1 (200 ng/ml) was bath applied for 5 min, and synaptic responses monitored for 40 min post-application. AMPAR- and NMDAR-mediated currents were evoked at ~70 mV and +40 mV, respectively, during baseline conditions and 20 min post-bath application of netrin-1. Input-output tests were conducted using increasing stimulus intensity from 0-200 μA in 25 μA increments. Access and input resistances were continually monitored throughout the recording through a 50 ms, 5 mV voltage step 150 ms prior to synaptic stimulation, and data were discarded if series resistance changed > 20%.

To assess HFS LTP in both NTN1 cKO and control littermates (Figure 2), as well as in the netrin-1 occlusion experiments (Figure 5), the recording mode of the cell was changed from voltage-clamp to current-clamp after a 5-10 min baseline period. Potentiation was induced by a single episode of a 1 s 100 Hz stimulation in current-clamp mode, which can elicit potentiation of evoked EPSCs at ~150%–200% of baseline values. Following stimulation, the cell was returned to voltage-clamp recording mode and held at ~70 mV to record evoked responses.

For rectification experiments following bath application of exogenous netrin-1 (200 ng/ml), AMPAR-mediated currents were evoked in the presence of PTX (100 μM) and D-(-)-2-Amino-5-phosphonopentanoic acid (50 μM, D-APV) at a range of voltages (~110 mV to +50 mV in 20 mV intervals). APV was validated by measuring the lack of NMDAR-mediated currents at +40 mV. QX-314 (5 mM) was also included in the intracellular solution to prevent action potential generation. All responses were normalized to peak AMPAR-mediated current. Rectification index was computed as a ratio of current amplitude of responses evoked at ~70 mV and +50 mV.

Miniature excitatory postsynaptic currents (mEPSCs) were recorded in voltage-clamp mode at a holding potential of ~70 mV in the presence of PTX (100 μM) to block GABAₐ-mediated synaptic currents and tetrodotoxin (1 μM, TTX) to block sodium currents. Currents were analyzed using MiniAnalysis (Specter). Events were detected using a threshold of 7 pA (> 3 pA root mean square of baseline noise levels). Cumulative distribution plots were generated using an equal number of events per condition (70 pA per event), which were randomly selected from each cell, rank-ordered, and averaged across each condition.

An Axopatch 200B or 700B amplifier (Molecular Devices) were used for recordings, and signals were digitized (Digidata 1322A or 1550A, Molecular Devices). Voltage-clamp recordings were sampled at 10 kHz, filtered at 2 kHz, and acquired using pClamp software (v9.0 or v10.4, Molecular Devices).

**Hippocampal culture electrophysiology**

Whole cell patch clamp recordings were made from embryonic rat hippocampal neurons (14 DIV) prepared as described above. Neurons were plated on glass coverslips at high density, and individual coverslips transferred to an upright SliceScope 2000 (Scientific), and perfused with ACSF containing (in mM): 135 NaCl, 3.5 KCl, 1.2 MgCl₂, 2 CaCl₂, 10 HEPES, and 20 D-Glucose (pH: 7.4, 300 mOsm). Current clamp recordings were performed using pipettes filled with (in mM): 120 K-glucosone, 20 KCl, 10 HEPES, 7 phosphocreatine di-Tris, 2 MgCl₂, 0.2 ethylene-glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 4 Na₂ATP, 0.3 Na₂GTP (pH: 7.2-7.26, 280-290 mOsm). Access resistance was monitored throughout the recording, and cells were held at resting membrane potential. Current-clamp recordings were sampled at 20 kHz and filtered at 10 kHz using pClamp (v10.4, Molecular Devices).

**Pharmacology**

Stocks were prepared at 1000x by dissolving tetrodotoxin citrate (T-550, Alomone labs), D-APV (0106, Tocris), BAPTA (ab120449, Abcam), IEM-1460 (ab141507, Abcam), Pep1-TGL (1601, Tocris), and clozapine-N-oxide (ab141704, Abcam) in water, and PTX (P1675, Sigma-Aldrich) and KN62 (ab120421, Abcam) in DMSO. All other salts and drugs were from Sigma-Aldrich unless otherwise noted. For certain experiments, pharmacological agents were included in the intracellular patch solution (Figures 6G-J and 7J-L). In these cases, cells were dialyzed with pipette solution for at least 15 min before beginning baseline recordings.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analyses on parametric data were assessed using repeated-measures ANOVAs, pairwise comparisons (Tukey), within-samples paired t tests, and independent t tests where appropriate. Normality, homoscedasticity, and outlier tests were performed on all datasets. Non-Gaussian distributed datasets were tested by Wilcoxon test for paired observations or Friedman’s repeated-measures ANOVA on ranks. For mEPSC cumulative distribution data, comparisons were performed using the Kolmogorov-Smirnov test. Significant interactions and main effects were assessed using pairwise comparisons and compensated using the Tukey method.
for parametric data. Statistical significance was determined with $p \leq 0.05$ using two-tailed tests. All data are presented as mean ± SEM. Data were analyzed using Clampfit 10.3 (Axon Instruments), MATLAB (Mathworks), Fiji (Schindelin et al., 2012), Photoshop (Adobe), MiniAnalysis (Synaptosoft), Prism 7 (Graphpad), and Sigmaplot 11 (Systat). Plotted data were then formatted in Adobe Illustrator CS6 (Adobe Systems).

DATA AND SOFTWARE AVAILABILITY

The custom MATLAB scripts used in this study are accessible at https://github.com/PDKlab/Exocytose-EventsDetection.