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RNA editing-mediated regulation of calcium-dependent activator protein for secretion (CAPS1) localization and its impact on synaptic transmission

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Abstract

Calcium-dependent activator protein for secretion 1 (CAPS1) is a SNARE accessory protein that facilitates formation of the SNARE complex to enable neurotransmitter release. Messenger RNAs encoding CAPS1 are subject to a site-specific adenosine-to-inosine (A-to-I) editing event resulting in a glutamate-to-glycine (E-to-G) substitution in the C-terminal domain of the encoded protein product. The C-terminal domain of CAPS1 is necessary for its synaptic enrichment and *Cadps* RNA editing has been shown previously to enhance the release of neuromodulatory transmitters. Using mutant mouse lines engineered to solely express CAPS1 protein isoforms encoded by either the non-edited or edited *Cadps* transcript, primary neuronal cultures from mouse hippocampus were used to explore the effect of *Cadps* editing on neurotransmission and CAPS1 synaptic localization at both glutamatergic and GABAergic synapses. While the editing of *Cadps* does not alter baseline evoked neurotransmission, it enhances short term synaptic plasticity, specifically short-term depression, at inhibitory synapses. *Cadps* editing also alters spontaneous inhibitory neurotransmission. Neurons that solely express edited *Cadps* have a greater proportion of synapses that contain CAPS1 than neurons that solely express non-edited *Cadps* for both glutamatergic and GABAergic synapses. Editing of *Cadps* transcripts is regulated by neuronal activity, as global network stimulation increases the extent of transcripts edited in wild-type hippocampal neurons, whereas chronic network silencing decreases the level of *Cadps* editing. Taken together, these results provide key insights into the importance of *Cadps* editing in modulating its own synaptic localization, as well as the modulation of neurotransmission at inhibitory synapses in hippocampal neurons.

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Conflicts of Interest Disclosure

The authors declare no conflicts of interest.

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Keywords

ADAR; glutamate; GABA; neurotransmitter

Introduction

Signal propagation between neurons relies on fast quantal release of neurotransmitters from the presynaptic terminal into the synaptic cleft. Action potential-mediated neuronal depolarization elicits the influx of Ca^{2+} into the nerve terminal, triggering the fusion of neurotransmitter-filled synaptic vesicles docked at the active zone of the presynaptic plasma membrane (Rothman, 2014; Südhof, 2013). Central to the exocytotic release of neurotransmitters is the interaction of four SNARE motifs in proteins on the synaptic vesicle membrane (synaptobrevin/VAMP) and on the plasma membrane (syntaxin and SNAP25) to facilitate fusion of opposing membranes. The regulated assembly of this SNARE complex is controlled by a cascade of proteins that can arrest or accelerate the formation of distinct intermediates and promote unidirectionality (Rizo, 2018; Brunger et al., 2019). Calcium-dependent activator protein for secretion 1 (CAPS1) is a SNARE accessory protein that facilitates formation of the SNARE complex through direct interactions with both SNARE and SNARE accessory proteins (Khodthong et al. 2011; Daily et al. 2010; Zhou et al. 2019; James et al. 2009). SNARE complex formation leads to the generation of a “readily-releasable” pool of synaptic vesicles which are located within a few nanometers of the active zone plasma membrane and can undergo rapid exocytosis upon Ca^{2+} influx into the synapse (Sudhof 2012). Simultaneous knockout of CAPS1, and its paralog CAPS2, reduces the number of vesicles located within 5 nm of the active zone (Imig et al. 2014), and knockout of CAPS1 alone reduces the size of the readily-releasable pool of vesicles (Jockusch et al. 2007; Eckenstaler et al. 2016). CAPS1-deficient neurons and neuroendocrine cells display reduced neurotransmitter and neuropeptide release (Rupnik et al. 2000; Speidel et al. 2005; Jockusch et al. 2007; Sadakata et al. 2013; Shaib et al. 2018) and global ablation of CAPS1 expression in mice results in perinatal lethality, albeit with normal development prior to birth (Speidel et al. 2005).

CAPS1 is a soluble, multi-domain protein that is expressed throughout the nervous and endocrine systems (Speidel et al. 2003; Sadakata et al. 2006; Sadakata et al. 2007b). In cultured neurons, CAPS1 is expressed diffusely in the soma (Eckenstaler et al. 2016) and in puncta along neurites which colocalize with several pre-synaptic proteins including the vesicular glutamate transporter 1 (vGlut1) (Farina et al. 2015), synaptobrevin-2 (Shaib et al. 2018), synapsin 1/2 (Shaib et al. 2018), and synaptophysin 1 (van Keimpema et al. 2017). The carboxyl-terminal domain of CAPS1 is necessary for its synaptic localization in cultured neurons, as deletion of the domain eliminates CAPS1 puncta in neurites and ablates its co-localization with synaptophysin (van Keimpema et al. 2017). Furthermore, neurons expressing a truncated CAPS1 protein lacking its C-terminus fail to support dense core vesicle (DCV) exocytosis (van Keimpema et al. 2017), demonstrating the significance of this domain for proper synaptic localization and function.

The role of CAPS1 in regulating excitatory spontaneous and evoked exocytosis has been studied extensively. CAPS1 deletion in primary hippocampal neurons decreases the frequency and amplitude of spontaneous release events, reduces the amplitude of evoked release events, and alters short term synaptic plasticity (Jockusch et al. 2007). These observations are further supported *in vivo,* as region-specific knockout of CAPS1 leads to a decrease in excitatory neurotransmission at hippocampal CA3-CA1 synapses (Shinoda et al. 2016) and cerebellar climbing fiber-Purkinje cell synapses (Sadakata et al. 2013). CAPS1-mediated short-term synaptic plasticity is physiologically significant, as mutant mice in which CAPS1 expression was selectively ablated from the thalamus exhibit altered short term synaptic depression at thalamocortical layer IV synapses (Nestvogel et al. 2020). Altered synaptic plasticity led to stronger adaptation to visual stimulation in anesthetized animals, illustrating the capacity for changes in pre-synaptic plasticity to have significant neuronal network effects. Thus, CAPS1 promotes excitatory spontaneous and evoked neurotransmission and alters short term synaptic plasticity using both *in vitro* and *in vivo* model systems.

Pre-mRNA transcripts encoding CAPS1 are subject to a highly conserved adenosine-to-inosine (A-to-I) RNA editing event within a region of the open reading frame encoding the carboxyl-terminal domain (Li et al. 2009). Since inosine is read as guanosine during translation (Basilio et al. 1962), this site-specific modification results in the conversion of a genomically-encoded glutamate (GAG) to a glycine (GIG) codon in mature *Cadps* mRNAs to produce a non-synonymous amino acid substitution in the protein (amino acid 1252 in mouse CAPS1). The conversion of A-to-I is mediated through the actions of a family of double-stranded RNA (dsRNA) binding proteins referred to as ADARs (adenosine deaminases acting on RNA) that selectively deaminate adenosine residue(s) in duplex regions of precursor and mature mRNA transcripts (Nishikura 2010). Two active members of the ADAR family, ADAR1 and ADAR2, are thought to be responsible for all mammalian A-to-I editing events and are essential for viability (Hartner et al. 2004; Higuchi et al. 2000; Wang et al. 2004). Levels of *Cadps* editing in the mouse brain vary in a region-specific manner, with approximately 20% of *Cadps* transcripts edited in whole brain (Miyake et al. 2016).The functional effect and physiological significance of *Cadps* editing have been studied using mice solely expressing edited *Cadps* transcripts encoding the glycine-containing CAPS1(G) isoform of the protein. Sole expression of CAPS1(G) results in leanness and hyperactivity in mutant animals and produces an increase in the evoked release of neuromodulatory transmitters, including norepinephrine from adrenal chromaffin cells and dopamine from striatal synaptosomes (Miyake et al. 2016). Additional studies suggested that *Cadps* editing-dependent enhancement of neurotransmission may result from increased binding between CAPS1(G) and the SNARE protein syntaxin-1 compared to the CAPS1(E) protein (Miyake et al. 2016).

The effect of *Cadps* RNA editing on exocytosis of synaptic vesicles containing the fast-acting neurotransmitters, glutamate and GABA, and a link between enhanced exocytosis and SNARE protein binding remain uncharacterized. In this study, we examined the effect of *Cadps* RNA editing on spontaneous and evoked neurotransmission at excitatory and inhibitory synapses using primary hippocampal neuron cultures isolated from mutant mice engineered to solely express either edited or non-edited *Cadps* mRNAs. These studies

indicate that *Cadps* RNA editing affects short-term synaptic plasticity at GABAergic synapses without altering baseline evoked neurotransmission. Analysis of spontaneous neurotransmission also reveals a change in inhibitory events. To further examine the mechanism(s) underlying enhanced exocytosis, we investigated the effect of RNA editing on CAPS1 synaptic localization to reveal that CAPS1(G) localizes to a greater proportion of both excitatory and inhibitory synapses than CAPS1(E). Additionally, we confirm that the extent of *Cadps* editing and *Adar1b* expression in wild-type neurons is regulated by neuronal activity.

Methods

Animal Information

Mutant mice in which the editing of *Cadps* transcripts was selectively ablated (*Cadps*^{em1Eme}), hereafter referred to as CAPS1(E) mice, were made using the CRISPR-cas9 system to delete the editing site complementary sequence in intron 27 of the *Cadps* gene (RRID:MGI:6506972). Two gRNAs (Sigma-Aldrich), and cas9 mRNA (Sigma-Aldrich, cat. CAS9MRNA, 2017) were injected into the cytoplasm of single cell embryos derived from C57Bl/6NHsd mice (Envigo, RRID:MGI:5655347). Embryos were transplanted into pseudo-pregnant foster dams and pups were screened for the desired deletion using both the CAPS1(E) genotyping protocol (*described below*) and direct sequencing of PCR amplicons. A founder animal, heterozygous for the mutant *Cadps* allele, was mated with wild-type C57Bl/6NHsd animals to generate heterozygous offspring. Mutant animals were backcrossed to wild-type C57Bl/6NHsd animals to eliminate potential off-target effects and all subsequent offspring were generated by heterozygous mating to maintain the homozygous mutant line and control littermates.

Mice solely expressing edited CAPS1 transcripts (*Cadps*^{tm1Osb}), hereafter referred to as CAPS1(G) mice, were developed using the C57Bl/6NJcl mouse strain and cryopreserved mouse embryos from this line were acquired from the Institute of Physical and Chemical Research (Riken; cat. CDB1091K, RRID:MGI:6506970). Mouse embryos were re-derived by implantation of embryos into pseudo-pregnant foster dams. Pups were screened for the mutant allele using the CAPS1(G) genotyping protocol (*described below*) and all subsequent offspring were generated by heterozygous mating to maintain the homozygous mutant line and control littermates.

As CAPS1(E) and CAPS1(G) mice were developed using slightly different background strains (C57Bl/6NHsd and C57Bl/6NJcl, respectively), all experiments were performed in both mutant and wild-type animals from each respective strain [referred to as CAPS1(E) WT and CAPS1(G) WT]. Comparisons between wild-type littermates for CAPS1(E) and CAPS1(G) mutant mice were made for every experiment to assess possible strain effects and are reported in the Supplementary Data.

All animal procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee (protocol #s M2000083 and M1500005). Animals were separated by sex at weaning and housed in cages with 2-5 mixed genotype littermates. *Ad libitum* access to food and water was provided with a 12-hour light/dark cycle and standard environmental

conditions. Neonatal animals were euthanized by rapid decapitation for neuron cultures and isoflurane overdose for adult tissue dissection. Twelve-week-old mice (3 females and 2 males/genotype; females 17-23 grams, males 23-29 grams, same initial and used quantities) were used for qRT-PCR and *Cadps* editing level quantification experiments. 12-week-old male mice (5 initial and used per group) were used for Western blotting analyses.

Mouse genotyping

Genomic DNA from tail and toe biopsy samples was prepared using REDExtract-N-Amp Tissue PCR Kit (Sigma-Aldrich, cat. XNAT, 2017-2020) according to the manufacturer's instructions. CAPS1(E) mice were genotyped by PCR amplification using forward (5'-TCCCACTTGTCCTCTCTCAGATG-3') and reverse (5'-GGAGGCCCCACTGGTGAGTT-3') primers to generate PCR amplicons that span the targeted deletion. Amplification products were resolved by 2% agarose gel electrophoresis to detect amplicons corresponding to the wild-type (177 bp) and CAPS1(E) (127 bp) alleles (Supplementary Figure S1). Mice bearing the CAPS1(G) allele were genotyped using direct sequence analysis of PCR amplicons containing the editing site, generated using forward (5'-GATGGACGTGGCCGACGCCTACG-3') and reverse (5'-CTGGGATGCAGACACAGCCACACC-3') primers.

Primary Hippocampal Cell Cultures

Primary dissociated hippocampal neuron cultures were prepared as described previously (Kavalali et al. 1999; Schoch et al. 2001). Briefly, hippocampi were dissected from 1- to 2-day-old homozygous CAPS1(E), CAPS1(G) mutant mice and wild-type littermates, with hippocampi from 1 to 4 animals pooled per culture. Dissected hippocampi were dissociated with a trypsin solution (10 mg/mL) for 10 minutes at 37°C. After mechanical trituration by pipetting, the cells were plated on 12 mm glass cover slips coated with Matrigel (Corning Biosciences, cat. 354234, 2018-2020) with a ratio of 3 cover slips per hippocampus. The growth medium contained MEM (without phenol red), 5 g/L D-glucose, 0.2 mg/L $NaHCO_3$, 100 mg/L transferrin (Sigma, cat. 616420-100MG, 2018-2020), 0.5 mM L-glutamine, 2% B27 supplement (Invitrogen, cat. 17504044, 2018-2020) and 5% fetal bovine serum (Fisher Scientific, cat. SH30070.03, 2018-2020). 2 μM cytosine arabinoside (Sigma, cat. C1768-100MG, 2018-2020) was added to the medium after 1 day *in vitro* (DIV 1) and the concentration was reduced to 1 μM at DIV 4. Cultures were incubated at 37°C in a humidified incubator with a 95% air/5% CO_2 environment and all experiments were performed at DIV 15-18.

Activity Modulation of Neurons

Primary hippocampal cell cultures from wild-type mice were treated with either 40 μM bicuculline (Sigma, cat. 14340, 2018-2020) or 2 μM TTX (Enzo Life Science, cat. BML-NA120-0001, 2018-2020), or an equal volume of 99.5% DMSO (Sigma, cat. D4540, 2016) as vehicle control on DIV 15 and were incubated for 48 hours at 37°C in a humidified incubator with a 95% air/5% CO_2 environment.

Quantification of CAPS1 RNA editing

RNA was extracted from flash frozen, sagittally bisected whole brain tissue using Trizol Reagent (Invitrogen, cat. 15596018, 2018) according to the manufacturer's instructions. RNA was isolated from neuron cultures using Trizol to harvest cells and phase separate the RNA according to manufacturer's instructions. The aqueous phase from the Trizol separation was added to an equal volume of 70% ethanol and loaded onto a purification column provided with the RNeasy Micro Kit (Qiagen, cat. 74004, 2019-2020), and the remaining steps of purification were carried out according to the manufacturer's instructions. The optional on-column DNase treatment step was performed using RNase-free DNase according to the manufacturer's instructions (Qiagen, cat. 79254, 2019-2020). cDNA was prepared from 2 μg (brain tissue) or 200 ng (neuron cultures) RNA using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, cat. 4368813). RT-PCR amplicons spanning the *Cadps* editing site were generated using forward (5'-GATGGACGTGGCCGACGCCTACG-3) and reverse (5'-CTGTCCTTCATGCTGATACCTTGTAAG-3') primers. PCR amplicons were visualized by ethidium bromide staining after agarose gel electrophoresis and purified using the SV Wizard PCR and Gel Clean-up Kit according to the manufacturer's instructions (Promega, cat. A9282, 2019-2020). Purified PCR amplicons were sequenced using the reverse primer indicated above. Relative peak heights in sequence electropherogram traces were used to quantify RNA editing, as previously described (Malik et al. 2021).

qRT-PCR

RNA was extracted and cDNA generated from bisected brain tissue and primary neuron cultures, as described above. Quantitative RT-PCR was performed using a *Cadps* probe/primer set (Applied Biosystems, assay Mm00488924_m1, 2017) with Eukaryotic 18S rRNA control probe/primer limited set (Applied Biosystems, cat. 4319413E, 2017), or *Adar* primer/probe set (Applied Biosystems, assay Mm00508001_m1, 2020) and *Adarb1* primer/probe set (Applied Biosystems, assay Mm00504621_m1, 2020) with control *Gapdh* primer/probe set (Applied Biosystems, assay Mm99999915_g1, 2020) and 2X Taqman Universal PCR Mastermix (Applied Biosystems, cat. 4304437, 2017, 2020). Data analysis was performed using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001).

High-Throughput Sequencing

Complementary DNA was generated as described above. A two-step RT-PCR strategy was employed to multiplex samples within one Illumina flow cell, as previously described (Hood et al. 2014), using forward (5'-ATTAACCCTCACTAAAGGGATTCTCAGGATGTCCTTCGTGATA-3) and reverse (5'-TAATACGACTCACTATAGGGTCAGCCACGTGCAGATGATG-3') primers that span the *Cadps* editing site. Data exclusion criteria were pre-defined as any reads that did not match the *Cadps* reference gene sequence in a region encompassing 15 nucleotides upstream to 15 nucleotides downstream from the editing site.

Western Blotting

Whole cell lysates from brain tissue were prepared using RIPA buffer supplemented with cOmplete™, Mini, EDTA-free protease inhibitor tablets (Roche, cat. 4693159001, 2017-2020). Equal concentrations of protein samples were resolved by SDS-PAGE electrophoresis using a 4-20% gradient gel (BioRad, cat. 4561094, 2017-2020). Proteins were transferred to a nitrocellulose membrane (Cytiva, cat. 10600004, 2018) using a semi-dry transfer apparatus. Membranes were air dried for at least 30 minutes, re-hydrated, and blocked for 1 hour with Intercept PBS blocking buffer (LI-COR, cat. 927-70001, 2017-2020). Primary and secondary antibodies (Table 1) were diluted in blocking buffer and blots were incubated overnight or for 2 hours with primary and secondary antibodies, respectively. Immunoblots were imaged using an Odyssey CLx infrared imaging system (LI-COR) and quantified using Image Studio Lite (LI-COR).

Immunocytochemistry and Quantitative Colocalization Analysis

DIV16-19 primary hippocampal neurons were washed in PBS and fixed in a buffer containing 1% PFA/7.5% sucrose in PBS (wt/vol). Autofluorescence was reduced with a 50mM glycine solution and cells were permeabilized in 0.0075% (wt/vol) digitonin buffer. Coverslips were blocked with 2% BSA and incubated overnight with primary antibodies diluted in blocking buffer. Primary antibodies used include rabbit anti-CAPS1 (Synaptic Systems, cat. 262 013, 1:200, RRID:AB_2619979), mouse anti-vGAT (Synaptic Systems, cat. 131 011, 1:200, RRID:AB_887872), and guinea pig anti-vGlut1 (Synaptic Systems, cat. 135 304, 1:1,000, RRID:AB_887878). After washing, secondary antibodies diluted in blocking buffer were added for 1-2 hours. Secondary antibodies used include goat anti-rabbit Alexa 568 (Invitrogen, cat. A11011, 1:500, RRID:AB_143157), donkey anti-mouse Alexa 488 (Invitrogen, cat. 21202, 1:500, RRID:AB_141607), and goat anti-guinea pig Alexa 647 (Invitrogen, cat. A21450, 1:1,000, RRID:AB_2735091). Coverslips were washed and imaged within 48 hours. Cells were imaged using a Zeiss LSM 510 Meta inverted confocal microscope. Image Z stacks were captured using a 63X (na 1.4) oil immersion objective. Images not used for analysis were pre-defined as those in which x,y drift occurred through the Z stack, or those in which obvious fluorescent artefacts (cell debris, aggregates of secondary antibody) were present that could skew proper threshold analysis.

Object-based colocalization was performed using the 3D object counter in Fiji (National Institutes of Health, RRID:SCR_002285), with slight modifications to the object overlap analysis method previously described (Bolte & Cordelieres 2006). Briefly, images were segmented using the Moments threshold analysis (Tsai 1985). Channels were split, and a shape filter (IJ Blob, elongation=0-0.6) (Wagner & Lipinski 2013) and water-shedding were applied to the green (vGAT) and blue (vGlut1) channels to select for individual, punctate-like structures. 3D objects were counted in the blue and green channels using the 3D object counter. Z stacks of each channel to be co-localized were multiplied (i.e. CAPS1 x vGAT and CAPS1 x vGlut1) to obtain a Z-stack of overlapping pixels. 3D objects were counted in each overlap stack using a size filter to define positive colocalization as an overlap of >15 voxels of two colors in the same object (calibration: 1 voxel = 0.143 × 0.143 × 1 μm). The number of CAPS1-containing puncta was determined by dividing the number of objects counted in the overlap z stack by the number of objects counted in the respective individual

z stack for vGlut1 or vGAT. A custom macro was written to automate these procedures and is available upon request. Line scan analysis was performed using a line thickness of 1.4 μm.

Electrophysiology

Electrophysiologic recordings were performed on pyramidal neurons from primary hippocampal cell cultures at room temperature. The external solution during the recordings was a modified Tyrode's solution containing (in mM) 150 NaCl, 4 KCl, 10 glucose, 10 HEPES, 2 $MgCl_2$, 2 $CaCl_2$ at pH 7.4 and 310 mOsm. The internal solution for the recording pipette contained (in mM) 15 Cs-$MeSO_3$, 10 CsCl, 5 NaCl, 10 HEPES, 0.6 EGTA, 20 Tetraethylammonium-Cl, 4 Mg-ATP, 0.3 Na_3GTP and 10 QX-314 [N-(2,6-dimethylphenylcarbamoylmethyl)-triethylammonium bromide] at pH 7.35 and 300 mOsm. D-AP5 (50 μM, Abcam, cat. 120003, 2019) was used to eliminate NMDA currents. Picrotoxin (50 μM, Sigma, cat. P1675, 2019) and CNQX (10 μM, Sigma, cat. C239, 2019) were used to isolate excitatory and inhibitory evoked postsynaptic currents, respectively. For miniature post-synaptic current recordings, TTX (1 μM, Enzo Life Science, cat. BML-NA120-0001, 2018-2020),) was added to block action potentials. The neurons were voltage-clamped at −70 mV and postsynaptic currents were measured using an Axon Instruments Axopatch 200B amplifier (Molecular Devices, San Jose, CA) with a 2 KHz filter. The signal was digitized using Axon Instruments Digidata 1550B data acquisition system (Molecular Devices) with a sampling rate of 10 kHz. The data was recorded using Clampex 11 software (Molecular Devices). Threshold for mEPSC and mIPSC amplitudes were 5 pA and the rise time threshold was set to 0.3 ms. For evoked experiments, any response where the stimulus was applied during a spontaneous action potential or any recording where the recorded minimum amplitude was higher than the starting baseline were discarded.

Study Design

This study was not preregistered. Unless otherwise stated, no randomization was performed to allocate subjects in the study. The experimenter was blinded to genotype during RNA and protein biochemical experiments. No blinding was performed during imaging or electrophysiology experiments or during statistical analyses. The study was exploratory as no primary or secondary endpoints were pre-specified. Unless otherwise stated, no exclusion criteria were pre-defined. All studies were performed between 08:00- 20:00, Central Standard Time.

Statistical Methods

Statistical analyses of data were performed using Prism software, version 9.0 (GraphPad, San Diego, CA). No sample size calculations were performed. Outliers were identified and excluded from the evoked IPSC and EPSC data sets using the ROUT Method, Q= 1%. Data normality was assessed using the Shapiro-Wilk Test, and non-parametric statistical tests were employed to compare non-normal data. Statistical tests are reported in each figure legend. Reported values represent the mean ± SEM unless otherwise stated.

Results

Activity-Dependent Modulation of *Cadps* Editing

Previous studies have demonstrated an increase in both dopamine release from striatal synaptosomes and DCV exocytosis from adrenal chromaffin cells prepared from mutant mice solely expressing the edited isoform of CAPS1 [CAPS1(G)] (Miyake et al. 2016). Given the broad expression of CAPS1 throughout the brain (Speidel et al. 2003; Wassenberg & Martin 2002), it is likely that editing-mediated alterations in neurotransmitter release are not limited to catecholaminergic systems. To examine the functional consequences of editing of *Cadps* mRNAs in the glutamatergic and GABAergic systems, primary hippocampal neuron cultures were selected as a model system. In wild-type neuronal cultures, an average of 29.9 ± 4.0% (n=6 cultures) of *Cadps* transcripts were edited. The variability in *Cadps* editing between cultures was not necessarily surprising, as neuronal activity has been shown to modulate RNA editing in primary cortical neurons and hippocampal brain slices for several ADAR targets (Sanjana et al. 2012; Balik et al. 2013). To examine whether *Cadps* editing was similarly modulated by neuronal activity in primary hippocampal neurons, cultures from wild-type mice were treated for 48 hours with tetrodotoxin (TTX)—a sodium channel blocker—to prevent action potential firing, or bicuculline—a $GABA_A$ receptor antagonist—to alleviate circuit inhibition and promote neuronal activation. *Cadps* editing was significantly decreased following TTX application (22.5 ± 2.1%, $p \leq 0.01$) compared to vehicle treated cultures (32.1 ±3.2%), and significantly increased following bicuculline application (40.8 ± 2.2%, $p \leq 0.01$) (Figure 1a, b). As *Cadps* editing is mediated by both ADAR1 and ADAR2 (Miyake et al. 2016), expression of RNA transcripts encoding these proteins also was quantified. While the expression of *Adarb1* RNA (encoding the ADAR2 protein) decreased with TTX application ($\Delta C_{t\ Adarb1\text{-}gapdh}$ TTX 6.1 ± 0.3, vehicle 5.3 ± 0.2, $p \leq 0.05$) and increased upon bicuculline treatment ($\Delta C_{t\ Adarb1\text{-}gapdh}$ BIC 5.0 ± 0.1, vehicle 5.3 ± 0.2, $p \leq 0.05$), the expression of *Adar* transcripts (encoding the ADAR1 protein) was not altered by pharmacologic manipulation of neuronal activity ($\Delta C_{t\ Adar\text{-}gapdh}$ TTX 9.6 ± 0.2, vehicle 9.9 ± 0.2, $p > 0.05$ and BIC 9.8 ± 0.3, $p > 0.05$ compared to vehicle) (Figure 1c). These findings suggest that *Cadps* editing is modulated by neuronal activity, potentially through changes in ADAR2 expression levels.

Generation of CAPS1(E) mice

To make direct functional comparisons between CAPS1 proteins encoded by non-edited and edited transcripts, it was essential to develop a model system in which the expression of the encoded CAPS1(E) and CAPS1(G) protein isoforms was limited to a single variant that could not be altered in response to neuronal activity. While mice solely expressing the CAPS1(G) isoform had been developed previously (Miyake et al. 2016), we engineered a mutant mouse model solely expressing the non-edited isoform of *Cadps*, encoding the CAPS1(E) protein variant. A CRISPR-Cas9 based approach using two single guide RNAs (sgRNAs) was employed to excise the editing site complementary sequence (ECS) in intron 27 of the mouse *Cadps* gene, a region critical for formation of the RNA duplex required for ADAR-mediated editing of *Cadps* transcripts (Figure 2a, b) (Miyake et al. 2016). Sequence analysis of genomic DNA-derived PCR amplicons generated from CAPS1(E) mice confirmed deletion of the expected 54 nucleotides and revealed an insertion of four

additional nucleotides within the intron, presumably resulting from non-homologous end joining (Figure 2b).

To confirm that mutant mice bearing the modified *Cadps* allele solely express transcripts encoding the CAPS1(E) isoform of the protein, RT-PCR amplification of the *Cadps* editing site from whole-brain derived RNA was performed and the resulting amplicons were sequenced directly. Complementary DNA from wild-type animals exhibited overlapping adenosine/guanosine peaks in electropherogram traces resulting from a mixture of non-edited and edited *Cadps* transcripts (16.7% editing), whereas cDNAs from CAPS1(E) animals showed only the non-edited adenosine nucleotide at the editing site (Figure 2c). To further confirm the absence of editing in CAPS1(E) animals, high-throughput sequencing was used to survey a large population of editing sites within RT-PCR amplicons generated from whole brain RNA. Of 737,158 total *Cadps* reads, 99.89% contained a non-edited adenosine residue at the editing site, while the remaining 0.11% of reads contained either G (0.04%), T (0.01%), or C (0.06%), likely representing experimental background created by reverse transcriptase or DNA polymerase errors made during generation and sequencing of the library. Offspring from the mating of heterozygous CAPS1(E) animals were genotyped at weaning (Supplementary Figure S1) and demonstrated a normal Mendelian distribution, indicating that the CAPS1(E) mutation does not result in embryonic or early postnatal lethality.

To assess whether the lack of editing of *Cadps* mRNAs affects steady-state *Cadps* mRNA or CAPS1 protein expression levels, quantitative RT-PCR and Western blotting strategies were used to compare brain samples from CAPS1(E) and wild-type mice. Results from these analyses revealed that sole expression of non-edited *Cadps* transcripts did not significantly alter either *Cadps* mRNA ($\Delta C_{t\ Cadps\text{-}18S}$ CAPS1(E) 12.0 ± 0.3, CAPS1(E) WT 12.0 ± 0.4, $p>0.05$) or CAPS1 protein expression in CAPS1(E) mice when compared to wild-type littermates (Figures 2 d–f). As we sought to use this mouse model to investigate pre-synaptic function, we also analyzed expression levels of canonical SNARE proteins (syntaxin-1, SNAP-25, synaptobrevin-2), a synaptic calcium sensor (synaptotagmin-1), and a SNARE accessory protein (munc18). Expression levels of all pre-synaptic proteins analyzed were unaltered in the CAPS1(E) mutant animals (Figure 2e, f, Supplementary Table 1). Analysis of CAPS1 and pre-synaptic protein expression levels also was performed in CAPS1(G) animals (Figures 2e, g) and while no changes in CAPS1 expression were found, altered expression of munc18-1 and SNAP-25 were identified (Supplementary Table 1).

Effect of *Cadps* Editing on Evoked Neurotransmission and Short-term Plasticity

Previous studies have demonstrated a role for CAPS1 in regulating glutamatergic neurotransmission and synaptic plasticity (Jockusch et al. 2007), however GABAergic neurotransmission has not been studied. As CAPS1 is present in 70-93% of GABAergic synapses (*see below*), we investigated the effect of *Cadps* editing on evoked inhibitory neurotransmission by analyzing whole cell recordings from pyramidal neurons in dissociated hippocampal cultures derived from CAPS1(E), CAPS1(G), and corresponding wild-type littermate animals. Biophysical properties including membrane resistance and capacitance were analyzed for all genotypes examined and no significant differences were

identified (Supplementary Figure 2). A train of 10 action potentials was delivered from 1 to 20 Hz to elicit multiple forms of short-term plasticity and the data was analyzed to study various aspects of evoked neurotransmission. There were no detectable changes in IPSC amplitudes measured from the initial stimulus (at 1 Hz) in CAPS1(G) neurons compared to CAPS(E) neurons (CAPS1(E) −2612 ± 350 pA, CAPS1(G) −2248 ± 276 pA, $p>0.05$) (Figure 3a, b). Paired-pulse ratios of the first two IPSC amplitudes were decreased in CAPS1(G) neurons compared to CAPS1(E) neurons, suggesting enhanced release probability in CAPS1(G) neurons (Two-way ANOVA, genotype effect $p\leq0.01$) (Figure 3c). All stimulation frequencies elicited short-term depression at inhibitory synaptic terminals, and CAPS1(G) neurons undergo enhanced synaptic depression compared to CAPS1(E) neurons during high frequency stimulation at 20 Hz (Mixed Effects analysis of 20 Hz, genotype effect $p\leq0.05$) (Figure 3d). No effect of strain was found in baseline neurotransmission, paired-pulse ratios, or short-term plasticity in comparisons of CAPS1(E) WT and CAPS1(G) WT neurons (Supplementary Figure S3a–c). Overall, these results are consistent with the premise that *Cadps* editing enhances release probability and short-term depression at inhibitory synapses.

In parallel electrophysiology studies, the effect of *Cadps* editing on glutamatergic neurotransmission also was assessed. Analysis of the response to the first stimulation at 1 Hz showed no change in EPSC amplitudes in CAPS1(G) neurons compared to CAPS1(E) neurons (CAPS1(E) −861.0 ± 150.7 pA, CAPS1(G) −860.0 ± 105.1 pA, $p>0.05$) (Figure 3e, f). Paired-pulse ratios of EPSC amplitudes from the first two responses show no difference between CAPS1(E) and CAPS1(G) neurons across all stimulation frequencies tested (Two-way ANOVA, genotype effect $p>0.05$) (Figure 3g), although a strain effect is noted when comparing paired-pulse ratios of CAPS1(E) WT to CAPS1(G) WT which prevents any conclusions from being drawn (Two-way ANOVA, genotype effect $p\leq0.05$) (Supplementary Figure S3e). No significant differences in short-term plasticity at excitatory synapses between CAPS1(E) and CAPS1(G) neurons were observed in response to a train of action potentials at 1- 20Hz (Figure 3h). No effect of strain was found in baseline neurotransmission or short-term plasticity in comparisons between CAPS1(E) WT and CAPS1(G) WT neurons (Supplementary Figure S3d, f). These results demonstrate that *Cadps* editing has no significant effect on baseline glutamatergic neurotransmission or high frequency stimulation-driven synaptic plasticity.

Effects of *Cadps* Editing on Spontaneous Neurotransmission

In addition to its role in evoked neurotransmission, CAPS1 also regulates spontaneous neurotransmission as deletion of the protein in cultured hippocampal neurons results in reduced mEPSC frequency and amplitude (Jockusch et al. 2007). To assess the effect of *Cadps* editing on spontaneous neurotransmission, miniature IPSCs and EPSCs were recorded from pyramidal neurons in hippocampal cultures derived from CAPS1(E), CAPS1(G), and wild-type littermate animals (Figure 4a, d). Analysis of mIPSC recordings found no change in frequency (CAPS1(E) 0.62 ±0.16 Hz, CAPS1(G) 0.46 ±0.07 Hz, $p>0.05$; Figure 4b), and an increase in mIPSC amplitudes in CAPS1(G) neurons compared to CAPS1(E) expressing neurons (CAPS1(E) 13.1 ±0.9 pA, CAPS1(G) 18.0 ±1.5 pA, $p\leq0.01$; Figure 4c). No strain effects were observed in mIPSC measurements

(Supplementary Figure S4b, c). While no *Cadps* editing-dependent changes were found in mEPSC frequency (CAPS1(E) 1.4 ±0.2 Hz, CAPS1(G) 1.9 ±0.2 Hz, $p>0.05$) or amplitude (CAPS1(E) 14.9 ±0.7 pA, CAPS1(G) 13.8 ±0.8 pA, $p>0.05$; Figure 4e, f), a strain effect was noted in which neurons from CAPS1(G) WT had reduced mEPSC amplitudes compared to those from CAPS1(E) WT neurons (CAPS1(E) WT 15.2 ±0.9 pA, CAPS1(G) WT 13.0 ±1.0 pA, $p\leq0.05$; Supplementary Figure S4f). Therefore, while a strain effect precludes drawing conclusions about *Cadps* editing-dependent effects on spontaneous glutamatergic neurotransmission, increased mIPSC amplitudes in CAPS1(G) neurons suggests *Cadps* editing alters spontaneous transmission at inhibitory synapses.

Effect *Cadps* Editing on Synaptic Localization

CAPS1 is selectively expressed in some, but not all, excitatory synapses in wild-type neurons (Farina et al. 2015) and the carboxyl-terminal region of CAPS1 (residues 654-1355) is essential for synaptic localization and subsequent vesicle exocytosis (van Keimpema et al. 2017). As editing of *Cadps* RNA results in a glutamate-to-glycine substitution ($E^{1252}G$) within the C-terminal domain, we hypothesized that *Cadps* RNA editing may play a role in modulating the targeting of CAPS1 to synapses. CAPS1 synaptic localization was examined by taking advantage of cultures of dissociated hippocampal neurons prepared from CAPS1(E), CAPS1(G), and corresponding wild-type littermates. CAPS1 synaptic localization was assessed by immunocytochemical analysis using antibodies targeting CAPS1 and both excitatory and inhibitory synapse markers, the vesicular glutamate transporter 1 (vGlut1) and the vesicular GABA transporter (vGAT), respectively. In agreement with previous studies, CAPS1 was expressed in puncta along neurites in cultures from wild-type neurons (Figure 5a), and similar localization was found in CAPS1(E) and CAPS1(G) neurons (Supplementary Figure S5a, b). As all synapses within a field of view were assessed for CAPS1 content, this analysis likely includes terminals from all cell types contained in the culture system, including excitatory pyramidal, granule and inhibitory neurons. CAPS1 co-localized with vGlut1 (Figures 5b, c) and vGAT (Figures 5d, e), and interestingly, much greater co-localization was seen in vGAT than vGlut1 synapses in primary hippocampal neurons.

The extent of CAPS1 synaptic localization was measured using object-based colocalization analysis applied to immunofluorescent images of CAPS1(E) and CAPS1(G) cultures (Bolte & Cordelieres 2006), allowing quantification of the number of vGlut1 and vGAT synapses that contained CAPS1 within a field of view. Results from this analysis showed an editing-dependent increase in CAPS1-containing vGlut1 puncta (Figure 5f). CAPS1(G) neurons had a significantly higher percentage of CAPS1-containing vGlut1 synapses (8.9 ±0.9%) compared to CAPS1(E) neurons (2.9 ±0.6%, $p\leq0.0001$). Furthermore, the percentage of $CAPS1^{+}/vGlut1^{+}$ puncta in wild-type neurons (4.0 ±0.8% CAPS1(E) WT, 6.0 ±0.9% CAPS1(G) WT) was intermediate to that observed in CAPS1(E) and CAPS1(G) neurons, presumably resulting from the intermediate level of editing (~30%) found in wild-type cultures. The change in co-localization of CAPS1 in excitatory pre-synapses did not result from an alteration in total vGlut1 puncta count (CAPS1(E) 731.1 ±80.5 puncta, CAPS1(G) 553.9 ±78.6 puncta, $p>0.05$; Figure 5g). Additionally, no strain effect was found when comparing the percentage of $CAPS1^{+}/vGlut1^{+}$ puncta from CAPS1(E) WT to CAPS1(G)

WT neurons (Supplementary Figure S5c). Together, these results demonstrate that *Cadps* editing enhances glutamatergic synaptic localization of CAPS1.

Parallel analysis of CAPS1 localization in GABAergic synapses also revealed an editing-dependent increase in the percentage of CAPS1-containing vGAT puncta (Figure 5h). In CAPS1(E) neurons, CAPS1 co-localized with an average of 70.0 ±4.8% vGAT puncta, whereas in CAPS1(G) neurons an average of 92.5 ±4.1% vGAT puncta contain CAPS1 ($p \leq 0.001$). Once again, the level of CAPS1 co-localization with the GABAergic synapse marker was at an intermediate level in neurons isolated from wild-type littermates, 87.3 ±3.1% and 81.7 ±2.7% from CAPS1(E) WT and CAPS1(G) WT neurons, respectively, when compared to neurons expressing CAPS1(E) and CAPS1(G). The change in percentage of $CAPS1^+$ puncta did not result from a change in the total number of vGAT synapses (CAPS1(E) 229.4 ±35.2 puncta, CAPS1(G) 298.0 ±30.0 puncta, $p > 0.05$; Figure 5i). As before, no strain effect was found when comparing the percentage of positive CAPS1 puncta in vGAT synapses between CAPS1(E) WT and CAPS1(G) WT neurons (Supplementary Figure S5d). These results show that CAPS1(G) exhibits increased GABAergic synaptic localization compared to CAPS1(E) in a manner like what is seen in glutamatergic synapses.

Discussion

In this report, a novel mouse model solely expressing the glutamate-containing isoform of the CAPS1 protein, CAPS1(E), was developed to assess the full extent of *Cadps* editing-dependent changes to synaptic localization and neurotransmission by comparison to mutant mice solely expressing the CAPS1(G) isoform. This experimental paradigm provides an advantage over the study of a single mutant mouse line in which comparisons are made to control animals, since neurons from wild-type mice exhibit an intermediate level of A-to-I conversion in *Cadps* transcripts and such post-transcriptional modifications can be modulated by neuronal activity (Figure 1a, b), thus making comparisons to wild-type neurons problematic. Additionally, this approach provides the greatest possible range in *Cadps* editing, either 0% or 100%, to distinguish functional differences between mutant mouse lines that express CAPS1 isoforms encoded by non-edited or edited *Cadps* transcripts, respectively.

The importance of CAPS1 as a SNARE accessory protein, to augment the release of neurotransmitters and peptide hormones, has been well-established (Tandon et al. 1998; Elhamdani et al. 1999; Rupnik et al. 2000; Speidel et al. 2005; Jockusch et al. 2007; Sadakata et al. 2013; Shaib et al. 2018; Nestvogel et al. 2020). *Cadps* RNA editing is known to enhance catecholaminergic neurotransmitter release in *ex vivo* synaptosome preparations and in cultured primary adrenal chromaffin cells (Miyake et al. 2016). Building on these findings, the effect of *Cadps* editing on neurotransmission was investigated further by exploring spontaneous and evoked neurotransmission and short-term plasticity at excitatory and inhibitory synapses. Taking advantage of cultured primary hippocampal neurons, our studies have indicated that *Cadps* editing does not have an appreciable effect on excitatory neurotransmission, which is not surprising given that our immunocytochemical analyses revealed CAPS1 expression is limited to an average of 6% of glutamatergic synapses. However, in GABAergic synapses, where CAPS1 is expressed in an average of 82% of

terminals, *Cadps* editing was found to alter paired-pulse ratios and short-term synaptic depression (Figure 3). *Cadps* editing enhanced synaptic depression in response to high frequency stimulation in inhibitory synapses while decreasing the paired pulse ratio, indicating an enhanced release probability. Short-term depression primarily is mediated pre-synaptically by depletion of the readily-releasable pool of vesicles, and enhanced synaptic depression is generally attributed to an increase in release probability or a decrease in the size of the readily-releasable pool of vesicles (Regehr 2012). While our data does not exclude an effect of RRP size on enhanced synaptic depression, the change in PPR in inhibitory synapses is consistent with a *Cadps*-editing dependent increase in release-probability, thus providing a likely explanation for increased short-term depression at these synapses. In agreement with previous studies that show CAPS1 mediates synaptic plasticity (Jockusch et al. 2007; Nestvogel et al. 2020), these studies further indicate that *Cadps* editing modulates a specific type of plasticity, short-term depression at inhibitory synapses. In addition, the observed change in evoked release probability, without a commensurate change in mIPSC frequency, may be attributed to the partial segregation of spontaneous and evoked neurotransmission at inhibitory synapses (Horvath et al. 2020).

Regarding spontaneous inhibitory neurotransmission, miniature IPSC amplitudes were increased in CAPS1(G) cultures with no change in event frequency (Figure 4). The factors most attributed to changes in mPSC amplitudes are post-synaptic receptor populations and vesicular neurotransmitter content (Nusser et al. 1997; Wojcik et al. 2004; Wojcik et al. 2006). Several studies have demonstrated a role of CAPS1 in regulating monoaminergic vesicle content through modulation of vMAT1/2 transporter function or intravesicular transmitter stability (Speidel et al. 2005; Brunk et al. 2009), although other studies have refuted these findings (Fujita et al. 2007). Additionally, a decrease in mEPSC amplitude but no change in amplitude generated in response to exogenously applied glutamate, kainate, or GABA were reported in CAPS1 knockout neurons (Jockusch et al. 2007). These observations indicate a deficiency in vesicle loading, not post-synaptic receptor populations, underlie decreased mEPSC amplitudes in these neurons. Therefore, while the effect CAPS1 in vGAT-mediated neurotransmitter loading has not been investigated explicitly, our studies suggest a possible modulatory role for *Cadps* editing in this process.

While we noted a significant increase in munc18-1 expression and a significant decrease in SNAP-25 expression in CAPS1(G) animals, these changes are unlikely to account for the observed alterations in neurotransmission. Previous studies have shown that munc18-1 overexpression alone does not alter miniature endplate potential amplitudes at the neuromuscular junction and causes an increase in the RRP size of cultured hippocampal neurons which would be predicted to blunt synaptic depression, an effect opposite to our results (Toonen et al. 2006). Similarly, hippocampal neurons with a 50% reduction in SNAP-25 expression have no changes in mEPSC amplitudes or frequencies and exhibit an increase in PPR, suggesting a decrease in release probability, which also contrasts with our results (Alten et al. 2021).

The C-terminal domain of CAPS1 is necessary for synapse localization and exocytosis of DCVs in cultured hippocampal neurons (van Keimpema et al. 2017). As editing in *Cadps* mRNA results in a non-synonymous E-to-G amino acid substitution within this C-terminal

region, the effect of *Cadps* editing on synaptic localization was investigated. CAPS1(G) localized to a significantly greater proportion of both glutamatergic and GABAergic synapses than the CAPS1(E) isoform (Figure 5 f, h). This effect is not explained by altered glutamatergic or GABAergic synapse count (Figure 5 g, i), suggesting the effect is solely driven by enhanced CAPS1 synaptic localization. Results from these studies also revealed that CAPS1 is more highly associated with GABAergic rather than glutamatergic synapses (Figure 5 f, h), which is consistent with electrophysiologic analyses where significant effects are seen in inhibitory but not excitatory neurotransmission (Figures 3, 4).

Previous *in vitro* studies have shown that the affinity of CAPS1(G) for syntaxin-1 is greater than that of CAPS1(E) (Miyake et al. 2016). While it is possible that interactions between syntaxin-1 and CAPS1 drive enhanced synaptic localization of CAPS1(G), as another SNARE accessory protein, munc18, is thought to traffic to synapses with syntaxin-1 (Cijsouw et al. 2014), these observations also may be correlative. In CAPS2—a CAPS1 paralog—the dynactin-binding domain is necessary for proper axonal trafficking (Sadakata et al. 2007a), whereas the synaptic enrichment of another family of SNARE priming proteins with a high homology to CAPS proteins—munc13s —is mediated by C2 domain interactions with RIM1s (Andrews-Zwilling et al. 2006) or an N-terminal coiled-coil domain interaction with ELK1 (Kawabe et al. 2017). As CAPS1 synaptic trafficking and enrichment is poorly understood beyond the identification of a critical role for the C-terminal domain in synapse localization (van Keimpema et al. 2017), additional studies will be required to examine whether the editing of *Cadps* transcripts alters interactions between CAPS1 and other key trafficking/enrichment proteins. More broadly, it remains to be determined whether the editing-dependent enhancement of neurotransmission mediated by CAPS1(G) is due to altered vesicle priming—as suggested by enhanced interactions with syntaxin-1 (Miyake et al. 2016) —or due to increased synaptic localization of CAPS1 (Figure 5 f, h), or both. Overexpression of CAPS1 in wild-type DRG neurons is sufficient to increase DCV release probability and exocytosis (Shaib et al. 2018), suggesting that increasing CAPS1 abundance and increased synaptic enrichment alone could drive enhanced neurotransmission. While the precise molecular mechanism(s) by which CAPS1(G) enhances neurotransmission remain unknown, the present studies reveal a possible role for editing-dependent enhancement of CAPS1 synaptic localization in this process.

ADAR-mediated RNA editing is subject to modulation by neuronal activity, with chronic activation generally leading to global increases in editing, whereas chronic silencing generally causes a global decrease in editing levels (Sanjana et al. 2012; Balik et al. 2013). Consistent with these observations, *Cadps* editing in wild-type hippocampal cultures increased after 48 hours of bicuculline treatment and decreased after 48 hours of TTX treatment (Figure 1a, b). While the direction of change for *Cadps* editing is consistent with previous reports, the magnitude of change in cultured hippocampal neurons (±10%) was far greater than previously observed in cultured cortical neurons (±2-3%) (Sanjana et al. 2012), despite comparable treatment conditions. Changes in the expression of *Adarb1*, but not *Adar*, were found in both model systems (Figure 1c) (Sanjana et al. 2012). Since *Cadps* RNAs are subject to editing by both enzymes (Miyake et al. 2016), alterations in *Adarb1* expression alone could account for the observed changes in A-to-I conversion. Alternatively, as suggested by differences in effect size, additional regulatory factors may be involved.

Numerous studies spanning the past decade have consistently concluded that changes in ADAR expression do not fully account for differences in the extent of A-to-I editing and the identification of such regulatory factors remains an active area of research for the field (Wahlstedt et al. 2009; Hood et al. 2014; Porath et al. 2019; Tan et al. 2017; Li & Church 2013; Schaffer et al. 2020; Sapiro et al. 2020).

Overall, these studies provide further insight into the regulation of *Cadps* editing and examine *Cadps* editing-dependent changes in neurotransmission and CAPS1 subcellular localization. Significant changes in brain *Cadps* editing levels have been reported in individuals with Fragile X syndrome (Tran et al. 2019) and in the hippocampus and frontal cortex of *Fmr1* knockout mice, a model of Fragile X syndrome (Filippini et al. 2017). Additionally, dysregulation of global RNA editing has been reported in several neurological disorders including Alzheimer's disease and amyotrophic lateral sclerosis (Khermesh et al. 2016; Hideyama et al. 2012). Expanding our knowledge of the functional outcomes of *Cadps* RNA editing in fast-acting neurotransmission provides insight into the potential role(s) that alterations in *Cadps* editing may play in such disease states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations:

ADAR
adenosine deaminase acting on RNA

A-to-I
adenosine to inosine

CAPS1
calcium-dependent activator protein for secretion 1

CRISPR
clustered regularly interspaced short palindromic repeats

DCV
dense core vesicle

ECS
editing site complementary sequence

EPSC

excitatory post synaptic current

GABA
γ-aminobutyric acid

IPSC
inhibitory post synaptic current

PCR
polymerase chain reaction

RRID, see scicrunch.org
Research Resource Identifier

RT-PCR
reverse transcription polymerase chain reaction

sgRNA
single guide ribonucleic acid

SNARE
soluble NSF attachment protein receptor

TTX
tetrodotoxin

VAMP
vesicle-associated membrane protein

vGAT
vesicular GABA transporter

vGlut1
vesicular glutamate transporter 1

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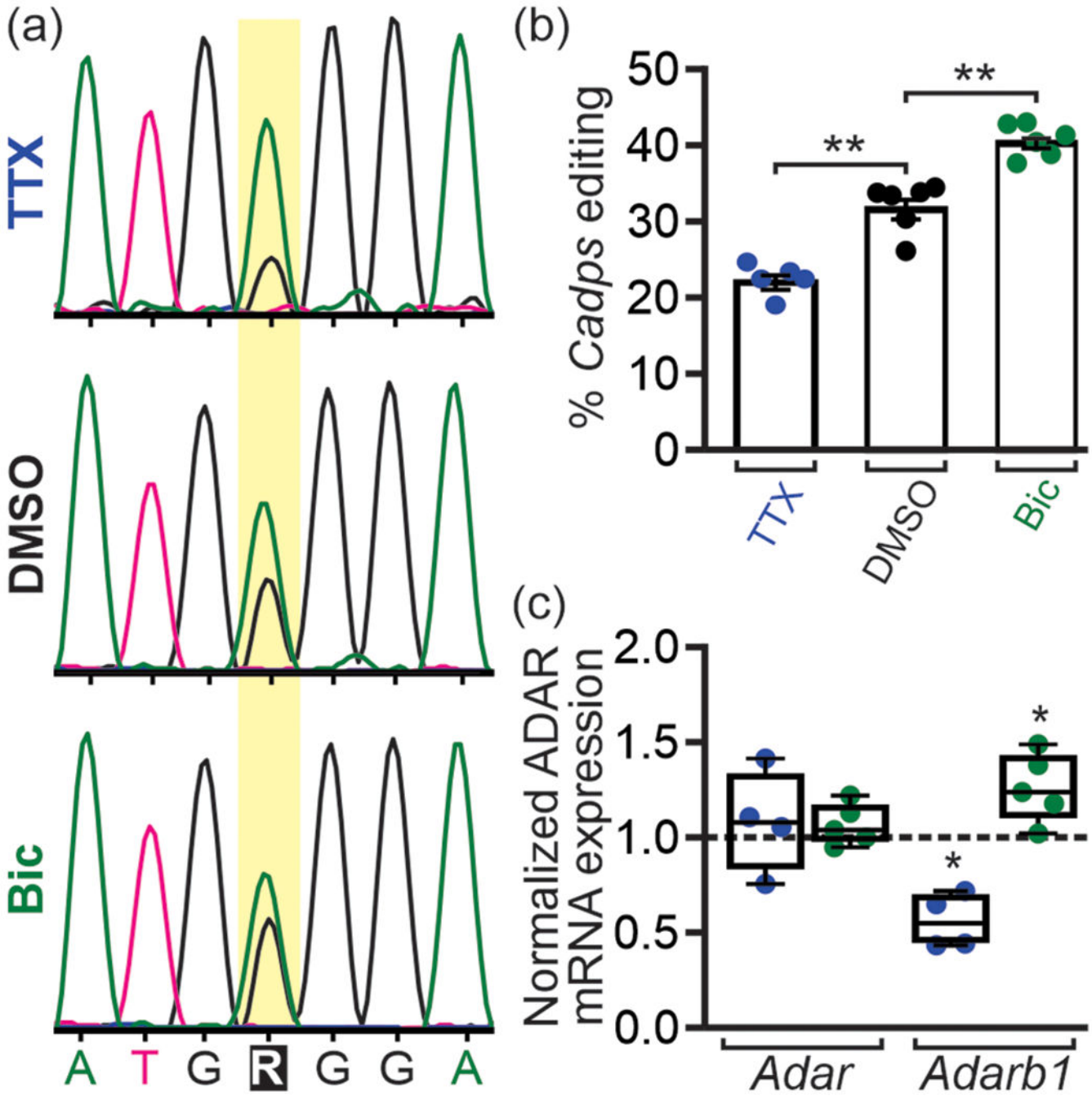

Figure 1. *Cadps* RNA editing is altered by neuronal activity.

(a) Electropherogram traces from Sanger sequencing of RT-PCR amplicons generated from wild-type neurons treated with tetrodotoxin (TTX), bicuculline (Bic), or vehicle (DMSO) are presented; the position of the editing site is indicated in yellow. **(b)** Quantification of *Cadps* RNA editing level in wild-type neurons treated with Bic (●), TTX (●), or vehicle (●) (n= 5-6 cell preparations per treatment, Holm-Sidak's Multiple Comparison Test, **p≤0.01). **(c)** Quantitative RT-PCR analysis of *Adar* and *Adarb1* mRNA levels in wild-type

neurons treated with Bic (●), TTX (●) normalized to vehicle is shown (n= 5-6 cell preparations per treatment, paired t-test of ΔC_t values, *p≤0.05).

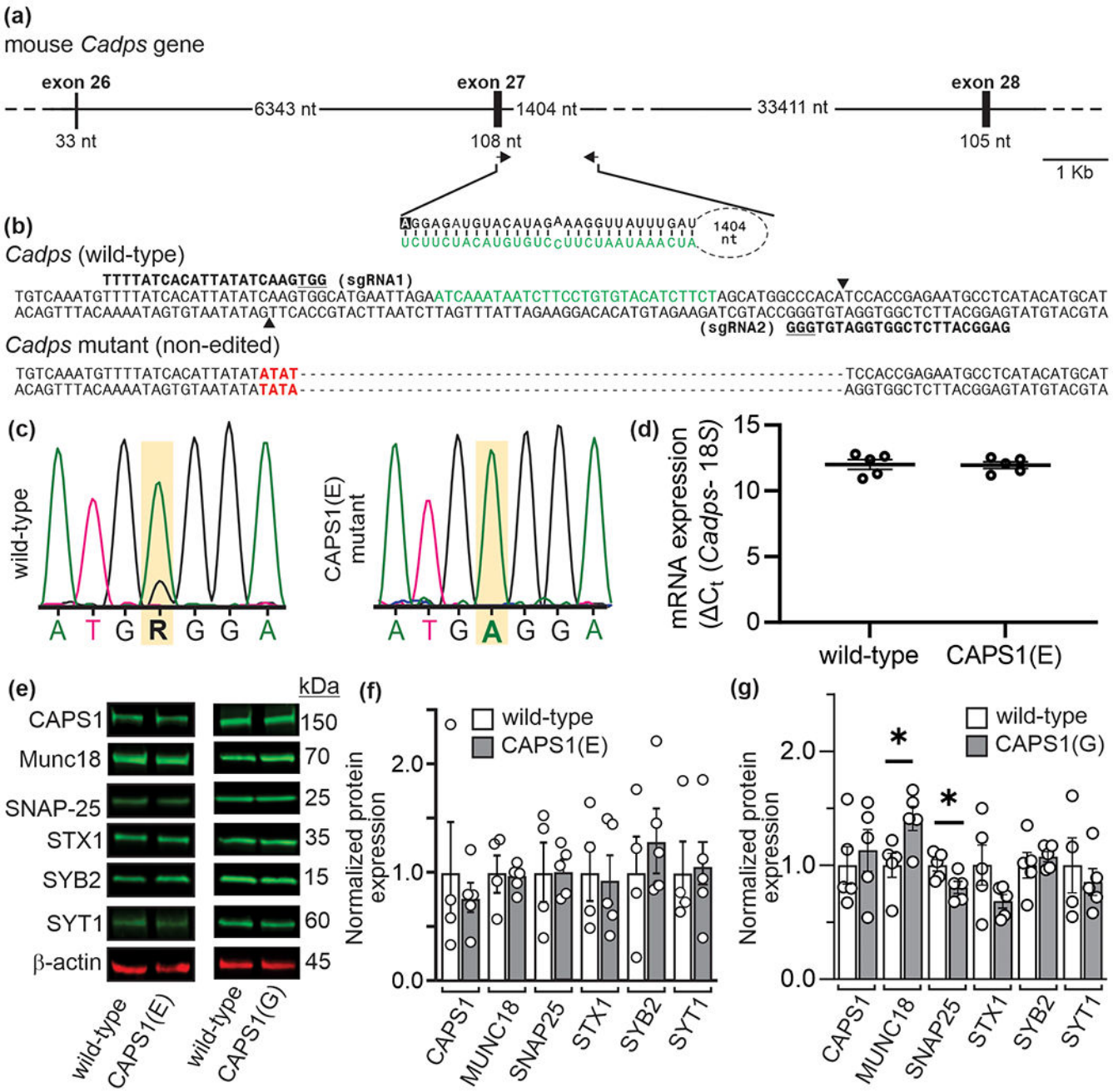

Figure 2. Generation of a mutant mouse line solely expressing the non-edited isoform of CAPS1, CAPS1(E).

(a) A schematic diagram for a portion of the mouse *Cadps* gene is presented illustrating the location of a dsRNA duplex formed by an inverted repeat (arrows) within exon 27 and intron 27 (*green lettering*). The adenosine subject to site-specific A-to-I RNA editing (*inverse lettering*) is located at the 5'-end of the duplex within exon 27. The lengths of the presented exons and introns in nucleotides (nt) are shown. **(b)** Two sgRNAs were used to direct CRISPR/Cas9-mediated excision of the editing site complementary sequence (ECS), the intronic portion of the dsRNA duplex (*green lettering*). The protospacer-adjacent motifs

(NGG) are underlined, and the predicted Cas9-mediated cleavage sites are indicated with arrowheads. The resulting mutant *Cadps* allele with the desired deletion (- - -) and an insertion of 4 random nucleotides (*red lettering*) by non-homologous end joining is shown. **(c)** Electropherogram traces from Sanger sequencing of RT-PCR amplicons generated from whole brain RNA are shown; the position of the editing site is indicated in yellow. **(d)** Quantitative RT-PCR analysis of *Cadps* mRNA levels in CAPS1(E) and wild-type mice (n= 5 animals/genotype, unpaired t-test, $p>0.05$). **(e)** Representative western blots and **(f, g)** quantitative analysis of the expression of major pre-synaptic exocytosis-regulating proteins, including CAPS1, munc-18, SNAP-25, syntaxin-1 (STX1), synaptobrevin-2 (SYB2), and synaptotagmin-1 (SYT1), in CAPS1(E) and CAPS1(E) WT mice and CAPS1(G) and CAPS1(G) WT mice, normalized to actin expression, set to WT= 1.0 (**f**) (n= 4-5 animals/genotype; Mann-Whitney tests, $p>0.05$). (**g**) (n= 5 animals/genotype; Unpaired t-tests, $*p\leq0.05$).

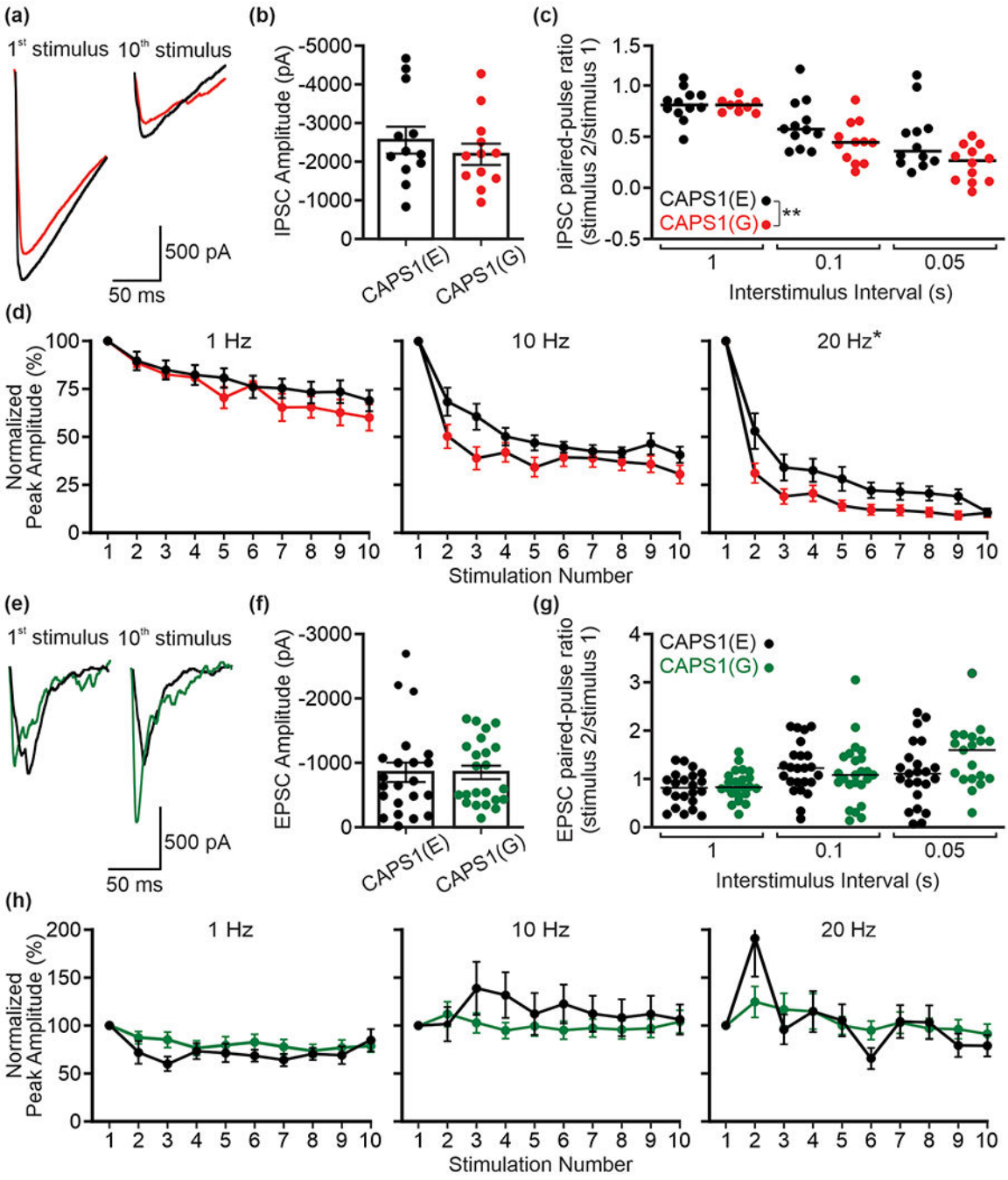

Figure 3. Effect of *Cadps* RNA editing on evoked neurotransmission.

(a) Representative IPSC traces of the first and last peak elicited by a train of 10 action potentials when delivered at 10 Hz, with CAPS1(E) (●) and CAPS1(G) (●) responses shown. **(b)** IPSC amplitudes elicited by a single action potential. (n= 12 neurons from 2 independent cell preparations; unpaired t-test, p>0.05). **(c)** Paired-pulse ratios calculated from IPSC amplitudes generated by stimulus 2/stimulus 1 responses with interstimulus intervals from 1- 0.05 seconds are presented. (n= 12 neurons per genotype from 2 cell preparations, Two-way ANOVA, genotype effect F (1, 63) = 7.642, **p≤0.01). **(d)**

Normalized peak amplitudes plotted by stimulation number elicited by a train of 10 action potentials delivered at 1 Hz, 10 Hz, and 20 Hz. (n= 12 neurons per genotype from 3 cell preparations; Mixed Effects Analysis, 1 Hz genotype effect $F(1, 22) = 1.839$, $p>0.05$, 10 Hz genotype effect $F(1, 22) = 3.514$, $p>0.05$, 20 Hz genotype effect $F(1, 22) = 4.695$, $^{*}p\leq0.05$). **(e)** Representative EPSC traces of the first and last peak elicited by a train of 10 action potentials when delivered at 10 Hz, with CAPS1(E) (●) and CAPS1(G) (●) responses shown. **(f)** EPSC amplitudes elicited by a single action potential (n= 24 neurons from 6 independent cell preparations; Mann-Whitney test, $p>0.05$). **(g)** Paired-pulse ratios calculated from EPSC amplitudes generated by stimulus 2/stimulus 1 with interstimulus intervals from 1- 0.05 seconds are presented (n= 24 neurons per genotype from 6 cell preparations, Two-way ANOVA, genotype effect $F(1, 129) = 1.383$, $p>0.05$). **(h)** Normalized peak amplitudes plotted by stimulation number elicited by a train of 10 action potentials delivered at 1 Hz, 10 Hz, and 20 Hz. (n= 24 neurons per genotype from 6 cell preparations; Mixed Effects Analysis, 1 Hz genotype effect $F(1, 46) = 0.6246$, $p>0.05$, 10 Hz genotype effect $F(1, 46) = 0.6580$, $p>0.05$, 20 Hz genotype effect $F(1, 46) = 0.4589$, $p>0.05$).

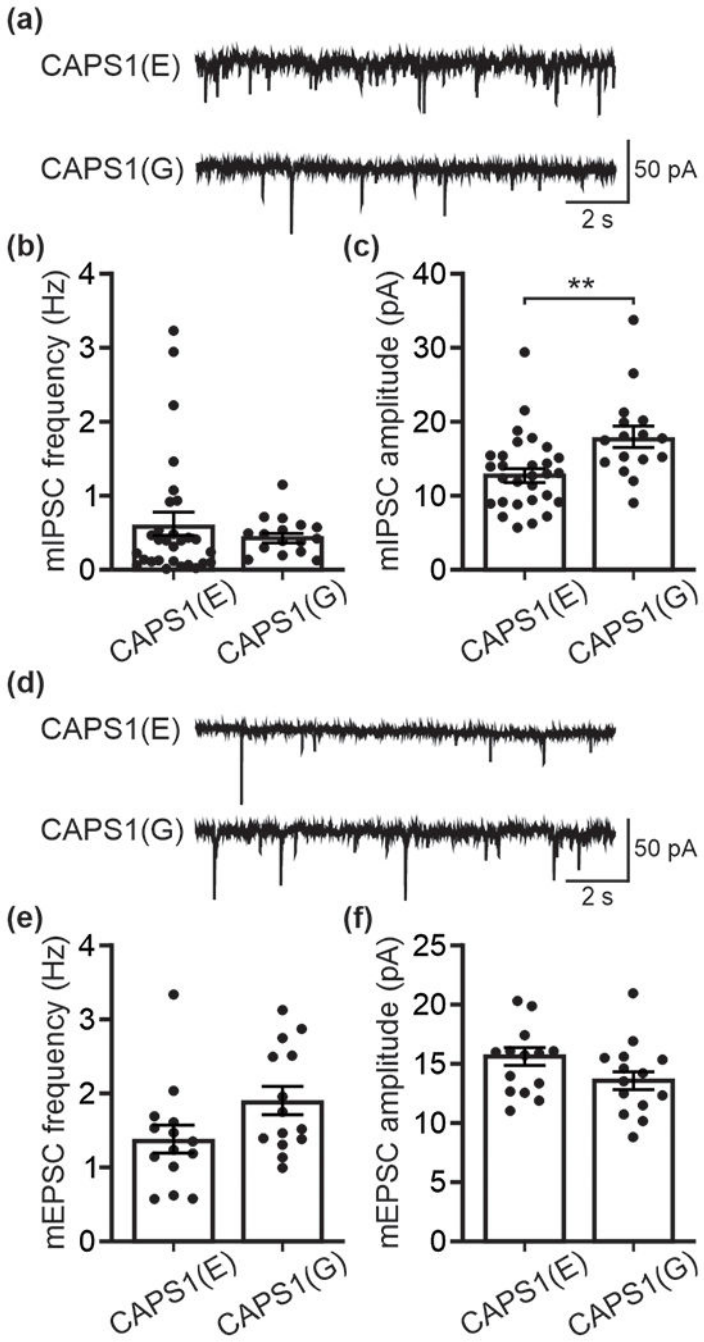

Figure 4. Effect of *Cadps* RNA editing on spontaneous neurotransmission.

(a) Representative mIPSC traces recorded from CAPS1(E) and CAPS1(G) expressing neurons. **(b)** An analysis of mIPSC frequency is shown. (n= 16-29 neurons per genotype from 8 cell preparations; Mann-Whitney test, $p>0.05$). **(c)** An analysis of mIPSC amplitudes is presented (n= 16-29 neurons per genotype from 8 cell preparations; Mann-Whitney test, $^{**}p\leq0.01$). **(d)** Representative mEPSC traces recorded from CAPS1(E) and CAPS1(G) expressing neurons. **(e)** An analysis of mEPSC frequency is shown (n= 14 neurons per genotype from 6 cell preparations; Mann-Whitney test, $p>0.05$). **(f)** An analysis of mEPSC

amplitude is presented. (n= 14 neurons per genotype from 6 cell preparations; Unpaired t test, $p>0.05$).

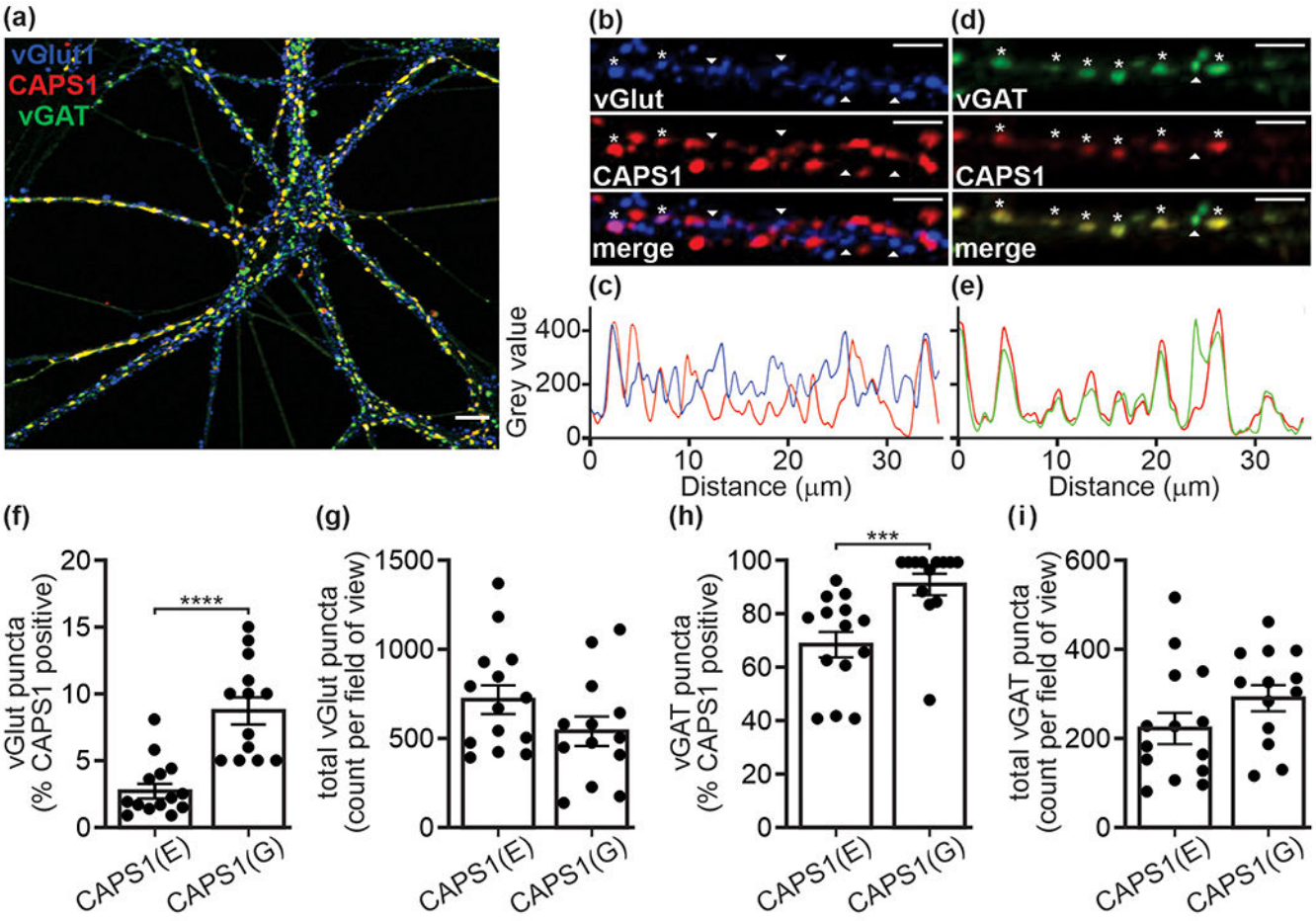

Figure 5. Enhancement of CAPS1(G) synaptic localization in cultured hippocampal neurons.

(a) Representative immunofluorescent image of cultured wild-type hippocampal neurons labelled using affinity purified antibodies targeting CAPS1 (*red*), a glutamatergic synapse marker, vGlut1 (*blue*), and a GABAergic synapse marker, vGAT (*green*); scale bar = 10 μm. **(b)** Magnified view of a neurite labelled for CAPS1 and vGlut1; vGlut1 and CAPS1 overlapping regions (*asterisks*) and vGlut1 puncta not enriched for CAPS1 (*arrowheads*) are shown; scale bar = 5 μm. **c)** Line scan of CAPS1 (*red*) and vGlut1 (*blue*) pixel intensity across the neurite shown in panel b. **(d)** Magnified view of a neurite labelled for CAPS1 and vGAT. vGAT and CAPS1 overlapping domains (*asterisks*) and vGAT puncta not enriched for CAPS1 (*arrowheads*) are shown; scale bar = 5 μm. **(e)** Line scan of CAPS1 (*red*) and vGAT (*green*) pixel intensity across the neurite shown in panel d. **(f)** Quantification of CAPS1 localization in vGlut1 puncta; CAPS1(E) 2.9 ± 0.6%, CAPS1(G) 8.9 ± 1.0% (n=13-14 fields of view from 2 independent cell preparations; Mann-Whitney test, ****p≤0.0001). **(g)** Quantification of total vGlut1 puncta; CAPS(E) 731.1 ± 80.5, CAPS1(G) 553.9 ± 78.6 (n= 13-14 fields of view from 2 independent cell preparations, unpaired t-test, p>0.05). **(h)** Quantification of CAPS1 localization in vGAT puncta; CAPS1(E) 69.9 ± 4.8%, CAPS1(G) 92.5 ± 4.1% (n=13-14 fields of view from 2 independent cell preparations; Mann-Whitney test, ***p≤0.001). **(i)** Quantification of total vGAT puncta; CAPS(E) 229.4 ± 35.2,

CAPS1(G) 298.0 ± 30.0 (n=13-14 fields of view in 2 independent cell preparations; unpaired t-test, $p>0.05$).

Table 1.

Primary and Secondary Antibodies used in Western Blotting

Antibody	Species	Dilution	Vendor	Catalog #	RRID
anti-β-actin	goat pab	1:1,000	Santa Cruz	sc-1616	RRID:AB_630836
anti-CAPS1	rabbit pab	1:1,000	Synaptic Systems	262 003	RRID:AB_2619978
anti-Munc18-1	rabbit mab	1:1,000	Cell Signaling Technology	13414	RRID:AB_2798213
anti-SNAP-25	rabbit pab	1:1,500	Abcam	ab41455	RRID:AB_945552
anti-synaptobrevin2	mouse mab	1:1,000	Synaptic Systems	104 211	RRID:AB_887811
anti-synaptotagmin1	mouse mab	1:5,000	Synaptic Systems	105 011	RRID:AB_887832
anti-syntaxin1a	rabbit mab	1:1,000	Abcam	ab170889	RRID:AB_2889824
anti-goat 680LT	donkey	1:50,000	LI-COR	926-68024	RRID:AB_10706168
anti-mouse Alexa 790	goat mab	1:15,000 to 1:50,000	Jackson Immuno	211-652-171	RRID:AB_2339174
anti-rabbit Alexa 790	mouse mab	1:15,000 to 1:50,000	Jackson Immuno	211-652-171	RRID:AB_2339174