INTRODUCTION

Calcium as a secondary messenger plays a significant role in cellular signaling and is intimately involved in the regulation of a variety of physiological activities (Barykina et al., 2017; Lin & Schnitzer, 2016). When voltage-gated calcium channels open following membrane depolarization, intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) in neurons, and other excitable cells is raised. This initiates a plethora of physiological responses, such as synaptic vesicle (SV) release and muscle contraction. Elevated intracellular ([Ca\textsuperscript{2+}]\textsubscript{i}) is quickly removed by intracellular buffering agents (e.g., endoplasmic reticulum and mitochondria) and extruded from the cell by membrane pumps and transporters (Catterall, 2011; Zamponi, Striessnig, Koschak, & Dolphin, 2015). As a result, calcium reporters, including genetically encoded calcium indicators (GECIs), have been widely used as a proxy to monitor synaptic and cellular activity in ensembles of neurons.
GECIs have a typical calcium binding domain, such as calmodulin or troponin C, and undergo conformational changes when bound to calcium, resulting in increased fluorescence of the fused protein probe (Barykina et al., 2017). The GCaMP family of proteins, in particular, is a widely utilized tool for calcium imaging (Akerboom et al., 2012; Badura, Sun, Giovannucci, Lynch, & Wang, 2014; Chen et al., 2013; Chisholm, Khovanov, Lopes, LaRusla, & McMahon, 2017; Xing & Wu, 2018). Targeted expression of these indicators has paved the way to more precisely explore the calcium activity in a variety of subcellular compartments like endoplasmic reticulum (ER; de Juan-Sanz et al., 2017), mitochondria (Zhang & Ding, 2018), and in neuronal synapses (Xing & Wu, 2018). Though development of calcium indicators has been rapidly pursued and fluorescence read-out is used to explain observed properties, little is known about the effects of GECIs on basal physiological function (Pérez Koldenkova & Nagai, 2013).

Expression of GECI probes may alter endogenous Ca\(^{2+}\) signaling within the cells or compartments where it is targeted. For example, the GCaMP family of GECI reporters have a range of binding affinities (150–700 nm) and decay kinetics (260–700 ms), which are presumed to affect basal and/or activity-dependent Ca\(^{2+}\) buffering proportionally (Badura et al., 2014; Mank & Griesbeck, 2008; Pérez Koldenkova & Nagai, 2013; Pologruto, Yasuda, & Svoboda, 2004). Similarly, the buffering capacity depends on the level of GCaMP expression, which is generally difficult to control in transgenic experimental models. Synaptic physiology and subsequent neuronal activity of cells may be affected by the inherent calcium buffering activity of GCaMP (Steinmetz et al., 2017). However, the extent to which expression of GCAMP indicators alters synaptic transmission and presynaptic short-term plasticity has not been examined directly.

In the present study, we evaluated the effect of virally expressed GCaMP6m on synaptic transmission properties in the presynaptic terminal at an identified central synapse, the calyx of Held. The calyx of Held synapse is one of the best-characterized central synapses, and its synaptic properties such as vesicle recycling, short-term depression, and Ca\(^{2+}\) buffering mechanisms have been carefully defined (Baydyuk, Xu, & Wu, 2016; Nakamura & Takahashi, 2007; Nakamura, Reva, & DiGregorio, 2018; Taschenberger, Leão, Rowland, Spirou, & von Gersdorff, 2002; Taschenberger, Woehler, & Neher, 2016; von Gersdorff & Borst, 2002). Thus, this synapse provides an excellent model for presynaptic-specific genetic manipulations and their effects on synaptic transmission. Our results show that presynaptic GCaMP has a profound effect on synaptic transmission by reducing SV release probability, including responses evoked at low frequency, where intracellular Ca\(^{2+}\) is highly spatially localized.

### 2 | METHODS

#### 2.1 | Animals

All animals in this study were used in accordance with animal welfare protocols approved by the Institutional Care and Use Committee at the University of Nevada, Reno, and in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals, 2011). C57bl/6 mice (Charles River Labs) postnatal day 20–25 of both sexes were used for this study. A total of 20 animals were used.

#### 2.2 | Viral-mediated presynaptic expression of GCaMP6m at calyx of Held

Adeno-associated virus encoding the GECI GCaMP6m (T. W. Chen et al., 2013) was procured from the Penn Vector Core in the Gene Therapy Program of the University of Pennsylvania (#AV-1-PV2823, 1.78 × 10\(^{13}\) GC/mL, in sterile PBS). This virus was injected into the ventral cochlear nucleus of neonatal (P1) mice, using procedures previously described (Chen, Cooper, Kalla, Varoqueaux, & Young, 2013; Lujan, Kushnerick, Das Banerjee, Dagda, & Renden, 2016; Pilpel, Landeck, Klugmann, Seeburg, & Schwarz, 2009). Briefly, mice were anesthetized via hypothermia on ice for 5 min until unresponsive, then fixed on a stereotaxic surgery platform. Anterior-posterior was aligned between lambda and bregma. Rostral-caudal axis was set so that the eyes and ears were aligned. Lateral roll was set so that ears were aligned at the same height. The mice were injected with 0.5 µl of GCaMP6m, using empirically determined stereotaxic coordinates targeting the ventral cochlear nucleus (VCN). Coordinates used were (in mm): \(x = +1.40–1.50\) (lateral), \(y = +2.3–2.5\) (caudal), \(z = -2.5–3.0\) (ventral), with \(x\) and \(y\) relative to lambda, and \(z\) relative to the surface of the skin. Afterward, mice were put on a heating pad (37 °C) to recover, and thereafter returned to their home cage. Animals were used for the experiments at 18–24 days after injection.

#### 2.3 | Slice preparation

Transverse brainstem slices containing the medial nucleus of the trapezoid body (MNTB) were cut at 200 µm using a vibratome (VT 1200S, Leica Microsystems, Germany), as previously described (Lujan et al., 2016; Singh, Miura, & Renden, 2018). The slicing solution contained (in mM): 85 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 7.5 sucrose, 0.5 CaCl\(_2\), 7 MgCl\(_2\), 3 myo-inositol, 2 Na-pyruvate, and 0.4 ascorbic acid; the pH was 7.4 when continuously bubbled with carbogen gas (95% O\(_2\)–5% CO\(_2\)). Slices were transferred to an incubation chamber containing recording ACSF (in mM): 125 NaCl, 2.5 KCl, 25 glucose, 25 NaHCO\(_3\), 1.25 NaH\(_2\)PO\(_4\), 2 CaCl\(_2\), 1 MgCl\(_2\), 3 myo-inositol, 2 Na-pyruvate, and 0.4 ascorbic
acid, 315–320 mOsm density. Slices were bubbled with carbogen gas for 30–45 min at 35 °C, and afterward maintained at room temperature (~23 °C) until used for recording.

2.4 | Electrophysiology

Slices were transferred to a recording chamber and continuously perfused with recording ACSF at a rate of 1–2 ml/min at room temperature (23 °C). Extracellular buffer was augmented with (in μM) 50 APV, 10 bicuculline, and 0.5 strychnine to isolate AMPA receptor currents. Patch pipettes were fabricated from 1.5 mm O.D. borosilicate capillary glass with open tip resistance of 2–3 MΩ. Postsynaptic intracellular recording solution contained (in mM): 130 cesium gluconate, 10 CsCl, 5 sodium phosphocreatine, 10 HEPES, 5 EGTA, 4 Mg-ATP, 0.5 GTP, and 5 QX-314, at pH 7.2 and 310–315 mOsm density. Voltage clamp recordings were made from the postsynaptic Principal cell of the MNTB using an Axon Multiclamp 700B amplifier (Molecular Devices, Sunnyvale CA). Voltage-clamped cells were held at −70 mV command voltage and not corrected for liquid junction potential (estimated at −11 mV). Series resistance (Rs) was in all cases <10 MΩ and compensated so that residual Rs was ~0.5 MΩ. Stimulation was delivered at low frequency (0.1–0.5 Hz) or high frequency at 100 Hz (400 ms) and 300 Hz (150 ms) via midline bipolar electrode with 100-μs biphasic pulses at 0.5–1V over the threshold voltage. Infected terminals were identified by GCaMP fluorescence in the calyx terminal.

2.5 | Fluorescence imaging of GCaMP

Infected terminals were identified by GCaMP fluorescence in the calyx terminal but were only visible in response to orthodromic stimulation in healthy synapses. Midline stimulation (100 Hz, 400 ms) was used to visually identify infected terminals and validate axonal connectivity (see Figure 6a–b). Synapses where GCaMP fluorescence was visible at rest were not used for recording. Widefield fluorescence illumination was provided by LED light engine (Lumencor SOLA). Illumination intensity was reduced 50% by a fluorescent attenuator to mitigate phototoxicity and bleaching. Light excitation filter (as middle wavelength/bandpass window) was 470/40 nm, with 495 nm beam splitter, and emission filter 525/50 nm, mounted on a Zeiss AXIO Examiner A1 upright microscope and visualized with 60x water immersion objective (1.0 NA). Ca2+ was imaged at 10 Hz during presynaptic depression trains, using an EM-CCD camera (Hamamatsu ImagEM X2; Bridgewater NJ) and controller software (HCI Image, RRID:SCR_015041) with EM sensitivity gain at 100x and 99 ms exposure. Images were analyzed offline using image analysis software (Velocity 6.3; RRID:SCR_002668). The maximum stimulated response was used to define the ROI. Activity-dependent change in fluorescence was reported as ∆F/F_base (%), normalized to the first frame at rest after background subtraction.

2.6 | Analysis

Data were analyzed and presented in IGOR Pro (RRID:SCR_000325) and Graphpad Prism (RRID:SCR_002798). Single exponential functions were used to fit depression curves. Data were considered significant at p < 0.05 and presented as mean ± SEM. The statistical test used was unpaired t test, unless otherwise noted.

Three methods were used to estimate readily releasable pool (RRP) size and release probability (Pves). Elmqvist and Quastel method (EQ plot): Individual responses in a train on the y-axis are plotted against the cumulative response on the x-axis. A straight line fitted to the initial responses in the curve and x-axis intercept is used as an estimate of the RRP. Elmqvist and Quastel (1965; Neher, 2015). SMN plot: The cumulative response during a high-frequency stimulus train is plotted against stimulus number. A straight line is fitted to the late points of the cumulative response during the depressed steady-state component of responses. Y-intercept of the line is used as an estimate of the RRP. Slope of the line is corrected for interstimulus interval and used to estimate the vesicle replenishment rate (Neher, 2015; Schneggenburger, Meyer, & Neher, 1999). Wesseling and Lo method (WL plot): The integral of responses during a 300-Hz stimulation train were measured after removing stimulus artifacts. RRP size at rest is determined numerically by comparing the measured RRP as a sum of all responses in a high-frequency train when the pool is stimulated to near depletion, similarly to the SMN plot. This number is then corrected for SV pool replenishment during the train. SV replenishment, estimated by a maximal unitary recruitment rate, is determined numerically by comparing the initial release probability to the release probability during steady-state responses (Mahfooz, Singh, Renden, & Wesseling, 2016; Wesseling & Lo, 2002).

3 | RESULTS

GECIs such as GCaMPs are widely employed for calcium imaging as they have distinct advantages over traditional calcium dyes such as ease of use, relatively consistent expression, fluorescence stability, and the ability to tag specific neuronal populations and subcellular domains (de Juan-Sanz et al., 2017; T. W. Chen et al., 2013; Xing & Wu, 2018; Zhang & Ding, 2018). However, expression at high levels may result in exogenous
calcium chelation, affecting synaptic transmission in undesirable ways (Steinmetz et al., 2017). In the present study, we expressed GCaMP6m specifically in the presynaptic terminal, and we investigated its effect on synaptic transmission.

### 3.1 | Virally expressed GCaMP6m at the calyx of Held follows stimulation with slow decay kinetics

Recombinant adeno-associated virus encoding GCaMP6m was used to infect bushy cells in the VCN of neonatal mice (see Methods) and visualized at postnatal day (P) 18–20. At rest, no GCaMP6m signal was visible. But GFP fluorescence was readily visible in response to a brief stimulus train, which was used to identify healthy connected calyx terminals (Figure 1a). First, we recorded GCaMP6m fluorescence in response to 200 ms trains at 100 Hz or 300 Hz, which provided a robust increase in cytosolic free calcium level in the presynaptic terminal ([Ca$^{2+}$]). We observed a strong but transient increase in GCaMP fluorescence following the stimulus trains, which decayed mono-exponentially (Figure 1b). Responses were graded and frequency dependent, as 300 Hz stimulation resulted in significantly higher GCaMP6m peak fluorescence (100 Hz: 59.2 ± 8.0%, n = 12; 300 Hz: 103.3 ± 11.4, n = 14; p = 0.005, Student’s t test, t = 3.085 df = 24, Figure 1b,c). The time course of Ca$^{2+}$-clearance was independent of the stimulation frequency for these trains, evidenced by similar decay constants ($\tau_{\text{decay}}$; 100 Hz: 1.63 ± 0.19 s, n = 15; 300 Hz: 1.86 ± 0.082 s, n = 14; P = 0.282, Student’s t test, t = 1.106 df = 19.19, Figure 6d). We do not think the GCaMP6m signal was saturated by these short HFS trains, as longer trains (e.g., 500 ms at 300 Hz) further increased the peak fluorescence to 141 ± 23% of baseline (N = 5, not shown).

After determining that cells were connected by visible fluorescence response to a short high-frequency train, we were also able to resolve Ca$^{2+}$ influx due to single AP events. After acquiring an image of the calyx during high-frequency stimulation to generate a region of interest, we then acquired sets of images at 70 Hz (no fluorescence attenuation, exposure 14 ms) and gave a single time-locked stimulus per image sequence. Multiple runs were acquired with 1–5 s between runs, and fluorescence intensity within the ROI generated from the response measured relative to baseline. Responses due to 200 runs per cell were averaged and corrected for photobleaching using an exponential fit to sequence. Multiple runs were acquired with 1–5 s between runs, and fluorescence intensity within the ROI generated from the response measured relative to baseline. Responses due to 200 runs per cell were averaged and corrected for photobleaching using an exponential fit to baseline prior to the electrical stimulus. GCaMP fluorescence change was very small in these experiments, increasing by 0.07 ± 0.01% on average, and it decayed with a time course of 126 ± 29 ms (N = 2 cells; Figure 1e). The kinetics of GCaMP6m decay were long enough to be resolved by 70 Hz imaging (42 ms minimum $\tau_{\text{decay}}$), and they did not match the known clearance time course of Ca$^{2+}$ from the calyx terminal ($\tau < 50$ ms, (Hosoi, Sakaba, & Neher, 2007; Müller, Felmy, Schwaller, & Schneggenburger, 2007). Thus, we suggest the events are likely limited by the binding kinetics of the GCaMP6m probe (T. W. Chen et al., 2013).

### 3.2 | Genetically encoded GCaMP does not affect quantal event frequency or kinetics

Spontaneous and evoked activity was recorded from principal neurons in MNTB principal cells postsynaptic to GCaMP-expressing calyx of Held terminals in juvenile mice. Only healthy connected neurons that showed negligible or no GCaMP signal at rest and responded to midline stimulation were used. We did not observe any change in the profile of spontaneous excitatory postsynaptic events (sEPSC) in GCaMP expressing synapses compared to control (nontransduced, GCaMP6m-negative) synapses (Figure 2 and Table 1). These results indicate that chronic GCaMP expression does not alter synaptic excitability, SV loading, or number and type of postsynaptic glutamate receptors at rest. Further, since sEPSC frequency was not altered and is in part dependent on free intracellular Ca$^{2+}$, these results suggest that that GCAMP does not dramatically alter free presynaptic [Ca$^{2+}$], at rest (<100 nM), which is below the GCaMP6m $K_{D}$ of 167 nm (T. W. Chen et al., 2013).

### 3.3 | GCaMP affects synaptic vesicle release probability and short-term depression

The calyx of Held has well-defined short-term depression (STD) due primarily to SV pool depletion (von Gersdorff & Borst, 2002). Fundamental properties of this decay, including the effective RRP size and initial release probability, are affected by external Ca$^{2+}$ and internal Ca$^{2+}$-buffering mechanisms (Eggermann, Bucurenciu, Goswami, & Jonas, 2012; Schneggenburger & Neher, 2000; Taschenberger, Scheuss, & Neher, 2005; Wang, Neher, & Taschenberger, 2008). We recorded EPSCs following high-frequency stimulus trains to better understand the effect of GCaMP expression on short-term plasticity, RRP size, and release probability (P$_{\text{ves}}$) at the calyx of Held synapse.

To test whether GCaMP has an effect on individual evoked events, we recorded evoked EPSCs in response to low (0.1 Hz) stimulation. EPSC amplitude in GCaMP-expressing synapses was robust but significantly decreased relative to control (Control: 14.28 ± 1.09, n = 12; GCaMP: 9.68 ± 1.91, n = 5; p = 0.043, Student’s t test, t = 2.05 df = 15, Figure 3), suggesting that even the rapid and highly localized Ca$^{2+}$ transient that occurs at these mature synapses was strongly attenuated by GCaMP6m, presumably by acting as a Ca$^{2+}$ buffer.

Short trains of 100 Hz and 300 Hz stimulation were used to induce STD, and EPSCs were recorded (Figures 4 and 5). In young animals, 100 Hz trains lead to significant evoked desensitization at the calyx of Held, but this is largely absent in older mice such as those used at this age (Renden et al., 2005; Wong, Graham, Billups, & Forsythe, 2003). A 100-Hz train significantly reduced STD in GCaMP-expressing synapses, evident in an increased decay time constant (Control: 25.7 ± 1.8 ms, n = 5; GCaMP: 61.4 ± 4.9 ms, n = 5; p = 0.008, Student’s t test, t = 6.823 df = 8, Figure 4b,d) and larger proportional steady-state response (37.8 ± 7.6% of initial value, n = 5) than in uninfected control (steady state: 14.1 ± 0.9% of initial value, n = 5; p = 0.014, Student’s t test, t = 3.108 df = 8, Figure 4e). This slower decay in transmission is evidence that GCaMP is likely...
buffering [Ca\(^{2+}\)] during the stimulus train. As a result, paired-pulse ratio of the second EPSC to the first EPSC in a train was increased significantly (Control: 0.70 ± 0.02, n = 5; GCaMP: 0.95 ± 0.05, n = 5; p = 0.001, Student’s t test, t = 4.903 df = 8, Figure 4f), again suggestive of decreased initial vesicle release probability.

In slice preparations the calyx of Held can reliably follow 300 Hz at room temperature, which is sufficient to nearly deplete the RRP and drives transmission to a strongly depressed steady state (Taschenberger et al., 2002; Wang et al., 1998; Figure 5a). This paradigm can be used to accurately estimate the RRP size, especially in older animals (Mahfooz et al., 2016). However, kynurenic acid was not used in the current study, so some receptor desensitization may be present in both control and GCAMP-expressing synapses. At 300 Hz, depression in GCaMP-expressing synapses was still significantly slower relative to control, exhibiting an increased decay time constant (Control: 14.6 ± 1.4, n = 7; GCaMP: 23.6 ± 2.6, n = 5; p = 0.008, Student’s t test, t = 3.288 df = 10, Figure 5d). The extent of depression measured at steady state was slightly higher in GCaMP-expressing synapses (13.2 ± 2.6%, n = 5), but was not statistically different from control (9.2 ± 1.3%, n = 7; p = 0.173, Student’s t test, t = 1.467 df = 10, Figure 5e), suggesting that the RRP of both synapses can eventually be depleted to similar levels at 300 Hz.

We estimated the RRP size using the data from 300 Hz depression trains with three different methods: forward extrapolation (EQ plot), back extrapolation (SMN plot), and the Wesseling and Lo method (WL; Elmqvist & Quastel, 1965; Kaeser & Regehr, 2017; Mahfooz et al., 2016; Neher, 2015; Schneggenburger et al., 1999). Estimation of the RRP is still a highly debatable subject, and all of the methods have caveats (Kaeser & Regehr, 2017; Neher, 2015). Therefore, we used all three methods to estimate RRP size and probability of release so that our results can be easily compared to existing reports (cf. Table 2). In addition, the SMN plot was used to calculate maximum refilling rate, and the WL method to calculate unitary refilling rate during the train.

A significant reduction in P\(_{ves}\) in GCaMP-expressing synapses was observed consistently among the calculation methods (Table 2, Figure 6b,f and Figure 7b). We did not observe any change in RRP size when calculated using SMN or WL (Table 2, Figures 6b and 7b); however, EQ plot showed a significant increase in RRP (Table 2, Figure 6g) for GCaMP-expressing synapses. This result from the EQ plot may be erroneous, as the method is heavily dependent on vesicular release probability, and accuracy is compromised by low P\(_{ves}\) (Kaeser & Regehr, 2017; Neher, 2015). Notably, GCaMP did not have any effect on maximum replenishment rate (Table 2, Figure 6d) or unitary refilling rate (Table 2, Figure 7d), suggesting that this GECI does not overtly affect RRP replenishment, which is also Ca\(^{2+}\)-dependent (Alabi & Tsien, 2012; de Jong & Fioravante, 2014).

**FIGURE 1** Activity-dependent GCaMP fluorescence at calyx of Held: (a) At rest, GCaMP signal is undetectable in healthy synapses (left panel). A train of stimulus transiently increased GCaMP signal at individual calyx of Held synapse (right panel). (b) Change in fluorescence intensity of GCaMP after HFS is activity-dependent. Stimulus (200 ms duration) is shown as gray bar at time = 0. (c) Summary plot of peak fluorescence in response to 100 Hz and 300 Hz stimulation. (d) Calcium clearance from the presynaptic cytosol is similar at 100 and 300 Hz HFS. (e) GCaMP6m can be used to resolve single AP-mediated events, with sufficient averaging. Two cells are shown, with exponential fit to decay when the two cells are averaged. Stimulus was delivered at time = 0.
Overall, our data suggest that GCaMP is a highly sensitive indicator for intracellular Ca\(^{2+}\), and is able to detect the rise in [Ca\(^{2+}\)]\(_i\), due to single AP-mediated events in the presynaptic terminal. However, our results show unequivocally that expression of this GECI comes at a cost, with a significant blunting of Ca\(^{2+}\)-dependent synaptic transmission, reducing access of intracellular Ca\(^{2+}\) for release of SVs both at rest and during high-frequency stimulation. Chronic expression of GCaMP6m does not seem to affect any homeostatic or other Ca\(^{2+}\)-dependent events not directly related to SV exocytosis. For example, quantal size, the ability to follow high-frequency repetitive stimulation, RRP size, and RRP replenishment were unaffected.

4.1 Possible mechanism underlying reduced vesicle release probability

Exogenous calcium buffering agents/chelators are routinely used to manipulate vesicle release probability by interfering with the coupling between endogenous calcium sensor and effector proteins (Eggermann et al., 2012), thereby affecting the synaptic transmission. The GCaMP family of proteins is a widely utilized tool in calcium imaging, and it has been optimized for sensitivity and speed (Akerboom et al., 2012; Badura et al., 2014; Chisholm et al., 2017; T. W. Chen et al., 2013; Xing & Wu, 2018). Unfortunately, GCaMP6m augments endogenous cytosolic Ca\(^{2+}\) buffering and alters the transmission profile of infected synapses. Several transgenic mouse lines expressing GCaMP6m have been shown to generate abnormal neuronal spiking in the cortex region; however, the authors used a combination of transgenic mice, and the aberrations were more prominent in the transgenic GCaMP6m mice expressing Cre recombinase, not ruling out the Cre toxicity as a causative factor (Steinmetz et al., 2017). Here, we expressed GCaMP6m at the calyx of Held presynapse using viral-mediated transduction of GCaMP6m expression, which was more tightly controlled; we may have expressed the probe at lower levels and for a shorter time period (less than three weeks). Still, we observed a significant change in evoked, activity-dependent synaptic transmission.

**Figure 2.** GCaMP does not influence spontaneous activity: (a) Example traces of spontaneous activity from control and GCaMP-expressing synapses. (b) Summary plot of sEPSC frequency, determined per cell. (c) Summary plot of sEPSC amplitude, averaged per cell. (d) Example traces, averaged from >1750 events in each condition. (e) Summary plot of 10–90% rise times, averaged per cell. (f) Summary plot of 90–10% decay times, averaged per cell.
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GCaMP6m-expressing synapses showed a significant reduction in synaptic depression and vesicle release probability. A stronger effect of GCaMP6m was seen on transmission at lower frequency stimulation (100 Hz) vs. higher (300 Hz) (Figures 4 and 5). We did not observe any difference in the decay of fluorescence kinetics due to 300 Hz frequency stimulation (Figure 1), suggesting endogenous buffering machinery (and GCaMP-mediated buffering) was not saturated to a greater extent due to increased accumulation of free calcium in the terminal. Thus, we attribute the loss of effect of GCaMP6m on depression at 300 Hz to increased depletion of the RRP, as approximately 50% of the RRP is not released by a 100-Hz train in mature mice (Mahfooz et al., 2016). Additionally, calcium channels are facilitated by free intracellular Ca2+ (Tsujimoto, Jeromin, Saitoh, Roder, & Takahashi, 2002). Reduced activation of these mechanisms by GCaMP6m is also likely to lead to less depression during high-frequency trains.

Exogenous Ca2+ chelators such as EGTA and BAPTA have been used extensively as a tool to suppress SV release probability. In calyx synapses from animals similar to the ages used in the current study, SVs are closely localized to Ca2+ channels, forming “nanodomains,” so that only very fast buffers are capable of intercepting Ca2+ prior to activating SV release machinery (Chen et al., 2015; Eggermann et al., 2012; Nakamura et al., 2015). For example, very high concentration of EGTA (>5 mM) must be used to reduce transmission amplitude in these terminals, while the fast Ca2+ buffer BAPTA is effective at 10-fold lower concentration (Chen, Das, Nakamura, DiGregorio, & Young, 2015; Fedchyshyn & Wang, 2005).

While it is difficult to estimate the concentration of GCaMP6m at the terminal, it acts similarly to ~12 mM exogenous EGTA, or <1 mM BAPTA, based on existing data from mature at calyx of Held terminals. Ca2+ binding to native calmodulin—which forms the core of GCaMP—is able to bind free Ca2+ faster than BAPTA, and acts as an effective endogenous buffer at uM concentration (Faas, Raghavachari, Lisman, & Mody, 2011). Thus, while the fluorescent signal from GCaMP6m is relatively slow, expression of this reporter clearly affects Pr, even at synapses with tightly coupled SV-Ca2+ interactions, such as the calyx of Held, due to the speed of Ca2+ binding prior to conformational folding and fluorescence increase. In other central synapses with loose spatial coupling, such as those in hippocampus and cortex, GCaMP6m would likely suppress Pr even further (Rozov, Burnashev, Sakmann, & Neher, 2001; Vyleta & Jonas, 2014).

SV priming is Ca2+-dependent, acting primarily through mUNC13-1, and is additionally affected by various kinase and phosphatase activity (Körber & Kuner, 2016; Lou, Korogod, Brose, & Schneggenburger, 2008; Nagy et al., 2004; Pang, Cao, Xu, & Südhof, 2010; Srinivasan, Kim, & Gersdorff, 2008). At the calyx of Held in particular, some vesicles show increased Ca2+-sensitivity via molecular “super-priming” (Lee, Ho, Neher, & Lee, 2013). While our current experiments cannot discriminate between these multiple mechanisms, it is possible that GCAMP6m expression may have also altered the extent of SV priming indirectly by limiting Ca2+-dependent kinase and phosphatase activity.

4.2 GCamp6 and RRP refilling

We observed no effect of GCAMP6m expression on the SV replenishment rate during a short stimulation train (Figures 6 and 7, and Table 2). However, RRP replenishment during periods of rest between activity bouts clearly has a Ca2+-dependent component, which has been well characterized at the calyx of Held (Sakaba & Neher, 2001; Lu Yang Wang & Kaczmarek, 1998). Our analysis used methods that evaluated the

| TABLE 1 Properties of sEPSC events at the calyx of Held |
|---------------------------------------------------------|
| Frequency (Hz) | Amplitude (pA) | Rise time (µs) | Decay time (µs) |
| Control (n = 12) | 3.01 ± 0.36† | 77.26 ± 4.93 | 126 ± 5 | 419 ± 31 |
| GCaMP (n = 6) | 5.63 ± 2.31 | 72.43 ± 7.76 | 139 ± 7 | 421 ± 50 |
| Student’s t-test | P = 0.135, t = 1.572, df = 16 | P = 0.593, t = 0.5452, df = 16 | P = 0.163, t = 1.462, df = 16 | P = 0.964, t = 0.04515, df = 16 |

† Reported as mean ± SEM

![FIGURE 3 Basal transmission is inhibited by GCaMP expression](image-url)

(a) Example traces of evoked EPSCs in control and GCaMP-expressing synapse (average of 10 individual responses from each cell).
(b) Summary plot showing a significant reduction in basal transmission
**FIGURE 4** Short-term plasticity at 100 Hz is altered by GCaMP: (a) Representative traces showing short-term depression in response to 100 Hz stimulation. (b) Averaged depression curves, showing EPSC amplitudes during the stimulus train. (c) EPSC amplitudes, normalized to the first response, to illustrate short-term depression and used to determine decay time constant and steady state. (d) Summary plot of decay time constant for depression, determined per cell. (e) Summary plot of steady-state current. (f) Summary plot of paired-pulse ratio.

**FIGURE 5** Short-term plasticity at 300 Hz is altered by GCaMP, but eventually depletes the readily releasable pool. (a) Representative traces showing STD in response to 300 Hz stimulation. (b) Averaged depression curves showing EPSC amplitudes during the stimulus train. (c) EPSC amplitudes, normalized to the first response, to illustrate short-term depression and used to determine decay time constant and steady state. (d) Summary plot of decay time constant for depression, determined per cell. (e) Summary plot of steady-state current. (f) Summary plot of paired-pulse ratio.
### TABLE 2  Estimates of RRP and P_v at the calyx of Held

|                          | SMN Plot                | EQ Plot                | WL Plot                |
|--------------------------|-------------------------|------------------------|------------------------|
| Probability of release (P_v) | Control (n = 7) 0.19 ± 0.02† | 0.18 ± 0.02            | 0.20 ± 0.02            |
|                          | GCaMP (n = 5) 0.14 ± 0.01* | 0.10 ± 0.01**          | 0.14 ± 0.01*          |
| Student’s t test         | P = 0.022, t = 2.694, df = 10 | P = 0.004, t = 3.703, df = 10 | P = 0.026, t = 2.600, df = 10 |
| Readily releasable pool (SV) | Control (n = 7) 937 ± 127 | 907 ± 91               | 910 ± 112              |
|                          | GCaMP (n = 5) 1195 ± 233 | 1493 ± 282*            | 1160 ± 236             |
| Student’s t test         | P = 0.319, t = 1.049, df = 10 | P = 0.046, t = 2.272, df = 10 | P = 0.317, t = 1.052, df = 10 |
| Maximum replenishment rate (SV/ms) | Control (n = 7) 15.9 ± 2.7 |            |                        |
|                          | GCaMP (n = 5) 22.7 ± 6.0 |            |                        |
| Student’s t test         | P = 0.279, t = 1.145, df = 10 |          |                        |
| Unitary refilling rate (s⁻¹) | Control (n = 7) 6.2 ± 0.6 |                        |                        |
|                          | GCaMP (n = 5) 7.0 ± 0.8 |                        |                        |
| Student’s t test         | P = 0.438, t = 0.8081, df = 10 |          |                        |

†Reported as mean ± SEM.
* p < 0.05; ** p < 0.01, by unpaired t test.

**FIGURE 6**  GCaMP reduces vesicle release probability at the calyx of Held synapse. (a) Cumulative amplitude plot of control versus GCAMP-expressing synapses in response to 300 Hz train. Data are the same as Figure 5. (b) Summary plot for P_{ves} estimated using SMN method, GCaMP reduced release probability. (c) Readily releasable pool, estimated using SMN method, is not affected by GCaMP expression. (d) Summary plot of replenishment rate, estimated using SMN method. (e) EQ-plot of cumulative EPSCs in response to 300 Hz train, illustrating estimate of RRP using initial response sizes. (f) Summary of P_{ves} estimate, using EQ method, measured per cell. Like SMN plot, P_{ves} is significantly reduced by GCaMP. (g) Summary of RRP, estimated per cell using EQ method, shows a significant increase in RRP for GCaMP-expressing synapses
replenishment rate during steady state at the end of 300 Hz stimulation trains, when the pool was depleted. SV replenishment quickly accelerates with activity and Ca$^{2+}$ influx (Lee, Ho, & Lee, 2010; Mahfooz et al., 2016; Stevens & Wesseling, 1999). Thus, it is likely that refilling rates—both bulk refilling and unitary refilling rates (SMN and WL plots, respectively)—are maximal at the end of the trains used here, and intraterminal free Ca$^{2+}$ quickly reaches micromolar levels, where buffering would be augmented by mitochondrial uptake (Billups & Forsythe, 2002; Kim, Korogod, Schneggenburger, Ho, & Lee, 2005).

4.3 Impact of GCaMP6m on recordings under physiological conditions

All the experiments in this study were performed under “standard” conditions: 2-mM extracellular Ca$^{2+}$ and ambient room temperature. But how might the results described in this study apply to in vivo recordings, or those performed under “physiological” conditions such as lower extracellular Ca$^{2+}$ (~1.2 mM) or higher temperature (35–37 ºC)? When external Ca$^{2+}$ is reduced to ~1.2 mM, Ca$^{2+}$ influx due to an AP-like stimulus is reduced nearly linearly (Chen et al., 2015; Yang & Wang, 2006), but EPSCs are about 30% of the amplitude when in 2 mM Ca$^{2+}$ (Körber et al., 2015; Thanawala & Regehr, 2013), and data not shown) due to the strong nonlinear dependence of SV release on Ca$^{2+}$ influx (Lou, Scheuss, & Schneggenburger, 2005). At higher temperature, instantaneous Ca$^{2+}$ influx is larger due to faster channel activation, but it may be balanced by increased Ca$^{2+}$ extrusion through Na-dependent calcium exchangers and ATP-dependent pump activity, as well as uptake by mitochondria (Elias et al., 2001; Kang, Carl, McHugh, Goff, & Kenyon, 2008; Kim, Korogod, Schneggenburger, Ho, & Lee, 2005). This may act to alleviate the cytosolic Ca$^{2+}$ load during a high-frequency train. Similarly, the RRP refilling rate is increased with temperature, resulting in less overall short-term depression (Kushmerick, Renden, & Gersdorff, 2006). How the Ca$^{2+}$ load in either condition is buffered by GCaMP6m is not clear, and not trivial.
to predict (Nakamura, Reva, & DiGregorio, 2018). The effect of GCaMP6m, as well as other calmodulin-based Ca\textsuperscript{2+} reporters with high sensitivity but different kinetics (e.g., GCaMP6f), on SV release under physiological conditions should be investigated further.

5 | CONCLUSION

The intent of this study was to emphasize the careful use of GCaMP when multiplexing synaptic transmission and Ca\textsuperscript{2+}-imaging experiments. We find that the synapse remains healthy up to three weeks after transduction and GCaMP6m expression. We did not observe any obvious homeostatic changes in RRP size or refilling. However, caveat emptor, as basal transmission properties, illustrated primarily as changes in release probability, are altered due to enhanced cytosolic Ca\textsuperscript{2+} buffering by GCaMP6m. We performed electrophysiology experiments where GCaMP6m was expressed exclusively in the presynaptic compartment, but Ca\textsuperscript{2+}-buffering by GCaMP is likely to affect postsynaptic Ca\textsuperscript{2+}-dependent processes as well. Given the relative similarity in binding affinity and off-rate, our findings are also likely to extend to other members of this class of GECIs.

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CONFLICT OF INTEREST

The authors declare that no conflict of interest exists for this work.

ORCID

Robert Renden http://orcid.org/0000-0001-7905-4789

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