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Graphical abstract

Deletion of synaptotagmin 2 from parvalbumin neurons in the cerebellar nuclei (CBN) results in action tremor of mice.

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Dysfunction of parvalbumin neurons in the cerebellar nuclei produces an action tremor

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Introduction

Essential tremor is the most common movement disorder of human patients (1). Clinically, essential tremor is characterized by a 4-Hz to 12-Hz action tremor (2) that affects different body parts, making basic daily activities difficult if not impossible in severe cases. Although no consensus has been reached on its exact prevalence, surveys show that the disease is present in 4% of individuals aged 40 and up (3). Essential tremor prevalence increases with age, such that up to 14% of people aged 65 and above exhibit essential tremor (4). Despite its widespread presence, our understanding of essential tremor’s biological mechanisms remains very limited (5), and few therapeutic options are available.

Studies of the etiology of essential tremor traditionally focused on the inferior olive (6–8). In an animal model of essential tremor induced by i.p. injections of harmaline, rhythmic burst-firing was detected in inferior olive neurons, and an action tremor was induced that exhibited the same frequency as the rhythmic firing in inferior olive neurons (6). Although these findings were promising, decades of clinical research found little evidence for a dysfunction or for pathological changes in the inferior olive in essential tremor patients (5). More recently, postmortem analyses and advanced neuroimaging techniques have identified pathological changes in the cerebellum, including Purkinje cells, basket cells, and the cerebellar nuclei (CBN) in essential tremor patients (9–13). In particular, degeneration of Purkinje cells has become a widely believed hypothesis to account for essential tremor (5). Yet, the data so far are correlative. There is no evidence to show the causality between cerebellar pathologies and essential tremor symptoms. More importantly, the physiological basis by which cerebellar dysfunction might lead to essential tremor remains unknown.

A major obstacle to a better understanding of the etiology of essential tremor is the lack of a reliable genetic animal model (14–16). The traditional harmaline-induced essential tremor model is useful for the preclinical testing of antitremor drugs. However, this model has limited value for translational research since its tremor symptoms spontaneously resolve in a few hours (6, 8). Recently, several genetic tremor animal models were described (15–18) that provided critical insights into the neural mechanisms of essential tremor. Nevertheless, these genetic models have limitations because they either exhibit nonspecific action tremor or produce a much higher tremor frequency than observed in essential tremor patients.

Synaptotagmin-2 (Syt2) is an ultrafast Ca2+ sensor for fast neurotransmitter release (19–21). We initially crossed Syt2 conditional knockout (Syt2fl/fl) mice with parvalbumin-Cre (PVcre) driver mice in order to study the functional roles of Syt2 at the calyx of Held synapse (21) and at cortical inhibitory synapses. Unexpectedly, we found that PVcre Syt2fl/fl mice exhibited a robust action tremor phenotype without other obvious behavioral abnormalities. This surprising discovery provided us a unique opportunity to examine the circuit components and pathological synaptic release properties that can underlie action tremor, the core symptom of the prevalent essential tremor disorder in human patients.

Combining region-, cell type-, and projection-specific deletions of Syt2, we identified the dysfunction of excitatory PV+ neurons in the CBN that project to neurons in the brainstem as the cause of action tremor. We determined that in PVcre Syt2fl/fl mice, fast synchronous synaptic neurotransmitter release in this cerebellum → brainstem circuit was converted into asynchronous neurotransmitter release, which then led to action tremor. We also found that blocking synaptic transmission in the CBN reversed the action tremor.

Conflict of Interest: The authors have declared that no conflict of interest exists.

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Figure 1. PV<sup>/cre</sup>-Syt2<sup>fl</sup> mice as an animal model for action tremor. (A–C) Exemplary tremor recordings on a force plate to illustrate the tremor observed in PV<sup>/cre</sup>-Syt2<sup>fl</sup> mice compared with PV<sup>/cre</sup>-Syt2<sup>wt</sup> mice. (A) Raw data of 3-second weight measurement using the force-plate actometer. (B) Spectrogram calculated from 20-minute force-plate measurements. (C) Averaged power spectrum from data in B. The tremor index was calculated by integrating the power in the 9-Hz to 12-Hz range and using the averaged power in the 3-Hz to 6-Hz range as baseline. (D) Correlation of the tremor index measured on a force plate and the tremor amplitude monitored simultaneously by video tracking in PV<sup>/cre</sup>-Syt2<sup>fl</sup> and control mice at different ages. (E) Power spectra of force-plate measurements from a representative PV<sup>/cre</sup>-Syt2<sup>fl</sup> mouse at different ages. (F) Summary plot of the tremor index of PV<sup>/cre</sup>-Syt2<sup>fl</sup>- and PV<sup>/cre</sup>-Syt2<sup>wt</sup>-mice as a function of age (n = 19 PV<sup>/cre</sup>-Syt2<sup>fl</sup>, n = 18 PV<sup>/cre</sup>-Syt2<sup>wt</sup>). (G) Summary plot of the tremor index of PV<sup>/cre</sup>-Syt2<sup>fl</sup> and control mice as a function of time after an s.c. injection of ethanol (EtOH); PV<sup>/cre</sup>-Syt2<sup>fl</sup> mice injected with saline used as a further control (n = 5 control + EtOH, n = 7 PV<sup>/cre</sup>-Syt2<sup>fl</sup> + saline, n = 6 PV<sup>/cre</sup>-Syt2<sup>fl</sup> + EtOH). (H) Exemplary simultaneous measurements of the movements and tremor in a PV<sup>/cre</sup>-Syt2<sup>fl</sup> mouse. Episodes longer than 3 seconds with speed less than 2.5 cm/s are indicated by vertical blue shaded bars. (I) Summary graph of the tremor index of PV<sup>/cre</sup>-Syt2<sup>fl</sup> mice before, during, and after periods of quiescence lasting longer than 3 seconds (n = 17). For F, G, and I, data are shown as means ± SEM from at least 3 independent litters. *P < 0.05; **P < 0.01; ***P < 0.001 by 2-sided, unpaired t test (F) or 1-way ANOVA (I). Scale bars: 20 g (A, vertical); 1 s (A, horizontal).
or phenotype in PV<sup>cre</sup> Syt2<sup>fl/fl</sup> mice, and that this rescue approach was also effective for harmaline-induced action tremor, the traditional animal model of essential tremor. Based on these data, we propose a conceptual framework to explain how temporally delayed synaptic transmission in the cerebellum → brainstem pathway could generate an action tremor. In summary, our study validated a promising genetic mouse model for action tremor and defined critical circuit components that generate such a tremor. The circuit components and synaptic defects we identified may provide significant insights into the etiology and therapeutic intervention of the widespread essential tremor brain disorder.

**Results**

**PV<sup>cre</sup> Syt2<sup>fl/fl</sup> mice are a promising animal model for action tremor.** By crossing PV<sup>cre/+</sup> Syt2<sup>fl/+</sup> mice with Syt2<sup>+/+</sup> mice, we generated mice with a deletion of Syt2 in PV neurons (PV<sup>cre/+</sup> Syt2<sup>fl/+</sup>, referred to as PV<sup>cre</sup> Syt2<sup>2</sup>) that exhibited a robust action tremor phenotype, while their heterozygous littermates (PV<sup>cre/+</sup> Syt2<sup>fl/+</sup>, referred to as PV<sup>cre</sup> Syt2<sup>wt</sup> or control) were normal (Supplemental Videos 1 and 2; supplemental material available online with this article; https://doi.org/10.1172/JCI35802DS1). Immunostaining confirmed the deletion of Syt2 from PV<sup>+</sup> synapses (Supplemental Figure 1).

To quantify the action tremor, we used a 28 × 28 cm force-plate actometer that was designed to measure the whole body weight at 100 Hz (22). PV<sup>cre</sup> Syt2<sup>fl</sup> mice exhibited an obvious tremor with an approximately 10-Hz rhythm, whereas their littermate PV<sup>cre</sup> Syt2<sup>wt</sup> controls did not (Figure 1, A and B). For each mouse, we plotted the averaged power spectrum and defined a “tremor index” as the summation of power in the 9-Hz to 12-Hz range, using the power in the 3-Hz to 6-Hz range as the baseline (Figure 1C). To cross-validate this tremor quantification method, we also directly measured the tremor amplitude of PV<sup>cre</sup> Syt2<sup>2</sup> and control mice using video tracking methods, while at the same time measuring their behavior on the force plate. Using a high-speed camera and video tracking software, the detailed movements of mice were recorded and band-pass filtered (9–12 Hz) to calculate the tremor amplitude (Supplemental Figure 2). This approach confirmed the tremor phenotype in PV<sup>cre</sup> Syt2<sup>2</sup> mice and validated the usage of the force-plate actometer to quantify the tremor phenotype (Figure 1D).

The tremor of PV<sup>cre</sup> Syt2<sup>2</sup> mice manifested at the time of weaning and became increasingly stronger as the mice grew older, as documented by force-plate quantifications as a function of age (Figure 1, E and F). Importantly, the observed age-dependent increase of tremor strength was not due to the increase in body size, as shown by plotting the tremor index normalized to the body weight (Supplemental Figure 3A). This age-dependent pattern of increasing tremor is consistent with clinical observations (2). Besides the tremor phenotype, PV<sup>cre</sup> Syt2<sup>2</sup> mice were grossly normal. Their body weight was similar to that of littermate controls (Supplemental Figure 3B). They were fertile and exhibited a normal lifespan, as followed up to 1.3 years, the oldest age we monitored. PV<sup>cre</sup> Syt2<sup>2</sup> mice likely had normal cognitive abilities as suggested by results from spontaneous alternating Y maze (Supplemental Figure 3C), novel object recognition (Supplemental Figure 3D), and fear conditioning assays (Supplemental Figure 3E). PV<sup>cre</sup> Syt2<sup>2</sup> mice did, however, show a significant deficit in the rotarod test (Supplemental Figure 3F), which was not surprising given their striking action tremor.

We next tested whether ethanol, which has been shown to temporarily alleviate essential tremor symptoms in human patients (2, 23), had a similar effect in PV<sup>cre</sup> Syt2<sup>2</sup> mice. A single s.c. injection of 2.5 g/kg ethanol dramatically but temporarily suppressed the tremor of PV<sup>cre</sup> Syt2<sup>2</sup> mice (Figure 1G and Supplemental Figure 3G). Importantly, the suppression of the tremor by ethanol could not be explained by the reduction in locomotion (Supplemental Figure 3, H and I). Moreover, we noticed that the PV<sup>cre</sup> Syt2<sup>2</sup> mice had little or no tremor when they were at rest and not moving (Supplemental Videos 2 and 3), suggesting that they had an action tremor phenotype. We quantified this aspect by measuring the moving speed and the instantaneous tremor index of PV<sup>cre</sup> Syt2<sup>2</sup> mice at the same time (Figure 1H). Although the mice usually continuously explored the novel environment and had few quiescent episodes, their action tremor phenotype became obvious when we selected all quiescent epochs of longer than 3 seconds and examined the tremor index before, during, and after these quiescent episodes (Figure 1I). Clearly, the tremor decreased during a mouse’s quiescent period. The action tremor phenotype of PV<sup>cre</sup> Syt2<sup>2</sup> mice was further illustrated in fear conditioning assays, during which PV<sup>cre</sup> Syt2<sup>2</sup> mice substantially froze to contextual cues (Supplemental Video 4), strongly suggesting lack of a resting tremor. Together, these results indicate that PV<sup>cre</sup> Syt2<sup>2</sup> mice are a reliable genetic animal model for action tremor and a promising candidate to model human essential tremor disorder (Table 1).

**Syt2 deletion from the cerebellum is sufficient to generate an action tremor.** To identify the brain region that is responsible for generating the action tremor in PV<sup>cre</sup> Syt2<sup>2</sup> mice, we first compared the Syt2 expression levels in PV<sup>cre</sup> and PV<sup>cre</sup> Syt2<sup>2</sup> mice across the entire brain (Figure 2, A and B, and Supplemental Figure 4). The PV<sup>cre</sup>-induced reduction of Syt2 signals was obvious in the cortex, hippocampus, and cerebellum (Figure 2, A and B). Since the reduction of Syt2 signals was most dramatic in cortical areas, likely due to the colocalization of Syt2 and PV expression (24), we analyzed the effect of the Syt2 deletion on inhibitory synaptic responses in the medial prefrontal cortex (mPFC) using whole-cell patch-clamp recordings in acute slices. We tested the change of spontaneous inhibitory postsynaptic currents (sIPSCs) by injecting adeno-associated viruses

### Table 1. Characteristics of PV<sup>cre</sup> Syt2<sup>2</sup> mouse model

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ET patients</th>
<th>Harmaline mouse model</th>
<th>PV&lt;sup&gt;cre&lt;/sup&gt; Syt2&lt;sup&gt;2&lt;/sup&gt; mouse model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency (Hz)</td>
<td>4–12</td>
<td>11–14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9–12</td>
</tr>
<tr>
<td>Acute/Chronic</td>
<td>Chronic</td>
<td>Acute&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Chronic</td>
</tr>
<tr>
<td>Progressive</td>
<td>Yes</td>
<td>No&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Yes</td>
</tr>
<tr>
<td>Alcohol</td>
<td>Effective</td>
<td>Effective</td>
<td>Effective</td>
</tr>
<tr>
<td>Tremor type</td>
<td>Action</td>
<td>Action</td>
<td>Action</td>
</tr>
<tr>
<td>Brain region</td>
<td>Cerebellum</td>
<td>Inferior olive&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Cerebellum</td>
</tr>
</tbody>
</table>

<sup>a</sup>Features different from human essential tremor patients are labeled red.
Figure 2. Syt2 is prominently expressed in cortical PV+ neurons, but deletion of Syt2 from mPFC PV+ neurons does not impair their synaptic releases. (A and B) Representative images of different coronal brain sections showing the immunostaining of Syt2 for PV+ (A) and PV+ Syt2fl (B) mice. (C and D) Deleting Syt2 from mPFC PV+ neurons did not affect sIPSCs received by pyramidal neurons. (C) Top, differential interference contrast (DIC) and fluorescence images showing the expression of EGFP-Cre in the mPFC of an Syt2fl/fl mouse. Slice is arranged upright; bottom, example traces showing the sIPSCs recorded from mPFC pyramidal neurons with (GFP side) and without (non-GFP side) deletion of Syt2 from PV+ neurons. (D) Summary graph of the sIPSC frequency (left) and amplitude (right) recorded from mPFC pyramidal neurons on GFP and non-GFP sides (n = 10 non-GFP, n = 10 GFP for both frequency and amplitude). (E and F) Deleting Syt2 from mPFC PV+ neurons did not affect evoked IPSCs received by pyramidal neurons. (E) Top, DIC and fluorescence images showing the expression of Cre-dependent CHIEF-tdTomato in mPFC PV+ neurons of a PVcre Syt2fl mouse. Slice is arranged upright; bottom, example traces showing the 1-ms, 45-Hz blue laser-evoked IPSCs (shown as blue vertical bars) recorded from mPFC pyramidal neurons in a PVcre Syt2fl mouse and a control mouse. (F) Summary graph of 45-Hz light-evoked IPSC amplitude (in response to the first 10 train stimuli) recorded from mPFC pyramidal neurons in control and PVcre Syt2fl mice (n = 9 control, n = 7 PVcre Syt2fl). For D and F, data are shown as means ± SEM from at least 3 independent litters. Scale bars: 1 mm (A); 0.5 mm (C, top); 50 pA (C, vertical); 0.5 s (C, bottom horizontal); 0.5 mm (E, top); 0.5 nA (E, vertical); 0.1 s (E, bottom horizontal).
Figure 3. Syt2 deletion from the cerebellum is sufficient to generate an action tremor. (A) Stereotactic injection strategy of AAVs encoding Cre-GFP into 1 of 4 brain regions: the motor cortex (MO), basal ganglia (BG), thalamus (TH), or cerebellum (CB). (B) Top, a representative image showing the expression of Cre-GFP in the cerebellum of Syt2fl/fl mice; bottom, power spectrum of force-plate measurements from the same mouse before and after viral injection. Also see Supplemental Video 5 and Supplemental Figure 5. (C) Summary graph of the tremor index before and after injections of AAV Cre-GFP into different brain regions (n = 5 MO, n = 6 BG, n = 5 TH, n = 7 CB). Please see the methods section for our approaches to infect these large brain areas. For C, data are shown as means ± SEM from at least 3 independent litters. ***P < 0.001 by 2-sided, paired t test. Scale bar: 1 mm (B).

(AAVs) expressing syn-EGFP-Cre unilaterally in the mPFC of Syt2fl/fl mice and recording sIPSCs from pyramidal neurons on either the GFP or the non-GFP side. Surprisingly, both the frequency and amplitude of recorded sIPSCs were normal on the GFP side, suggesting that Syt2 knockout did not change sIPSCs in the cortex (Figure 2, C and D). We further recorded optogenetically evoked IPSCs by expressing DIO-CHiEF-tetTomato unilaterally in the mPFC of PVcre Syt2fl and control mice. In PVcre Syt2fl slices, 1-ms, 45-Hz laser-evoked IPSCs were also normal (Figure 2, E and F). These results suggest that synaptic release by cortical PV+ neurons was not affected by the Syt2 deletion, potentially due to the compensatory effect of synaptotagmin-1 (25), which could also explain the lack of other striking phenotypes besides tremor in PVcre Syt2fl mice.

Next, we employed a more direct approach to delete Syt2 from different brain regions, and tried to identify the specific brain area in which the Syt2 deletion is sufficient to generate an action tremor. We tested 4 candidate brain areas implicated in motor behaviors for this purpose: the motor cortex, basal ganglia, thalamus, and cerebellum. The motor cortex, including the primary and secondary motor cortex, provides command signals for voluntary movements. The basal ganglia are involved in movement initiation, and have been hypothesized to mediate the resting tremor that is a key symptom of Parkinson’s disease. The thalamus includes relay centers for motor pathways, and the ventral intermediate nucleus of the thalamus is targeted by deep brain stimulation (DBS) to treat essential tremor (26–28). The cerebellum plays a critical role in fine motor control, and pathological studies have detected cerebellar degeneration as a hallmark of essential tremor (5). Strikingly, removing Syt2 expression from the motor cortex, basal ganglia, or thalamus did not induce an action tremor (Supplemental Figure 5), whereas removing Syt2 from the cerebellum replicated the action tremor observed in PVcre Syt2fl mice (Figure 3, A–C, Supplemental Figure 5E, and Supplemental Video 5). These results indicate that removing Syt2 from neurons in the cerebellum was sufficient to generate an action tremor.

Syt2 deletion from PV+ neurons in the CBN is sufficient to generate an action tremor. To pinpoint the cell types in the cerebellum that generate the action tremor in PVcre Syt2fl mice, we took advantage of multiple Cre mouse lines that target different PV+ cell types in the cerebellum (ref. 29, Table 2, and Supplemental Figure 6, A and B). We first crossed Syt2fl/fl mice with L7cre mice, which expressed Cre recombinase exclusively in Purkinje cells in the cerebellar cortex (Supplemental Figure 6, A and B). Degeneration of Purkinje cells has been hypothesized to be the cause of essential tremor (5). However, L7cre Syt2fl mice exhibited no action tremor (Figure 4, A and C). Next, we crossed Syt2fl/fl mice with Prkcdcre mice (30), which expressed Cre recombinase in the molecular layer inhibitory neurons and in some Purkinje cells in the cerebellar cortex (ref. 31 and Supplemental Figure 6, A and B). Prkcdcre Syt2fl mice were also normal and had no tremor phenotype (Figure 4C and Supplemental Figure 6C).

To test the roles of neurons in the CBN, we crossed Syt2fl/fl mice with either Vglut2cre or Gad2cre mice, which expressed Cre recombinase in excitatory and inhibitory neurons, respectively (Supplemental Figure 6, A and B). Surprisingly, Vglut2cre Syt2fl mice exhibited a robust tremor phenotype, whereas Gad2cre mice did not (Figure 4, B and C, and Supplemental Figure 6D). Besides the tremor phenotype, Vglut2cre Syt2fl mice also had a significantly reduced body weight (Supplemental Figure 6E), presumably due to knockout of Syt2 from

Table 2. Cerebellar cell type expression patterns for 5 mouse Cre driver lines

<table>
<thead>
<tr>
<th>Mouse line</th>
<th>Stellate cells</th>
<th>Basket cells</th>
<th>Purkinje cells</th>
<th>CBN cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVcre</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>L7cre</td>
<td>Yes</td>
<td></td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Prkcdcre</td>
<td>Yes</td>
<td>Yes</td>
<td>Some</td>
<td></td>
</tr>
<tr>
<td>Vglut2cre</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>GABAergic</td>
</tr>
<tr>
<td>Gad2cre</td>
<td>Yes</td>
<td>Yes</td>
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</table>
Vglut2+ excitatory neurons in brain regions other than the CBN that were not targeted by the PVcre driver line. Since at least in cortical areas, PV+ neurons are primarily inhibitory (32), we further crossed PVcre mice with a nucleus-localized tdTomato reporter line (Ai75) and immunostained for Vglut2 in the CBN. Indeed, the majority of PV+ neurons in the CBN colocalized with Vglut2+ neurons, indicating that PV+ neurons in the CBN were mostly glutamatergic (Figure 4D).

Both PVcre and Vglut2cre mice expressed Cre in many other brain regions outside the cerebellum. Therefore, we further tested whether removing Syt2 from the CBN alone is sufficient to generate an action tremor. Indeed, bilaterally injecting AAVs expressing Cre into the CBN of Syt2fl/fl mice induced an action tremor with the same frequency range as observed in PVcre Syt2fl mice (Figure 4, E and F), whereas expressing Cre in either the medial or lateral cerebellar cortex of Syt2fl/fl mice failed to replicate the tremor (Figure 4F and Supplemental Figure 6, F and G). Furthermore, the age-dependent tremor progression of PVcre Syt2fl mice was significantly rescued by overexpression of Syt2 in the CBN (Figure 4, G and H, and Supplemental Figure 6, H and I). Together, these results indicate that the deletion of Syt2 from the CBN was sufficient to generate an action tremor.

**Figure 4. Syt2 deletion from PV+ neurons in the CBN is sufficient to generate an action tremor.** (A and B) Power spectra of force-plate measurements from representative control and L7cre Syt2fl mice (A) or control and Vglut2cre Syt2fl mice (B). (C) Summary graph of the tremor index of Syt2fl/fl mice after crossing with the 5 different Cre mouse lines (n = 19 PV-control, n = 18 PV-mutant, n = 8 L7-control, n = 7 L7-mutant, n = 8 Prkcd-control, n = 5 Prkcd-mutant, n = 12 Vglut2-control, n = 9 Vglut2-fl/mut, n = 7 GAD2-control, n = 7 GAD2-fl/mut). (D) Representative images of the CBN section from a PVcre Ai75 mouse showing the colocalization of antibody-labeled Vglut2 neurons and genetically labeled PV+ neurons in the CBN. (E) Left, stereotactic injection strategy of AAVs encoding Cre-GFP into the CBN or the cerebellar cortex of Syt2fl/fl mice; middle, a representative image showing the bilateral expression of Cre-GFP in the CBN; right, power spectrum of force-plate measurements from the same Syt2fl/fl mouse before and after Cre-GFP expression in the CBN. (F) Summary graph of tremor index before and after Cre-GFP expression in the CBN or cerebellar cortex of Syt2fl/fl mice (n = 6 CB cortex, n = 10 CBN). (G and H) Figure panels arranged the same way as in E and F, except that AAVs encoding GFP or Syt2-2A-GFP were injected into the CBN of PVcre Syt2fl mice (n = 5 GFP, n = 6 Syt2). For C, F, and H, data are shown as means ± SEM from at least 3 independent litters. *P < 0.05; ***P < 0.001 by 2-sided, unpaired t test (C) or 2-sided, paired t test (F and H). Scale bars: 100 μm (D); 1 mm (E).
Loss of fast synchronous neurotransmitter release at CBN → GRN synapses may induce the action tremor. To identify the downstream targets of PV' neurons in the CBN that generate the action tremor, we injected AAVs expressing Cre-dependent mCherry (DIO-mCherry) into the CBN of PVcre mice. Three weeks later, robust mCherry signals were observed in the thalamus (ventral anterior-lateral complex of the thalamus, VAL; ventral medial nucleus of the thalamus, VM), the midbrain (red nucleus; midbrain reticular nucleus; periaqueductal gray), and the brainstem (giganto-cellular reticular nucleus, GRN; parvicular cell reticular nucleus; vestibular nuclei; Figure 5A). Among these brain regions, the VAL and VM may be analogous to the human ventral intermediate nucleus of the thalamus, which has been targeted for DBS treatment (26–28). The red nucleus and VAL were previously proposed to constitute an essential tremor pathway outside of the cerebellum (12). Moreover, the GRN has been shown to directly innervate motor neurons in the spinal cord, and therefore is in a good position to control fine motor movement (33, 34). We also performed more restricted injections of AAVs expressing DIO-mCherry into each individual nucleus of the CBN (fastigial nucleus; dentate nucleus; Supplemental Figure 7, A–C), and found that although all 3 nuclei projected to the thalamus, the midbrain, and the thalamus in general, there were some differences. For example, mainly the fastigial nucleus and dentate nucleus projected to the GRN (Supplemental Figure 7, A–C). Using retrograde tracing, we further confirmed that GRN neurons received robust inputs from the fastigial nucleus and dentate nucleus (Supplemental Figure 7, D and E).

To determine whether the same group of CBN neurons project to the GRN and to more rostral brain structures (red nucleus and VAL/VM), we injected retro-AAVs encoding mCherry into the GRN and retro-AAVs encoding GFP into either the red nucleus or the VAL/VM (Supplemental Figure 8, A and C). Subsequent imaging of labeled CBN neurons suggested that distinct CBN neurons projected to the GRN and red nucleus or VAL/VM (Supplemental Figure 8, B and D). To test the roles of different CBN projections in action tremor, we bilaterally injected AAVs expressing wheat germ agglutinin–conjugated Cre (WGA-Cre) into the VAL/VM, red nucleus, or GRN of Syt2fl/fl mice that had been crossed with Cre-dependent EYFP reporter (Ai3; Supplemental Figure 8, E and F).

Figure 5. Identification of CBN downstream targets that induce the action tremor. (A) Representative images of anterograde tracing experiments of projections from PV' neurons in the CBN to other brain regions (left, stereotactic injection strategy of AAVs encoding DIO-mCherry into the CBN of PVcre mice; right images, coronal slices arranged in a caudal → rostral direction showing mCherry expression in the indicated brain regions [note that the 3 fluorescence images on the right were overexposed to reveal the axon terminal signals]). (B) Stereotactic injection strategies of AAVs encoding flipase-dependent Cre (Frt-Cre) in the CBN (most left) or both Frt-Cre in the CBN and AAV retro-flipase in the VAL/VM, red nucleus, or GRN of Syt2fl/fl crossed with EYFP reporter (Syt2 Ai3) mice (3 on the right). (C) Representative images showing the expression of EYFP in CBN for each injection experiment depicted in B. (D) Power spectra of force-plate measurements from representative Syt2 Ai3 mice before and after the corresponding injections shown in B (n = 5 Frt-Cre, n = 5 VAL/VM, n = 6 RN, n = 5 GRN). For E, data are shown as means ± SEM from at least 3 independent litters. *P < 0.05 by Mann-Whitney test (E). Scale bars: 1 mm (A); 1 mm (C).
postsynaptic currents (sEPSCs), which are largely equivalent to miniature EPSCs (mEPSCs), was significantly increased, whereas the amplitude of sEPSCs was unchanged (Figure 6, B and C). Increases in mEPSC frequency are a typical phenotype induced by deletion of fast synaptotagmins (21). We next stimulated synaptic inputs to the GRN using blue laser light at 50 Hz, and observed that fast synchronous neurotransmitter release was abolished in some of the GRN neurons recorded, whereas asynchronous neurotransmitter release appeared to be enhanced (Figure 6, D and E, and Supplemental Figure 8J). Moreover, we applied rabies tracing to label CBN neurons projecting to the GRN. Imaging results suggested that there were no obvious morphological differences between CBN neurons in PV cre Syt2 fl and PV cre Syt2 wt mice (Supplemental Figure 8, K–M). Together, these results suggest that the deletion of Syt2 from CBN neurons caused a loss of fast synchronous but not slower forms of neurotransmitter release, suggesting that a shift in the pattern of evoked neurotransmitter release in CBN → GRN synapses may be the cause of the action tremor observed in PVcre Syt2fl mice.

Blocking neurotransmitter release in CBN neurons rescues the action tremor of PV cre Syt2 fl mice and of harmaline-injected mice. To test whether the remaining asynchronous neurotransmitter release at CBN → GRN synapses of PVcre Syt2 wt mice induce the action tremor, we expressed Cre-dependent tetanus toxin (DIO-TetTox) in CBN neurons of PV cre Syt2 fl mice, which will block all synaptic release from PV + neurons in the CBN (ref. 37 and Figure 7A). Surprisingly, TetTox expression rescued instead
of aggravated the age-dependent action tremor in PVcre Syt2fl mice (Figure 7B and C, and Supplemental Figure 9A and B), suggesting that the action tremor of PVcre Syt2fl mice was generated de novo. To understand the nature of this approximately 8-Hz tremor, we expressed DIO-TetTox in the CBN of PVcre Syt2fl mice (Supplemental Figure 9D), which did not exhibit visible tremor. Interestingly, completely blocking the synaptic release in CBN PV+ neurons in PVcre mice induced tremor at approximately 8 Hz (Supplemental Figure 9E and F), the same frequency range as the physiological tremor in PVcre mice (Supplemental Figure 9E and G). This result suggests that the remaining approximately 8-Hz tremor after blocking all synaptic release from PV+ neurons in the CBN of PVcre Syt2fl mice was not due to a frequency shift of the 9-Hz to 12-Hz action tremor.

Next, we tested whether completely blocking synaptic transmission in CBN PV+ neurons (Figure 8A) could also rescue the tremor phenotype in PVcre Syt2fl mice, resulting in expression of GFP only or TetTox light chain fused to GFP only in PV+ neurons; right, a representative image showing the bilateral expression of GFP in the CBN. Averaged power spectrum of force-plate measurements from PVcre Syt2fl mice before and after injection of AAVs encoding GFP or TetTox-GFP (n = 6 GFP, n = 7 TetTox). Note that after TetTox expression, the tremor not only decreased, but the peak tremor frequency also shifted to approximately 8 Hz. (C) Summary graph of the tremor index before and after injection of AAVs encoding GFP or TetTox-GFP in the CBN of PVcre Syt2fl mice (n = 6 GFP, n = 7 TetTox). For B and C, data are shown as means ± SEM from at least 2 independent litters. ***P < 0.001 by 2-sided, paired t test. Scale bar: 1 mm (A).

**Figure 7. Blocking neurotransmitter release in CBN neurons rescues the action tremor of PVcre Syt2fl mice.** (A) Left, stereotactic injection strategy of AAVs encoding DIO-GFP or DIO-TetTox-GFP into the CBN of PVcre Syt2fl mice, resulting in expression of GFP only or TetTox light chain fused to GFP only in PV+ neurons; right, a representative image showing the bilateral expression of GFP in the CBN. (B) Averaged power spectrum of force-plate measurements from PVcre Syt2fl mice before and after injection of AAVs encoding GFP or TetTox-GFP (n = 6 GFP, n = 7 TetTox). Note that after TetTox expression, the tremor not only decreased, but the peak tremor frequency also shifted to approximately 8 Hz. (C) Summary graph of the tremor index before and after injection of AAVs encoding GFP or TetTox-GFP in the CBN of PVcre Syt2fl mice (n = 6 GFP, n = 7 TetTox). For B and C, data are shown as means ± SEM from at least 2 independent litters. ***P < 0.001 by 2-sided, paired t test. Scale bar: 1 mm (A).
previous studies showed that cooling the dentate nucleus could induce an action tremor (42, 43). CBN neurons projecting to the red nucleus and VAL/VM were largely distinct from GRN-projecting neurons (Supplemental Figure 8, A–D). We speculate that these CBN \( \rightarrow \) thalamus projection neurons could be responsible for suppressing action tremor. From a clinical perspective, in the future it will be important to apply advanced neuroimaging techniques to record whether activities in the brainstem regions are correlated with the action tremor of essential tremor patients. It will also be

Figure 8. Blocking neurotransmitter release in CBN neurons rescues the action tremor of harmaline-injected mice. (A) Left, stereotactic injection strategy of AAVs encoding DIO-TetTox-GFP into the CBN of PV\(^{cre}\) mice; right, a representative image showing the bilateral expression of GFP in the CBN. (B) Averaged power spectrum of force-plate measurements from WT mice before and 5 minutes after harmaline injection (\( n = 7 \)). (C) Averaged power spectrum of force-plate measurements from TetTox-injected mice before and 5 minutes after harmaline injection (\( n = 7 \)). (D) Summary of tremor index in the 11-Hz to 14-Hz range (see methods) before and 5 minutes after harmaline i.p. injection in WT and TetTox-injected mice (\( n = 7 \) WT, \( n = 7 \) TetTox). (E) Left and middle, stereotactic injection strategy of AAV-retro-Cre into the GRN and AAV DIO-TetTox-GFP into the CBN of WT mice; right, a representative image showing the bilateral expression of GFP in the CBN. (F) The same as in E, except that the AAV-retro-Cre virus was injected into the VAL/VM. (G) Averaged power spectrum of force-plate measurements from GRN-injected mice before and 5 minutes after harmaline injection (\( n = 6 \)). (H) Averaged power spectrum of force-plate measurements from VAL/VM-injected mice before and 5 minutes after harmaline injection (\( n = 6 \)). (I) Summary of tremor index in the 11-Hz to 14-Hz range before and 5 minutes after harmaline i.p. injection in GRN- and VAL/VM-injected mice (\( n = 6 \) GRN, \( n = 6 \) VAL/VM). For B–D and G–I, data are shown as means ± SEM from at least 2 independent litters. *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \) by 2-sided, paired t test. Scale bars: 1 mm (A); 1 mm (E); 1 mm (F).
In this simplified simulation, as the duration of the fixed temporal delay 

delayed 
y 
tially compensated by the delayed correction signal –1. Continuing with this 
and continues on the wrong trajectory to (3, 2), where it starts to be par-

in red at the bottom. The deviated movement at (2, 1) is not compensated 
to move between 2 ticks on the x time and continues its planned trajectory. In action tremor animals, we 
movement is compensated by the axis “correction” signal “–1” in real 
planned path is a straight line from (0, 0) to (13, 0); assuming movement 
correction could generate oscillatory movement. Here the 
B command signals. (Figure 9A) Simplified simulation showing how delayed online 
gram of the neural circuit controlling the real-time correction of movement 
Figure 9. Model for the generation of action tremor. (A) Schematic dia-

Figure 6, B–E) are consistent with previous studies of the role 
synaptotagmins in clamping and mediating fast synchronous 
transmitter release (25, 37), as supported by 
other synaptotagmin isoforms that mediate fast and intermediate 
synchronous neurotransmitter release (20, 21, 44, 45). These synaptic changes 
correlate well with the tremor and provide interesting insights into 
the roles of the cerebellum in action tremor. In our working 
model (Figure 9A), we propose that the cerebellum receives a copy 
of the motor command and another copy of the motor execution 
feedback on a moment-by-moment basis. If these 2 signals are not 
perfectly matched, the cerebellar circuits will calculate the real-
time “correction” signal needed to adjust and smoothen the ongo-
ing movement (46). This hypothesized correction signal likely 
flows from the glutamatergic PV+ neurons in the CBN to GRN 
neurons, which are directly connected to motor neurons in the 
spinal cord (33, 34), thereby adjusting the motor command sig-
nals in real time. If Syt2 is removed from glutamatergic neurons in the 
CBN, this correction signal becomes temporally delayed 
and causes oscillations of the motor command signals, thereby 
inducing tremor (Figure 9B). Since this delayed correction signal 
only applies to the movement phase, the resulting tremor would 
be specifically an action tremor, as opposed to a resting tremor. 
Completely abolishing this pathologically delayed correction sig-
nal, as by TetTox treatment, removed the oscillation of the motor 
command and blocked the 9-Hz to 12-Hz action tremor (Figure 
9B). In general, our working model is consistent with the previous 
understanding of the role of the cerebellum in motor learning (47). 
Future studies will be needed to identify the neural circuits that 
transmit the motor command and execution feedback signals into 
the cerebellum, to characterize the local cerebellar circuit com-
puting the correction signal, and to determine how oscillations 
are generated in downstream targets and how the frequency and 
amplitude of the resulting tremor are determined.

In this study, we used Syt2 as a molecular tool to identify the 
circuit components and synaptic defects underlying an action 
tremor. Given the wide distribution of PV+ cells in the nervous 
system and the critical role of Syt2 as a fast Ca2+ sensor for neu-
rotransmitter release (19, 20, 45, 48), it is somewhat unexpected 
that PV+ Syt2fl mice displayed a specific action tremor phenotype 
without exhibiting other dramatic behavioral defects (Supplemen-
tal Figure 3, B–E). We think there are 2 reasons for this phenotypic 
specificity. First, the function of Syt2 could be compensated by 
other synaptotagmin isoforms that mediate fast and intermediate 
synchronous neurotransmitter release (25, 37), as supported by 
our recording results in the cortex (Figure 2, C–F). Second, many 
cells expressing Syt2 as the only fast Ca2+ sensor are PV negative, 
as supported by the lethality in Syt2 constitutive knockout mice 
(19) and the dramatic weight loss in Vglut2+ Syt2fl mice (Supple-
mental Figure 6E). Therefore, PV+ Syt2fl mice serendipitously 
targeted, among others, a specific population of PV+ neurons in the 
CBN (49) that use Syt2 as the only fast Ca2+ sensor and play critical 
roles in action tremor.

A limitation to our study is that Syt2 has not been shown as an 
essential tremor risk gene. Although the genetics of essential 
tremor are still poorly understood (50, 51), we do not think Syt2 
will be identified as a risk gene for essential tremor patients, 
because of the lethality in Syt2 constitutive knockout mice, which 
reveals its central importance in brain function. Nevertheless, we 
found that PV+ Syt2fl mice reproduced the major symptoms of 
essential tremor patients. The critical neural pathway and synap-
tic release defect we identified using this mouse model are likely 
downstream of the initial genetic cause and are probably shared by 
different animal models and human patients of essential tremor. 
Indeed, we found that blocking the abnormal neurotransmitter 
release in CBN PV+ neurons with TetTox could also rescue the 
action tremor of harmaline-injected mice (Figure 8, C and D). Our 
data thus suggest that PV+ Syt2fl mice are a reliable action tremor 
model that promises to be of use for studying the etiology of essen-
tial tremor and for developing therapeutic interventions in essen-
tial tremor patients.

Methods

Animals. Syt2fl/fl mice were designed to have Syt2 exon 2 flanked by 
loxP sites and were generated at the Janelia gene targeting and trans-
genics facility (21). They were backcrossed to C57BL/6J WT mice at 
least 7 times. C57BL/6J, PV+V, L7+V, Vglut2+V, GAD2+V, Ai3, Ai4l, 
and Ai75 transgenic mice were purchased from The Jackson Labora-
tory. Prkcdcre mice were provided by David Anderson’s laboratory at 
The California Institute of Technology, Pasadena, California. Syt2fl/fl 
mice were crossed with each different Cre driver line 2 generations to
generate Drivercre Syt2m 

One week later, mice were perfused and processed for imaging areas. Coordinates were the following: mPFC (1.25, 0.3, 1.4 μL); dentate nucleus (–6, 2.2, 2.5; 0.05 μL). For each large volume: CBN (–6.2, 1.75, 2.5; 0.2 μL); red nucleus (–3.38, 0.75, 4.3; 0.2 μL); thalamus (–0.8, ±0.75, 4 → 3; 1 μL), (–1.31, ±4.3 → 3.3; 2 μL), and (–1.9, ±1, 4 → 3; 2 μL); cerebellum (–5.8, ±0.75, 2.3 → 0.7; 1.5 μL), (–5.8, ±2.25, 2.3 → 0.7; 1.5 μL), (–6.35, 0.2 → 0.7; 1.5 μL), (–6.35, ±1.5, 2.3 → 0.7; 1.5 μL), (–6.35, ±3, 2.3 → 0.7, 1.5 μL), (–7, ±0.75, 2.1 → 0.7; 1.2 μL), and (–7, ±2.25, 2.1 → 0.7; 1.2 μL); lateral cerebellar cortex (–5.8, ±2.75, 1 μL), (–6.35, ±3, 1; 1 μL) and (–7, ±2.5, 1; 1 μL); medial cerebellar cortex (–5.8, ±0.5, 1; 1 μL), (–6.35, ±0.5, 1; 1 μL), and (–7, ±0.5, 1; 1 μL).

**Behavior.** Male and female mice 65 to 80 days old were used for all behavior tests unless noted otherwise. The genetic background of the mice and type of virus injected were coded and blinded to the experimenters.

**Force plate.** The design and applications of the same force-plate actometer device were described in detail in a previous study (22). We took advantage of this device’s high sampling rate (100 Hz) of weight measurement and used a custom MATLAB script for data analysis. Mice were individually placed on the 28 × 28 cm plate and allowed to freely explore for 5 minutes unless noted otherwise. The raw data were divided into 3-second segments and fast Fourier transformation was performed for each 3-second segment. The power spectra were then averaged (Figure 1C). A “tremor index” was calculated by integrating the power value in the 9-Hz to 12-Hz window. Power value in the 3-Hz to 6-Hz window was used as the baseline. Real-time locomotion distance information could be derived from the same force-plate raw data using the Pascal programs that came with the force-plate device. For ethanol injection experiments, animals were first measured on the force plate for 5 minutes, then given s.c. injections of 2.5 g/kg ethanol or saline and measured on the force plate for another 60 minutes. For harmaline injection experiments, 1 mg/mL harmaline solution was prepared fresh by dissolving harmaline hydrochloride (Millipore-Sigma, H1392) in saline. Animals were first measured on the force plate for 5 minutes, then given i.p. injections of 20 mg/kg harmaline and measured on the force plate for another 20 minutes. The action tremor was induced approximately 5 minutes after harmaline injection. The 5- to 10-minute window was analyzed for harmaline-induced tremor.

**Video analysis of tremor.** To simultaneous collect video and force-plate data, mice were placed on the force plate and surrounded by a small (13.35 × 8.40 × 20 cm) custom floorless acrylic chamber (TAP Plastic) suspended over the force plate. Video recording was performed with an iPhone X mounted on top of the acrylic chamber using slow-motion mode (240 frames per second). Each behavioral session consisted of 2 minutes of free exploration, and the raw force-plate data and video were saved for offline analysis. Using a tracking software View III (BIOSERVE), we extracted the x-y position of the animal’s nose over time (Supplemental Figure 2, A and B). With a custom MATLAB script, these coordinates were bandpass filtered (9–12 Hz) to produce the filtered x and filtered y signals. The envelopes (instantaneous amplitude) of these 2 signals were then calculated using the Hilbert transform (Supplemental Figure 2C and Supplemental Videos 1 and 2). Instantaneous tremor amplitude was calculated by taking the Euclidean norm of individual x and y instantaneous amplitude. This was then averaged to calculate the average tremor amplitude for the session.

**Spontaneous alternation Y maze.** A light gray plastic Y maze was used to evaluate spatial working memory. The maze consisted of 3 arms separated by 120° (dimensions of each arm: 40 × 10 × 17 cm).
Mice around 4 weeks old were individually placed in the distal end of one arm and allowed to freely explore the whole maze for 10 minutes. Completed arm entry was defined as the entering of a whole arm including its tail into an arm. The sequences and total numbers of arm entries were recorded and analyzed with the Viewer III tracking system. Visiting all 3 different arms consecutively was termed a “correct” trial, and visiting one arm twice or more in 3 consecutive entries was termed “wrong” trial. We calculated the correct alternation percentage as (number of correct trials/total number of correct and wrong trials) × 100.

Novel object recognition. The same boxes used for open field test were used. On day 1, mice around 1 month old were given 10 minutes of habituation time individually in an empty box. On day 2, mice were individually placed in the box for 10 minutes with 2 identical objects, either T75 cell culture flask filled with bedding material or blocks of LEGO®. On day 3, one object was replaced with a novel object. Object location in chamber was randomized. Exploration behavior was recorded and analyzed with the Viewer III tracking system. The recognition index was defined as the time spent on the novel object (or the left object for training phase) divided by the time spent on both objects.

Rotarod. An accelerating rotarod designed for mice (IITC Life Science) was used. The test consisted of 3 trials per day over the course of 3 days. The rotarod was activated after placing mice on the motionless rod. The rod accelerated from 4 to 40 revolutions per minute in 5 minutes. Each trial ended when a mouse fell off, made 1 complete revolution while hanging on, or reached 300 seconds.

Fear conditioning. On training day, mice were individually placed in a fear-conditioning chamber (Coulbourn Instruments) located in the center of a sound-attenuating cubicle. The conditioning chamber was cleaned with 10% ethanol to provide a background odor. A ventilation fan provided a background noise at approximately 55 dB. After a 2-minute exploration period, 3 tone-foot shock pairings separated by 1-minute intervals were delivered. The 85–dB, 2-kHz tone lasted for 30 seconds and the foot shock was 0.75 mA and lasted for 2 seconds. The foot shocks coterminated with the tone. The mice remained in the training chamber for another 60 seconds before being returned to home cages. In the context test, mice were placed back into the original conditioning chamber for 5 minutes. The behavior of the mice was recorded with Freezeframe software and analyzed with Freezview software (Coulbourn Instruments). The running speed of the mice was analyzed offline with Viewer III software. The average running speeds of mice in the 2-minute exploration period and 1-minute period after each foot shock were summarized as an indication of contextual fear memory acquisition. The average running speed of mice in the 5-minute period during the context recall test was summarized as an indication of contextual fear memory retrieval.

Acute brain slice electrophysiology. P21 mice were used for viral injections, and slice recordings were performed 20 to 30 days later. To increase visibility and obtain better recording quality of GRN neurons from adult brainstem slices, we slightly modified an N-methyl-D-glucamine–based (NMDG-based) protocol (52). Specifically, 160-μm coronal brainstem slices were cut with a vibrotome (VT1200S, Leica) in chilled cutting solution (in mM): 92 NMDG, 2.5 KCl, 1.25 NaH2PO4, 20 HEPES, 10 MgSO4, 0.5 CaCl2, 30 NaHCO3, 25 D-glucose, 2 thiourea, 5 Na-aspartate, 3 Na-pyruvate (pH 7.3, adjusted with HCl) saturated with 95% O2/5% CO2 and recovered in the same NMDG-based cutting solution at 33°C for 10 to 15 minutes. Slices were then transferred to room temperature oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM): 119 NaCl, 2.5 KCl, 1.25 NaH2PO4, 2 MgSO4, 2 CaCl2, 26 NaHCO3, 12.5 D-glucose, 2 thiourea, 5 Na-aspartate, 3 Na-pyruvate. After slices were transferred to a recording chamber, oxygenated ACSF was continuously perfused. Whole-cell voltage-clamp recordings were made with 3- to 4-MΩ pipettes filled with internal solution containing (in mM): 140 CsMeSO4, 2 CsCl, 10 HEPES, 10 EGTA, 0.3 Na2-GTP, 2 Mg-ATP, 7 phosphocreatine, 5 TEA-Cl, 1 QX314 (pH 7.3, adjusted with CsOH). The whole-cell current signals were recorded with MultiClamp700B and Clampex 10.4 data acquisition software (Molecular Devices). Recordings were made from neurons in the GRN under bright field visualization with an upright microscope (BX51WI, Olympus). After establishment of the whole-cell configuration and equilibration of the intracellular pipette solution with the cytoplasm, sEPSCs were recorded by holding the cell at –70 mV. Spontaneous sEPSCs were recorded for 5 minutes. A 473-nm laser (OEM Laser Systems) was used for light-evoked EPSC recordings. Light was delivered through an optic fiber (200 μm, 0.22 NA) pointing toward the recorded area. Laser intensity was adjusted to get a maximal response without overstimulation; 50-Hz, 1-ms blue light was applied in 3 repetitions at 20-second intervals. Synaptic currents were analyzed offline using Clampfit 9 (Molecular Devices) software. Spontaneous events were analyzed using the template matching search and a minimum threshold of 5 pA, and each event was visually inspected by an experimenter blind to the experiment conditions. Slice recordings for mPCF neurons were performed similarly, except that a regular sucrose-based slice cutting protocol was used (35). sIPSCs were recorded by using a regular high-chloride internal solution (36), including 20 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 50 μM (2R)-amino-5-phosphonovaleric acid (APV) in the ACSF, and clamping the neurons at –70 mV. Evoked IPSCs were recorded by using the same internal solution as GRN recordings, including 20 μM CNQX, 50 μM APV in the ACSF, and clamping the neurons at 0 mV.

Histology. Mice were deeply anesthetized with tribromoethanol and perfused with 10 mL of PBS followed by 10 mL of fixative (4% paraformaldehyde diluted in PBS). The brains were removed and postfixed in 4°C overnight, and then immersed in 30% sucrose solution for 2 days before being sectioned at 50-μm thicknesses on a cryostat (Leica CM3050 S). The free-floating brain sections were collected in PBS. For injection site verification, the sections were directly mounted onto glass slides with Vectashield mounting medium with DAPI, except for results shown in Supplemental Figure 7D, in which mounting medium without DAPI was used. For tracing experiments, 1 out of every 5 sections was collected for the whole brain. For immunohistochemistry, standard procedures were followed (36). We used primary antibodies to Syt2 (rabbit, A320, 1:1000, ref. 19), Vglut1 (guinea pig, Millipore AB5905, 1:1000), and Vglut2 (guinea pig, Millipore AB2251, 1:1000). A scanning microscope (BX61VS, Olympus) was used to scan fluorescence images for whole brain slices, and a confocal microscope (Nikon A1) was used for higher resolution imaging.

Statistics. All results are presented as mean ± SEM and were analyzed by OriginPro 8 software (OriginLab Corp.). No statistical methods were used to predetermine sample sizes, but our sample sizes were similar to those reported in previous publications. Normality tests and F tests for equality of variance were performed before choosing the
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