

# 1 **Novel genetically encoded tools for imaging or silencing neuropeptide** 2 **release from presynaptic terminals *in vivo***

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## 12 **SUMMARY**

13 Neurons produce and release neuropeptides to communicate with one another. Despite their profound  
14 impact on critical brain functions, circuit-based mechanisms of peptidergic transmission are poorly  
15 understood, primarily due to the lack of tools for monitoring and manipulating neuropeptide release *in vivo*.  
16 Here, we report the development of two genetically encoded tools for investigating peptidergic transmission  
17 in behaving mice: a genetically encoded large dense core vesicle (LDCV) sensor that detects the  
18 neuropeptides release presynaptically, and a genetically encoded silencer that specifically degrades  
19 neuropeptides inside the LDCV. Monitoring and silencing peptidergic and glutamatergic transmissions  
20 from presynaptic terminals using our newly developed tools and existing genetic tools, respectively, reveal  
21 that neuropeptides, not glutamate, are the primary transmitter in encoding unconditioned stimulus during  
22 Pavlovian threat learning. These results show that our sensor and silencer for peptidergic transmission are  
23 reliable tools to investigate neuropeptidergic systems in awake behaving animals.

24

## 25 **INTRODUCTION**

26 Two types of transmitters are found within synaptic terminals for neuronal communication: classical  
27 neurotransmitters and neuromodulators. Classical fast-acting neurotransmitters, including glutamate,  
28 acetylcholine, GABA, and glycine, are packaged into synaptic vesicles (SVs), which are released from  
29 synaptic terminals in an activity-dependent manner (Sudhof, 2012). By contrast, neuromodulators, such as  
30 neuropeptides and monoamines, are stored in large dense-core vesicles (LDCVs), which are released in  
31 response to a train of high-frequency action potentials (Cifuentes et al., 2008; Martinez-Rodriguez and  
32 Martinez-Murillo, 1994; Salio et al., 2006; Silm et al., 2019). The unique functions and distinct release

33 properties of neuromodulators suggest that they can function as the main neurotransmitter in neurons, like  
34 dopamine and norepinephrine (Eskenazi et al., 2021; Hnasko and Edwards, 2012; Vaaga et al., 2014).  
35 Neuropeptides are by far the most diverse class of neuromodulators. Currently, more than 100  
36 neuropeptides and their postsynaptic receptors have been discovered, with each neuropeptide exhibiting  
37 unique functions, such as arousal, sleep/wake, reproduction, feeding, reward, learning/memory, and threat  
38 perception (van den Pol, 2012). Furthermore, dysregulation of neuropeptides has been closely associated  
39 with many neurological and neuropsychological disorders (Beal and Martin, 1986). Thus, elucidating the  
40 mechanism by which neuropeptidergic systems act in brain circuits is critical for understanding brain  
41 function and associated disorders, and it requires the tools that monitor and manipulate peptidergic  
42 transmissions in a temporally precise manner in behaving animals. To date, this is a technical feat with very  
43 limited availability.

44         Recent progress in developing neuromodulator sensors by genetically modifying their postsynaptic  
45 G-protein-coupled receptors (GPCR) allow researchers to monitor the release of many monoamine and  
46 catecholamine neuromodulators in behaving animals (Sabatini and Tian, 2020; Wu et al., 2022). The  
47 development of neuropeptide sensors is also progressing rapidly (Melzer et al., 2021; Qian et al., 2022; Wu  
48 et al., 2022). However, it is impossible to develop a universal neuropeptide sensor by engineering  
49 postsynaptic GPCRs due to their diversity. Furthermore, despite their fundamental contribution on systemic  
50 understanding of peptidergic circuits in the brain, the postsynaptic GPCR-based neuropeptide sensors has  
51 some inherent limitations primarily because their site of action is postsynaptic (Rusakov, 2022). To resolve  
52 these issues, several attempts have been made to develop presynaptic neuropeptide sensors by genetically  
53 labeling neuropeptides with a fluorescent protein (Ding et al., 2019; Shaib et al., 2018; Taraska et al., 2003).  
54 However, this approach has never been applied to awake behaving animals, because fluorescently labeled  
55 neuropeptides are rapidly depleted after release. Here, we report a new genetically encoded fluorescent  
56 LDCV release sensor that directly monitors the release of neuropeptides from presynaptic terminals in  
57 behaving mice. The sensor uses a pH-sensitive variant of GFP, superecliptic pHluorin (SEP)  
58 (Sankaranarayanan et al., 2000), to detect pH changes inside the lumen of LDCVs during release events.  
59 We targeted SEP to the luminal membrane of the LDCV by incorporating it into the luminal loop of  
60 cytochrome b561 (CYB561), a LDCV-specific membrane protein (Birinci et al., 2020; Perin et al., 1988).  
61 We validated and optimized the CYB561-SEP fusion protein as a LDCV sensor (CybSEP) in differentiated  
62 PC12 pheochromocytoma cell lines, mouse brain slices, and awake behaving mice.

63         Peptidergic neurons co-express fast-acting transmitters and neuropeptides, and they are packaged  
64 in different vesicles that have distinct release properties suggesting that each transmitter may shape the  
65 function of these neurons differentially. However, circuit-specific dissection of functional roles played by  
66 each transmitter is impossible due to the lack of tools that specifically manipulate peptidergic transmissions

67 leaving fast neurotransmission unaltered. Here, we report a novel genetically encoded silencer that  
68 specifically blocks peptidergic transmissions without changing fast neurotransmission. The silencer uses a  
69 neuropeptide-specific peptidase, neutral endopeptidase (NEP), also called neprilysin or enkephalinase,  
70 which specifically inactivates many neuropeptides, including but not limited to enkephalin, bradykinin,  
71 calcitonin gene-related peptide (CGRP), substance P, neurotensin, and oxytocin by cleaving their  
72 hydrophobic amino acid chains (Gourlet et al., 1997; Katayama et al., 1991; Scholzen and Luger, 2004;  
73 Skidgel et al., 1984; Stancampiano et al., 1991; Turner et al., 1985a, b). We targeted the NEP to the luminal  
74 LDCV by combining it with the LDCV-targeting signal peptide, then validated this LDCV-targeted NEP  
75 as a silencer of peptidergic transmission electrophysiologically in mouse brain slices, and behaviorally in  
76 awake behaving mice. Our study, using novel neuropeptide release sensor and silencer, demonstrates that  
77 neuropeptides, not glutamate, are shown to be essential for conveying aversive unconditioned stimuli during  
78 Pavlovian threat learning.

79

## 80 **RESULTS**

### 81 **Design and characterization of the LDCV sensor**

82 SEP was originally engineered to detect synaptic transmission by targeting it to the inside of SVs by  
83 incorporating it into SV-specific membrane proteins, such as synaptobrevin or synaptophysin (Miesenböck  
84 et al., 1998; Zhu et al., 2009). We re-purposed this proven system to monitor LDCV release by fusing SEP  
85 to CYB561. The CYB561 is a transmembrane electron transport protein unique to LDCVs (Fleming and  
86 Kent, 1991; Perin et al., 1988). It mediates transmembrane electron transport to regenerate ascorbic acid  
87 inside the lumen of LDCVs and plays a critical role in the biosynthesis of neuropeptides by  
88 supplying reducing equivalents to  $\alpha$ -amidase (Lu et al., 2014). We first substituted two histidines for  
89 alanines at positions 86 and 159. These histidines are essential sites for ascorbate binding, and therefore  
90 replacing them with alanines eliminated CYB561's electron transporter activity (Kipp et al., 2001; Lu et  
91 al., 2014). We then inserted SEP coding sequence into the luminal domain of CYB561, between  
92 transmembrane domain 3 and 4 (CybSEP) (Figure 1A). In addition, we created a control fusion protein by  
93 inserting gamillus, an acid-tolerant monomeric GFP, into the same position of Cyb561 (CybGam) (Shinoda  
94 et al., 2018). To determine whether CybSEP alters fluorescence in response to changes in pH, we expressed  
95 CybSEP in PC12 cells differentiated by nerve growth factor and perfused acidic solution (pH 5.5) followed  
96 by  $\text{NH}_4\text{Cl}$  treatment to deacidify intracellular compartments. We observed almost a complete loss of  
97 CybSEP fluorescence in the acidic condition. The signal returned following  $\text{NH}_4\text{Cl}$  perfusion. In contrast,  
98 CybGam showed no changes in fluorescence (Figures 1B-1D) under the same conditions. Bath application  
99 of 70 mM KCl, which leads to membrane depolarization, induced robust increases in CybSEP fluorescence,  
100 whereas CybGam exhibited no detectable changes in fluorescence in response to KCl (Figures 1E, 1F). We

101 further engineered the CybSEP to increase fluorescence intensity by incorporating two SEPs into CYB561  
102 (CybSEP2). Evoked changes in fluorescence were 30% larger for CybSEP2 compared with CybSEP  
103 (Figures S1A and S1B). Thus, CybSEP2 was used for the rest of the experiments.

104 Electrical stimulation of differentiated PC12 cells at various frequencies (10, 25, 50, and 100 Hz)  
105 via a glass pipet showed frequency-dependent increases in fluorescent signal (the maximum response was  
106 observed at 100 Hz). Moreover, EGTA treatment abolished the fluorescent signal evoked by 100-Hz  
107 stimulation, indicating that the response depended on calcium (Figures 1G-1H) (Nakamura, 2019). To test  
108 whether the fluorescent response resulted from LDCV release events, we inhibited vesicular fusion using  
109 the tetanus toxin light chain (TetTox), which disrupts the release of both SVs and LDCVs by cleaving  
110 synaptobrevin (McMahon et al., 1992). Expressing TetTox-mCherry with CybSEP2 resulted in a greatly  
111 reduced fluorescent signal in response to electrical stimulation (100 Hz), compared with mCherry  
112 expressing controls (Figures 1I-1K). These data indicate that fluorescent responses from CybSEP2 reflect  
113 LDCV release events.

#### 114 **Imaging presynaptic LDCV release in acute brain slices**

115 After successfully validating that CybSEP2 can be used to detect LDCV release in the differentiated PC12  
116 cells, we tested it in intact brain slices. The parabrachio-amygdaloid pathway is a well-described peptidergic  
117 circuit that encodes aversive unconditioned sensory stimuli (US) during Pavlovian threat learning (Nagase  
118 et al., 2019). Previous studies have shown that peptidergic neurons expressing CGRP (encoded by the *Calca*  
119 gene) in the external lateral parabrachial nucleus (PBel) and their direct downstream CGRP receptor-  
120 expressing neurons in the lateral subdivision of the central amygdala (CeAl) plays important roles in pain  
121 perception and threat learning in mice (Han et al., 2005; Han et al., 2015; Salmon et al., 2001; Sato et al.,  
122 2015). In addition to glutamate, CGRP neurons in the PBel (CGRP<sup>PBel</sup>) co-express various neuropeptides,  
123 such as substance P (SP), pituitary adenylyl cyclase-activating peptide (PACAP), and neurotensin (NTS)  
124 (Kang et al., 2020; Palmiter, 2018; Pauli et al., 2022). However, the main transmitter that relays the US  
125 information in this peptidergic circuit was not known. Therefore, this circuit is ideal for functionally  
126 validating CybSEP2 in the brain. To monitor peptidergic transmissions in this circuit, recombinant adeno-  
127 associated viruses (AAVs) Cre-dependently encoding CybSEP2 or CybGam (AAV<sub>DJ</sub>-DIO-CybSEP2 or  
128 AAV<sub>DJ</sub>-DIO-CybGam) were bilaterally injected into the PBel of *Calca*<sup>Cre/+</sup> mice (Figures 2A and S2A).  
129 Four weeks after the injection, we confirmed that CybSEP2 and CybGam were robustly expressed in  
130 neuronal cell bodies within the PBel, and in axons projecting to the CeAl (Figures 2B and S2A). A high-  
131 speed fluorescence imaging system was used to monitor the CybSEP2 fluorescence changes in acutely  
132 dissociated brain slices. Electrical stimulation of CGRP<sup>PBel</sup> axonal terminals in the CeAl via a glass pipet  
133 resulted in frequency-dependent increases in fluorescence, with a maximum response at 100 Hz. These  
134 responses were abolished by bath application of calcium chelator, EGTA. No significant increases in

135 fluorescence were observed in brain slices expressing CybGam (Figures 2C-2E). The rise time ( $\tau_{on}$ ) of these  
136 responses was faster than the decay time ( $\tau_{off}$ ) at 50 and 100 Hz (Figure 2F). Interestingly, significant  
137 fluorescence was also detected near the CGRP<sup>PBel</sup> neuronal cell bodies and dendrites that express CybSEP2  
138 (Figures S2B-S2D), an intriguing observation that the CGRP signaling might be functionally relevant in  
139 the PBel (Ludwig and Leng, 2006; van den Pol, 2012). Repeated stimulation of CGRP<sup>PBel</sup> axonal terminals  
140 in the CeAl 4 times over 20 min with 5-min intervals evoked similar fluorescence signal, indicating that the  
141 CybSEP2 fluorescence signal was not depleted nor quenched under these conditions (Figures 2G and 2H).  
142 Inhibiting vesicular release by co-expressing TetTox in CGRP<sup>PBel</sup> neurons of the *Calca*<sup>Cre/+</sup> mice  
143 substantially attenuated 100 Hz-evoked changes in fluorescence in their axonal terminals within the CeAl  
144 (Figures 2I and 2J). By contrast, no noticeable decreases in fluorescence were observed in mCherry-  
145 expressing control brain slices (Figures 2K and 2L). Taken together, these results suggest that CybSEP2  
146 can be used to reliably detect LDCV release events at axonal terminals in acutely dissociated brain slices,  
147 making it a useful tool to study neuropeptide release from presynaptic terminals.

#### 148 **Monitoring presynaptic LDCV release in freely moving mice**

149 The CGRP<sup>PBel</sup> neurons are activated by unconditioned stimulus (electric foot shock), but not by the  
150 conditioned stimulus (tone) during Pavlovian threat conditioning (Kang et al., 2022). We therefore  
151 examined whether CybSEP2 can be used as a LDCV sensor to detect electric foot shock-evoked  
152 neuropeptide release from CGRP<sup>PBel→CeAl</sup> terminals by monitoring fluorescence changes in the CeAl of  
153 freely moving mice during threat conditioning. We stereotaxically injected AAV<sub>DJ</sub>-DIO-CybSEP2 or  
154 AAV<sub>DJ</sub>-DIO-CybGam into the PBel of *Calca*<sup>Cre/+</sup> mice, and then implanted a fiberoptic cannula into the  
155 CeAl (Figure 3B). A CMOS fiber photometry system was used to monitor LDCV release in freely moving  
156 mice (Figure 3A). Following 4 weeks of recovery from the stereotaxic surgery, CMOS fiber photometry  
157 revealed that a mild electric footshock (0.3 mA, 2 s) triggered a sharp increase in fluorescence in the  
158 CGRP<sup>PBel→CeAl</sup> terminals of CybSEP2-expressing *Calca*<sup>Cre/+</sup> mice, but not in CybGam-expressing control  
159 mice (Figures 3C and 3D). Since the CGRP<sup>PBel</sup> neurons are also activated by painful stimuli, we monitored  
160 the neuropeptide releases evoked by noxious heat. In the hot plate test, 52°C thermal stimulus significantly  
161 increased the fluorescence signal in CGRP<sup>PBel→CeAl</sup> terminals, whereas 42°C thermal stimulus did not. No  
162 changes in fluorescence were observed in response to both thermal stimuli (42 and 52°C) in CybGam-  
163 expressing mice (Figures 3E and 3F). We also tested quinine as another aversive sensory stimulus, since  
164 previous studies have shown that the CGRP<sup>PBel→CeAl</sup> pathway is activated by quinine consumption (Kang et  
165 al., 2022). Fluorescence intensity rapidly increased at the onset of quinine consumption in CGRP<sup>PBel→CeAl</sup>  
166 terminals of CybSEP2-expressing *Calca*<sup>Cre/+</sup> mice, but not in CybGam-expressing control mice. Water  
167 consumption failed to evoke fluorescence changes in both groups of mice (Figures 3G and 3H). Taken

168 together, CybSEP2 imaging in freely moving mice shows that aversive sensory stimuli induce robust signals  
169 from the LDCV sensor, indicative of neuropeptide release.

170 CGRP<sup>PBel</sup> neurons express multiple neuropeptides, but they also express vesicular glutamate  
171 transporter type 2 (Vglut2) and make glutamatergic synapses onto neuronal targets in the CeAl (Carter et  
172 al., 2013; Huang et al., 2021). To test how glutamatergic and peptidergic transmissions interact in  
173 CGRP<sup>PBel→CeAl</sup> terminals in response to aversive sensory stimuli, we monitored fast neurotransmitter release  
174 from axonal terminals in response to the aversive sensory stimuli. To monitor glutamatergic transmissions  
175 in CGRP<sup>PBel→CeAl</sup> terminals, we fused the SEP with the SV-specific protein, synaptophysin (SypSEP), and  
176 packaged it into an AAV vector with a DIO cassette (AAV<sub>DJ</sub>-DIO-SypSEP) (Granseth et al., 2006; Zhu et  
177 al., 2009). We expressed SypSEP in the PBel of *Calca*<sup>Cre/+</sup> mice (Figure S3A) and examined fluorescence  
178 response evoked by various aversive stimuli. In contrast to CybSEP, SypSEP fluorescence did not increase,  
179 but rather slightly decreased from the baseline when mice were exposed to aversive sensory stimuli (Figures  
180 S3B-S3D). However, the SypSEP fluorescence was robustly increased as these mice consumed high-  
181 nutrient liquid formula (Ensure, Abbott) (Figure S3E). These results indicate that SEP-based LDCV or SV  
182 release sensors can detect peptidergic and glutamatergic transmissions independently, and together they can  
183 be used to distinguish between behaviors that result from LDCV vs. SV transmissions from presynaptic  
184 terminals of peptidergic neurons in freely moving mice.

### 185 **Inhibiting peptidergic transmission attenuates threat learning**

186 Monitoring LDCV and SV releases from the CGRP<sup>PBel→CeAl</sup> terminals showed that aversive sensory stimuli  
187 triggers the release of neuropeptides. To investigate whether neuropeptides play a pivotal role in  
188 transmitting aversive sensory stimuli to the amygdala during threat learning, we engineered a peptidase that  
189 selectively degrades neuropeptides by targeting it specifically into the luminal side of the LDCV. Neutral  
190 endopeptidase (NEP) is a transmembrane protease present at the cell surface which cleaves a broad range  
191 of neuropeptides that contain hydrophobic amino acid in the extracellular space (Helin et al., 1994; Hui,  
192 2007; Katayama et al., 1991). The CGRP<sup>PBel</sup> neurons co-express multiple neuropeptides including CGRP,  
193 neurotensin, substance P, and pituitary adenylate cyclase-activating peptide (Kang et al., 2020), all of which  
194 can be degraded by the NEP (Gourlet et al., 1997; Katayama et al., 1991; Skidgel et al., 1984). Therefore,  
195 we utilized the NEP to degrade active neuropeptides packaged inside the LDCVs of the CGRP<sup>PBel</sup> neurons.  
196 We incorporated the LDCV targeting signal peptide from pro-opiomelanocortin (POMC) into the NEP to  
197 selectively translocate it to the luminal side of LDCV (Cool et al., 1995). We then constructed P2A-  
198 mediated bicistronic AAV vector encoding LDCV-targeted NEP (NEP<sub>LDCV</sub>), as well as the cytosolic  
199 mRuby3 (AAV<sub>DJ</sub>-DIO-NEP<sub>LDCV</sub>-P2A-mRuby3) (Figure 4A). To validate its expression in the mouse brain,  
200 AAV<sub>DJ</sub>-DIO-NEP<sub>LDCV</sub>-P2A-mRuby3 or AAV<sub>DJ</sub>-DIO-mCherry was bilaterally delivered into the PBel of

201 *Calca*<sup>Cre/+</sup> mice (Figure 4B). After confirming the expression of mRuby3 in the CGRP<sup>PBel</sup> neurons, we  
202 immunostained the PBel-containing coronal brain sections with antisera against CGRP to evaluate the  
203 degradation of CGRP in the presence of the NEP<sub>LDCV</sub>. We found that most mCherry-expressing neurons  
204 were co-labeled with CGRP-immunofluorescent signals while CGRP-immunoreactivity were barely  
205 detected in NEP<sub>LDCV</sub>-expressing neurons suggesting efficient proteolytic degradation of CGRP by the  
206 NEP<sub>LDCV</sub> (Figure 4C and 4D). We next sought to determine whether the loss of neuropeptides in the  
207 CGRP<sup>PBel</sup> neurons affects peptidergic transmission in CGRP<sup>PBel→CeAl</sup> synapses by electrophysiological  
208 recording of the postsynaptic CeAl neurons in brain slices. AAVs Cre-dependently expressing Chr2 were  
209 injected into the PBel of *Calca*<sup>Cre/+</sup> mice and AAVs Cre-dependently encoding NEP<sub>LDCV</sub>-P2A-mRuby or  
210 mCherry were co-injected in the same mice. Four weeks after the injections, the optogenetically evoked  
211 excitatory postsynaptic currents (oEPSCs) and potentials (oEPSPs) were recorded in the CeAl neurons that  
212 were surrounded by the CGRP<sup>PBel→CeAl</sup> perisomatic synaptic terminals with mCherry or mRuby  
213 fluorescence to validate whether the NEP<sub>LDCV</sub> selectively silence peptidergic transmission without altering  
214 glutamatergic transmissions in CGRP<sup>PBel→CeAl</sup> synapses (Figure 4E). The oEPSCs were recorded to monitor  
215 the glutamatergic transmission, then the oEPSP were recorded in the same neuron to monitor long-lasting  
216 resting membrane potential changes induced by trains of 40-Hz stimulation, which is the characteristic  
217 response of peptidergic transmission. The whole-cell, patch-clamp recording results revealed that the  
218 NEP<sub>LDCV</sub> had no effect on oEPSCs compared to controls (Figure 4F), whereas it significantly attenuated the  
219 oEPSP induced by 40-Hz stimulation in CEAl neurons compared to controls (Figure 4G). These results  
220 indicate that the NEP<sub>LDCV</sub> selectively attenuated the peptidergic transmission leaving the glutamatergic  
221 transmission unaltered.

222 We then investigated whether the degradation of neuropeptides by the NEP<sub>LDCV</sub> has an impact on  
223 Pavlovian threat learning. In the auditory fear conditioning (Figure 4H), we found that the gradual increase  
224 of freezing behaviors was observed in the mCherry-expressing control, while NEP<sub>LDCV</sub>-expressing group  
225 exhibited a significant reduction of freezing behavior compared to the control group (Figure 4I). In memory  
226 tests 24 hr later, mice expressing the NEP<sub>LDCV</sub> exhibited marked suppression of freezing behaviors in  
227 response to the context cue (Figure 4J) and auditory cues (Figure 4K) as compared with the mCherry-  
228 expressing group. In addition, we examined whether lowering neuropeptide release by NEP<sub>LDCV</sub> in  
229 CGRP<sup>PBel</sup> neurons affects animals' responses to formalin-induced inflammatory pain and quinine, a bitter  
230 tastant (Kang et al., 2022). In the formalin assay, formalin-induced licking behaviors were significantly  
231 reduced during acute and inflammatory phases (Figure S4A-C). Furthermore, the NEP<sub>LDCV</sub> group showed  
232 significantly increased quinine consumption as compared with mCherry control group (Figure S4D).

233 Overall, these results demonstrated that the NEP<sub>LDCV</sub> efficiently and selectively lowered neuropeptide  
234 release in behaving mice making it an ideal tool to study the direct involvement of peptidergic transmission  
235 in the peptidergic circuits.

### 236 **Glutamatergic transmission is not involved in threat learning**

237 Since aversive sensory stimuli failed to trigger the SypSEP fluorescence increase at CGRP<sup>PBel→CeAl</sup>  
238 terminals, we investigated whether the glutamate release by CGRP<sup>PBel</sup> is dispensable in Pavlovian treat  
239 learning. To disturb glutamate release, we edited the *Slc17a6* gene, which encodes Vglut2, using the  
240 CRISPR/saCas9 system (Hunker et al., 2020), based on a report showing that the Vglut2 is the main  
241 glutamate transporter in the PBel (Pauli et al., 2022). AAVs that express Cas9 and sgRNAs for *Slc17a6* or  
242 *Rosa26* (as control) in a Cre-dependent fashion were bilaterally injected into the PBel of *Calca*<sup>Cre/+</sup> mice.  
243 Note that *Rosa26* expression has no biological effect since it does not encode a functional protein. Therefore,  
244 sg*Rosa26* is used as a control for sg*Slc17a6* (Figure 5A). *In situ* hybridization for *Slc17a6* and *Calca* genes  
245 in PBel showed that *Slc17a6* mRNA was depleted in CGRP neurons of sg*Slc17a6*-injected mice but not in  
246 sg*Rosa26*-injected mice (Figure 5B).

247 To confirm knockdown of glutamatergic transmission, slice electrophysiology was used to measure  
248 oEPSCs in postsynaptic CeAl neurons. AAVs Cre-dependently expressing ChR2 and sg*Slc17a6* were  
249 stereotaxically injected into the PBel of *Calca*<sup>Cre/+</sup> mice. Three weeks after surgery, whole-cell, patch-clamp  
250 recording of CeAl neurons showed that sg*Slc17a6* resulted in a significant reduction of oEPSCs compared  
251 to controls; responses were mediated by monosynaptic glutamatergic transmission (Figure 5C lower trace).  
252 The average amplitude of oEPSCs exhibited by CeAl neurons showed 78.3 % of reduction in sg*Slc17a6*-  
253 expressing mice compared to controls. Majority of neurons (21/30) did not show any oEPSCs and 30 % of  
254 remaining neurons showed reduced oEPSCs (Figure 5C upper trace, and 5D). We then performed Pavlovian  
255 threat conditioning with sg*Slc17a6* and control groups (Figure 5E). Animals in both groups showed a  
256 gradual increase in freezing behavior as the number of pairings increased (Figure S5A). Furthermore, both  
257 groups of mice showed similar levels of freezing in contextual (Figure 5F) and auditory cue-induced (Figure  
258 5G) threat memory tests.

259 To further test the contribution of glutamatergic transmission in threat learning, *Slc17a6* conditional  
260 knockout (cKO) mice were produced by crossing the *Slc17a6* floxed mice (*Slc17a6*<sup>lox/lox</sup>) with mice that  
261 express Cre-recombinase specifically in CGRP neurons in a Flp-dependent manner (*Calca*<sup>FrtCre/+</sup>). Bilateral  
262 injection of AAV encoding Flp-recombinase into the PBel of *Calca*<sup>FrtCre/+::Slc17a6</sup><sup>lox/lox</sup> mice induced  
263 conditional knockout of the *Slc17a6* gene specifically in CGRP<sup>PBel</sup> neurons (Figure 5H). Both cKO and  
264 control mice displayed similar freezing responses during threat learning (Figure S5B), as well as contextual-



265 (Figure 5I) and cue-dependent retrieval tests (Figure 5J). These results show that lowering glutamate release  
266 from CGRP<sup>PBel</sup> neurons had no effect on threat learning.

267

## 268 **DISCUSSION**

269 Here we report the development of two new genetically encoded tools that can monitor and silence the  
270 release neuropeptides in awake behaving mice: The CybSEP2, a genetically encoded fluorescent LDCV  
271 sensor that reliably detects LDCV release presynaptically in freely moving mice, and the NEP<sub>LDCV</sub>, a  
272 genetically encoded peptidase that attenuates neuropeptide release by degrading neuropeptides inside the  
273 LDCVs in freely moving mice.

274 Recent progress in developing several neuropeptide sensors that utilize postsynaptic neuropeptide  
275 receptors now allows one to monitor the release of some neuropeptides in behaving animals. These  
276 technologies will provide important insights into the function of neuropeptides in brain circuits (Sabatini  
277 and Tian, 2020; Wu et al., 2022), but they have some limitations. First, the GPCR-based sensor is expressed  
278 in postsynaptic neurons and detects the release of neuropeptides indirectly, regardless of their source. In  
279 cases where a postsynaptic neuron receives peptidergic inputs from different brain areas, it is not possible  
280 to distinguish which synaptic terminal evoked the release event using a postsynaptic sensor. In addition, it  
281 has been suggested that neuropeptides can be released from anywhere in a neuron, including the cell body,  
282 dendrites, and axonal terminals (Ludwig and Leng, 2006; van den Pol, 2012). Thus, GPCR-based sensors  
283 cannot pinpoint the exact neuropeptide release site. Second, the receptors for some neuropeptides, such as  
284 cocaine and amphetamine-related transcript (CART), have not yet been discovered (Ahmadian-Moghadam  
285 et al., 2018). Thus, GPCR-based sensors cannot be applied to these neuropeptides. Third, the GPCR-based  
286 sensors are artificially expressed in postsynaptic neurons at high level, and therefore can act as competitive  
287 inhibitors of the endogenous GPCR by capturing endogenous ligands even if their downstream signaling  
288 actions are eliminated by genetic modification. This could potentially cause abnormal behavior in animals.  
289 Finally, one GPCR-based sensor usually detects the release of only one neuropeptide, which provides high  
290 sensitivity for a specific neuropeptide release event but limits its versatility. Considering more than a  
291 hundred neuropeptides and their postsynaptic GPCRs have been discovered, more than a hundred  
292 individually developed GPCR-based sensors are needed. These limitations would be resolved by  
293 developing a presynaptic neuropeptide release sensor. Our CybSEP2 system: 1) can pinpoint exactly where  
294 the neuropeptides are released from, 2) can be used to monitor essentially any neuropeptide by driving the  
295 expression of CybSEP2 via a neuropeptide-specific Cre-driver mouse line, and 3) does not interfere with  
296 endogenous peptidergic signaling. Given the fact that only a handful of GPCR-based neuropeptide sensors  
297 are currently available (Dong et al., 2022; Qian et al., 2022; Wang et al., 2022), CybSEP2 can serve as an

298 alternative tool for monitoring the release of neuropeptides for which postsynaptic sensors are not yet  
299 available. However, this versatility limits the specificity of the CybSEP2 system. Considering that multiple  
300 neuropeptides can be co-packaged into the same LDCV, monitoring LDCV release events as a proxy for  
301 neuropeptide release cannot define which neuropeptide has been released. Therefore, current pre- and post-  
302 synaptic neuropeptide sensors each have limitations, but when used in combination their unique properties  
303 could lead to transformative discoveries.

304 Most peptidergic neurons are multi-transmitter neurons (Hokfelt, 1991; Lundberg, 1996; Merighi,  
305 2002). Therefore, to tease apart the roles played by each transmitter in single neurons, a silencing tool that  
306 selectively inhibits the release of specific transmitter in a defined peptidergic circuit is critically required,  
307 but such a tool is currently unavailable. Genetic knock-out of the neuropeptide encoding gene is one way  
308 to interrogate the role of certain neuropeptide. Yet, considering most peptidergic neurons co-express and  
309 co-package multiple neuropeptides in a single LDCV, knocking-out one neuropeptide gene may be  
310 insufficient to produce phenotypic changes due to the compensatory actions of other neuropeptides co-  
311 expressed in the same neurons (Zajdel et al., 2021). Therefore, developing a tool that silences the release  
312 of all neuropeptides in a genetically defined neuronal population is required to interrogate peptidergic  
313 transmission systemically. In this study, we selectively silenced the peptidergic transmission by targeting  
314 the NEP into the luminal side of the LDCVs that proteolytically degrade neuropeptides inside the LDCVs  
315 leaving fast synaptic transmission unaltered. The NEP, a zinc-dependent metalloprotease, is an integral  
316 plasma membrane protein that primarily degrades neuropeptides from the extracellular surface (Booth and  
317 Kenny, 1980). The enzymatic activity of the NEP at pH 5.0 is comparable to its activity at pH7.4 (Kerr and  
318 Kenny, 1974). Therefore, the LDCV-targeted NEP (NEP<sub>LDCV</sub>) can reliably and selectively degrade  
319 neuropeptides at low pH in the LDCV. Our results showed that the cell-type-specific expression of the  
320 NEP<sub>LDCV</sub> in the CGRP<sup>PBel→CeA</sup> peptidergic circuit substantially reduced peptidergic transmission without  
321 altering glutamatergic transmission by selectively degrading neuropeptides in the CGRP<sup>PBel</sup> neurons, which  
322 resulted in impaired responses to innately aversive sensory stimuli, such as electric footshock, quinine  
323 consumption, and the plantar injection of formalin (Figure 4). These results indicate that the AAV-DIO-  
324 NEP<sub>LDCV</sub>-P2A-mRuby3 can be used as the specific silencer for the transmission of many neuropeptides that  
325 contain hydrophobic amino acid in awake behaving animals.

326 With the novel sensor and silencer for neuropeptide release at hand, we asked whether  
327 neuropeptides can function as the primary transmitter in mediating a major output, or whether they  
328 invariably act as a co-transmitter to modulate classical neurotransmission. Pharmacological blockade of  
329 postsynaptic neuropeptide receptors substantially affected neuronal outputs and behavior, indicating that  
330 peptidergic transmissions play major roles in certain peptidergic systems in the brain (Hokfelt et al., 2003;  
331 Salio et al., 2006). Yet, it remained unclear whether neuropeptides can be the only released transmitter or

332 whether they are always co-released with other transmitters to affect behavior or physiology in mammals  
333 (Salio et al., 2006). To address this question, we monitored the release of two types of transmitter vesicles,  
334 LDCVs and SVs, using our newly developed CybSEP2 together with a previously developed SV sensor,  
335 SypSEP, during threat learning. We investigated the CGRP<sup>PBel→CeA</sup> peptidergic circuit since our previous  
336 analyses have shown that CGRP<sup>PBel</sup> neurons and their direct downstream targets (neurons in the CeA that  
337 express the CGRP receptor) are critically involved in affective pain transmission and aversive memory  
338 formation (Han et al., 2015). Using a CMOS-coupled fiber photometry system, we observed that noxious  
339 stimuli such as an electric footshock, exposure to a hot plate, or quinine consumption triggered the release  
340 of neuropeptides including CGRP in the CGRP<sup>PBel→CeA</sup> peptidergic pathway, but not glutamate (Fig. 3).  
341 Interestingly, noxious, and aversive taste stimuli decreased SypSEP fluorescence below the baseline in the  
342 same circuit (Fig. S3). These results suggest that only neuropeptides are released from CGRP<sup>PBel→CeA</sup>  
343 terminals evoked by aversive sensory stimuli. Furthermore, functional silencing of peptidergic or  
344 glutamatergic transmissions in this pathway using the NEP<sub>LDCV</sub>, or the *Slc16a7* gene disruption further  
345 confirmed that peptidergic, but not glutamatergic transmission is required for conveying aversive US from  
346 the PBel to the amygdala during Pavlovian threat learning. It is surprising that glutamatergic transmission  
347 is dispensable for relaying aversive sensory cues during threat learning. Previous studies showed that  
348 glutamatergic transmission in the CGRP<sup>PBel→CeA</sup> peptidergic pathway mediate hypercapnic arousal and  
349 sleep regulation (Kaur et al., 2013; Kaur et al., 2017). These results suggest that different transmitters in  
350 the same neural circuit may play different roles, which raises an intriguing question whether single  
351 CGRP<sup>PBel</sup> neurons can encode distinct information by releasing different transmitters, or two distinct  
352 CGRP<sup>PBel</sup> subpopulations exclusively use different transmitters to mediate distinct functions. Further  
353 investigation is required to address this question.

354 Taken together, our results show that the CybSEP2 sensor and the NEP<sub>LDCV</sub> silencer are reliable  
355 tools for monitoring and silencing presynaptic neuropeptide release in awake behaving mice as they  
356 experience sensory or emotional stimuli. Furthermore, these new tools will allow researchers to investigate  
357 molecular and cellular mechanisms of peptidergic transmission more thoroughly, which has been largely  
358 neglected compared to the fast synaptic transmission.

359

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365

366 **Author Contributions**

367 S.H. conceived of the idea. S.H., R.D.P and L.S.Z. secured funding. S.H., D-I.K., S.P., and J.Y.C. designed  
368 the experiments and wrote the manuscript. D-I.K. cloned the sensor and silencer of peptidergic transmission  
369 and performed most of the experiments. S.P. performed the *Slc17a6* loss-of-function experiments. M.Y.  
370 and J.Y.C. performed the slice electrophysiology associated with the NEP<sub>LDCV</sub>, and *Slc17a6* loss-of-  
371 function experiments. J.J. built the custom-made CMOS fiber photometry system. A.C.H, and L.S.Z.  
372 provided AAVs Cre-dependently expressing Cas9 and sgRNAs for *Slc17a6* or *Rosa26*.

373

374 **Competing Interests**

375 The authors declare no competing interests.

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- 528



529 **STAR METHODS**

530 **EXPERIMENTAL MODEL AND SUBJECT DETAILS**

531 **Mouse lines**

532 All protocols for animal experiments were approved by the IACUC of the Salk Institute for Biological  
533 Studies and University of Washington according to NIH guidelines for animal experimentation. *Calca<sup>cre</sup>*,  
534 *Slc17a6<sup>lox/lox</sup>*, and *Calca<sup>FrtCre</sup>* transgenic mouse lines used in this study are all C57BL/6J background, and  
535 generated from the Palmiter lab (Hnasko et al., 2010; Carter et al., 2013; Chen et al., 2018). 3–4-month-old  
536 heterozygous mice were used for all experiments except for the *Slc17a6* experiments where homozygous  
537 for *Slc17a6<sup>lox/lox</sup>* were used. Animals were randomized to experimental groups, and no sex differences were  
538 noted. Mice were maintained on a standard 12-hour light/dark cycle and provided with food and water *ad*  
539 *libitum*.

540 **METHOD DETAILS**

541 **Construct design and molecular cloning**

542 To generate fusion constructs, cytochrome b561-superecliptic pHluorin (CybSEP), mouse cytochrome  
543 b561 (NM\_007805.4, Genscript) and pcDNA3-SypHluorin2 (#37005, Addgene) were used for this  
544 construct. We first created point mutated cytochrome b561 by substituting two extracellular histidines (His  
545 86 and 159) by two alanines, which are a critical role in binding of ascorbic acid. Then, pHluorin was  
546 inserted into the lumen domain at position 339-340 between transmembrane domain 3 and 4 of cytochrome  
547 b561. For two copies of pHluorin, the second one was subsequently inserted into the backbone vector. For  
548 cloning cytochrome b561-gamillus construction (124837, Addgene), pHluorin was replaced with gamillus.  
549 For adeno-associated virus (AAV) constructs, we used rAAV-hSyn backbone (51509, Addgene) and *AscI*  
550 and *FseI* were used to excise the insert of the backbone for replacement of fusion constructs. For LDCV  
551 targeted peptidase (NEP<sub>LDCV</sub>) generation, the signal peptide (0-26 amino acids) of pro-opiomelanocortin  
552 (#176704, Addgene) and ectodomain (52-750 amino acids) of the NEP (#7283, Addgene) were used for  
553 the fusion protein and then inserted in rAAA-FLEX-axonGCaMP6s-P2A-mRUBY3 vector (#112008,  
554 Addgene) used as a backbone after excising axonGCaMP6s by *BamHI* and *NheI*. All construct generation  
555 was performed by In-Fusion HD cloning kit (638920, Takara).

556

557 **Cell culture and live-cell imaging**

558 PC-12 cells (CRL-1721, ATCC) were maintained in DMEM high glucose (#11995065, Invitrogen)  
559 supplemented with 10% fetal bovine serum (#10437028, Hyclone) and 5% horse serum (16050130, GIBCO)  
560 at 37°C incubator with 5% CO<sub>2</sub>. For imaging, cells were plated on the poly-L-lysine (Sigma) coated

561 coverslips in 24-well plates and following day plasmids were transfected using by lipofectamin 3000  
562 (#L3000015, Invitrogen) and experiments were performed 48 hr after transfection. To differentiate cells,  
563 50 ng/ml nerve growth factor (NC010, Sigma) was added to the plates 12 hours after transfection. For pH-  
564 dependent experiments, CybSEP or CybGam-expressing cells were perfused with extracellular bath  
565 solution and with acidic extracellular solution at pH5.5 to quench the pHluorin. 50 mM NH<sub>4</sub>Cl was then  
566 added to the chamber to neutralize the cellular environment. To elicit the membrane fusion of large dense  
567 core vesicles by depolarization, 70 mM KCl was added on the cells grown on coverslips. For the electrical  
568 stimulation, broken glass pipettes were pulled from borosilicate glass (G150TF-4, Warner Instruments)  
569 with pipette puller (SU-P97, Shutter instrument) and filled with the extracellular bath solution. For  
570 stimulation, they were positioned near fluorescence expressing cells and were evoked by electrical  
571 stimulator (Model 2100 Isolated Pulse Stimulator, A-M systems). The stimulation voltage was set at 4-5 V  
572 (3-ms pulse width) with various frequencies to elicit release of neuropeptides from LDCVs. During  
573 perfusion, the temperature of the bath chamber was maintained at 32°C by in-line solution heater (TC-324C,  
574 Warne Instruments). The extracellular bath solution contained (in mM) 130 NaCl, 2.8 KCl, 2 CaCl<sub>2</sub>, 1  
575 MgCl<sub>2</sub>, 10 HEPES and 10 glucose; for cellular acidification the acid solution contained (in mM) 90 NaCl,  
576 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 60 Na-acetate and 10 HEPES. For the cellular neutralization, the 40 mM NaCl of bath  
577 solution was replaced with 40 mM NH<sub>4</sub>Cl. To test the role of calcium in the release of neuropeptides, 5  
578 mM EGTA was replaced with CaCl<sub>2</sub>.

579 Imaging was carried out in upright scope (Slice scope Scientifica) with water immersion objective  
580 lens (5X, 0.1 NA; 40X, 0.9 NA, LUMPLFLN-W, Olympus), LED illumination (GFP, 490-nm; mCherry,  
581 580-nm) (pE-4000, CoolLED) and a multiband filter set (89402, Chroma). Images were acquired at 10 Hz  
582 (2 × 2 digital binning, 1,024 × 1,024-pixel resolution) equipped with an sCMOS camera (Prime 95B,  
583 Teledyne Photometrics) using micro-manager open-source software. Quantification and statics analysis  
584 image data from cultured cells and brain slices were processed with Image J or Prism (Graphad). ROIs were  
585 manually selected by live scanning function of ImageJ after photobleach correction. The fluorescent signal  
586 from each cell was obtained by averaging fluorescent changes ( $\Delta F/F_0$ ) of individual cells. The fluorescence  
587 responses ( $\Delta F/F_0$ ) were calculated as  $(F_{\text{raw}} - F_{\text{baseline}})/F_{\text{baseline}}$ .

588

### 589 **AAV viral preparation**

590 All AAV viruses used in the study were generated in the lab as previously described with a minor  
591 modification except AAV-DIO-mCherry (#50459, Addgene), AAV<sub>DJ</sub>-EF1a-DIO-hChR2(H134R)-EYFP-  
592 WPRE-pA (# 20298, Addgene), AAV1-DIO-saCas9-sg*Slc17a6* (The Zweifel Lab), AAV1-DIO-saCas9-  
593 sg*Rosa26* (The Zweifel Lab), AAV1-CBA-FLPo-dsRed (The Palmiter Lab) and AAV1-hSyn-DIO-YFP  
594 (The Palmiter Lab). In brief, constructs cloned in AAV-hSyn vectors were transfected with RC/DJ and

595 adenovirus-helper plasmid into the AAV-293 cells (#240073, Agilent) using calcium phosphate  
596 precipitation method. 72 hr after transfection, cells were harvested, lysed, and collected by centrifugation  
597 to remove debris. AAV particles were subsequently purified using a HiTrap heparin column (GE healthcare,  
598 UK) and concentrated by Amicon ultra-4 centrifugal filter (UFC801008, MilliporeSigma).

599

## 600 **Stereotaxic surgery**

601 All surgeries were carried out under 1.5%-2% isoflurane anesthesia (Dräger Vapor® 2000; Draegar) and  
602 kept on the water circulating heating pad to maintain body temperature during surgery. Mice were placed in  
603 a stereotaxic frame (David Kopf Instruments). Viral injections were unilaterally or bilaterally delivered  
604 using a syringe (65458-01, Hamilton, USA) controlled by an ultra-micropump (UMP-3, World Precision  
605 Instruments, USA) at a rate of 0.1  $\mu$ l/min (total volume of 0.6  $\mu$ l for slice imaging and 0.4  $\mu$ l for fiber  
606 photometry). For slice imaging, AAVDJ-hSyn-DIO-CybSEP, AAV<sub>DJ</sub>-hSyn-DIO-CybGam, AAVDJ-hSyn-  
607 DIO-mCherry, or/and AAV<sub>DJ</sub>-hSyn-DIO-TetTox-mCherry were bilaterally injected into the PBN of  
608 *Calca*<sup>Cre/+</sup> (AP: -5.2 mm, ML:  $\pm$ 1.5 mm, DV: -3.6 mm). For fiber photometry recording, AAV<sub>DJ</sub>-hSyn-DIO-  
609 CybSEP or AAV<sub>DJ</sub>-hSyn-DIO-CybGam was unilaterally injected into the PBN of *Calca*<sup>Cre/+</sup> and a custom-  
610 made optic ferrule (0.4  $\mu$ M, 0.5 NA) was implanted into the CeA of *Calca*<sup>Cre/+</sup> (AP: -1.2 mm, ML: -2.85  
611 mm, DV: -4.5 mm). Optic ferrule was then implanted above the injection site (AP: -1.2 mm, ML: -2.85 mm,  
612 DV: -4.5 mm). Mice were allowed to recover for at least 3 weeks following viral infection and fiber  
613 implantation before behavioral testing. For NEP<sub>LDCV</sub> expression, AAV<sub>DJ</sub>-hSyn-DIO-NEP<sub>LDCV</sub>-P2A-  
614 mRuby2 or AAV<sub>DJ</sub>-hSyn-DIO-mCherry were injected bilaterally into the PBN of *Calca*<sup>Cre/+</sup>. For patch  
615 clamp, AAV<sub>DJ</sub>-hSyn-DIO-NEP<sub>LDCV</sub>-P2A-mRuby2 or AAV<sub>DJ</sub>-hSyn-DIO-mCherry with AAV1-DIO-ChR2-  
616 YFP were bilaterally injected into the PBN of *Calca*<sup>Cre/+</sup>. For *Slc17a6* gene inactivation, AAV1-DIO-  
617 saCas9-sg*Slc17a6* or AAV1-DIO-saCas9-sg*Rosa26* were bilaterally injected into the PBel (AP, -4.9 mm;  
618 ML,  $\pm$ 1.35 mm; DV, 3.5 mm) at a rate of 0.1  $\mu$ l/min (total 0.5  $\mu$ l). For patch clamp, AAV1-DIO-saCas9-  
619 sg*Slc17a6* and AAV1-DIO-ChR2-YFP were bilaterally injected into the PBel.

620

## 621 **Histology and imaging**

622 Mice were euthanized with CO<sub>2</sub> at a flow rate of 3 L/min (LPM) and transcardially perfused with cold  
623 paraformaldehyde (PFA) (4% in PB). Brains were kept in PFA overnight at 4 °C and dehydrated in 30%  
624 sucrose (in PBS) for at least 18 hours before vibratome sectioning (VT1000s, Leica). Brains were cut into  
625 50  $\mu$ m coronal section using cryostat (CM 1950, Leica), collected in PBS and mounted on Superfrost  
626 microscopic slides (Fisher Scientific) with DAPI Fluoromount-G mounting media (Southern Biotech) for  
627 imaging. For immunostaining with CGRP antibody, coronal slices were obtained from AAV<sub>DJ</sub>-hSyn-DIO-  
628 NEP<sub>LDCV</sub>-P2A-mRuby2 or AAV<sub>DJ</sub>-hSyn-DIO-mCherry expressing mice. Slices then were washed with

629 PBST containing 0.1% Tween-20s and blocked with 3% normal donkey serum for 1 hour at room  
630 temperature. After rinsing with PBST, slices were incubated with rabbit anti-CGRP (1;1000) at 4 °C  
631 overnight. Next day, slices were rinsed with PBST and then incubated with anti-rabbit Alexa Fluor® 488-  
632 secondary antibody for 1 hour. Images of slices were acquired using all-in-one fluorescence microscope  
633 (BZ-X710, Keyence) with objective lens (10X, 0.40 NA; 20X, 0.75 NA, Olympus) with the BZ-X viewer  
634 software.

635

### 636 **Fluorescence imaging in acute brain slices**

637 Three weeks after viral injection into PBN, acute brain slices containing PBN (200 µm) and CeA (250 µm)  
638 were prepared for imaging. Mice were deeply anesthetized by isoflurane prior to decapitation and  
639 transcardial perfusions were performed with 50 mL of ice-cold carbogenated (95% O<sub>2</sub> :5% CO<sub>2</sub>) cutting  
640 solution (110.0 mM choline chloride, 25.0 mM NaHCO<sub>3</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 2.5 mM KCl, 0.5 mM  
641 CaCl<sub>2</sub>, 7.0mM MgCl<sub>2</sub>, 25.0 mM glucose, 5.0 mM ascorbic acid and 3.0 mM pyruvic acid). Brains were  
642 immediately removed and mounted on the chamber of a VT 1200S Vibratome (Leica) followed by 200 µm  
643 thick sections by vibratome in the same solution. Slices were transferred recovery chamber containing  
644 carbogenated artificial cerebrospinal fluid (aCSF; 124 mM NaCl, 2.5 mM KCl, 26.2 mM NaHCO<sub>3</sub>, 1.2  
645 mM NaH<sub>2</sub>PO<sub>4</sub>, 13 mM glucose, 2 mM MgSO<sub>4</sub> and 2 mM CaCl<sub>2</sub>). After recovery at 34°C water bath for  
646 15 min, slices were transferred to room temperature for at least 30 min before imaging and then slices were  
647 transferred to the imaging chamber and perfused with carbogenated aCSF solution with the flow rate of 2  
648 ml/ min and the temperature of the chamber was maintained at 30–34°C by a temperature controller (TC-  
649 324C, Warne Instruments). For fluorescence imaging in cell bodies or axonal terminals, the electrical  
650 stimulation and acquisition were applied the same condition as in the cultured cells.

651

### 652 **Slice electrophysiology**

653 Mice were anesthetized with pentobarbital sodium and phenytoin sodium solution (Euthasol, 0.2 ml, i.p.)  
654 and transcardially perfused with ice-cold cutting solution (92 mM N-methyl-D-glucamine, 2.5 mM KCl,  
655 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 30 mM NaHCO<sub>3</sub>, 20 mM HEPES, 25 mM D-glucose, 2 mM thiourea, 5 mM Na-  
656 ascorbate, 3 mM Na-pyruvate, 0.5 mM CaCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>). Mice were decapitated, brains quickly  
657 removed and chilled in ice-cold cutting solution. Coronal slices (300 µm) were cut with a vibratome (Leica  
658 VT1200) and incubating in the same cutting solution at 33°C for 12 min. Slices were transferred to a storage  
659 chamber containing recovery solution at room temperature (124 mM NaCl, 2.5 mM KCl, 1.25 mM  
660 NaH<sub>2</sub>PO<sub>4</sub>, 24 mM NaHCO<sub>3</sub>, 5 mM HEPES, 13 mM D-glucose, 2 mM CaCl<sub>2</sub>, 2 mM MgSO<sub>4</sub>). Slices were  
661 transferred into the recording chamber maintained at 33 °C and perfused with artificial cerebral spinal fluid  
662 (126 NaCl, 2.5 KCl, 1.2 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 11 D-glucose, 2.4 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>). Blockers were

663 added to the recording solution for a final concentration of: 10  $\mu$ M DNQX, 1  $\mu$ M TTX, and 50  $\mu$ M 4-AP  
664 (Tocris Bioscience). All solutions were continuously bubbled with 95% O<sub>2</sub>-5% CO<sub>2</sub> with pH 7.3-7.4 and  
665 300-310 mOsm. Whole cell patch-clamp recordings were obtained using an amplifier (Molecular Devices,  
666 MultiClamp 700B) and filtered at 2 kHz. Patch electrodes (3-5 M $\Omega$ ) were filled with a cesium-  
667 methanesulfonate internal solution (117 mM Cs-methanesulfonate, 20 mM HEPES, 0.4 mM EGTA, 2.8  
668 mM NaCl, 5 mM TEA, 5 mM ATP, 0.5 mM GTP; pH 7.35, 280 mOsm). Only cells with access resistance  
669 <20 MOhms throughout the recording were used in the analysis. Peak amplitudes of evoked responses were  
670 calculated with the average of 10 traces using Clampfit in the pClamp 11 software suite (Molecular Devices).

671

### 672 **Fiber photometry recording**

673 CybSEP2 response in axonal terminals were recorded through bundle-imaging fiber photometry system  
674 equipped with CMOS sensor (Doric lenses) and acquired by a LabVIEW (National Instrument) based  
675 platform. To measure fluorescence signals, 470-nm LED was used for inducing Ca<sup>2+</sup> dependent  
676 fluorescence signals and 405-nm LED was used for Ca<sup>2+</sup> independent (isosbestic control) fluorescence  
677 signals at a sampling rate of 10 Hz. Both LEDs were bandpass filtered and passed through a 20X/0.4 NA  
678 objective lens (Olympus) coupled with optic ferrule implanted on mice by a custom patchcord (400  $\mu$ m,  
679 0.48 NA). Photometry data were analyzed using Python script. 405-nm channel ( $F_{405fit}$ ) was fit with least  
680 mean squares that was scaled to the 470-nm channel ( $F_{470}$ ). Motion corrected  $\Delta F/F$  was calculated as  $\Delta F/F$   
681 =  $(F_{470} - F_{405fit}) / F_{405fit}$ .  $\Delta F/F$  was then z-scored relative to the mean and SD of the fluorescence signal and  
682 smoothed by 2nd-order Savitzky–Golay filter using Prism 8 (GraphPad software).

683

### 684 **Behavioral experiments**

#### 685 **Hot plate test**

686 For recording fluorescent activity in response to noxious thermal stimulus, mice were tethered to a patch  
687 cord and placed inside a transparent Plexiglas cylinder (D = 11 cm, H = 15 cm). The bottom of the cylinder  
688 was wrapped by thin foil to facilitate the cylinder transfer with minimizing movement effect. Mice were  
689 allowed to freely move in the cylinder at room temperature (RT) for 30 min before measurement. Then the  
690 baseline was measured at RT and cylinder was transferred to 42°C or 52°C hot plate (PE34, IITC Life  
691 Science) to measure the activity of CybSEP2 during noxious thermal stimulus (cutoff time of 20 s).

#### 692 **Quinine-induced taste aversion test**

693 For recording fluorescent activity in response to aversive taste stimulus, mice were tethered to a patch cord  
694 and placed inside a plastic cylinder (11-cm diameter, 15 cm height) with 2-cm diameter hole to deliver  
695 water or 0.5 mM quinine. For the first 2 days, mice were habituated in the cylinder. The day before  
696 measurement, mice were water deprived at the home cage overnight. The following day, mice were placed

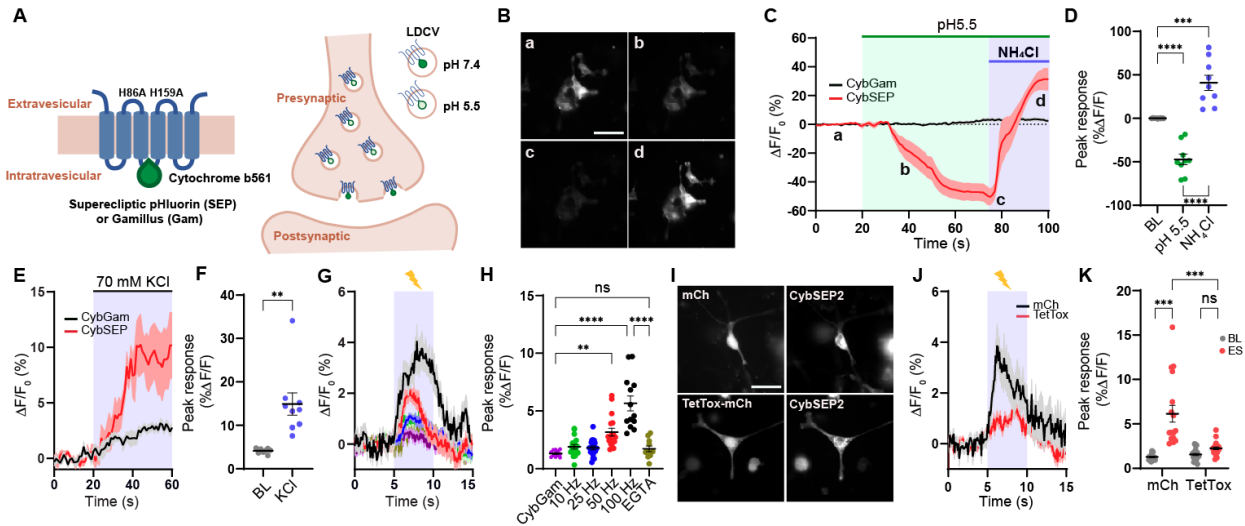
697 to the cylinder for 30 min prior to recording and then fluorescent signal of the sensors was recorded during  
698 licking water or quinine. In the test with NEP<sub>LDCV</sub> expressing mice, they consumed quinine for 10 min after  
699 overnight water deprivation and amount consumed was calculated. For taste preference test, a two-bottle  
700 choice test between water and quinine was performed.

### 701 **Foot shock fear conditioning**

702 A fear conditioning chamber (26 × 30 × 33 cm, ENV-007CT, MED Associates) consisted of a metal grid  
703 floor (ENV-005, MED Associates) and standalone aversive electric shock stimulator (ENV-414S, MED  
704 Associates) was employed for footshock (unconditioned stimulus: US) fear conditioning. To deliver a tone  
705 (conditioned stimuli; CS+), two speakers (AX210, Dell) were placed right next to the foot shock chamber.  
706 On day 1, mice were tethered to the patch cord and habituated inside the foot-shock chamber, which  
707 involved six CS+ (30-sec, 2-kHz pure tone) with random inter-event intervals. On day 2, mice were placed  
708 to the same chamber and underwent footshock fear conditioning, which involved five CS+ that co-  
709 terminated with footshock (0.2 mA, 2 sec) over random inter-event intervals. For contextual fear  
710 conditioning on the third day, mice were placed to the foot-shock chamber for 3 min. For the cue test, mice  
711 were placed to the new context (a glass cylinder wrapped with a non-transparent material; 20-cm diameter,  
712 15-cm height) and then CS+ was delivered three times without US. EthoVision XT 12 software (Noldus)  
713 with GigE USB camera (Imagine Source) was used for video recording, foot shock delivery, and analysis  
714 of freezing behavior.

715 For the fear conditioning experiments in Figure 5 and S5, mice were habituated to the context  
716 (28×28×25 cm chamber with metal walls and electric grid bottom, MedAssociate) and a 30 s CS (10 kHz,  
717 70 dB tone) on Day 1. After 2 min of baseline, 6 CS were introduced with randomized intervals (60 s – 180  
718 s). The next day, they were exposed to the same context and the same number of tones co-terminated with  
719 foot shock (0.3 mA, 0.5 s). On Day 3, mice were exposed to the same context for 5 min to test context-  
720 dependent fear memory. The cue-dependent fear memory test was conducted in a different context  
721 (28×28×25 cm chamber with white acrylic panel walls). Mice placed in the different context received 3 CS  
722 following of 2 min baseline. Freezing behavior was measured using Ethovision XT 15 software (Noldus).  
723 Freezing was determined as the time when the velocity of center point of mouse was under 0.75 cm/s.  
724 Freezing levels during the 30 s of CS were counted for day 1 and 3. For the contextual fear memory test,  
725 freezing time was measured for last 3 min of the session. The average of 3 CS was calculated to obtain the  
726 freezing level for the cue-induced fear memory test.

727



728

729 **Figure 1. Design and characterization of CybSEP as an LDCV sensor**

730 (A) Schematic of constructs and working principle.

731 (B) Representative images of CybSEP expression in differentiated PC12 cells when perfused with acidic  
 732 and NH<sub>4</sub>Cl solutions, described in (C) (a, bath solution; b and c, acidic solution; d, NH<sub>4</sub>Cl; Scale bar, 100  
 733 μm).

734 (C) The traces of CybSEP and CybGam fluorescence change during application of various extracellular  
 735 solutions.

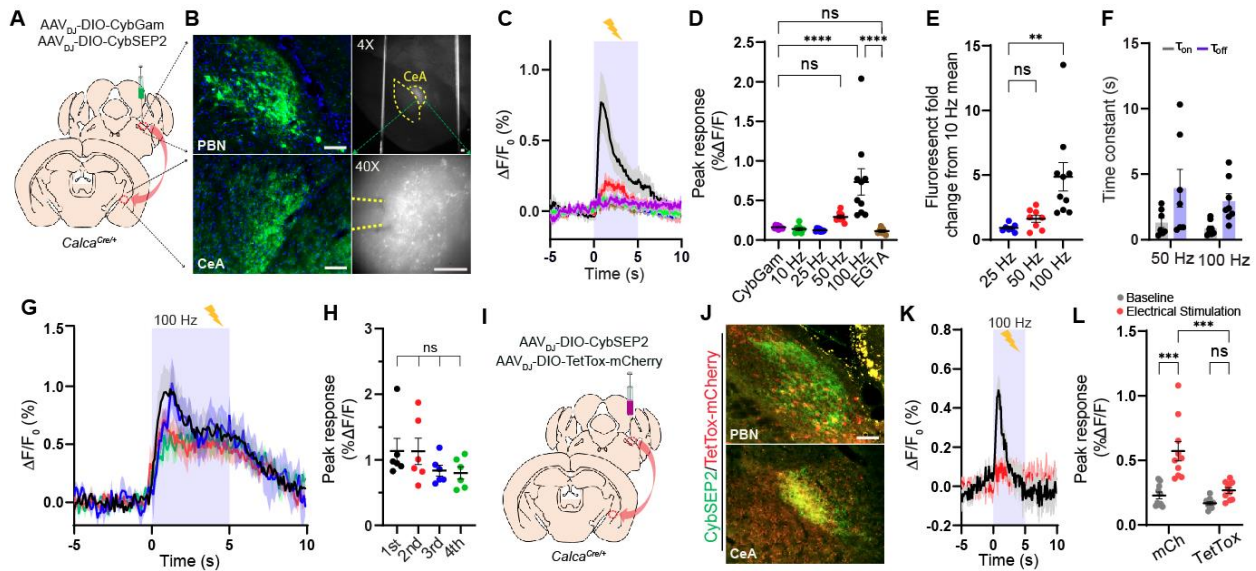
736 (D) Quantification of percent  $\Delta F/F_0$  peak intensity in CybSEP expressing PC12 cells (n= 9 over 3  
 737 experimental replicates; \*\*\*p < 0.001, \*\*\*\*p < 0.0001 via one-way ANOVA followed by Tukey's multiple  
 738 comparisons). BL indicates baseline.

739 (E and F) Average trace of fluorescence change during 70 mM KCl treatment and quantification of percent  
 740  $\Delta F/F_0$  peak intensity in (E) (n=8-10 over 2 experimental replicates; \*\*p < 0.01 via paired t test to the  
 741 baseline).

742 (G and H) Average traces of fluorescence change during various electrical stimulation and quantification  
 743 of percent  $\Delta F/F_0$  peak intensity in (G). For extracellular calcium removal, 5 mM EGTA was used instead  
 744 of CaCl<sub>2</sub> (n=11-19, over 3 experimental replicates; \*\*p < 0.001, \*\*\*\*p < 0.0001 via one-way ANOVA  
 745 followed by Tukey's multiple comparisons).

746 (I) Representative images of CybSEP2 with mCherry (mCh) or TetTox-mCh expressed in PC12 cells  
 747 (Scale bar, 100 μm).

748 (J and K) Average traces of fluorescence change in CybSEP2 co-expressed with mCh or TetTox-mCh  
 749 during electrical stimulation at 100 Hz and quantification of percent  $\Delta F/F_0$  peak intensity in (J) (n=19-23,  
 750 3 over 3 experimental replicates; \*\*\*p < 0.0001 via two-way ANOVA followed by Šidák multiple  
 751 comparisons). Data are represented as mean ± SEM.



752

753 **Figure 2. Imaging the LDCV release in brain slices**

754 (A and B) Schematic and representative images showing CybSEP2 targeted region, its projection to the  
 755 CeA and expression of CybSEP2 in the PBN and the CeA of the *Calca<sup>Cre/+</sup>* brain slices. Images in the right  
 756 panel (B) show slices containing the CeA for slice imaging experiment (Scale bar, 100  $\mu$ m).

757 (C and D) Average traces of fluorescence change in response to various electrical stimulation and  
 758 quantification of the data (C). For extracellular calcium removal, 5 mM EGTA was used instead of  $\text{CaCl}_2$ .  
 759 Each trace is the average of 7-9 trials in 24 slice slices prepared from 4 mice (\*\*\*\* $p < 0.0001$  via one-way  
 760 ANOVA followed by Šidák multiple comparisons).

761 (E) Quantification of fold change from percent  $\Delta F/F_0$  peak intensity compared to 10 Hz in (D) (\*\* $p < 0.01$   
 762 via one-way ANOVA followed by Tukey's multiple comparisons).

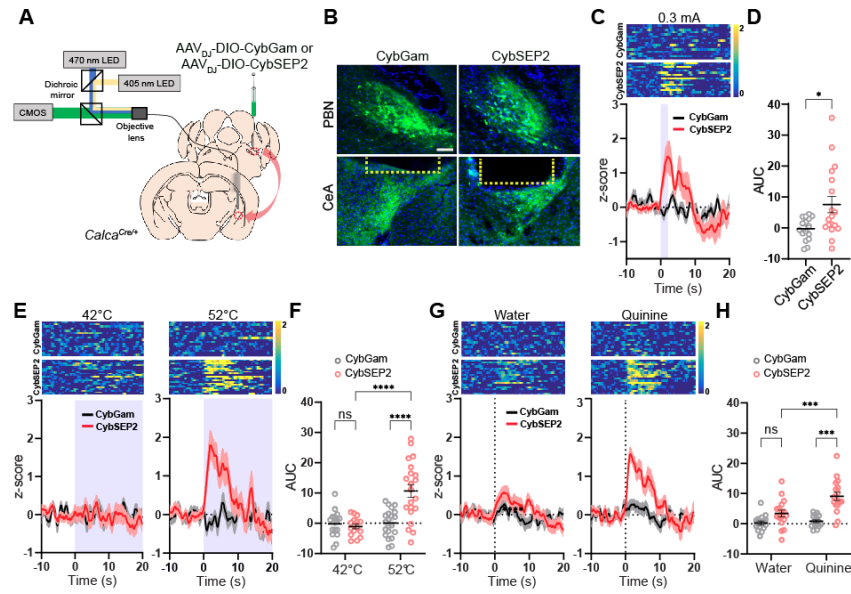
763 (F) Time constant ( $\tau$ ) of CybSEP2 expressing neurons during electrical stimulation at 50 Hz (n=7) and 100  
 764 Hz (n=8). The rising ( $\tau_{on}$ ) and decay ( $\tau_{off}$ ) phases were determined by fitting across an entire stimulation  
 765 period ( $\tau_{on} = 1.30 \pm 0.37$ ,  $\tau_{off} = 3.93 \pm 1.44$  at 50 Hz;  $\tau_{on} = 0.85 \pm 0.18$ ,  $\tau_{off} = 2.93 \pm 0.55$  at 100 Hz).

766 (G and H) Average traces of fluorescence change in response to repeated electrical stimulation at 100 Hz  
 767 and quantification of percent  $\Delta F/F_0$  peak intensity in (G). Each trial was measured at 5 min interval between  
 768 trials (n=6; ns, not significant via one-way ANOVA followed by Tukey's multiple comparisons).

769 (I and J) Schematic brain region targeted for viral injection and co-expression of CybSEP2 and TetTox-  
 770 mCherry in the PBN and in the CeA of *Calca<sup>Cre/+</sup>* (Scale bar, 100  $\mu$ m).

771 (K and L) The trace of fluorescence change in CybSEP2 with mCherry (n=10 slices from 3 mice) or TetTox-  
 772 mCherry (n=10 slices from 3 mice) expressing neurons during electrical stimulation at 100 Hz and  
 773 quantification of date in (K) (\*\*\* $p < 0.0001$  via two-way ANOVA followed by Šidák multiple  
 774 comparisons). Data are represented as mean  $\pm$  SEM.





775

776 **Figure 3. Monitoring LDCV release from the synaptic terminals in behaving mice**

777 (A) Schematic illustration of fiber photometry system used for CybSEP2 response recording in the  
778 *Calca<sup>Cre/+</sup>* mice.

779 (B) Expression images of CybGam and CybSEP2 in the PBN and the CeA of *Calca<sup>Cre/+</sup>* mice with an optic  
780 fiber implanted over the CeA. Yellow dot line represents the location of optic fiber (Scale bar, 100  $\mu$ m).

781 (C) Heat map and average traces of fluorescence change elicited by footshock (0.3 mA) in CybGam (16  
782 traces from 4 mice) and CybSEP2 (18 traces from 5 mice) expressing mice.

783 (D) Quantification of data in (C) by area under curve (AUC) for 0-10 s (\*p < 0.005 via unpaired t-test  
784 comparisons to control).

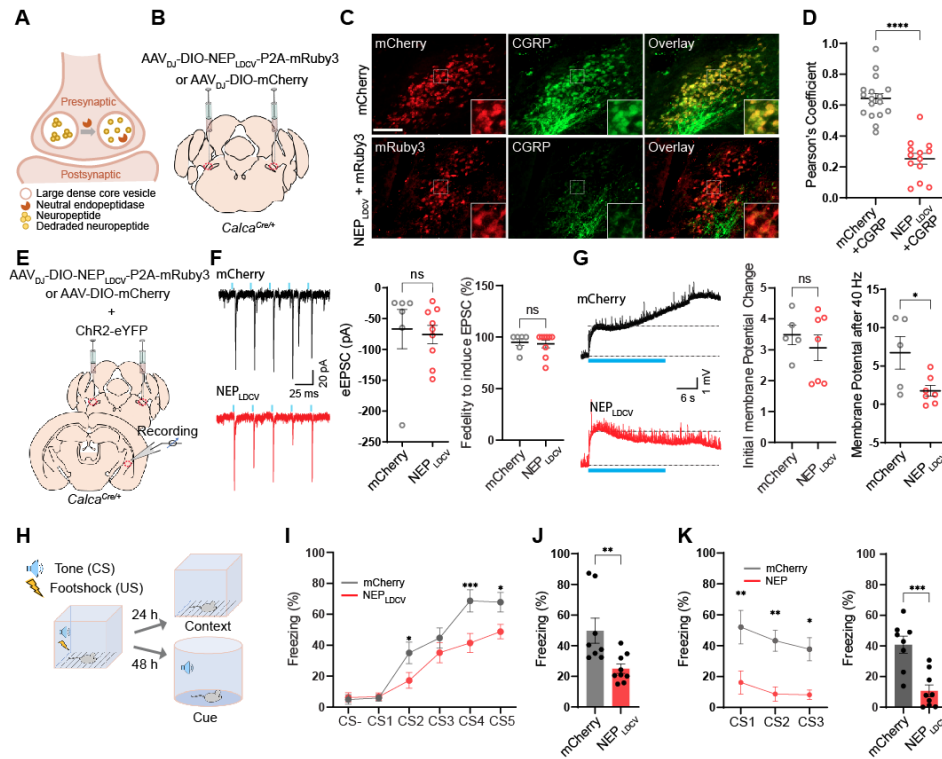
785 (E) Heat map and average traces of fluorescence change during thermal stimulus in CybGam (16 traces  
786 from 4 mice) and CybSEP2 (21 traces from 5 mice) expressing mice at 42 or 52°C hot plate with a cutoff  
787 time of 20 s.

788 (F) Quantification of data in (E) by AUC for 0-10 s (\*\*\*\*p < 0.0001 via two-way ANOVA followed by  
789 Tukey's multiple comparisons).

790 (G) Heat map and average traces of fluorescence change during aversive taste stimulus in CybGam (16  
791 traces from 4 mice) and CybSEP2 (17 traces from 5 mice) expressing mice elicited by 0.5 mM quinine  
792 solution or water.

793 (H) Quantification of data in (G) by AUC for 0-10 s (\*\*\*p < 0.001 via two-way ANOVA followed by  
794 Tukey's multiple comparisons to the CybGam or CybSEP2). Data are represented as mean  $\pm$  SEM.

795



796

797 **Figure 4. NEP<sub>LDCV</sub> lowers neuropeptide release and attenuates threat learning.**

798 (A) Schematic illustrating working principle of LDCV targeted NEP (NEP<sub>LDCV</sub>).

799 (B) Schematic of bilateral stereotaxic injection of NEP<sub>LDCV</sub> and mCherry into the PBN of *Calca*<sup>Cre/+</sup> mice.  
800 Scale bar is 100  $\mu$ m.

801 (C) Representative images showing mCherry or mRuby3 expressing neurons co-labeling CGRP positive  
802 neurons (green) (Scale bar, 100  $\mu$ m).

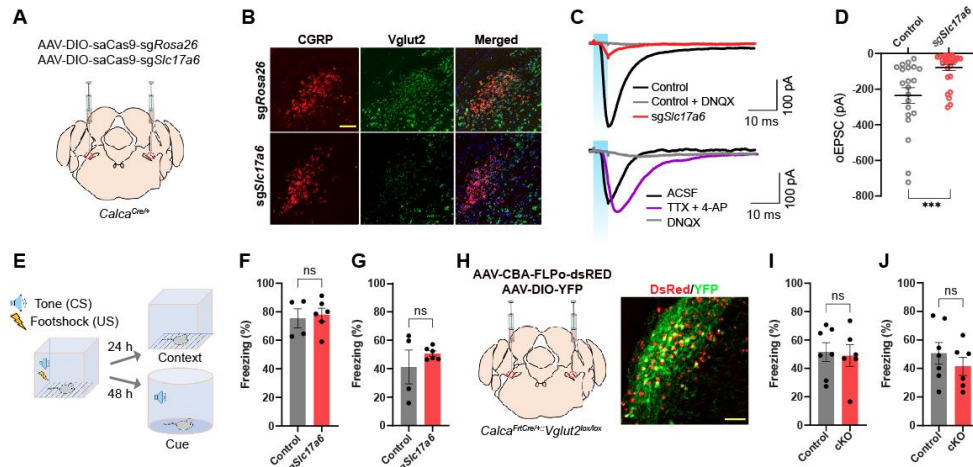
803 (D) Quantification of CGRP co-localization in the NEP<sub>LDCV</sub> sections (n=13 sections from three mice), and  
804 mCherry sections (n=17 sections from three mice) by Pearson's coefficient. \*\*\*\*p < 0.0001 via Two-tailed  
805 unpaired t-test comparisons. Data are represented as mean  $\pm$  SEM.

806 (E) Schematic of viral injection and whole cell recording of *Calca*<sup>Cre/+</sup> PBN slices expressing ChR2 and  
807 mCherry or NEP<sub>LDCV</sub>.

808 (F) Example traces (left; Top, ChR2 + mCherry slice. Bottom, ChR2 + NEP<sub>LDCV</sub> slice.), amplitude (right),  
809 and fidelity (right) of oEPSCs in CeA neurons elicited by photostimulation of ChR2-expressing CGRP<sup>PBel</sup>  
810 axonal terminals. n=6 for mCherry, n=9 for NEP<sub>LDCV</sub>. ns, not significant. Data are represented as mean  $\pm$   
811 SEM.

812 (G) Example traces of oEPSP (left; Top, ChR2 + mCherry slice. Bottom, ChR2 + NEP<sub>LDCV</sub> slice.), initial  
813 oEPSP amplitude (middle), and sustained oEPSP amplitude (right) after 40-Hz photostimulation. ns, not

814 significant. \* $p < 0.05$  via two-tailed unpaired t-test comparisons. Photostimulation onset is indicated by  
815 blue line.  $n=5$  for mCherry,  $n=7$  for NEP<sub>LDCV</sub>. Data are represented as mean  $\pm$  SEM.  
816 (H) Schematic illustration of auditory fear conditioning experiment.  
817 (I) Freezing during fear conditioning in mice expressing mCherry ( $n = 8$ ) and NEP<sub>LDCV</sub> ( $n = 9$ ). \* $P < 0.05$ ,  
818 \*\*\* $P < 0.001$  via repeated measures two-way ANOVA with Sidak's multiple comparisons test. Data are  
819 represented as mean  $\pm$  SEM.  
820 (J) Freezing at the same context 24 hr after fear conditioning. \*\* $p < 0.01$  via unpaired t-test comparisons to  
821 mCherry. Data are represented as mean  $\pm$  SEM.  
822 (K) Freezing to the tone 48 hr after fear conditioning. Left, \* $P < 0.05$ , \*\* $P < 0.01$  via repeated measures one-  
823 way ANOVA. Right: \*\*\* $P < 0.001$  via unpaired t-test comparisons. Data are represented as mean  $\pm$  SEM.



824

825 **Figure 5. CRISPR and genetic disruption of glutamate release by CGRP<sup>PBeI</sup> does not influence on**  
 826 **Pavlovian threat conditioning.**

827 (A) Schematic depiction of guide RNAs and saCas9 expression in CGRP<sup>PBeI</sup> neurons.

828 (B) Representative images of *in situ* hybridization detecting *Calca* (encodes CGRP) and *Slc17a6* (encodes  
 829 *Vglut2*) in PBL.

830 (C) Example traces of evoked EPSCs in CeA neurons elicited by optogenetic stimulation of axonal  
 831 terminals of Chr2-expressing CGRP<sup>PBeI</sup> neurons. Top, EPSC traces of control (*sgRosa26*) and *sgSlc17a6*  
 832 group. Bottom, EPSC traces with the bath application of TTX and 4-AP or DNQX in *sgSlc17a6* group.

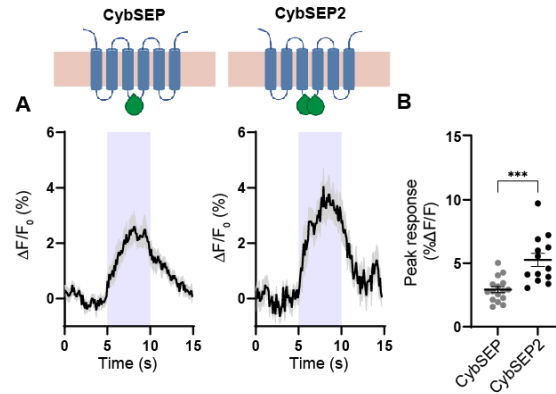
833 (D) Amplitudes of EPSCs (n = 21 for control, n = 30 for *sgSlc17a6*). \*\*\*\*p < 0.0001 via Two-tailed  
 834 unpaired t-test comparisons. Data are presented as mean ± SEM.

835 (E) Freezing during fear conditioning in control (n = 4) and *sgSlc17a6* mice (n = 6). Scale bars are 100 μm.

836 (F and G) Percent of time to spend freezing at the same context 24 hr (F) and to the tone 48 hr (G) after  
 837 learning in mice expressing *sgSlc17a6* (n = 4) or *sgRosa26* (n = 6). ns via Two-tailed unpaired t-test  
 838 comparisons. Data are presented as mean ± SEM.

839 (H) Schematic and histology of *Slc17a6* conditional knockout in CGRP<sup>PBeI</sup> neurons.

840 (I and J) Percent of time to spend freezing at the same context 24 hr (I) and to the tone 48 hr (J) after  
 841 learning in control (n = 7) and cKO (n = 6) mice. ns via Two-tailed unpaired t-test comparisons. Data are  
 842 presented as mean ± SEM.

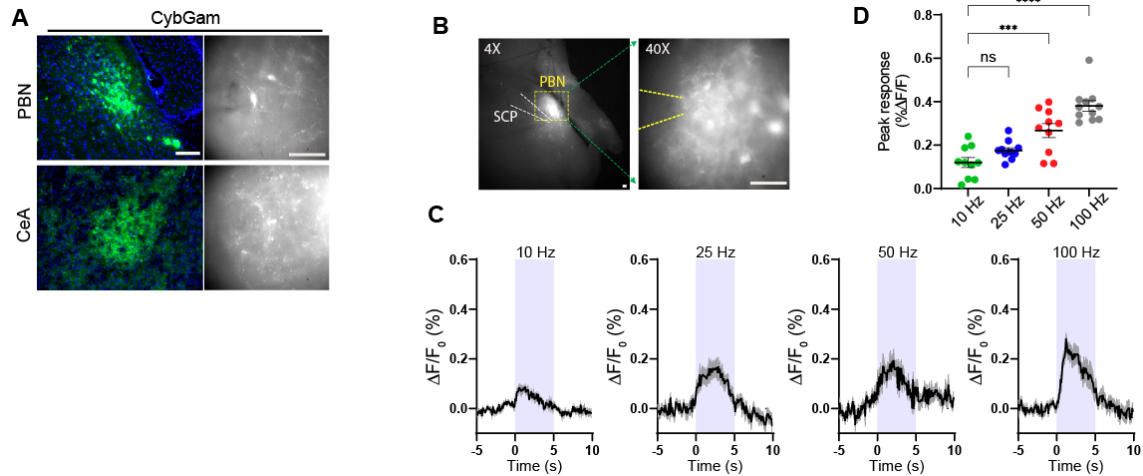


843

844 **Figure S1. Comparison of fluorescence levels of CybSEP and CybSEP2 evoked by electrical**  
845 **stimulation**

846 (A and B) Schematic and average traces showing comparison between CybSEP (n = 18) and CybSEP2 (n  
847 = 13) expressing cells (duplicated from Figures 1G) during electrical stimulation at 100 Hz and  
848 quantification of percent  $\Delta F/F_0$  peak intensity in (A) (\*\*\*)  $p < 0.001$  via unpaired t test). Data are represented  
849 as mean  $\pm$  SEM.

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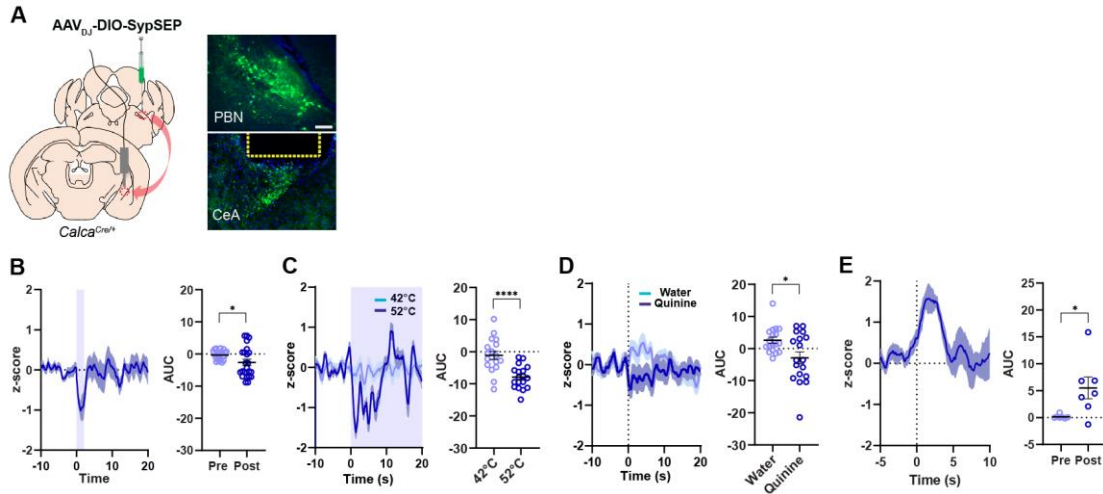
852 **Figure S2. Expression of CybGam and imaging CybSEP2 in acute brain slice containing the PBN**

853 (A) Schematic and images showing expression of CybGam in the PBN and the CeA of *Calca<sup>Cre/+</sup>*.

854 (B) Images of PBN showing CybSEP2 expression for slice imaging. SCP indicates superior cerebellar  
855 peduncle (Scale bar, 100 μm).

856 (C and D) Average traces of fluorescence change in response to various electrical stimulation and  
857 quantification of data in (C) (10-12 traces from 18 slices prepared from 3 mice; \*\*\*p < 0.001, \*\*\*\*p <  
858 0.0001 via one way ANOVA followed by Tukey's multiple comparisons to the 10 Hz). Data are represented  
859 as mean ± SEM.

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862 **Figure S3. Deep brain recording of the SV sensor in the synaptic terminals of freely moving mice**

863 (A) Schematic illustration of viral injection and images showing expression of SypSEP in the PBN and in  
864 the CeA of *Calca*<sup>Cre/+</sup>. Yellow dot line represents the location of optic fiber (Scale bar, 100  $\mu$ m).

865 (B) Average trace of fluorescence change on footshock and quantification of data 10 s before and after  
866 footshock (23 traces from 4 mice; \* $p < 0.05$  via paired t test).

867 (C) Average traces of fluorescence change during thermal stimulus and quantification of data for 0-10 s (18  
868 traces from 4 mice; \*\*\*\* $p < 0.0001$  via unpaired t test).

869 (D) Average traces of fluorescence change during quinine intake and quantification of data for 0-10 s (18  
870 traces from 4 mice \* $p < 0.05$  via unpaired t test). Data are represented as mean  $\pm$  SEM.

871 (E) Average traces of fluorescence change during Ensure intake and quantification of data for 0-10 s (7  
872 traces from 3 mice \* $p < 0.05$  via unpaired t test). Data are represented as mean  $\pm$  SEM.

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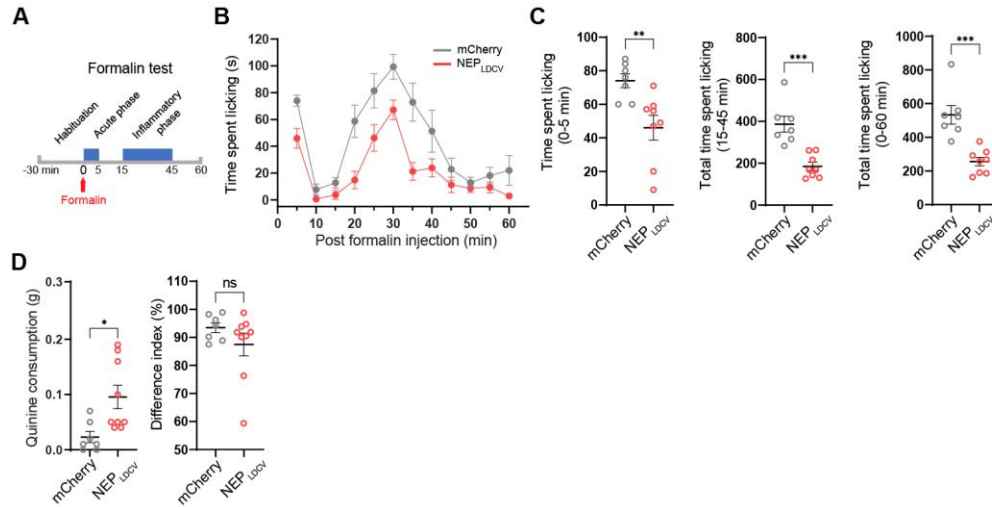
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**Figure S4. The effect of the NEP<sub>LDCV</sub> on pain behavior by sensory stimulus and formalin injection.**

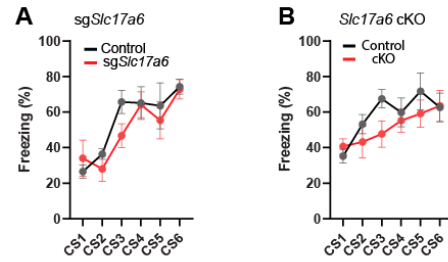
(A) Schematic of formalin assay for acute and inflammatory pain tests.

(B) Time course of formalin-induced nociceptive responses in the mice expressing mCherry (n=7 for mCherry, n=9 for NEP<sub>LDCV</sub>).

(C) Quantification of acute phase (0-5 min, left), inflammatory phase (15-45 min, middle), and a total spent time for locking (0-60 min). \*\*P<0.01, \*\*\*P<0.001 via unpaired t-test comparisons to mCherry. Data are represented as mean ± SEM.

(D) Quinine consumption (left) and two bottle (right) tests. in the mice expressing mCherry (n = 7) and NEP<sub>LDCV</sub> (n = 9). \*P<0.05, ns via unpaired t-test comparisons to mCherry group.





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892 **Figure S5** Learning curves during fear conditioning.

893 (A) Freezing during tone (CS) in learning sessions (n = 4 for control, n = 6 for *sgSlc17a6*).

894 (B) Freezing during tone (CS) in learning sessions (n = 7 for control, n = 6 for cKO).

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