

Protein 4.1N Plays a Cell Type-Specific Role in Hippocampal Glutamatergic Synapse Regulation

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Many glutamatergic synapse proteins contain a 4.1N protein binding domain. However, a role for 4.1N in the regulation of glutamatergic neurotransmission has been controversial. Here, we observe significantly higher expression of protein 4.1N in granule neurons of the dentate gyrus (DG granule neurons) compared with other hippocampal regions. We discover that reducing 4.1N expression in rat DG granule neurons of either sex results in a significant reduction in glutamatergic synapse function that is caused by a decrease in the number of glutamatergic synapses. By contrast, we find reduction of 4.1N expression in hippocampal CA1 pyramidal neurons has no impact on basal glutamatergic neurotransmission. We also find 4.1N's C-terminal domain (CTD) to be nonessential to its role in the regulation of glutamatergic synapses of DG granule neurons. Instead, we show that 4.1N's four-point-one, ezrin, radixin, and moesin (FERM) domain is essential for supporting synaptic AMPA receptor (AMPA) function in these neurons. Altogether, this work demonstrates a novel, cell type-specific role for protein 4.1N in governing glutamatergic synapse function.

Key words: 4.1N; AMPA receptor; dendritic spines; dentate gyrus; synapse

Significance Statement

Glutamatergic synapses exhibit immense molecular diversity. In comparison to heavily studied Schaffer collateral, CA1 glutamatergic synapses, significantly less is known about perforant path-dentate gyrus (DG) synapses. Our data demonstrate that compromising 4.1N function in CA1 pyramidal neurons produces no alteration in basal glutamatergic synaptic transmission. However, in DG granule neurons, compromising 4.1N function leads to a significant decrease in the strength of glutamatergic neurotransmission at perforant pathway synapses. Together, our data identifies 4.1N as a cell type-specific regulator of synaptic transmission within the hippocampus and reveals a unique molecular program that governs perforant pathway synapse function.

Introduction

Neurons are specialized cells within the central nervous system that transmit information between each other at synapses. Formation and maintenance of a synapse is dependent on a broad range of neuropeptides and selectively expressed molecules (Lüscher et al., 2000; Huganir and Nicoll, 2013; Südhof, 2018). Historically, methodological limitations have restricted studies of synaptic function to one cell type. Results obtained

from these isolated investigations are often extrapolated to deduce whole-brain synaptic regulatory mechanisms. Recent technological advances have allowed for deeper investigations into the inner workings of the trillions of synapses within our brains. Whole-brain synaptome cartography, 3D electron microscopy, and proteomic mass-spectrometry have uncovered a plethora of brain region-specific synaptic subtypes, each showing unique morphologic and molecular signatures (Roy et al., 2018; Cizeron et al., 2020; Zhu et al., 2021). While more work remains to be done, it is clear that population extrapolation undoubtedly masks individual influence when attempting to elucidate function of an individual protein.

There has been considerable interest in understanding the role protein 4.1N plays in regulating neuronal function (Shen et al., 2000; Biederer and Südhof, 2001; D.T. Lin et al., 2009; Wozny et al., 2009). 4.1N belongs to a larger 4.1 family of proteins, and is the predominant 4.1 isoform expressed in the brain (Walensky et al., 1999). Biochemical evidence shows protein 4.1N binds to many glutamatergic synapse regulatory proteins, resulting in 4.1N being deemed a potential hub protein and central organizer of synaptic

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membrane proteins (Baines et al., 2014; Yang et al., 2021). In dissociated hippocampal cultures, 4.1N is enriched along neuronal dendrites, colocalizing with important glutamatergic synapse proteins (e.g., GluA1 and PSD95; Walensky et al., 1999). Protein 4.1N's ability to interact with neuronal membrane proteins is made possible through its four-point-one, ezrin, radixin, and moesin (FERM) domain and C-terminal domain (CTDs; Scott et al., 2001), and many of 4.1N's binding partners show partial or exclusive postsynaptic protein localization. For example, studies using dissociated rat neurons show that the AMPA receptor (AMPA) subunits GluA1 binds to 4.1N (Shen et al., 2000). Further, 4.1N interacts with SynCAM1, a synaptic cell adhesion molecule that facilitates the recruitment of AMPARs to glutamatergic synapses (Hoy et al., 2009). However, a role for 4.1N in synaptic function has been controversial. A germline knock-out model of 4.1N and 4.1G produced no disruptions to basal synaptic transmission or long-term potentiation (LTP) in hippocampal CA1 pyramidal neurons of juvenile mice (Wozny et al., 2009). An independent study, using acute lentiviral knock-down of 4.1N in adult mice, found no effect on basal glutamatergic neurotransmission in CA1 pyramidal neurons, but did observe a reduction in LTP maintenance (D.T. Lin et al., 2009). Based on these studies in CA1 pyramidal neurons, 4.1N's role in governing glutamatergic synapse function appears rather modest.

In this study, we investigate whether 4.1N plays a pathway-specific role in regulating synaptic transmission within the hippocampus. In accordance with previous work, we find that knock-down of protein 4.1N has no effect on basal glutamatergic neurotransmission in CA1 pyramidal neurons. Our immunohistochemical analysis suggests this lack of a phenotype is because of very low, if any, protein 4.1N expression in these neurons. Conversely, we observe robust dendritic protein 4.1N immunoreactivity in granule neurons of the dentate gyrus (DG granule neurons), and we find that inhibition of protein 4.1N expression in DG granule neurons produces a marked disruption of both AMPA receptor-mediated and NMDA receptor-mediated neurotransmission. This disruption of normal synaptic transmission is accompanied by a significant decrease in dendritic spine density in DG granule neurons. Our molecular replacement experiments demonstrate that 4.1N's CTD is nonessential to its function at perforant pathway synapses, while 4.1N's FERM domain is critical for the observed synaptic AMPAR-mediated deficit. Altogether, our work demonstrates a novel, cell type-specific function for protein 4.1N in regulating glutamatergic neurotransmission at perforant pathway synapses and further confirms these synapses are governed by a unique set of molecular regulators.

Materials and Methods

Experimental constructs

A previously validated 4.1N-shRNA target sequence against rat 4.1N was used for all knock-down experiments (5'-AGGAGAGGGATGCGG TATT-3'; D.T. Lin et al., 2009). The 4.1N-shRNA was subcloned behind the H1 promoter region of a GFP-expressing pFUGW expression vector. Rat 4.1N cDNA sequence was acquired from Genscript (clone ID: ORa13272D). An shRNA-resistant 4.1N was generated by introducing five silent point mutations within the RNAi target sequence (AGAA GGGGTATGCGATACT; underlined nucleotides indicate mismatches in the target region). All cloning was performed using overlap-extension PCR followed by Clontech In-Fusion Cloning (TaKaRa Bio). Both 4.1N cDNA and the shRNA-resistant 4.1N cDNA were cloned into a pCAGGS-IRES-mCherry expression vector. The shRNA-resistant 4.1NACTD mutant was generated in-house using the method described above, by deleting the last 144 amino acids corresponding to the CTD domain (Lys1408-Ser1551).

The shRNA-resistant 4.1NΔFERM mutant was generated by deleting the residue corresponding to the FERM domain (Tyr283-Leu376) and was obtained from Genscript (catalog #SC1626). All plasmids were confirmed by DNA sequencing. A pFUGW vector expressing only GFP was co-expressed with pCAGGS-IRES-mCherry constructs to enhance identification of transfected neurons and was also used as a control vector in spine imaging experiments.

Electrophysiology

All experiments were performed in accordance with National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals*, and all procedures were approved by the Institutional Animal Care and Use Committee of the University of Southern California. Rat organotypic entorhino-hippocampal slice cultures were prepared from both male and female postnatal day (P)6–P8 Sprague Dawley rats as previously described (Stoppini et al., 1991; Prang et al., 2001; Bonnici and Kapfhammer, 2009; Sadybekov et al., 2017; Tian et al., 2018). Tissue was isolated and a MX-TS tissue slicer (Siskiyou) was used to make 400- μ m transverse sections. Tissue slices were placed on squares of Biopore Membrane Filter Roll (Millipore) and placed on Millicell Cell Culture inserts (Millipore) in 35 mm dishes. Slices were fed 1 ml of culture media containing MEM + HEPES (Invitrogen, catalog #12360-038), horse serum (25%), HBSS (25%), and L-glutamine (1 mM). Media was exchanged every other day. Slices with large portions of entorhinal cortex were visually identified after slicing. These slices were selected and plated for use in our experiments, and presence of entorhinal cortex was again confirmed when selecting slices appropriate for data acquisition. Whole-cell recordings were performed on day *in vitro* (DIV)7–DIV8. During recordings, slices were maintained in room-temperature artificial CSF (aCSF) containing 119 mM NaCl, 2.5 mM KCl, 1 mM NaH₂PO₄, 26.2 mM NaHCO₃, 11 mM glucose, 4 mM CaCl₂, and 4 mM MgSO₄. We added 5 μ M 2-chloroadenosine and 0.1 mM picrotoxin to the aCSF to dampen epileptiform activity and block GABA_A receptor activity, respectively. AMPAR-miniature EPSCs (AMPA-mEPSCs) were isolated by the addition of 0.5 μ M tetrodotoxin (TTX) and 0.1 mM picrotoxin to the aCSF. Osmolarity was adjusted to 310–315 mOsm. aCSF was saturated with 95% O₂/5% CO₂ throughout the recording. Borosilicate recording electrodes were filled with an internal whole-cell recording solution containing 135 mM CsMeSO₄, 8 mM NaCl, 10 mM HEPES, 0.3 mM EGTA, 5 mM QX-314, 4 mM Mg-ATP, and 0.3 mM Na-GTP. Osmolarity was adjusted to 290–298 mOsm, and pH-buffered at 7.3–7.4.

DG granule neurons and CA1 pyramidal neurons were identified using differential interference phase contrast microscopy, while GFP-expressing transfected neurons identified using epifluorescence microscopy. Dual whole-cell recordings of either neuronal subtype were made through simultaneous recordings from a transfected neuron and a neighboring, untransfected control neuron. Synaptic responses were evoked by stimulating with a monopolar glass electrode filled with aCSF in the stratum radiatum for CA1 recordings and the perforant pathway for DG granule neuron recordings. Membrane holding current, pipette series resistance, and input resistance were monitored throughout recording sessions. Data were acquired using a Multiclamp 700B amplifier (Molecular Devices), filtered at 2 kHz, and digitized at 10 kHz. AMPAR-evoked EPSCs (eEPSCs) were measured at –70 mV. NMDAR-eEPSCs were measured at +40 mV and were temporally isolated by measuring amplitudes 150 ms following the stimulus, at which point the AMPAR-eEPSC has completely decayed. Paired-pulse ratio was recorded by delivering two stimuli at varying intervals of 20, 40, 70, and 100 ms and dividing the peak response of stimulus 2 by the peak response of stimulus 1. Data analysis was performed using Igor Pro (Wavemetrics). In the scatter plots for simultaneous dual whole-cell recordings, each open circle represents one paired recording, and the closed circle represents the average of all paired recordings. No more than one paired recording was performed on any given entorhino-hippocampal slice.

Biolistic transfection

Sparse biolistic transfections were performed on DIV1 as previously described (Stoppini et al., 1991; Schnell et al., 2002; Lu et al., 2009). A total of 50 μ g of mixed plasmid DNA was coated on 1- μ m-diameter

gold particles in 0.5 mM spermidine, precipitated with 0.1 mM CaCl_2 , and washed four times in pure ethanol. The DNA-coated gold particles were then coated onto PVC tubing, dried briefly using ultra-pure N_2 gas, and stored at 4°C in desiccant. Before use, the gold particles were brought up to room temperature and delivered to slice cultures via a Helios Gene Gun (Bio-Rad). Construct expression was confirmed by GFP or mCherry epifluorescence.

Immunohistochemistry

P15 Sprague Dawley rats of both sexes were transcardially perfused with 11 ml of cold PBS and 25 ml of cold 4% PFA in PBS at a flow rate of 3 ml/min. The hippocampi were immediately dissected and postfixed overnight at 4°C in 4% PFA. After three brief washes in PBS, the hippocampi were sliced using a vibratome at 100- μm thickness. Slices were placed into 24-well culture plates containing PBS and stained within the wells. Slices were blocked in PBST (PBS + 0.25% Triton X-100) with 10% goat serum for 1 h at room temperature, rinsed in PBST, and incubated with primary antibody diluted in PBST overnight at 4°C. The next day, slices were thoroughly washed in PBST and stained with secondary antibody diluted in PBST for 2 h at room temperature. After this, slices were mounted onto slides, dried for 15 min, and mounted with either Fluoromount-G (SouthernBiotech, catalog #0100-01) or Fluoroshield with DAPI (Sigma-Aldrich, catalog #F6057). Slides were imaged and tiled using a Zeiss 880 Confocal Microscope using a 10 $\times$ objective. Antibodies used are as follows: monoclonal mouse anti-4.1N (1:1000, BD Transduction Laboratories, catalog #611836, RRID:AB_2098366), goat anti-mouse Alexa Fluor 488 (1:1000, Thermo Fisher Scientific, catalog #A32723, RRID:AB_2633275).

Western blotting

Embryonic day (E)16.5 rat hippocampi of both sexes were dissected, dissociated, and cultured in DMEM with 10% FBS. Neurons were plated into 24-well plates and treated with Lipofectamine 2000 (Invitrogen). Plasmid transfections were performed on DIV2 per manufacturer's protocol. A total of 25 μl of transfection complex was added to each well. Lysates were prepared at DIV5 in RIPA buffer containing protease inhibitor mix (Halt Protease Inhibitor Cocktail, EDTA-free (100 $\times$), Thermo Fisher Scientific, catalog #78425). Proteins were resolved by SDS-PAGE. Following the transfer, membranes were cut and analyzed by Western blotting with a monoclonal mouse anti-4.1N antibody (1:1000, BD Transduction Laboratories, catalog #611836, RRID:AB_2098366) or a polyclonal rabbit anti-GAPDH antibody (1:1000, Thermo Fisher Scientific, catalog #PA1-987, RRID:AB_2107311). Goat anti-rabbit IgG (H+L)-HRP Conjugate secondary antibody (1:5000, Bio-Rad, catalog #170-6515, RRID:AB_11125142) or goat anti-mouse IgG (H+L)-HRP Conjugate secondary antibody (1:5000, Thermo Fisher Scientific, catalog #31430, RRID:AB_228307) were used for all immunoblotting experiments described. All lysates were run on a 4–15% Mini-PROTEAN TGX Precast Protein Gel (Bio-Rad) with 50 μg of protein loaded per lane. Membranes were scanned using the Bio-Rad Chemidoc Imaging System.

Spine density analysis

Cultured entorhino-hippocampal slices were transfected on DIV1 with pFUGW-GFP construct, pFHUGW-GFP-shRNA construct, or pFHUGW-GFP-shRNA + pCAGGS-mCherry-cDNA constructs. Slices were fixed in 4% PFA, 4% sucrose in PBS, and washed three times with PBS, then cleared with an abbreviated SeeDB-based protocol (Ke et al., 2013) and mounted on microscope slides. Images were acquired at DIV7 using super-resolution microscopy (Carl Zeiss). High-resolution confocal z-stacks of spine-containing DG granule neuron secondary apical dendrites were acquired on a Zeiss 880 using an EC Plan-Neofluar 40 $\times$ /1.3 oil-immersion DIC M27 objective. Approximately 60- μm sections of secondary apical dendrites were manually selected for analysis. Z-stacks were collected at maximum X–Y pixel dimensions (512 $\times$ 512 pixels) at eight bits with a 488-nm laser excitation wavelength. An experimenter, blinded to the experimental condition, performed spine density analysis on sections using the Dendritic Spine Counter plug-in on ImageJ to count spines extending laterally from the dendrite. The ImageJ plug-In

SpineJ was used to obtain values for the following spine morphology metrics: spine head area, total spine length, neck width, and head width (used for spine type classification; Lev et al., 2020). Dendritic processes are commonly classified into the following categories: stubby, mushroom, and thin. Based on previous literature, we categorized dendrites into three aforementioned categories using the following criteria. Stubby spines are those which lack a visible neck region but have a bulbous head. Mushroom spines, often viewed as mature spines, are those with a short length (<2 μm), a clearly defined neck region, and a head which is >50% as wide as the neck. Thin spines are those with a long length (>2 μm) as well as a head (H. Lin et al., 2004; Mattison et al., 2014).

Experimental design and statistical analyses

All electrophysiological data are expressed as mean $\pm$ SEM. Imaging analysis was performed blind to experimental condition. Statistical significance was determined using: Wilcoxon signed-rank test for paired dual whole-cell patch-clamp data, Wilcoxon rank-sum test for electrophysiological data across independent conditions as well as imaging data, and Student's *t* test for paired-pulse facilitation data. Data were analyzed using IGOR Pro (Wavemetrics RRID:SCR_000325) or KaleidaGraph (Synergy Software RRID:SCR_014980), and graphed using Microsoft Excel (RRID:SCR_016137) or GraphPad Prism (RRID:SCR_002798). All *p*-values < 0.05 were considered significant and denoted with a single asterisk. All error bars represent standard error measurement. For all experiments, at least four male and female rat pups were used. Sample sizes in the present study are similar to those reported in the literature (Herring and Nicoll, 2016; Incontro et al., 2018).

Coefficient of variation (CV) analysis was performed on AMPAR-eEPSCs by comparing the change in eEPSC variance with the change in mean amplitude as previously described (Del Castillo and Katz, 1954; Bekkers and Stevens, 1990; Malinow and Tsien, 1990; Xiang et al., 1994). CV was calculated as SD/M (SD = standard deviation; M = mean). The SD and M were measured, normalized, and plotted for a concurrent set of stimuli from a control and its neighboring, transfected cell. Theoretical and experimental work shows that CV^{-2} (M^2/SD^2) is invariant with changes in quantal size (i.e., the number of AMPARs at all synapses), while CV^{-2} varies predictably with changes in quantal content (i.e., the number of functional synapses containing AMPARs) according to the following equation: $\text{CV}^{-2} = n \times P_r / (1 - P_r)$. In this equation, *n* is the number of vesicle release sites and *P_r* is the presynaptic release probability. CV^{-2} values for transfected and control cells were plotted on the *y*-axis, and mean eEPSC amplitude values for transfected and control cells were plotted on the *x*-axis. Values on or near the 45° (*y* = *x*) line indicate changes in quantal content, while values approaching the horizontal line (*y* = 1) indicate a change in quantal size. Filled circles represent the mean $\pm$ SEM of the entire dataset.

Failure analysis was performed by analyzing AMPAR-eEPSCs from dual whole-cell patch-clamp recordings where stimulation levels elicited failures visually distinguishable from synaptic currents. Stimulation events were assigned as failures if their absolute magnitudes were less than or equal to noise for each sweep. The number of failures for each cell was estimated as the number of stimulation events with absolute current amplitude not greater than noise divided by the total number of stimulation events to yield the percentage failure rate.

Data, materials, and software availability

All study data are provided within the paper. All additional information will be made available on reasonable request to the authors.

Results

Protein 4.1N is required for the structure and function of glutamatergic synapses in DG granule neurons

Previous work has shown that knocking out protein 4.1N expression in CA1 pyramidal neurons of the hippocampus does not affect basal glutamatergic synapse function (D.T. Lin et al., 2009; Wozny et al., 2009). However, gene expression profiles of different hippocampal subregions show extensive differences in RNA and synaptic protein expression (Datson et al., 2009; Rao et al.,

2019; Kay et al., 2022). To determine the expression profile of protein 4.1N, we performed immunohistochemical analysis on hippocampal slices from rats of either sex. This analysis revealed a marked contrast between the expression of protein 4.1N in the DG compared with other hippocampal subregions. The inner and outer molecular layer of the DG display robust expression, whereas the stratum oriens and stratum radiatum of CA3 and CA1 exhibit little, if any, 4.1N expression (Fig. 1A).

Given the DG granule neuron-specific 4.1N expression we observe in the hippocampus, we sought to determine whether protein 4.1N has a functional role at DG granule neuron synapses. To accomplish this, we generated a 4.1N-shRNA construct using a previously published shRNA sequence against 4.1N (D.T. Lin et al., 2009). We then independently verified the validity of our knock-down in dissociated hippocampal neurons using a well-established antibody against protein 4.1N (D.T. Lin et al., 2009; Fig. 1B). Western blot analysis of hippocampal neuron homogenates revealed that expression of this 4.1N-shRNA reduces 4.1N expression by ~70%. Following this, we employed a biolistic transfection method to express our protein 4.1N-shRNA in neurons in rat organotypic entorhino-hippocampal slice cultures (Elias et al., 2008; Paskus et al., 2019; Tian et al., 2021). These slices allow us to examine the impact genetic modifications have on hippocampal neurons within their native circuitry (Stoppini et al., 1991; Schnell et al., 2002). Six days after transfection, we record AMPA receptor- and NMDA receptor-evoked EPSCs (AMPA-eEPSCs and NMDAR-eEPSCs) from transfected and neighboring, untransfected control DG granule neurons simultaneously during perforant pathway stimulation (Fig. 1C). This approach allows for a pairwise, internally controlled comparison of the consequences of our acute genetic manipulation. We find that knock-down of protein 4.1N in DG granule neurons produces a ~60% reduction in both AMPAR-eEPSC amplitude ($n = 11$ pairs, $p = 0.032$, Wilcoxon signed-rank test; Fig. 1D) and NMDAR-eEPSC amplitude ($n = 10$ pairs, $p = 0.0019$, Wilcoxon signed-rank test; Fig. 1E). To determine whether this reduction is because of a disruption in presynaptic neurotransmitter release, we performed paired-pulse facilitation (PPF) experiments in DG granule neurons. We observe no changes in PPF at a range of interstimulus intervals (ISIs) between neurons transfected with a 4.1N-shRNA when compared with untransfected, control neurons ($n = 6$ pairs, ISI: 20 ms: $p = 0.62$, 40 ms: $p = 0.90$, 70 ms: $p = 0.47$, 100 ms: $p = 0.50$, Student's t test; Fig. 1F). Thus, the observed deficits in glutamatergic synapse function seen with knock-down of 4.1N are because of a reduction in postsynaptic function.

Most excitatory synapses are formed on dendritic spines. Observed reductions in both AMPAR-eEPSC and NMDAR-eEPSC amplitude are often associated with a reduction in dendritic spine density. Therefore, we performed dendritic spine density analysis to determine whether the observed deficits in AMPA receptor-mediated and NMDA receptor-mediated transmission caused by 4.1N knock-down are produced by a loss of spines. We compared the density of dendritic spines on secondary apical dendrites of DG granule neurons expressing the 4.1N-shRNA or a control GFP construct (GFP: $n = 31$ segments, 4.1N-shRNA: $n = 37$ segments, $p < 0.0001$, Wilcoxon rank-sum test; Fig. 1G). We find that knocking down protein 4.1N produces a ~50% reduction in dendritic spine density in DG granule neurons. The magnitude of this reduction in spine density is comparable to the percent reduction we see in AMPAR-eEPSC and NMDAR-eEPSC amplitude following knock-down of protein 4.1N expression (Fig. 1D,E). We also examined the total

spine length, head area, and proportion of different spine types (mushroom, thin, stubby) in 4.1N-shRNA-expressing neurons compared with GFP-expressing control neurons, and found no significant differences in these spine parameters in 4.1N-shRNA-expressing DG granule neurons (GFP: $n = 5$ cells, 4.1N-shRNA: $n = 4$ cells, $p = 0.90$ for spine length, $p = 1$ for head area, $p = 0.56$ for stubby spines, $p = 0.90$ for thin spines, $p = 0.29$ for mushroom spines, Wilcoxon rank-sum test; Fig. 1G). Taken together, these data demonstrate that 4.1N is essential for maintaining both the structure and function of glutamatergic synapses in DG granule neurons.

We also examined whether knock-down of 4.1N alters single synaptic events in DG granule neurons by recording AMPAR-mediated miniature EPSCs (AMPA-mEPSCs). Consistent with the reduction in dendritic spine number we observe, we find that the frequency of AMPAR-mEPSCs is reduced by ~75% in 4.1N knock-down neurons (control: $n = 6$ cells, 4.1N-shRNA: $n = 6$ cells, control = 0.62 ± 0.11 Hz, 4.1N-shRNA = 0.14 ± 0.02 Hz, $p = 0.002$, Wilcoxon rank-sum test; Fig. 1H). The amplitude of the remaining AMPAR-mEPSCs in 4.1N-shRNA-expressing neurons was unchanged (control: $n = 6$ cells, 4.1N-shRNA: $n = 6$ cells, control = 14.33 ± 1.12 pA, 4.1N-shRNA = 20.01 ± 2.38 pA, $p = 0.13$, Wilcoxon rank-sum test; Fig. 1H). These data provide additional confirmation that reducing 4.1N protein expression results in a substantial loss of functional glutamatergic synapses in DG granule neurons.

It has been shown previously that 4.1N preferentially binds to the AMPAR subunit GluA1, but not to GluA2 or GluA3 (Shen et al., 2000; Coleman et al., 2003). Previous work has also shown that decay kinetics of synaptic AMPAR currents vary by subunit composition, with GluA2/A3 heteromers deactivating more quickly than GluA1/A2 heteromers (Lu et al., 2009; Herring et al., 2013). Therefore, the loss of GluA1-containing AMPARs at synapses produces a hallmark increase in the decay rate of synaptic AMPAR currents caused by a higher GluA2/A3 to GluA1/A2 synaptic AMPAR ratio (Lu et al., 2009; Herring et al., 2013). To determine whether the synaptic AMPAR deficit seen with loss of 4.1N is accompanied by a change in synaptic AMPAR subunit composition, we examined the kinetics of AMPAR-eEPSCs. We find no differences in the AMPAR-eEPSC rate of decay between 4.1N-shRNA-expressing and control DG granule neurons ($n = 9$ pairs, $p = 0.73$, Wilcoxon signed-rank test; Fig. 1I). Altogether, these data demonstrate that reducing 4.1N expression in DG granule neurons results in a substantial reduction in glutamatergic synapse number in DG granule neurons.

4.1N knock-down does not perturb basal glutamatergic synapse function in CA1 pyramidal neurons

Our immunohistochemical analysis reveals considerably lower expression of protein 4.1N in the dendrites of CA3 and CA1 pyramidal neurons when compared with the dendrites of DG granule neurons (Fig. 2A). Such data suggest that the synaptic expression of protein 4.1N is very low in hippocampal subregions outside of the DG, and therefore may play a DG-specific role in synaptic regulation within the hippocampus. To test this hypothesis, we examined the glutamatergic synapse function of CA1 pyramidal neurons transfected with our 4.1N-shRNA construct compared with that of neighboring, untransfected control neurons (Fig. 2B). Consistent with our immunohistochemical data, we observe no significant change in either AMPAR-eEPSC ($n = 8$ pairs, $p = 0.55$, Wilcoxon signed-rank test; Fig. 2C) or NMDAR-eEPSC amplitude in CA1 pyramidal neurons ($n = 8$ pairs, $p = 0.95$, Wilcoxon signed-rank test; Fig. 2D). We also examined whether presynaptic neurotransmitter

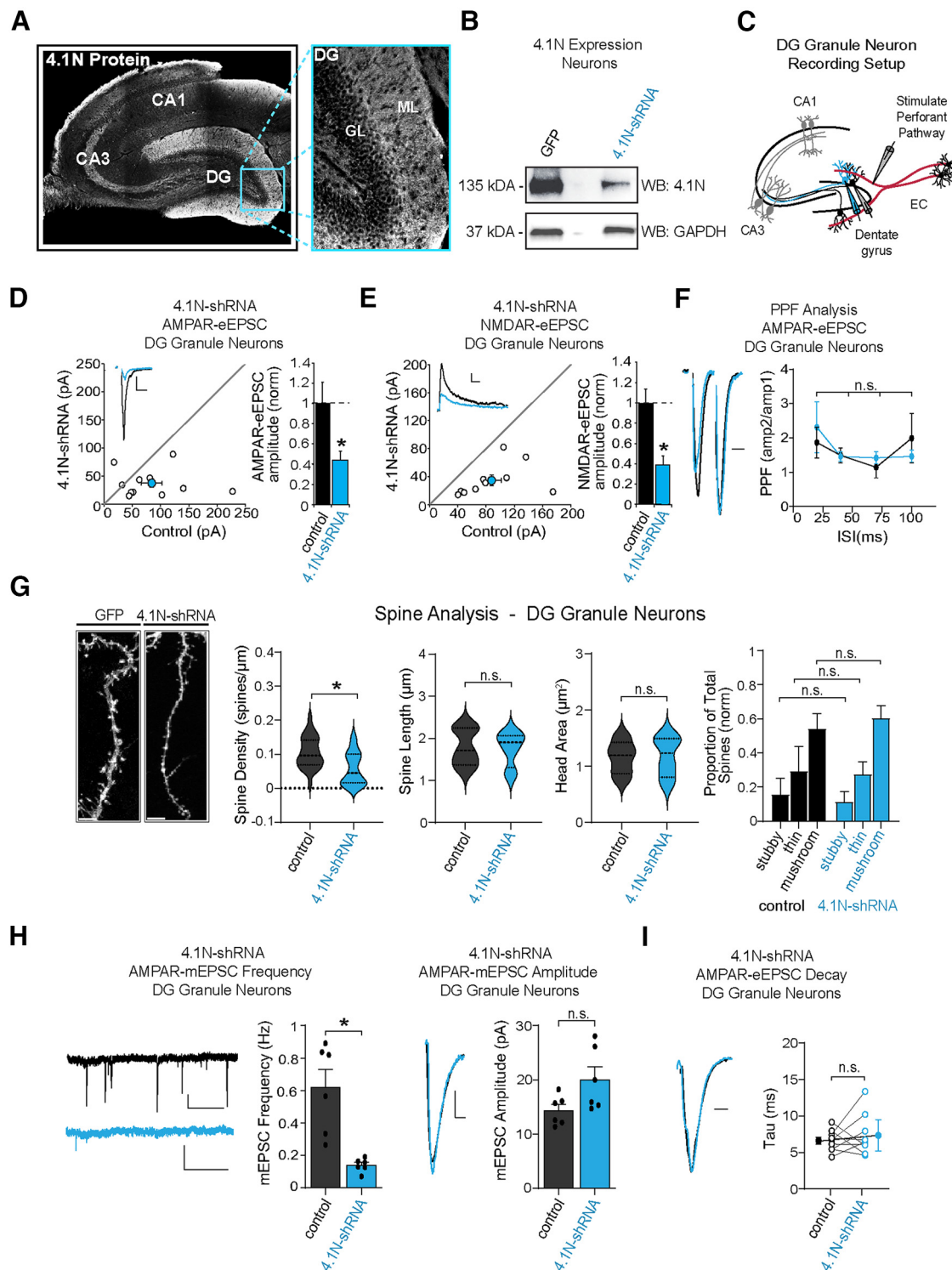


Figure 1. Protein 4.1N is required for glutamatergic synapse structure and function in DG granule neurons. **A**, Representative immunolabeling showing enrichment of protein 4.1N in the molecular layer of the DG in a rat hippocampal slice. Blue box shows enlarged DG region. GL: granule layer, ML: molecular layer. **B**, Western blotting showing shRNA-mediated reduction of 4.1N protein in dissociated hippocampal neurons. **C**, Schematic representation of electrophysiological recording setup for DG granule neurons. **D**, Knocking down 4.1N significantly decreases both AMPAR-eEPSC ($n = 11$ pairs) and **E**, NMDAR-eEPSC amplitudes ($n = 10$ pairs) in DG granule neurons. **D**, **E**, Scatterplots show eEPSC amplitudes for pairs of untransfected and transfected cells (open circles) with corresponding mean $\pm$ SEM (filled circles). Insets show representative current traces from control and transfected (blue) neurons with stimulation artifacts removed. Scale bars: 20 ms, 20 pA for both AMPAR-eEPSCs and NMDAR-eEPSCs. Bar graphs show the average AMPAR-eEPSC and NMDAR-eEPSC amplitudes ($\pm$ SEM) of DG granule neurons expressing the 4.1N-shRNA (blue) normalized to their respective control cell average eEPSC amplitudes (black). **F**, Paired-pulse facilitation (PPF) ratios (mean $\pm$ SEM) for 4.1N-shRNA-expressing DG granule neurons and paired control neurons show no detectable differences in facilitation at a variety of interstimulus intervals (ISIs; $n = 6$ pairs, ISI: 20, 40, 70, and 100 ms). Peak 2-scaled current traces from control (black) and transfected (blue) neurons. Scale bars: 20 ms. **G**, 4.1N-shRNA-expressing DG granule neurons have significantly lower dendritic spine density in comparison to GFP-expressing control neurons. Leftmost images display representative dendritic segments of GFP-expressing (left) and 4.1N-shRNA-expressing (right) DG granule neurons. Scale bars: 10 μm . Violin plots show a significant difference in the spine density of DG granule neurons expressing the 4.1N-shRNA when compared with GFP-expressing control neurons (GFP: $n = 31$ segments, 4.1N-shRNA: $n = 37$ segments), but no differences in total spine length or head area. Bar graph (right) shows no changes to the proportion of spine types between 4.1N-shRNA-expressing

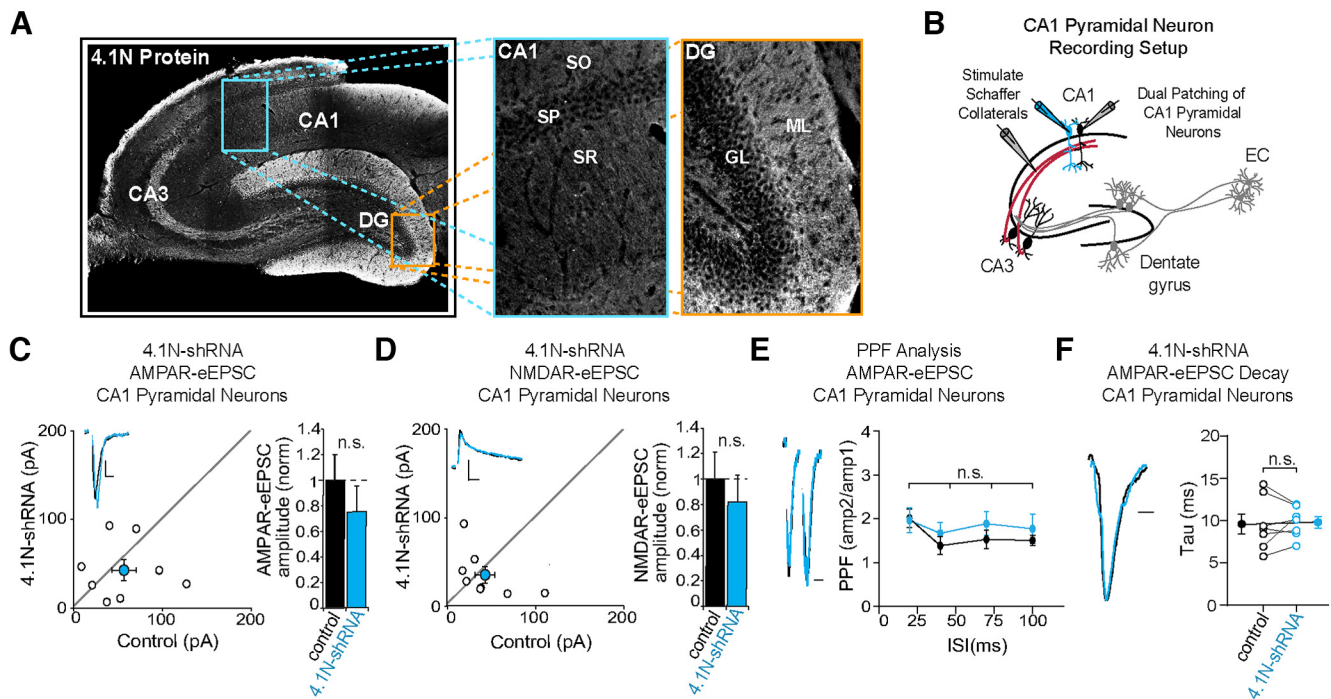


Figure 2. Protein 4.1N is not required for glutamatergic neurotransmission in CA1 pyramidal neurons. **A**, Representative immunolabeling showing minimal 4.1N expression in the CA1 region of a rat hippocampal slice. Blue box shows enlarged CA1 region. SO: stratum oriens, SP: stratum pyramidale, SR: stratum radiatum. Orange box shows enlarged DG region. GL: granule layer, ML: molecular layer. **B**, Schematic representation of electrophysiological recording setup for CA1 pyramidal neurons. **C**, Knock-down of 4.1N in CA1 pyramidal neurons does not significantly affect AMPAR-eEPSC ($n = 8$ pairs) or **D** NMDAR-eEPSC amplitudes for pairs of untransfected and transfected cells (open circles) with corresponding mean $\pm$ SEM (filled circles). Insets show representative current traces from control (black) and transfected (blue) neurons with stimulation artifacts removed. Scale bars: 20 ms, 20 pA for AMPAR-eEPSCs; 50 ms, 50 pA for NMDAR-eEPSCs. Bar graphs show the average AMPAR-eEPSC and NMDAR-eEPSC amplitudes ($\pm$ SEM) of CA1 pyramidal neurons expressing the 4.1N-shRNA (blue) normalized to their respective control cell average eEPSC amplitudes (black). **E**, Paired-pulse facilitation ratios (mean $\pm$ SEM) for 4.1N-shRNA-expressing CA1 pyramidal neurons and paired control neurons show no detectable differences in facilitation at a variety of interstimulus intervals ($n = 6$ pairs, ISIs: 20, 40, 70, and 100 ms). Peak 2-scaled current traces from control (black) and transfected (blue) neurons. Scale bars: 20 ms. **F**, Paired scatterplot shows no differences in decay kinetics between averaged AMPAR-eEPSCs from 4.1N-shRNA-expressing and paired control neurons ($n = 7$ pairs). Inset shows peak-normalized sample traces from control (black) and transfected (blue) neurons. Scale bar: 10 ms; n.s., not significant.

release is altered in 4.1N knock-down neurons. We observe no change in PPF at a range of paired stimulus intervals between CA1 pyramidal neurons transfected with our 4.1N-shRNA when compared with untransfected, control neurons ($n = 6$ pairs, ISI: 20 ms: $p = 0.91$, 40 ms: $p = 0.41$, 70 ms: $p = 0.32$, 100 ms: $p = 0.48$, Student's t test; Fig. 2E). Because of 4.1N's proposed interaction with the AMPAR subunit GluA1, we again examined AMPAR-eEPSC decay rate in CA1 pyramidal neurons (Shen et al., 2000). We find the decay rates of AMPAR-eEPSCs from 4.1N-shRNA-expressing neurons to not vary from that of control neurons ($n = 7$ pairs, $p = 0.94$, Wilcoxon signed-rank test; Fig. 2F). Akin to other studies, we conclude that 4.1N has no role in basal synaptic transmission in CA1

pyramidal neurons (D.T. Lin et al., 2009; Wozny et al., 2009). Together, these data demonstrate that 4.1N plays a cell type-specific role in regulating glutamatergic neurotransmission in the hippocampus.

Protein 4.1N's C-terminal domain is not necessary for its function at DG granule synapses

To further confirm the specificity of our protein 4.1N-shRNA, we generated a recombinant shRNA-resistant 4.1N cDNA expression construct. We then co-expressed the 4.1N-shRNA with the shRNA-resistant 4.1N cDNA (4.1N Rescue) in DG granule neurons and performed paired recordings. We find that molecularly replacing endogenous 4.1N with recombinant 4.1N rescues the reductions in AMPAR-eEPSC ($n = 9$ pairs, $p = 1$, Wilcoxon signed-rank test; Fig. 3B,D) and NMDAR-eEPSC amplitude caused by expression of 4.1N-shRNA ($n = 9$ pairs, $p = 0.57$, Wilcoxon signed-rank test; Fig. 3E,G). We also examined spine density phenotypes of the 4.1N rescue construct in comparison to 4.1N-shRNA-expressing and GFP-expressing control DG granule neurons. Co-expression of 4.1N-shRNA with 4.1N-shRNA-resistant cDNA rescues the reduction in dendritic spine density produced by our 4.1N-shRNA [GFP: $n = 31$ segments, 4.1N-shRNA and 4.1N-shRNA-resistant cDNA (4.1N Rescue): $n = 48$ segments, $p = 0.27$, Wilcoxon rank-sum test; Fig. 3I]. Further, we find no differences in any measures of spine morphology between 4.1N Rescue-expressing DG granule neurons and control, GFP-expressing DG granule neurons (GFP: $n = 5$ cells, 4.1N Rescue: $n = 6$ cells, $p = 0.66$ for spine length, $p = 0.54$ for head area, $p = 0.41$ for stubby spines, $p = 0.66$

neurons compared with GFP-expressing control neurons (GFP: $n = 5$ cells, 4.1N-shRNA: $n = 4$ cells). **H**, AMPAR-mEPSC analysis reveals a significant reduction in the frequency, but not the amplitude, of AMPAR-mEPSCs in 4.1N-shRNA-expressing DG granule neurons compared with control DG granule neurons (control: $n = 6$ cells, 4.1N-shRNA: $n = 6$ cells). Bar graphs show the averaged frequency and amplitude of AMPAR-mEPSC events $\pm$ SEM, with each point representing the averaged value from one neuron. Leftmost panel shows sample traces of control AMPAR-mEPSC events (black/top), compared with 4.1N-shRNA AMPAR-mEPSC events (blue/bottom). Scale bars: 1 s, 20 pA. Left of the amplitude bar graph displays an averaged representative trace from a control (black) and transfected (blue) neuron. Scale bars: 5 ms, 5 pA. **I**, Paired scatterplot shows no differences in decay kinetics between averaged AMPAR-eEPSCs from 4.1N-shRNA-expressing and control DG granule neurons ($n = 9$ pairs). Inset shows peak-normalized sample traces from control (black) and transfected (blue) neurons. Scale bar: 10 ms. * $p < 0.05$; n.s., not significant.

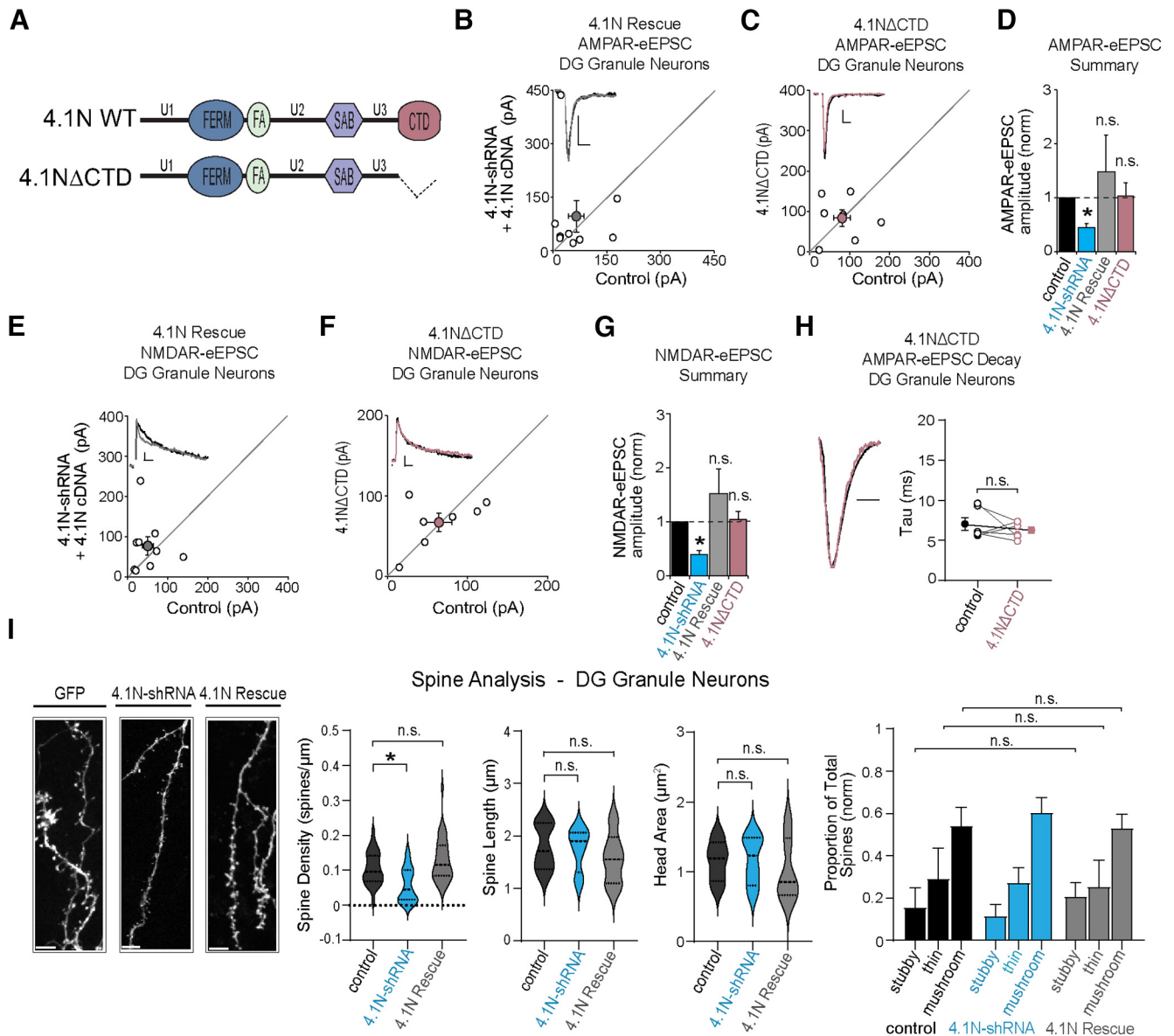


Figure 3. Protein 4.1N's C-Terminal domain is not required for glutamatergic synapse function in DG granule neurons. **A**, Schematic depicting the domain structure of full length 4.1N protein, followed by the domain structure of 4.1NΔCTD. **B**, Molecular replacement of endogenous 4.1N with shRNA-resistant 4.1N cDNA (4.1N Rescue) rescues both AMPAR-eEPSC ($n = 9$ pairs) and (**E**) NMDAR-eEPSC amplitudes ($n = 9$ pairs) in DG granule neurons. Molecular replacement of endogenous 4.1N with 4.1NΔCTD also rescues both (**C**) AMPAR-eEPSC ($n = 7$ pairs) and (**F**) NMDAR-eEPSC amplitudes ($n = 7$ pairs) in DG granule neurons. **B**, **C**, **E**, **F**, Scatterplots show eEPSC amplitudes for pairs of untransfected and transfected cells (open circles) with corresponding mean $\pm$ SEM (filled circles). Insets show representative current traces from control (black) and transfected (4.1N Rescue: gray, 4.1NΔCTD: red) neurons with stimulation artifacts removed. Scale bars: 20 ms, 20 pA for both AMPAR-eEPSCs; 50 ms, 20 pA for 4.1N Rescue NMDAR-eEPSCs; 20 ms, 50 pA for 4.1NΔCTD NMDAR-eEPSCs. **D**, **G**, Bar graphs show the average AMPAR-eEPSC and NMDAR-eEPSC amplitudes ($\pm$ SEM) of DG granule neurons co-expressing: 4.1N-shRNA and 4.1N-shRNA-resistant cDNA (gray) or 4.1N-shRNA and 4.1NΔCTD (red) normalized to their respective control cell average eEPSC amplitudes (black). **H**, Paired scatterplot shows no differences in the decay kinetics of averaged AMPAR-eEPSCs from 4.1NΔCTD-expressing compared with control DG granule neurons ($n = 6$ pairs). Inset shows peak-normalized sample traces from control (black) and transfected (red) neurons. Scale bar: 10 ms. **I**, Co-transfection of DG granule neurons with 4.1N-shRNA and 4.1N-shRNA-resistant cDNA (4.1N Rescue) rescues the 4.1N-shRNA-mediated reduction in dendritic spine density. Leftmost images display representative dendritic segments of GFP-expressing (left), 4.1N-shRNA-expressing (middle), and 4.1N Rescue-expressing (right) DG granule neurons. Scale bars: 10 μ m. Violin plots show no significant differences in dendritic spine density, spine length, or head area in DG granule neurons co-expressing the 4.1N-shRNA with 4.1N-shRNA-resistant cDNA when compared with GFP-expressing control neurons (GFP: $n = 31$ segments, 4.1N Rescue: $n = 48$ segments). Bar graph (right) shows no significant differences in proportion of spine types between neurons co-expressing 4.1N-shRNA and 4.1N-shRNA-resistant cDNA compared with GFP-expressing control neurons (GFP: $n = 5$ cells, 4.1N Rescue: $n = 6$ cells). * $p < 0.05$; n.s., not significant.

for thin spines, $p = 1$ for mushroom spines, Wilcoxon rank-sum test; Fig. 3I). We, therefore, conclude that the deficit in glutamatergic synapse density and function we observe resulting from expression of our 4.1N-shRNA is because of reduced protein 4.1N expression.

Having confirmed that the observed synaptic deficit seen in DG granule neurons following loss of 4.1N is rescued by recombinant 4.1N, we sought to understand the 4.1N domain(s) responsible for producing these marked decreases in AMPA

receptor-mediated and NMDA receptor-mediated currents. Initially, we hypothesized that 4.1N function at DG granule synapses was dependent on its C-Terminal domain (CTD), given that this domain was previously reported to interact with the AMPAR subunit GluA1 (Shen et al., 2000). Based on this reported interaction, we hypothesized that molecular replacement of endogenous 4.1N with a truncated variant missing its CTD (4.1NΔCTD) would fail to rescue synaptic deficits seen

with knock-down of full length 4.1N (Fig. 3A). Surprisingly, molecular replacement of endogenous 4.1N with 4.1N Δ CTD rescues both AMPAR-eEPSC ($n = 7$ pairs, $p = 0.94$, Wilcoxon signed-rank test; Fig. 3C,D) and NMDAR-eEPSC ($n = 7$ pairs, $p = 0.69$, Wilcoxon signed-rank test; Fig. 3F,G) amplitudes in DG granule neurons. Given this unexpected finding, we sought to examine whether this rescue of synaptic function results from a compensatory effect of remaining GluA2/A3 subunits. As previously mentioned, GluA2/A3 heteromers deactivate at faster rates than GluA1/A2 heteromers (Lu et al., 2009; Herring et al., 2013). However, we find the kinetics of AMPAR-eEPSCs from 4.1N Δ CTD-expressing neurons to not vary from that of control neurons ($n = 6$ pairs, $p = 0.56$, Wilcoxon signed-rank test; Fig. 3H). These data strongly suggest that the cell type-specific synaptic deficits in DG granule neurons following 4.1N knock-down are not because of a loss of protein-protein interactions supported by 4.1N's CTD.

Protein 4.1N's FERM domain is required for synaptic AMPA receptor function in DG granule neurons

4.1N's remaining protein binding domain, the FERM domain, binds a vast array of transmembrane proteins (Biederer and Südhof, 2001; Scott et al., 2001; Li et al., 2007; Hoy et al., 2009). Given that elimination of 4.1N's CTD does not affect 4.1N function, we hypothesized that the FERM domain may play an important role in supporting protein 4.1N's function at glutamatergic synapses in DG granule neurons. To determine the importance of protein 4.1N's FERM domain, we molecularly replaced endogenous 4.1N with a mutant variant lacking its FERM domain (4.1N Δ FERM; Fig. 4A). We find that replacing endogenous 4.1N with 4.1N Δ FERM fails to rescue the reduction in AMPAR-eEPSC amplitude caused by 4.1N knock-down ($n = 13$ pairs, $p = 0.021$, Wilcoxon signed-rank test; Fig. 4B,C). However, 4.1N Δ FERM is able to rescue the reduction in NMDAR-eEPSC amplitude caused by 4.1N knock-down ($n = 12$ pairs, $p = 0.52$, Wilcoxon signed-rank test; Fig. 4D,E). As such, we examined whether removal of 4.1N's FERM domain is accompanied by a change in the number and/or morphology of dendritic spines at DG granule synapses. We find that co-expression of 4.1N-shRNA with 4.1N Δ FERM cDNA rescues the reduction in spine density produced by reducing protein 4.1N expression (GFP: $n = 31$ segments, 4.1N Δ FERM: $n = 31$ segments, $p = 0.59$, Wilcoxon rank-sum test; Fig. 4F). Further, we find no significant differences in any spine parameters assessed in DG granule neurons co-expressing 4.1N-shRNA and 4.1N Δ FERM compared with GFP-expressing control neurons (GFP: $n = 5$ cells, 4.1N Δ FERM: $n = 6$ cells, $p = 0.79$ for spine length, $p = 0.43$ for head area, $p = 0.36$ for stubby spines, $p = 0.66$ for thin spines, $p = 0.93$ for mushroom spines, Wilcoxon rank-sum test; Fig. 4F). Next, we examined the frequency and amplitude of AMPAR-mEPSCs to assess whether removal of the FERM domain produces an alteration in single synaptic events. We find that AMPAR-mEPSC frequency is reduced by $\sim 65\%$ in 4.1N Δ FERM mutants (control: $n = 6$ cells, 4.1N Δ FERM: $n = 6$ cells, control = 1.31 ± 0.28 Hz, 4.1N Δ FERM = 0.45 ± 0.09 Hz, $p = 0.041$, Wilcoxon rank-sum test; Fig. 4G), while the amplitude of remaining AMPAR-mEPSCs is comparable to that of control DG granule neurons (control: $n = 6$ cells, 4.1N Δ FERM: $n = 6$ cells, control = 14.28 ± 1.67 pA, 4.1N Δ FERM = 19.17 ± 3.54 pA; $p = 0.39$, Wilcoxon rank-sum test; Fig. 4G). Together, these data demonstrate that protein 4.1N's FERM domain plays a specialized role in synaptic AMPAR function in DG granule neurons.

Loss of 4.1N's FERM domain results in a selective deficit in AMPAR, but not NMDAR, function. Loss of 4.1N's FERM

domain also results in a reduction in AMPAR-mEPSC frequency, but AMPAR-mEPSC amplitude is unchanged. Such data are consistent with the deletion of 4.1N's FERM domain causing a loss of all AMPARs at a subset of existing synapses, resulting in an increase in the number of "silent synapses." To further examine this possibility, we performed coefficient of variation (CV^{-2}) analysis on AMPAR-eEPSC amplitudes (Del Castillo and Katz, 1954; Malinow and Tsien, 1990). By comparing the normalized variance in AMPAR-eEPSC amplitudes from control and transfected neurons receiving the same stimulus, we are able to estimate the relative quantal size and quantal content. Changes in quantal size modify the mean eEPSC amplitude and variance such that the CV^{-2} remains constant, and indicate a change in the number of glutamate receptors at all synapses. In contrast, changes in quantal content produce proportional changes of equal magnitude in CV^{-2} and mean eEPSC amplitudes which cause the marker of the mean to fall on the diagonal ($y = x$) line. Changes in quantal content indicate a change in the number of synapses expressing glutamatergic receptors. CV^{-2} analysis reveals that the reduction in AMPAR-eEPSC amplitude caused by loss of the FERM domain is because of a reduction in quantal content and, therefore, because of an increase in the number of silent synapses in these neurons ($n = 13$ pairs; Fig. 4H). An increase in silent synapse number is also accompanied by an increase in the probability that a given axonal stimulation fails to elicit an AMPAR-eEPSC (Goold and Nicoll, 2010; Gray et al., 2011). We, therefore, examined the frequency at which presynaptic stimulation fails to elicit an AMPAR-eEPSC when 4.1N's FERM domain is deleted. Consistent with our AMPAR-mEPSC data and CV^{-2} analysis, we find that removal of 4.1N's FERM domain results in a significant increase in failures ($n = 13$ pairs, $p = 0.035$, Student's t test; Fig. 4I). Altogether, our data demonstrate that 4.1N's FERM domain is critical for synaptic AMPAR expression and that loss of 4.1N's FERM domain increases the percentage of synapses that lack functional AMPARs.

It has been shown previously that loss of GluA1-containing AMPARs results in a significant reduction in AMPAR-eEPSC amplitude, no change in NMDAR-eEPSC amplitude, and a substantial reduction in AMPAR-mEPSC frequency (Lu et al., 2009; Herring et al., 2013). Removal of 4.1N's FERM domain produces a similar synaptic phenotype, and 4.1N has been proposed to support the trafficking of GluA1-containing AMPARs (D.T. Lin et al., 2009; Bonnet et al., 2023). Given that the loss of GluA1-containing AMPARs also produces a hallmark speeding of synaptic AMPAR current decay kinetics (Lu et al., 2009; Herring et al., 2013), we examined AMPAR-eEPSC decay kinetics in the context of a selective deletion of 4.1N's FERM domain. Indeed, we find that removal of 4.1N's FERM domain produces a marked speeding in the decay of AMPAR-eEPSCs ($n = 9$ pairs, $p = 0.039$, Wilcoxon signed-rank test; Fig. 4J). Thus, the loss of 4.1N's FERM domain phenocopies the loss of the GluA1 subunit in neurons and strongly suggests that 4.1N's FERM domain, rather than its CTD, plays a critical role in the expression of functional GluA1-containing AMPARs in a subpopulation of DG granule neuron synapses.

Discussion

We have identified, for the first time, a postsynaptic role for protein 4.1N in regulating basal glutamatergic neurotransmission. Within the hippocampus, this function is cell type-specific. Consistent with previous work, we show that knock-down of protein 4.1N in CA1 pyramidal neurons has no effect on basal

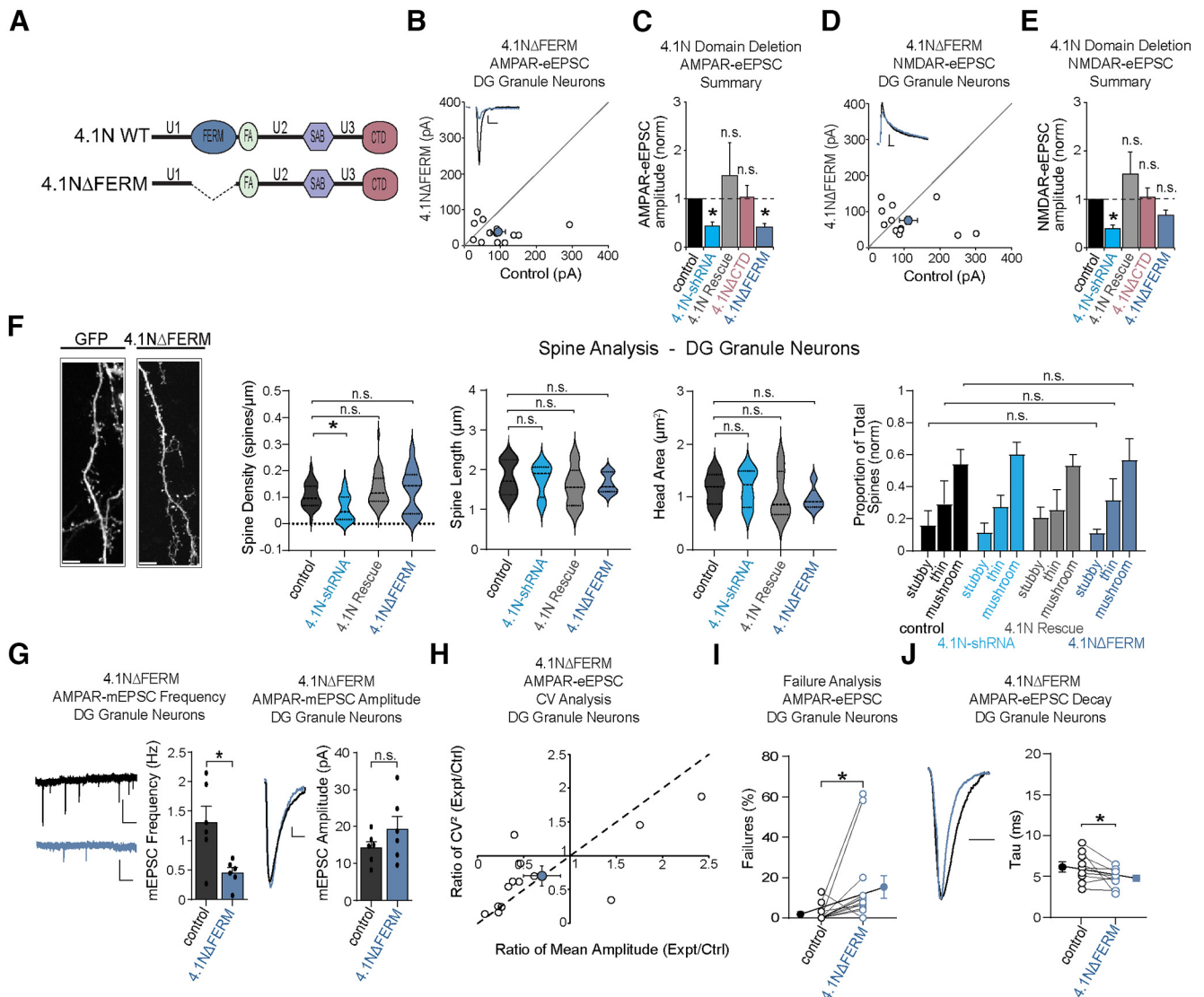


Figure 4. Protein 4.1N's FERM domain is required for maintaining synaptic AMPA receptor function in DG granule neurons. **A**, Schematic depicting the domain structure of 4.1NΔFERM. Molecular replacement of endogenous 4.1N with 4.1NΔFERM rescues **(D)** NMDAR-eEPSC ($n = 12$ pairs) but fails to rescue **(B)** AMPAR-eEPSC amplitudes ($n = 13$ pairs) in DG granule neurons. **B, D**, Scatterplots show eEPSC amplitudes for pairs of untransfected and transfected cells (open circles) with corresponding mean $\pm$ SEM (filled circles). Insets show representative current traces from control (black) and transfected (deep blue) neurons with stimulation artifacts removed. Scale bars: 20 ms, 20 pA for AMPAR-eEPSCs; 50 ms, 50 pA for NMDAR-eEPSCs. **C, E**, Bar graphs show the average AMPAR-eEPSC and NMDAR-eEPSC amplitudes ($\pm$ SEM) of DG granule neurons co-expressing 4.1N-shRNA and 4.1NΔFERM (deep blue) normalized to their respective control cell average eEPSC amplitudes (black). **F**, Molecular replacement of endogenous 4.1N with 4.1NΔFERM rescues the 4.1N-shRNA-mediated reduction in dendritic spine density. Representative dendritic segment of GFP-expressing (left) and 4.1N-shRNA + 4.1NΔFERM-expressing (right) DG granule neurons. Scale bars: 10 μm. Violin plots show no significant differences in dendritic spine density, spine length, or head area in DG granule neurons co-expressing the 4.1N-shRNA with 4.1NΔFERM in comparison to GFP-expressing control neurons (GFP: $n = 31$ segments, 4.1NΔFERM: $n = 31$ segments). Bar graph (right) shows no significant differences in proportion of spine types between neurons co-expressing 4.1N-shRNA and 4.1NΔFERM compared with GFP-expressing control neurons (GFP: $n = 5$ cells, 4.1NΔFERM construct: $n = 6$ cells). **G**, AMPAR-mEPSC analysis reveals a significant reduction in the frequency, but not the amplitude, of AMPAR-mEPSCs in 4.1NΔFERM-expressing DG granule neurons compared with control DG granule neurons (control: $n = 6$ cells, 4.1NΔFERM: $n = 6$ cells). Bar graphs show the averaged frequency and amplitude of AMPAR-mEPSCs $\pm$ SEM, with each point representing the averaged value of one neuron. Leftmost panel shows sample traces of control AMPAR-mEPSC events (black/top), compared with 4.1NΔFERM AMPAR-mEPSC events (deep blue/bottom). Scale bars: 500 ms, 20 pA. Left of the amplitude bar graph displays an averaged representative trace from a control (black) and transfected (deep blue) neuron. Scale bars: 5 ms, 2 pA. **H**, Coefficient of variation analysis of AMPAR-eEPSCs from pairs of control and 4.1NΔFERM-expressing DG granule neurons. Coefficient of variation analysis reveals the reduction in AMPAR-eEPSC amplitude caused by the loss of the FERM domain is because of a reduction in quantal content ($n = 13$ pairs). CV^{-2} values are plotted against corresponding ratios of mean amplitudes within each pair (open circles) with mean $\pm$ SEM (filled circle). **I**, Failure analysis of AMPAR-eEPSCs reveals that 4.1NΔFERM-expressing DG granule neurons exhibit significantly higher rates of failure compared with control DG granule neurons ($n = 13$ pairs). **J**, Paired scatterplot shows a significant speeding in the decay kinetics of 4.1NΔFERM AMPAR-eEPSCs when compared with control DG granule neurons ($n = 9$ pairs). Inset shows peak-normalized sample traces from control (black) and transfected (deep blue) neurons. Scale bar: 10 ms. * $p < 0.05$; n.s., not significant.

glutamatergic neurotransmission (D.T. Lin et al., 2009; Wozny et al., 2009). By contrast, we find that reducing protein 4.1N expression in DG granule neurons leads to a substantial reduction in basal glutamatergic synapse function. Immunohistochemical analysis supports the validity of these results, showcasing 4.1N protein expression levels to be markedly greater in DG granule

neurons in comparison to the pyramidal neurons of CA1 and CA3.

Here, we show that knock-down of protein 4.1N in DG granule neurons results in a loss of glutamatergic synapses. Reduced protein 4.1N expression in DG granule neurons produces significant reductions in both AMPAR-eEPSC and NMDAR-eEPSC

amplitude. These deficits in synaptic function are accompanied by a comparable reduction in dendritic spine density. Spine morphology analysis and AMPAR-mEPSC data suggest that those synapses which remain following 4.1N knock-down are normal, and are potentially supported by residual endogenous 4.1N expression. Both the structural and functional synaptic deficits produced by reducing protein 4.1N expression in DG granule neurons are rescued with the expression of recombinant protein 4.1N. Based on these data, we conclude that protein 4.1N plays a major and cell type-specific role in supporting glutamatergic synapse structure and function in DG granule neurons of the hippocampus.

Having identified a major role for 4.1N in DG granule neuron glutamatergic synapse regulation, we examined which domains of protein 4.1N support glutamatergic synapse function in these neurons. Protein 4.1N contains two established domains capable of protein-protein interactions: the C-terminal domain (CTD) and the FERM domain (Baines et al., 2014; Yang et al., 2021). Initially, we hypothesized that 4.1N's postsynaptic function at glutamatergic synapses in DG granule neurons was dependent on its CTD. Previous biochemical studies have reported that 4.1N's CTD binds to the AMPAR subunit, GluA1 (Shen et al., 2000). In dissociated neuronal cultures, 4.1N has been implicated in synaptic GluA1 trafficking, suggesting a noteworthy role for 4.1N in AMPAR regulation (D.T. Lin et al., 2009; Bonnet et al., 2023). However, the 4.1N domain required for this role was not examined. Surprisingly, we find that expression of recombinant 4.1N lacking its CTD in DG granule neurons completely rescues the 4.1N knock-down phenotype. Based on these data, we conclude that 4.1N's CTD is not necessary for the role protein 4.1N plays in supporting synaptic transmission at perforant pathway synapses.

Having found the CTD to be dispensable for 4.1N's synaptic regulatory role in DG granule neurons, we examined the role of the FERM domain. This domain has been shown to bind to a large number of postsynaptic regulatory proteins implicated in the assembly, maintenance, and plasticity of synapses (Cohen et al., 1998; Biederer and Südhof, 2001; Li et al., 2007; Hoy et al., 2009). Molecular replacement of endogenous 4.1N with a mutant lacking the FERM domain (4.1N Δ FERM) only partially restores synaptic deficits seen with knock-down of 4.1N. While NMDAR function and dendritic spine density are restored to baseline, synaptic AMPAR function remains markedly reduced with the 4.1N Δ FERM mutant. Our AMPAR-mEPSC, quantal analysis, and failure analysis data reveal that the selective reduction in AMPAR-eEPSC amplitude is because of a reduction in the number of glutamatergic synapses that express functional AMPARs. Together, these data demonstrate that 4.1N's FERM domain is critical for supporting synaptic AMPAR expression in DG granule neurons and that its dysfunction results in an increase in the number of silent synapses.

As stated, protein 4.1N has been implicated in the trafficking of GluA1-containing AMPARs (D.T. Lin et al., 2009; Bonnet et al., 2023). It has been shown that knocking out GluA1 and preventing the trafficking of GluA1-containing receptors results in a marked reduction in AMPAR-eEPSC amplitude and AMPAR-mEPSC frequency (Lu et al., 2009; Herring et al., 2013). We find that deletion of 4.1N's FERM domain produces a similar synaptic phenotype. Additionally, it has been shown that preventing GluA1 expression and trafficking results in a marked speeding of synaptic AMPAR current decay kinetics. This effect is attributable to the loss of GluA1/A2 receptors, which deactivate more slowly than GluA2/A3 receptors (Lu et al., 2009; Herring et al., 2013). We, therefore, examined the impact of

4.1N's FERM domain on AMPAR-eEPSC decay. Remarkably, we find that deletion of 4.1N's FERM domain produces a significant increase in the decay rate of AMPAR-eEPSCs, similar to that observed with the loss of GluA1 expression/trafficking. Thus, loss of 4.1N's FERM domain phenocopies the synaptic alterations observed when GluA1-containing AMPARs are compromised in neurons (Lu et al., 2009; Herring et al., 2013). Together, these results suggest that, in DG granule neurons, 4.1N's FERM domain, as opposed to its CTD, is responsible for GluA1-containing AMPAR trafficking to synapses.

In the present study, we show that knocking down 4.1N expression in DG granule neurons results in a reduction in AMPAR- and NMDAR-eEPSC amplitudes that is caused by a loss of dendritic spines. Molecular replacement of 4.1N with 4.1N Δ FERM rescues dendritic spine loss and supports synapses that contain NMDARs but lack AMPARs. Thus, 4.1N's FERM domain is necessary for the presence of functional AMPARs at synapses. However, the domain(s) required for 4.1N's role in supporting glutamatergic synapse structure remains unknown. We hypothesize that a yet to be identified domain of 4.1N promotes the recruitment of actin regulatory proteins that are required for the development or maintenance of dendritic spines. Alternatively, it is possible that 4.1N directly supports dendritic NMDAR recruitment and/or clustering, which in turn facilitates NMDAR-dependent synaptogenesis (Kwon and Sabatini, 2011). At present, a direct interaction between 4.1N and NMDARs has yet to be observed. Nevertheless, it is plausible that 4.1N promotes dendritic NMDAR recruitment/clustering through an intermediate protein – and that the loss of this indirect interaction following 4.1N knock-down inhibits NMDAR-mediated synaptogenesis. Protein 4.1 is often described as a hub protein with the ability to associate and organize many synaptic regulatory proteins (Baines et al., 2014; Yang et al., 2021). Going forward, it will be important to carry out thorough investigations of candidate binding proteins to identify the protein-protein interactions necessary for the role 4.1N plays in supporting the structure and function of glutamatergic synapses in DG granule neurons.

In the present work, we do not observe a role for 4.1N in supporting basal glutamatergic synapse function in CA1 pyramidal neurons. Historically, a synaptic regulatory role for 4.1N in CA1 pyramidal neurons has been controversial. Germline knock-out of 4.1N and 4.1G, another 4.1 family member, in a juvenile mouse model produced no changes in baseline synaptic transmission or in LTP in CA1 pyramidal neurons (Wozny et al., 2009). In an independent study, lentiviral knock-down of 4.1N in CA1 pyramidal neurons of adult mice resulted in no change to basal glutamatergic synapse function, but LTP maintenance was found to be inhibited (D.T. Lin et al., 2009). These seemingly discrepant results may suggest that 4.1N has a developmentally regulated role in LTP maintenance in CA1 pyramidal neurons. In our study we observe little, if any, 4.1N expression in the CA1 hippocampal subregion of juvenile rats. However, protein 4.1N expression within CA1 may achieve appreciable levels later in adulthood. Going forward, it will be important to examine cell type-specific roles for 4.1N in synaptic function and plasticity at different stages of development.

In conclusion, we demonstrate a major role for 4.1N in supporting basal glutamatergic synapse structure and function, and we find that this role is cell type-specific within the hippocampus. In contrast to a possible modulatory role in LTP maintenance observed in CA1 pyramidal neurons (D.T. Lin et al., 2009), we believe that 4.1N serves as a master regulator of synaptic development in DG granule neurons. With this study, 4.1N

joins a growing list of synaptic proteins that play cell type-specific roles in regulating perforant pathway synapses (Roy et al., 2018; Rao et al., 2019; Grant and Fransén, 2020; Kay et al., 2022). For example, we have recently shown that schizophrenia-associated mutations in the synaptic scaffolding protein SAP97 produce dramatic and likely pathologic elevations in glutamatergic synapse strength specifically in DG granule neurons (Kay et al., 2022). Glutamatergic synapses onto DG granule neurons act as the gateway for information flow into the hippocampus. Regulation of the number and strength of these synapses underlie the ability of the dentate gyrus to separate similar information into distinct representations in a process called pattern separation (Leutgeb et al., 2007; McHugh et al., 2007). It is generally held that pattern separation underlies our ability to distinguish a memory from similar stored memories. Thus, functional deficits in proteins governing dentate gyrus synaptic function stand to have profound consequences on how we perceive the external world. Going forward, it will be important to understand how the unique synaptic proteins present at perforant pathway synapses support information processing in the dentate gyrus, and how deficits in the function of these proteins contribute to the development of complex brain disorders.

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