

# Transcutaneous auricular vagus nerve stimulation alleviates pain–depression comorbidity in mice through dorsal raphe–central amygdala serotonergic signaling

**Haohan Zhu**

China Academy of Chinese Medical Sciences

**Zijie Chen**

China Academy of Chinese Medical Sciences

**Xingke Song**

China Academy of Chinese Medical Sciences

**Yifei Wang**

China Academy of Chinese Medical Sciences

**Xue Pu**

China Academy of Chinese Medical Sciences

**Jinling Zhang**

China Academy of Chinese Medical Sciences

**Xianghong Jing**

China Academy of Chinese Medical Sciences

**Hanlai Zhang**

China Academy of Chinese Medical Sciences

**Peijing Rong**

**[drorongpj@163.com](mailto:drorongpj@163.com)**

China Academy of Chinese Medical Sciences

**Junying Wang**

China Academy of Chinese Medical Sciences

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## Research Article

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**Transcutaneous auricular vagus nerve stimulation alleviates pain–  
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amygdala serotonergic signaling**

*Haohan Zhu<sup>1,2#</sup>, Zijie Chen<sup>1#</sup>, Xingke Song<sup>1#</sup>, Yifei Wang<sup>1</sup>, Xue Pu<sup>1</sup>, Jinling Zhang<sup>1</sup>,  
Xianghong Jing<sup>1</sup>, Hanlai Zhang<sup>1</sup>, Peijing Rong<sup>1,2\*</sup>, Junying Wang<sup>1\*</sup>*

1.Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical  
Sciences, Beijing, China

2.Institute of Basic Research in Clinical Medicine, China Academy of Chinese  
Medical Sciences, Beijing, China

Correspondence:

Peijing Rong: drrongpj@163.com; Junying Wang: wjyanguning@aliyun.com;

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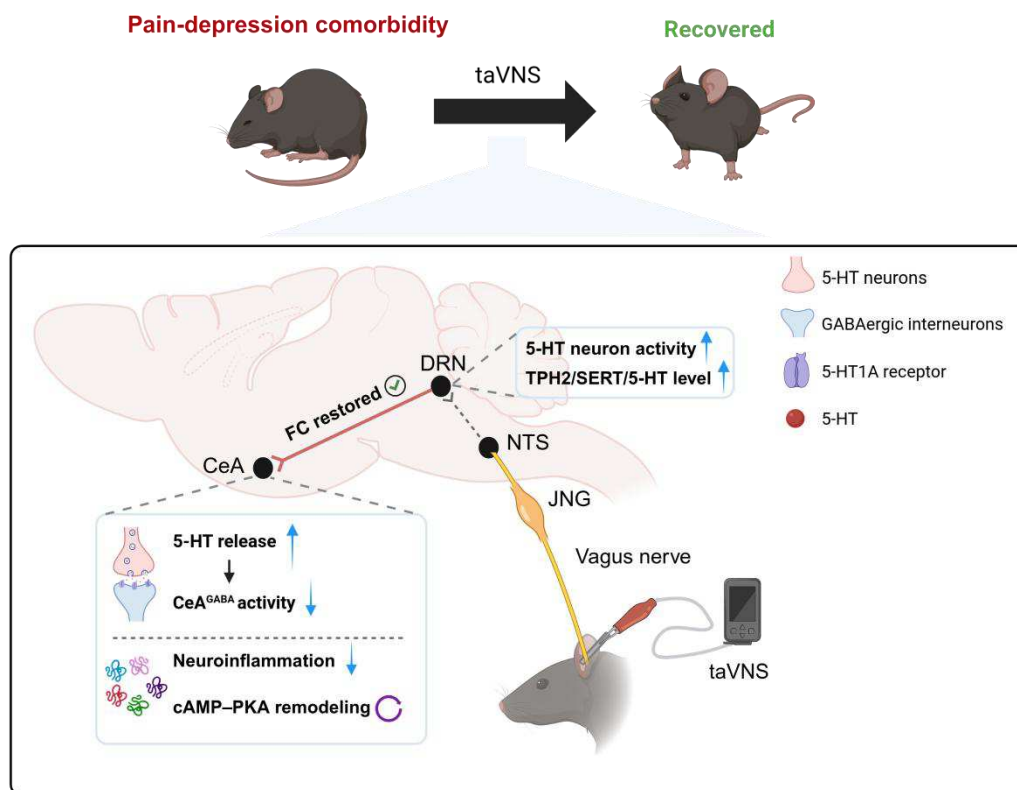
**Abstract**

**Background:** Chronic pain and depression frequently co-occur, creating a need for  
interventions that address both sensory and affective disturbances. Transcutaneous  
auricular vagus nerve stimulation (taVNS) offers a non-invasive therapeutic approach,  
but the neural mechanisms underlying its effects on pain–depression comorbidity  
remain unclear. We investigated whether dorsal raphe nucleus (DRN)–central  
amygdala (CeA) serotonergic signalling mediates the effects of taVNS on neuropathic  
pain and associated depressive-like behaviours.

**Methods:** Male C57BL/6 and Pet1-Cre mice underwent chronic constriction injury  
(CCI) of the sciatic nerve, followed four weeks later by daily taVNS for 28 days.  
Mechanical hypersensitivity and depressive-like behaviours were assessed using  
mechanical withdrawal, sucrose preference, tail suspension, and open-field tests. We  
combined resting-state functional magnetic resonance imaging, anatomical tracing,  
calcium and serotonin photometry, in vivo electrophysiology, and microdialysis to  
characterise DRN–CeA connectivity, neuronal activity, and serotonin transmission.  
Chemogenetic inhibition of DRN serotonergic terminals in the CeA, inhibition of  
DRN serotonin synthesis, and local blockade of CeA 5-HT<sub>1A</sub> receptors were used to  
test the contribution of this pathway to treatment effects. CeA proteomic and  
phosphoproteomic profiling characterised treatment-associated molecular adaptations.

**Results:** Repeated taVNS alleviated mechanical hypersensitivity and depressive-like behaviours, shifted DRN–CeA functional connectivity towards control values, and restored extracellular serotonin concentrations in the CeA. Anatomical tracing supported an auricular concha–jugular–nodose ganglion–nucleus tractus solitarius–DRN relay and identified serotonergic projections from the DRN to the CeA. Acute taVNS recruited DRN serotonergic neurones and increased CeA serotonin signals, with stronger responses in CCI mice. CeA neuronal responses were state dependent, being predominantly excitatory in control mice and predominantly inhibitory after CCI. Chemogenetic inhibition of DRN serotonergic terminals, suppression of DRN serotonin synthesis, and CeA 5-HT<sub>1A</sub> receptor blockade each attenuated the behavioural benefits of taVNS. Proteomic analysis revealed reversal of inflammation-associated protein signatures, whereas phosphoproteomic analysis identified cAMP/PKA-associated changes involving synaptic plasticity, calcium handling, and inhibitory-synapse organisation.

**Conclusions:** taVNS alleviates neuropathic pain and associated depressive-like behaviours in mice through recruitment of DRN–CeA serotonergic signalling involving CeA 5-HT<sub>1A</sub> receptors. These findings link non-invasive peripheral stimulation to state-dependent limbic circuit responses and coordinated molecular adaptations, providing a mechanistic basis for developing neuromodulation strategies targeting both the sensory and affective dimensions of chronic pain.



58 #These authors contributed equally to this work.

59 \*These authors jointly supervised this work and share corresponding authorship.

## 60     **Introduction**

61     Chronic pain and depression are two debilitating conditions that frequently co-occur.  
62     A recent systematic review and meta-analysis reported that approximately 39.3% of  
63     adults with chronic pain have comorbid depression, a prevalence significantly higher  
64     than in pain-free populations[1]. Epidemiological data from the United States indicate  
65     that approximately 12 million adults experience co-occurring chronic pain and  
66     anxiety/depression symptoms[2]. Rather than simply coexisting, pain and depression  
67     mutually reinforce each other in a vicious cycle: chronic pain exacerbates depressive  
68     symptoms, while depression lowers pain thresholds and amplifies pain perception[3].  
69     This comorbidity leads to more severe functional impairment, poorer treatment  
70     outcomes, and a substantial economic burden on patients and society[2,4]. Current  
71     pharmacological treatments for pain – depression comorbidity, such as selective  
72     serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake  
73     inhibitors (SNRIs), offer limited efficacy, are slow to take effect, carry significant  
74     side effects, and approximately one-third of patients fail to respond to multiple  
75     antidepressants[5,6]. Therefore, there is an urgent need to explore novel therapeutic  
76     strategies that can simultaneously address both the sensory and affective dimensions  
77     of this comorbidity.

78     Vagus nerve stimulation (VNS) was approved by the U.S. Food and Drug  
79     Administration as an adjunctive treatment for treatment-resistant depression[7], but its  
80     invasive nature and potential side effects, including cough, hoarseness, and  
81     gastrointestinal discomfort, have limited clinical adoption[8]. Transcutaneous  
82     auricular vagus nerve stimulation (taVNS), a non-invasive alternative, delivers  
83     electrical stimulation to the auricular branch of the vagus nerve (ABVN) distributed in  
84     the auricular concha and has been shown to effectively alleviate depressive  
85     symptoms[9]. Systematic reviews have confirmed that taVNS significantly reduces  
86     depression scores with a favorable safety profile[10]. Moreover, recent clinical studies  
87     and meta-analyses have demonstrated that taVNS also shows positive efficacy in  
88     relieving various chronic pain syndromes[11,12]. Our previous research indicates that  
89     taVNS effectively alleviates symptoms of pain – depression comorbidity[13,14].  
90     These findings suggest that taVNS may act on shared neural substrates underlying  
91     both pain and depression, yet the central mechanisms mediating its dual analgesic and  
92     antidepressant effects remain incompletely understood.

93     From an anatomical perspective, the auricular concha is the only body surface  
94     distribution area of the ABVN. A growing body of evidence has established that the  
95     ABVN conveys sensory signals from the auricular concha through the jugular-nodose  
96     ganglion (JNG) to the nucleus tractus solitarius (NTS) in the brainstem[15–17], and  
97     functional magnetic resonance imaging studies in humans have confirmed that taVNS  
98     significantly activates the ipsilateral NTS[18]. Recent circuit-level analyses have  
99     further demonstrated that this peripheral afferent pathway engages the descending  
100     pain modulatory system through NTS projections to brainstem regions such as the

periaqueductal gray[17]. While these advances have clarified the peripheral afferent route and its role in taVNS-induced analgesia, the downstream circuit through which taVNS simultaneously exerts its antidepressant effects—and how vagal signals reaching the NTS gain access to the neural systems governing mood regulation—remains to be determined.

The dorsal raphe nucleus (DRN) is the largest serotonergic output nucleus in the brain, projecting extensively to the limbic system, prefrontal cortex, and brainstem[19]. The serotonergic system has been extensively implicated in the neurobiology of both depression and chronic pain: dysfunction of DRN serotonergic signaling to limbic regions is a key pathological feature of depression[20], while DRN serotonergic neurons simultaneously participate in descending pain modulation, and their activity is closely associated with chronic pain states[21]. Notably, anatomical studies have demonstrated projections from the NTS to the DRN[22], raising the possibility that vagal afferent signals could access the central serotonergic system via this route. The DRN may thus serve as a critical relay downstream of the NTS through which taVNS engages neural circuits relevant to both pain and depression.

As the primary output nucleus of the amygdaloid complex, the central amygdala (CeA) projects to multiple downstream structures and serves as a critical hub for integrating nociceptive information and negative emotions[23]. The CeA is predominantly composed of GABAergic neurons, including molecularly defined subpopulations such as SOM<sup>+</sup>, Crh<sup>+</sup>, and PKC $\delta$ <sup>+</sup> neurons, whose aberrant activity is closely linked to chronic pain and emotional disorders[24]. Recent studies have revealed that DRN serotonergic neurons regulate CeA function through cell-type-specific receptor signaling. Zhou et al.[25] demonstrated that in a chronic pain model, DRN 5-HT inhibits CeA SOM<sup>+</sup> neurons via 5-HT<sub>1A</sub> receptors; chronic pain weakens this inhibition, leading to disinhibition of SOM<sup>+</sup> neurons projecting to the lateral habenula and driving depression-like behavior. Activation of 5-HT<sub>1A</sub> receptors reversed the depressive phenotype, whereas the SSRI paroxetine was ineffective. In a complementary study, Wang et al.[26] showed that during acute stress, DRN 5-HT excites CeA Crh<sup>+</sup> neurons via 5-HT<sub>2A</sub> receptors, which in turn suppress the nociceptive responses of SOM<sup>+</sup> neurons through local GABAergic transmission, mediating stress-induced analgesia. Additional studies have further implicated CeA output pathways, including CeA–vIPAG projections, in pain- and affect-related behavioral regulation[27]. Together, these findings demonstrate that DRN → CeA serotonergic signaling fine-tunes both nociceptive and emotional processing through distinct receptor subtypes acting on different CeA cell populations, positioning this circuit as a compelling candidate substrate for the dual analgesic and antidepressant effects of taVNS.

Despite these important advances, several key questions remain. It is not yet established how the peripheral signal of taVNS reaches the DRN to engage this serotonergic circuit. Whether taVNS can alleviate pain – depression comorbidity by modulating the DRN → CeA serotonergic pathway—and whether this pathway is

causally necessary for its dual behavioral effects—has not been tested. Furthermore, beyond circuit-level alterations, the molecular consequences of disrupted serotonergic signaling in the CeA during pain – depression comorbidity—and whether taVNS can reverse these molecular changes—remain largely unexplored.

In this study, using a chronic constriction injury (CCI) mouse model of pain – depression comorbidity, we systematically define a peripheral-to-central serotonergic signaling pathway—auricular concha → JNG → NTS → DRN → CeA—through which taVNS simultaneously alleviates pain and depression. We provide convergent evidence across anatomical, functional, causal, and molecular levels that this pathway is both engaged by taVNS and necessary for its dual behavioral effects, and further characterize the downstream molecular changes in the CeA that accompany taVNS-mediated recovery. These results provide a mechanistic basis for the therapeutic application of taVNS in depression and pain comorbidity.

## Results

### taVNS alleviates pain sensitization, depressive-like behaviors, and restores DRN–CeA functional connectivity in CCI model mice

CCI model mice were established and received 28 days of taVNS intervention starting at week 4 post-surgery (Fig. 1A–B). At 4W, both the CCI and taVNS groups exhibited significant mechanical allodynia (decreased PWT,  $P < 0.001$ ), behavioral despair (prolonged TST immobility, CCI  $P < 0.001$ , taVNS  $P < 0.01$ ), reduced exploratory motivation (decreased OFT center time,  $P < 0.01$ ), and anhedonia (decreased sucrose preference,  $P < 0.05$ ) compared with the Normal group, with no significant differences between the two groups, confirming stable comorbidity phenotypes prior to intervention. Following 28 days of taVNS, the taVNS group showed significantly increased PWT ( $P < 0.01$ ), reduced TST immobility ( $P < 0.001$ ), increased OFT center time ( $P < 0.01$ ), and restored sucrose preference ( $P < 0.05$ ) compared with the CCI group (Fig. 1C–F).

Resting-state fMRI revealed significant alterations in DRN–CeA functional connectivity across the three groups. In normal mice, DRN and CeA exhibited negative functional connectivity, indicating an inverse temporal relationship between the two regions at the network level. CCI induced a polarity reversal to positive correlation ( $P < 0.001$ ), indicating aberrant functional synchronization. taVNS reversed this abnormal connectivity ( $P < 0.05$ ), restoring it toward baseline levels (Fig. 1G–H). ROI-to-ROI analysis across 13 pain- and emotion-related brain regions further confirmed that DRN–CeA was among the most significantly affected connections, and identified additional alterations in prefrontal–striatal and hypothalamic–cortical connectivity (Supp. Fig. 1). These imaging findings identify DRN–CeA functional coupling as a key locus of network-level dysfunction in pain – depression comorbidity. Across CCI and taVNS-treated mice, more positive DRN–CeA connectivity was associated with poorer behavioral outcomes (Supp. Fig. 5A,

5E–G).

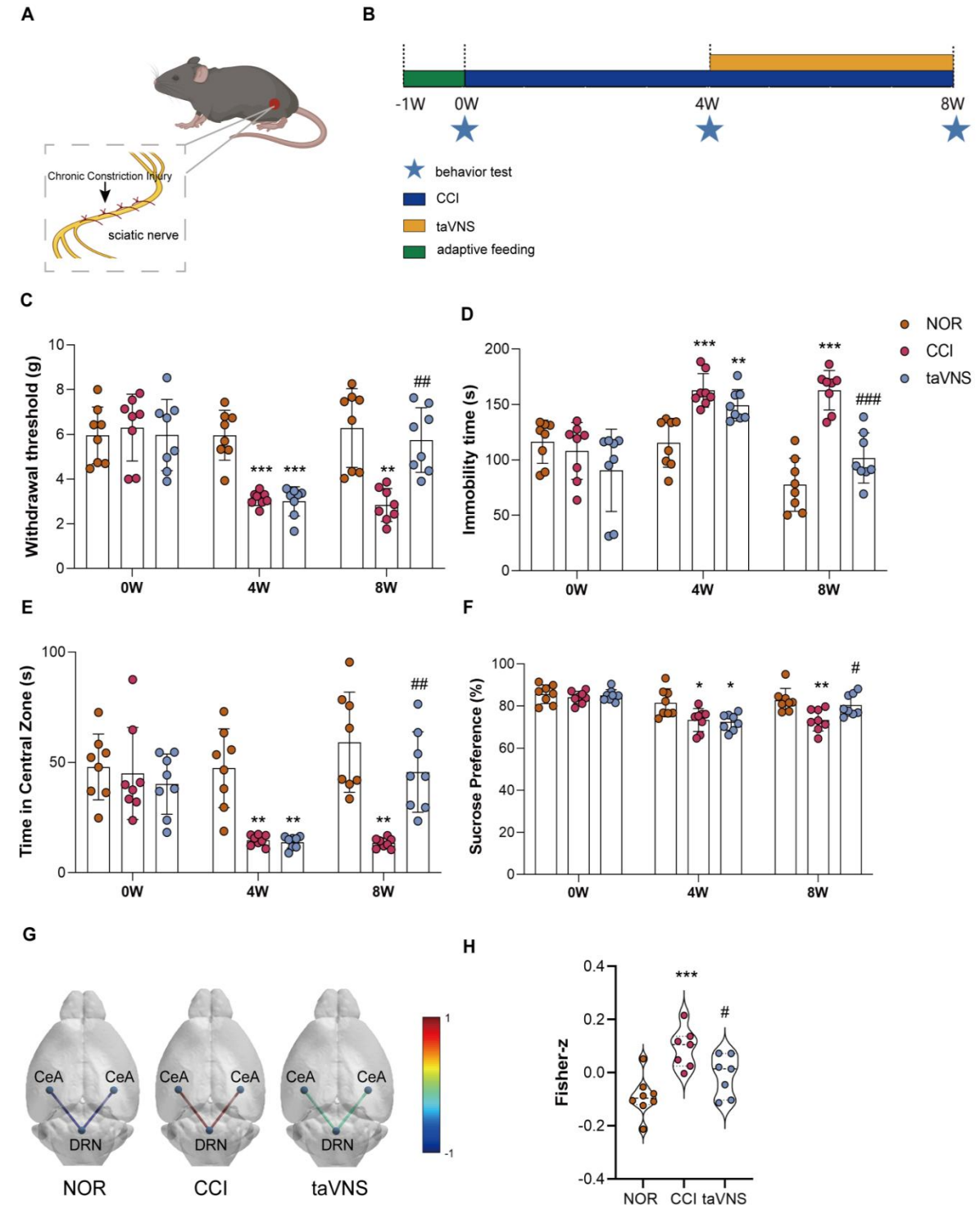


**Figure 1. taVNS alleviates pain sensitization and depression-related behavioral abnormalities and normalizes altered DRN–CeA functional connectivity in CCI mice.**

(A) Schematic of the chronic constriction injury (CCI) procedure performed on the sciatic nerve. (B) Experimental timeline. (C) Paw withdrawal threshold (PWT) measured at 0W, 4W, and 8W in the Normal, CCI, and taVNS groups. (D) Immobility

time in the tail suspension test (TST). (E) Time spent in the center zone in the open field test (OFT). (F) Sucrose preference in the sucrose preference test (SPT). Data are presented as mean  $\pm$  SD;  $n = 8$  per group.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  versus Normal;  $\#P < 0.05$ ,  $##P < 0.01$ ,  $###P < 0.001$  versus CCI. (G) Three-dimensional visualization of DRN–CeA resting-state functional connectivity in the three groups. Line color represents Fisher  $z$ -transformed correlation coefficients (color bar:  $-1$  to  $+1$ ). (H) Quantification of DRN–CeA functional connectivity (Fisher  $z$  values).  $P < 0.001$  versus Normal;  $\#P < 0.05$  versus CCI.

## **Auricular concha sensory afferents preferentially access the DRN through a vagal–NTS relay**

To map the central afferent pathway of taVNS, we employed dual retrograde tracing to compare the vagal and trigeminal pathways. Retro-rAAV-EGFP was injected into the NTS and retro-rAAV-mCherry into the auricular concha (Fig. 2A). In the JNG, a substantial population of neurons co-expressed both fluorescent markers, with approximately 50% of NTS-projecting (EGFP<sup>+</sup>) neurons also innervating the auricular concha (mCherry<sup>+</sup>), and vice versa (Fig. 2B–C). In a parallel experiment targeting the Sp5 (Fig. 2D), co-labeling in the JNG was markedly lower ( $\sim 10$ – $20\%$ ), despite the presence of EGFP-positive neurons in both Sp5 and TG (Fig. 2E–F). Notably, both experiments revealed co-labeled fluorescence in the TG, indicating that the auricular concha also receives minor trigeminal innervation; however, the predominant sensory input originates from the auricular branch of the vagus nerve. These results demonstrate that auricular concha sensory afferents are predominantly relayed through the vagal JNG–NTS pathway rather than the JNG–Sp5 pathway.

To further determine whether the NTS relays signals to the DRN, we performed H129 $\Delta$ TK-tdTomato-based anterograde transsynaptic tracing by co-injecting AAV-TK-GFP (helper) and H129 $\Delta$ TK-tdTomato into the NTS (Fig. 2G). tdTomato-positive neurons were detected in the DRN (Fig. 2H), confirming a direct NTS–DRN projection. Together with the retrograde tracing data, these findings establish the complete anatomical relay from the auricular concha through the JNG and NTS to the DRN, providing the structural basis for taVNS signal entry into the central serotonergic system.

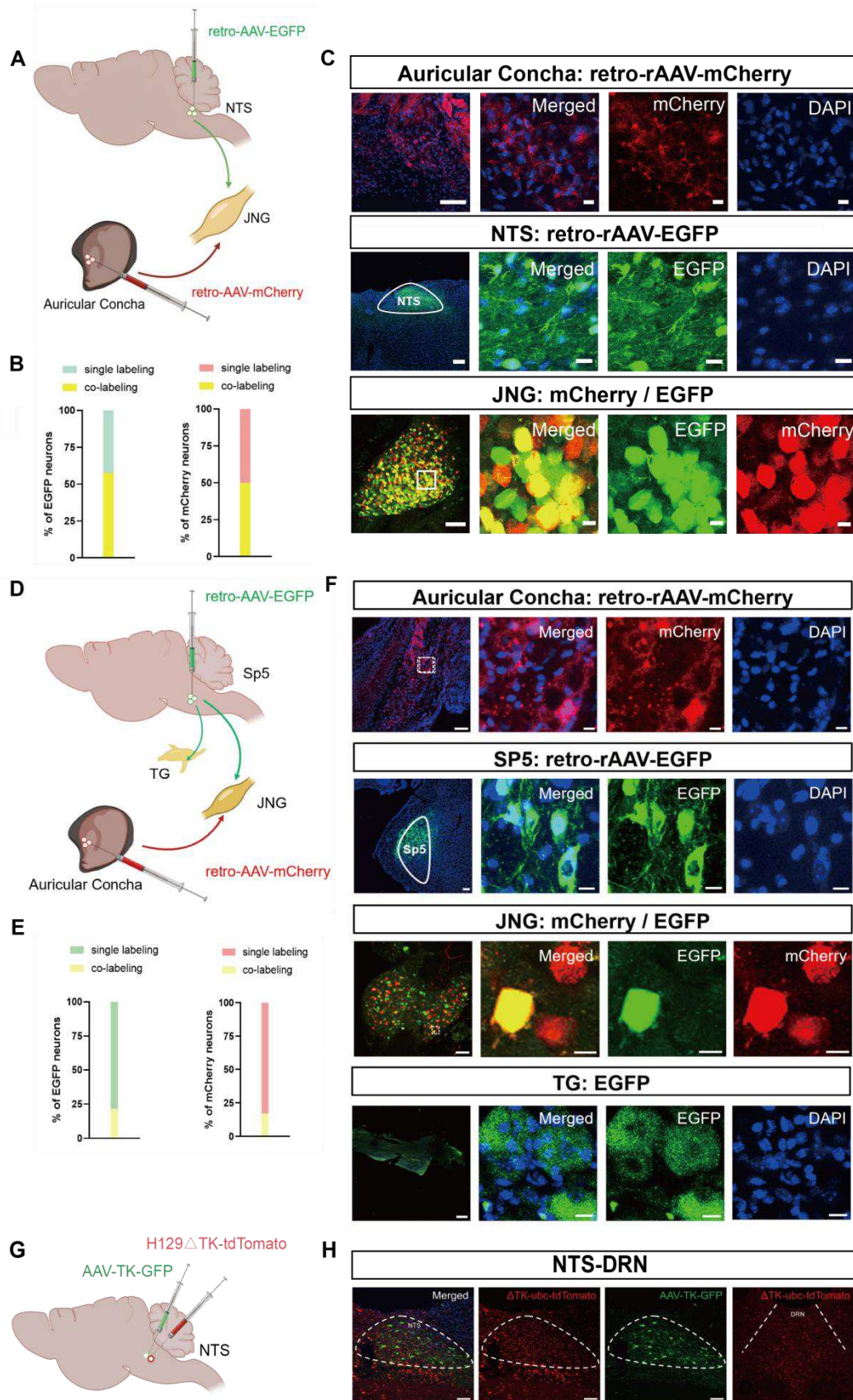


**Figure 2. Auricular concha sensory afferents preferentially access the DRN through a vagal–NTS relay.**

(A) Schematic of the dual retrograde tracing strategy for the vagal pathway. Retro-rAAV-EGFP (green) was injected into the NTS and retro-rAAV-mCherry (red) into the auricular concha; co-labeling was assessed in the JNG. (B) Quantification of co-labeling in the JNG for the NTS experiment. Left, percentage of EGFP<sup>+</sup> neurons co-expressing mCherry; right, percentage of mCherry<sup>+</sup> neurons co-expressing EGFP. (C) Representative fluorescence images showing mCherry expression in the auricular concha, EGFP expression in the NTS, and co-labeling in the JNG. (D) Schematic of the dual retrograde tracing strategy for the trigeminal pathway. Retro-rAAV-EGFP was injected into Sp5 and retro-rAAV-mCherry into the auricular concha; co-labeling was assessed in the JNG and TG. (E) Quantification of co-labeling in the JNG for the Sp5 experiment. (F) Representative fluorescence images showing mCherry labeling in the auricular concha, EGFP labeling in Sp5, co-labeling in the JNG, and EGFP labeling in the TG. (G) Schematic of H129ΔTK-tdTomato anterograde transsynaptic tracing from the NTS. AAV-TK-GFP (helper) and H129ΔTK-tdTomato were co-injected into the NTS. (H) Left, injection site verification showing co-expression of tdTomato (red) and GFP (green) in the NTS. Right, tdTomato-positive signals detected in the DRN. Scale bars are indicated in each panel.

#### **taVNS activates DRN serotonergic neurons and restores serotonergic marker expression in the DRN**

To verify whether taVNS functionally activates DRN serotonergic neurons, AAV-DIO-GCaMP6s or EGFP (control) was injected into the DRN of Pet1-Cre mice, and fiber photometry recordings were performed during taVNS (Fig. 3A). Post-hoc immunohistochemistry confirmed that approximately 90% of TPH2-immunoreactive neurons in the DRN co-expressed GCaMP6s, verifying the cell-type specificity of our recordings (Fig. 3E, F). Trial-by-trial heatmaps and group-averaged traces showed that taVNS evoked robust calcium responses in DRN 5-HT neurons in both Normal and CCI mice, with no response in EGFP controls (Fig. 3B, C). Notably, the taVNS-evoked calcium response was significantly greater in CCI mice than in Normal mice (AUC quantification, Fig. 3D), suggesting that DRN serotonergic neurons are more strongly recruited by taVNS under the pain – depression comorbidity state.

Changes in key serotonergic marker proteins in the DRN were further examined by Western blot. CCI significantly reduced the protein expression of both SERT (Fig. 3G) and TPH2 (Fig. 3H) in the DRN, and taVNS treatment significantly reversed these reductions, restoring SERT and TPH2 expression to near-Normal levels. The concurrent restoration of TPH2—the rate-limiting enzyme for 5-HT synthesis—and SERT—the principal 5-HT reuptake transporter—indicates that taVNS comprehensively enhances the synthetic and turnover capacity of DRN serotonergic neurons.

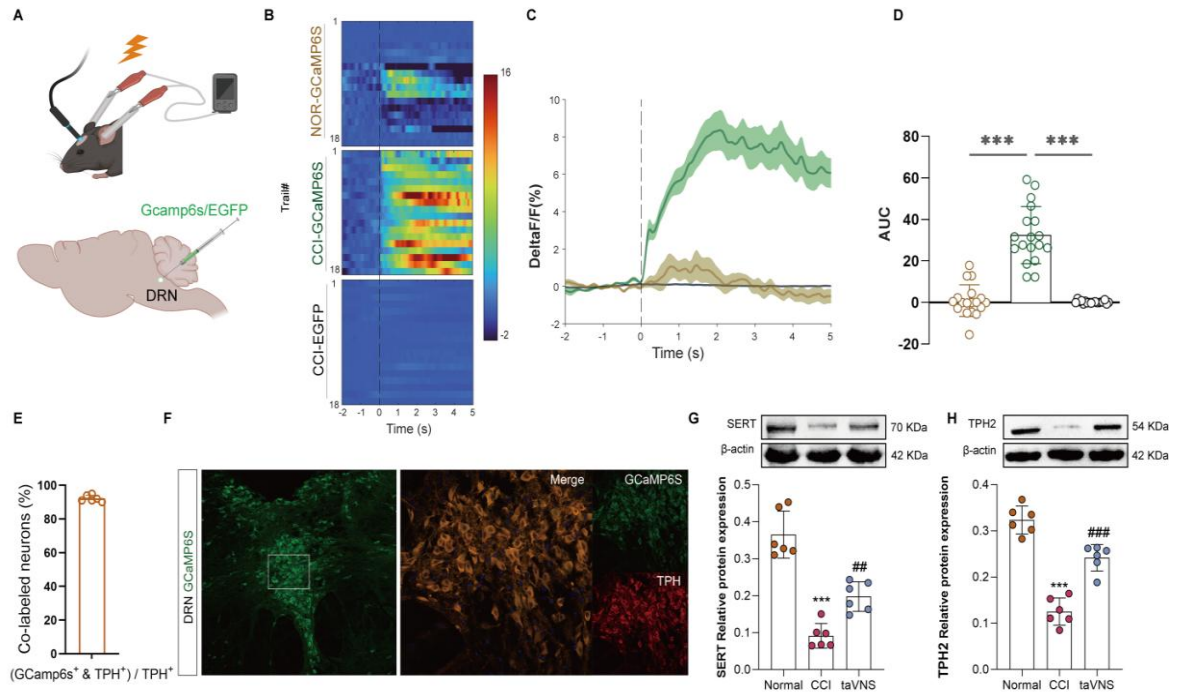


**Figure 3. taVNS activates DRN serotonergic neurons and restores serotonergic marker expression in the DRN.**

(A) Schematic of the experimental design. AAV-DIO-GCaMP6s or AAV-DIO-EGFP (control) was injected into the DRN of Pet1-Cre mice, and fiber photometry signals were recorded during taVNS. (B) Trial-by-trial heatmaps showing calcium responses of DRN 5-HT neurons during taVNS. Top, NOR-GCaMP6s; middle, CCI-GCaMP6s; bottom, CCI-EGFP. Time 0 (dashed line) indicates taVNS onset. Color scale indicates  $\Delta F/F$  (%). (C) Group-averaged  $\Delta F/F$  traces (mean  $\pm$  SD) of taVNS-evoked calcium responses. (D) Quantification of the area under the curve (AUC) of taVNS-evoked calcium responses.  $P < 0.001$ ;  $n = 6$  mice per group. (E) Quantification of GCaMP6s and TPH2 co-localization in the DRN, expressed as the percentage of TPH2-positive neurons co-expressing GCaMP6s.  $n = 6$  mice. (F) Representative immunofluorescence images of the DRN showing GCaMP6s expression and co-localization with TPH2. (G) Representative Western blot images and quantification of SERT protein expression in the DRN across Normal, CCI, and taVNS groups.  $\beta$ -Actin served as the loading control. (H) Representative Western blot images and quantification of TPH2 protein expression in the DRN. Data are presented as mean  $\pm$  SD. Statistical comparisons were performed using one-way ANOVA followed by Tukey's post hoc test.  $P < 0.001$  versus Normal;  $##P < 0.01$  or  $###P < 0.001$  versus CCI, as indicated.

### taVNS enhances CeA serotonin signaling and induces state-dependent modulation of CeA neuronal firing

WGA-FLP-based transsynaptic tracing in Pet1-Cre mice confirmed that DRN 5-HT neurons send direct monosynaptic projections to the CeA, as evidenced by

EGFP-positive fiber-like signals surrounding neuronal soma in the CeA (Supp. Fig. 2A–B).

To determine whether taVNS promotes 5-HT release in the CeA, the genetically encoded 5-HT fluorescent sensor GRAB-5-HT3.0 or its non-functional mutant (MUT, control) was injected into the CeA, and fluorescence signals were recorded during taVNS (Fig. 4A). Heatmaps and group-averaged traces showed that taVNS evoked significant increases in CeA 5-HT fluorescence in both Normal and CCI mice, with no response in MUT controls (Fig. 4A, B). Consistent with the DRN fiber photometry results (Fig. 3), the CeA 5-HT release response was significantly greater in CCI mice than in Normal mice (AUC quantification, Fig. 4C), further supporting enhanced recruitment of the DRN → CeA serotonergic pathway by taVNS under the comorbidity state.

To characterize the modulatory effects of taVNS on CeA neuronal activity, in vivo electrophysiological recordings were performed. Waveform analysis confirmed that the recorded neurons exhibited characteristics consistent with putative GABAergic interneurons (Supp. Fig. 2C–E). In Normal mice, taVNS predominantly produced excitatory responses: 94.0% of 50 recorded units showed increased firing rates, with only 6.0% showing decreases ( $P < 0.001$ ) (Fig. 4D–F). In CCI mice, the effect was reversed: 71.4% of 42 recorded units showed decreased firing rates, 26.2% showed increases, and 2.4% showed no change ( $P < 0.01$ ) (Fig. 4G–I), demonstrating that taVNS produces opposite net effects on CeA neurons depending on the pathological state. To verify whether this inhibitory effect depends on DRN serotonergic input, DRN serotonergic neurons were chemogenetically inhibited (hM4Di) in CCI mice before taVNS administration. Under this condition, taVNS-evoked responses shifted: 62.5% of 48 recorded units showed increased firing and 37.5% showed decreases, with no significant directional bias ( $P = 0.11$ ) (Fig. 4J–L), confirming that the inhibitory modulation of CeA by taVNS under the CCI state depends on DRN serotonergic input.

Whereas GRAB-5-HT3.0 photometry captures rapid, phasic 5-HT dynamics during acute stimulation, we further asked whether taVNS also produces a sustained biochemical restoration of 5-HT tone after a full course of treatment. To address this, in vivo microdialysis was performed in the CeA following the complete 28-day taVNS intervention, and dialysates were collected for HPLC quantification (Fig. 4M). Extracellular 5-HT levels were markedly reduced in CCI mice compared with Normal controls and were restored to Normal levels after long-term taVNS (Fig. 4O). Across CCI and taVNS-treated mice, higher CeA 5-HT was associated with better behavioral outcomes (Supp. Fig. 5A, 5B–D). In contrast, levels of the 5-HT metabolite 5-HIAA showed no significant differences among groups (Fig. 4N), indicating that the cumulative taVNS effect is specifically attributable to enhanced 5-HT availability rather than altered bulk 5-HT turnover. Together with the acute sensor recordings, these results establish that taVNS engages the DRN → CeA serotonergic pathway

333 across both acute and sustained time scales to replenish CeA 5-HT tone under the  
 334 pain–depression comorbidity state.

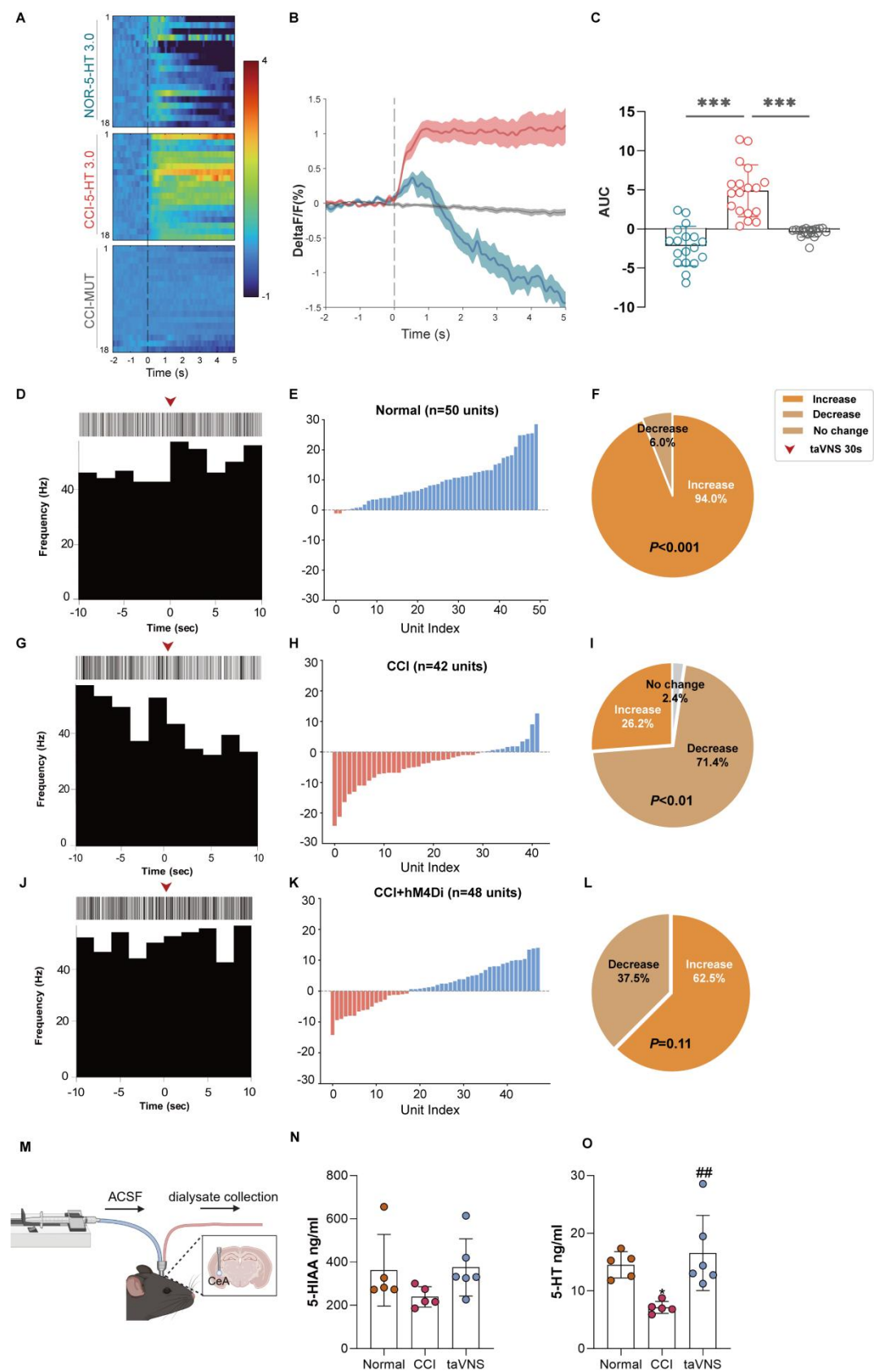


**Figure 4. taVNS enhances CeA serotonin signaling and induces state-dependent modulation of CeA neuronal firing.**

(A) Heatmaps showing trial-by-trial CeA serotonin sensor responses during taVNS after expression of GRAB-5-HT3.0 or mutant control sensor in the CeA. Top, Normal-5-HT3.0 group; middle, CCI-5-HT3.0 group; bottom, CCI-MUT group. Time 0 indicates taVNS onset. (B) Group-averaged  $\Delta F/F$  traces (mean  $\pm$  SD) of taVNS-evoked CeA serotonin sensor responses. (C) Quantification of the area under the curve (AUC) of CeA serotonin sensor responses.  $P < 0.001$ ;  $n = 6$  mice per group. (D) Representative CeA neuronal firing response to taVNS in a Normal mouse. Top, spike raster; bottom, time–frequency plot. Red arrowhead indicates taVNS onset. (E) taVNS-evoked firing rate changes ( $\Delta$ Hz) for all recorded units in the Normal group, ranked by magnitude.  $n = 50$  units. (F) Proportional distribution of CeA unit response directions in the Normal group. (G) Representative CeA neuronal firing response to taVNS in a CCI mouse. (H) taVNS-evoked firing rate changes ( $\Delta$ Hz) for all recorded units in the CCI group, ranked by magnitude.  $n = 42$  units. (I) Proportional distribution of CeA unit response directions in the CCI group. (J) Representative CeA neuronal firing response to taVNS in a CCI+hM4Di mouse. (K) taVNS-evoked firing rate changes ( $\Delta$ Hz) for all recorded units in the CCI+hM4Di group, ranked by magnitude.  $n = 48$  units. (L) Proportional distribution of CeA unit response directions in the CCI+hM4Di group. (M) Schematic of in vivo microdialysis in the CeA. ACSF was perfused through a microdialysis probe implanted in the CeA, and dialysates were collected after completion of the 28-day taVNS intervention for HPLC quantification of 5-HT and 5-HIAA. (N) Extracellular 5-HIAA concentrations in the CeA across Normal, CCI, and taVNS groups. (O) Extracellular 5-HT concentrations in the CeA across Normal, CCI, and taVNS groups. Data are presented as mean  $\pm$  SD. Group comparisons of serotonin sensor signals and microdialysis measurements were performed using one-way ANOVA followed by Tukey's post hoc test; directionality of electrophysiological responses was assessed by binomial test.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ .

**Disruption of DRN serotonergic signaling or CeA 5-HT1A receptors attenuates the behavioral effects of taVNS**

To causally validate the necessity of the DRN 5-HT–CeA pathway in mediating the therapeutic effects of taVNS, three independent loss-of-function strategies were employed: chemogenetic inhibition of DRN serotonergic projections to the CeA, pharmacological suppression of 5-HT synthesis in the DRN, and blockade of 5-HT1A receptors in the CeA.

In the chemogenetic experiment (Fig. 5A), at 4W post-CCI, the taVNS, taVNS-hM4Di, and taVNS-mCherry groups exhibited comparable behavioral phenotypes across all measures. Following 28 days of taVNS with concurrent CeA-targeted CNO delivery, both the taVNS and taVNS-mCherry groups showed significant behavioral recovery at 8W. In contrast, the taVNS-hM4Di group exhibited significantly lower PWT ( $P < 0.001$ ), reduced OFT center time ( $P < 0.01$ ), prolonged

378 TST immobility ( $P < 0.001$ ), and decreased sucrose preference ( $P < 0.05$ ) compared  
379 with the taVNS group (Fig. 5B–E). The taVNS-mCherry group showed no significant  
380 difference from the taVNS group, confirming that viral infection itself did not affect  
381 the results.

382 In the pharmacological experiments, DRN microinjection of the 5-HT synthesis  
383 inhibitor PCPA (Fig. 5F) abolished the therapeutic effects of taVNS: the DRN-PCPA  
384 group showed significantly lower PWT ( $P < 0.01$ ), reduced OFT center time ( $P <$   
385  $0.001$ ), prolonged TST immobility ( $P < 0.01$ ), and decreased sucrose preference ( $P <$   
386  $0.05$ ) compared with the DRN-ACSF group at 8W (Fig. 5G–J). Similarly, CeA  
387 microinjection of the 5-HT<sub>1A</sub> antagonist WAY-100635 (Fig. 5K) blocked all taVNS  
388 effects: the CeA-WAY group exhibited significantly lower PWT ( $P < 0.01$ ), reduced  
389 OFT center time ( $P < 0.001$ ), prolonged TST immobility ( $P < 0.001$ ), and decreased  
390 sucrose preference ( $P < 0.05$ ) compared with the CeA-ACSF group (Fig. 5L–O).  
391 These converging loss-of-function results demonstrate that the DRN serotonergic  
392 input and CeA 5-HT<sub>1A</sub> receptor activation are both necessary for the analgesic and  
393 antidepressant effects of taVNS.

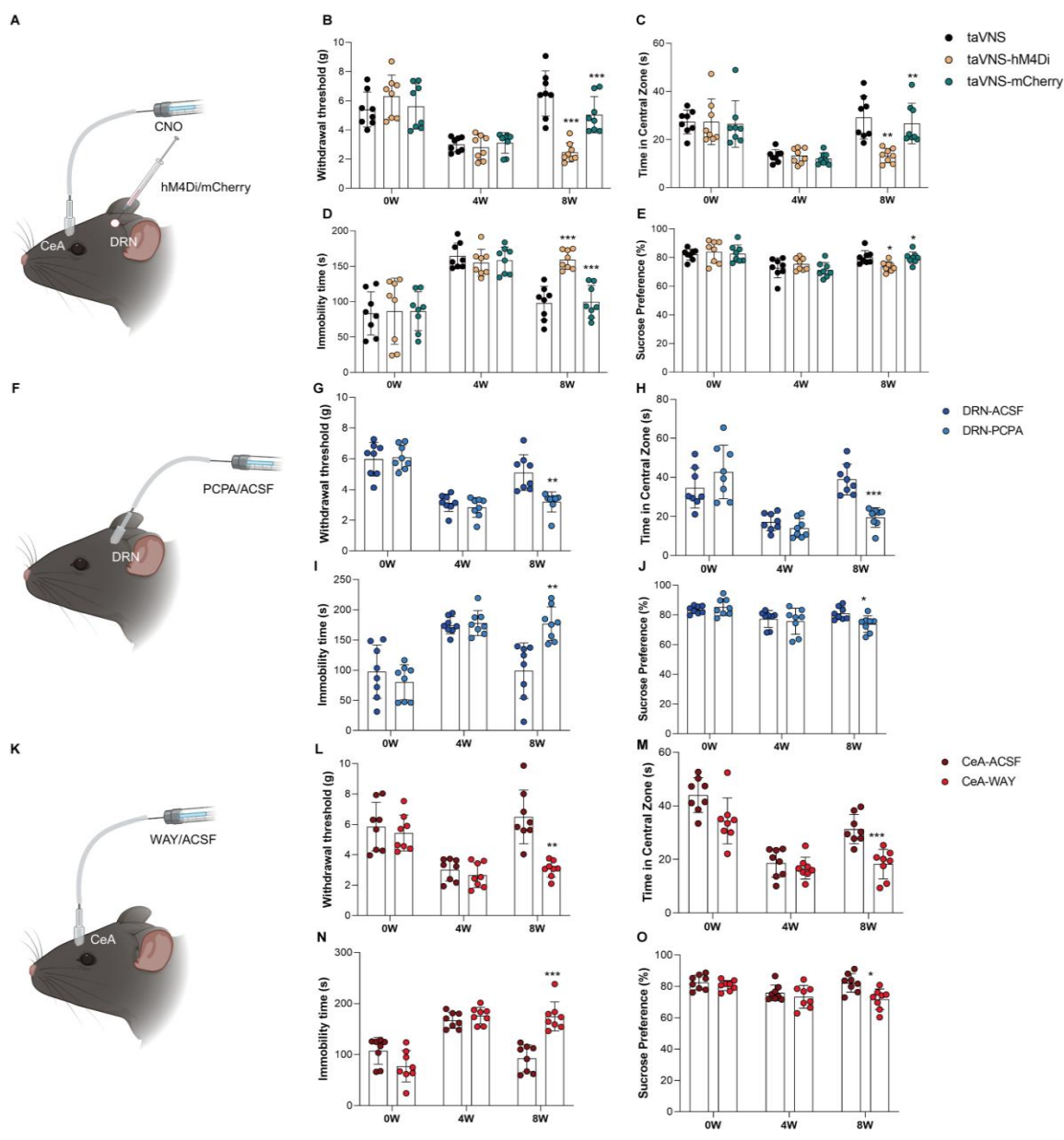


**Figure 5. Disruption of DRN serotonergic signaling or CeA 5-HT1A receptors attenuates the behavioral effects of taVNS.**

(A) Schematic of the chemogenetic experiment. AAV-DIO-hM4Di-mCherry or AAV-DIO-mCherry was injected into the DRN of Pet1-Cre mice, and a cannula was implanted above the CeA for local CNO administration. (B) Paw withdrawal threshold (PWT) across 0W, 4W, and 8W in the taVNS, taVNS-hM4Di, and taVNS-mCherry groups. (C) OFT center time. (D) TST immobility time. (E) Sucrose preference in the SPT. (F) Schematic of the DRN-PCPA experiment. PCPA or ACSF was microinjected into the DRN before each taVNS session. (G) PWT in the DRN-ACSF and DRN-PCPA groups. (H) OFT center time. (I) TST immobility time. (J) Sucrose preference. (K) Schematic of the CeA-WAY-100635 experiment. WAY-100635 or ACSF was microinjected into the CeA before each taVNS session. (L) PWT in the CeA-ACSF and CeA-WAY groups. (M) OFT center time. (N) TST

immobility time. (O) Sucrose preference. Data are presented as mean  $\pm$  SD;  $n = 8$  per group. Statistical comparisons were performed using two-way repeated-measures ANOVA followed by Tukey's post hoc test.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$ , as indicated in the panels.

### **Quantitative proteomics identifies a taVNS-reversible inflammation-associated molecular signature in the CeA**

To characterize the molecular landscape of CeA under pain – depression comorbidity and taVNS intervention, we performed quantitative proteomics on CeA tissue from Normal, CCI, and taVNS groups ( $n = 6$  per group). PCA revealed distinct separation among the three groups, with the taVNS group occupying a unique molecular space rather than reverting to the Normal cluster (Fig. 6A). Differential expression analysis identified 466 DEPs between CCI and Normal (350 upregulated, 116 downregulated) and 762 DEPs between taVNS and CCI (271 upregulated, 491 downregulated) (Fig. 6B–C). Four-quadrant directional analysis of proteins differentially expressed in both comparisons revealed that the majority (164/194, 84.5%) exhibited a reversal pattern, including 125 reversed-down and 39 reversed-up proteins, whereas only 30 showed consistent directional changes (Fig. 6D), indicating that taVNS predominantly reverses rather than compounds CCI-induced proteomic alterations.

GO and KEGG enrichment analyses of the 125 reversed-down proteins consistently identified neuroinflammation-related pathways, including immune response, classical complement pathway activation, defense response, and antigen binding (Fig. 6E; Supp. Fig. 3A). PPI network analysis of the 125 reversed-down proteins revealed a multi-modular topology comprising 63 interacting proteins and 103 edges (Supp. Fig. 3C). The network core was dominated by a neuroimmune inflammation hub containing acute-phase proteins (Ahsg, Anxa2, Anxa1), immune adhesion molecules (Icam1), and complement/coagulation components (C7, C8b), confirming neuroinflammation as the central pathological program established by CCI in the CeA. Additional modules included a BBB/vascular permeability cluster (Slc4a1, Spta1), an ECM remodeling module (Bgn, Fmod), antigen presentation (H2-Q10, Tapbp, Rfxap), and gap junction components (Gjb1/2/6). Beyond reversal of CCI-induced changes, 568 proteins were differentially expressed exclusively in the taVNS versus CCI comparison, enriched in ECM remodeling and TGF- $\beta$  signaling (Supp. Fig. 3B), suggesting that taVNS actively initiates structural remodeling programs beyond simple pathology reversal.

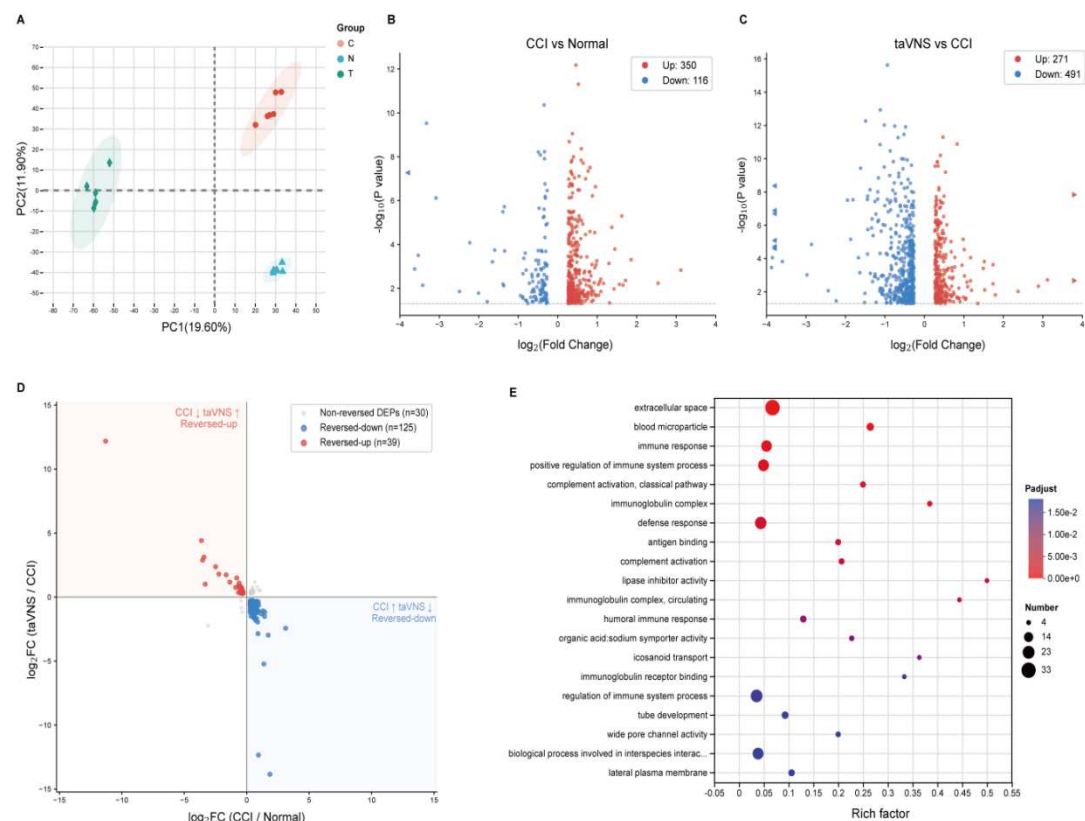


**Figure 6. Quantitative proteomics identifies a taVNS-reversible inflammation-associated molecular signature in the CeA.**

(A) PCA of proteomic profiles from the Normal (N), CCI (C), and taVNS (T) groups ( $n = 6$  per group). (B) Volcano plot of differentially expressed proteins (DEPs) for CCI versus Normal (350 upregulated, 116 downregulated). (C) Volcano plot of DEPs for taVNS versus CCI (271 upregulated, 491 downregulated). (D) Four-quadrant directional analysis of proteins differentially expressed in both comparisons. X-axis,  $\log_2FC$  (CCI/Normal); Y-axis,  $\log_2FC$  (taVNS/CCI). Blue, reversed-down; red, reversed-up; gray, non-reversed. (E) GO enrichment analysis of the 125 reversed-down proteins. Bubble size represents gene count, and color indicates adjusted  $P$  value.

### Phosphoproteomics and KSEA identify cAMP/PKA-associated synaptic phosphorylation remodeling in the CeA

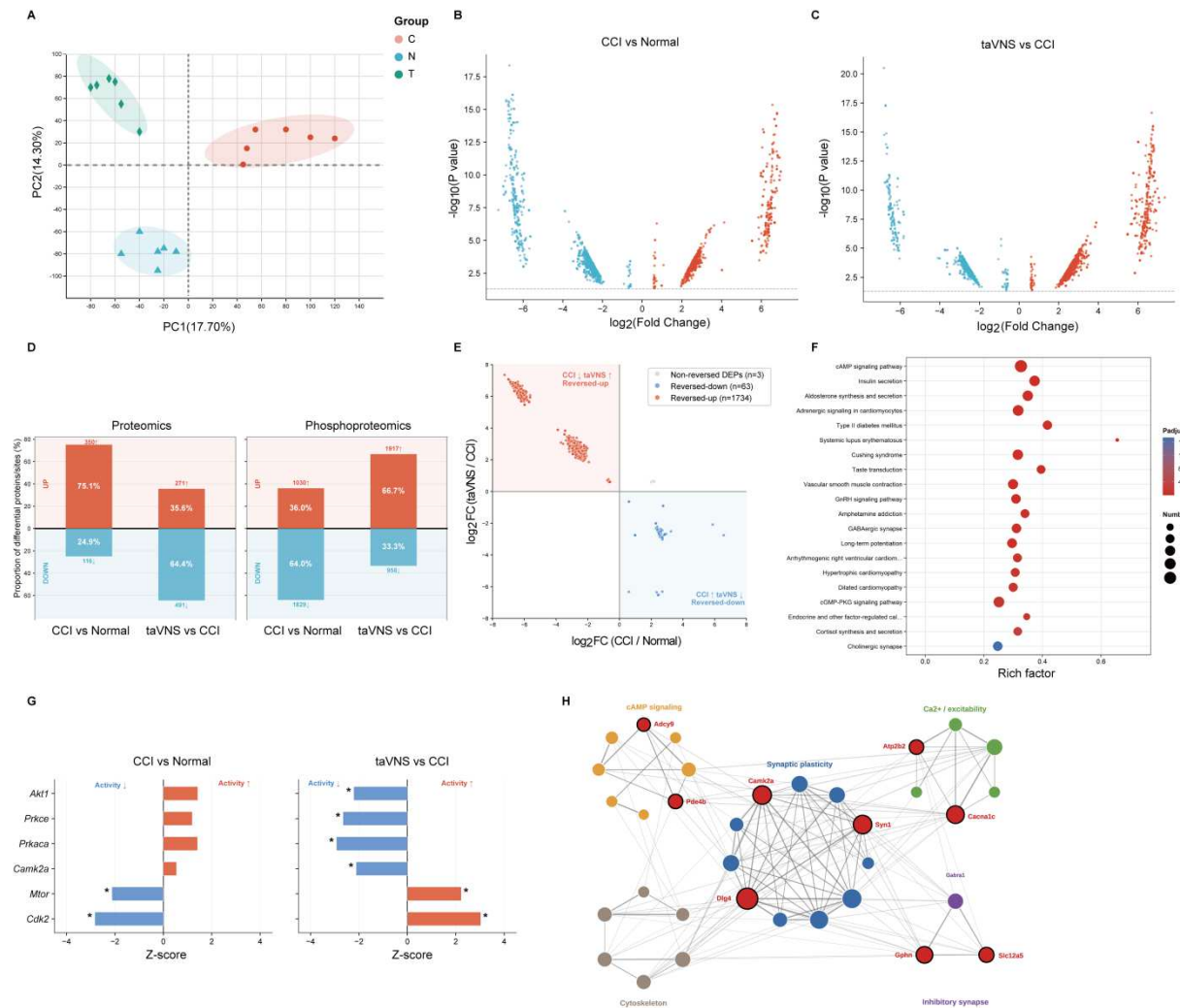
To explore the molecular changes underlying taVNS-induced normalization of CeA function, we performed quantitative phosphoproteomic profiling of the CeA across the Normal, CCI, and taVNS groups. Principal-component analysis demonstrated clear separation among the three groups (Fig. 7A). Differential analysis identified 2,859 phosphosites between CCI and Normal, including 1,030 upregulated and 1,829 downregulated sites, and 2,875 phosphosites between taVNS and CCI, including 1,917 upregulated and 958 downregulated sites (Fig. 7B–C). Notably, the phosphoproteome exhibited a directional pattern complementary to the proteome: whereas CCI predominantly increased protein abundance (75.1%), it predominantly

decreased phosphorylation (64.0%); conversely, taVNS reversed both trends (Fig. 7D). These results suggest that CCI and taVNS engage opposing molecular remodeling programs across both protein abundance and phosphorylation levels in the CeA.

Directional analysis of phosphosites differentially expressed in both comparisons revealed a marked asymmetric reversal pattern: 1,734 sites were classified as reversed-up, compared with 63 reversed-down sites and 3 non-reversed sites (Fig. 7E), indicating that taVNS primarily restored phosphosites suppressed under CCI. KEGG and GO enrichment analyses of the 1,734 reversed-up sites showed significant enrichment of pathways related to cAMP signaling, GABAergic synapse, long-term potentiation, and cGMP-PKG signaling (Fig. 7F). Of note, GABAergic synapse was enriched at the phosphorylation level but not at the protein abundance level, suggesting that taVNS may regulate CeA inhibitory synaptic function primarily through phosphorylation remodeling rather than through changes in protein abundance. Beyond reversal of CCI-induced changes, approximately 1,075 phosphosites were differentially expressed exclusively in the taVNS versus CCI comparison and were enriched in dendritic spine morphogenesis, synapse protein localization, and TGF- $\beta$  signaling (Supp.Fig. 4A–B).

To examine upstream kinase activities associated with these phosphoproteomic changes, we performed kinase–substrate enrichment analysis (KSEA). KSEA revealed CCI-induced alterations in several kinase activities, including Prkaca/PKA, Prkce/PKC $\epsilon$ , Camk2a/CaMKII $\alpha$ , and Akt1, which were reversed or shifted toward the Normal pattern by taVNS (Fig. 7G). Mtor and Cdk2 activities showed the opposite pattern. Together, the pathway- and kinase-level analyses suggest that taVNS normalizes a CCI-induced multi-kinase phosphorylation program in the CeA centered on cAMP/PKA-associated signaling and related synaptic kinases, rather than acting through a single kinase.

To further organize these pathway- and kinase-level findings, we mapped representative reversed-up phosphoproteins onto a STRING protein–protein interaction network (Fig. 7H). The affected phosphoproteins clustered into functional modules related to synaptic plasticity and scaffolding, cAMP signaling and turnover, Ca<sup>2+</sup> handling and excitability, and inhibitory-synapse organization, represented by Dlg4/PSD-95, Camk2a, Syn1, Adcy9, Pde4b, Cacna1c, Atp2b2, Gphn, Gabra1, and Slc12a5/KCC2. Together, these findings suggest that taVNS restores a functionally connected synaptic phosphorylation program in the CeA.



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**Figure 7. Phosphoproteomics and KSEA identify cAMP signaling and kinase remodeling associated with taVNS in the CeA.**

(A) PCA of phosphoproteomic profiles from the Normal (N), CCI (C), and taVNS (T) groups ( $n = 6$  per group). (B) Volcano plot of differentially expressed phosphosites for CCI versus Normal (1,030 upregulated, 1,829 downregulated). (C) Volcano plot for taVNS versus CCI (1,917 upregulated, 958 downregulated). (D) Comparison of the directional patterns observed in the proteomic and phosphoproteomic datasets. (E) Four-quadrant directional analysis of phosphosites differentially expressed in both comparisons. Red, reversed-up; blue, reversed-down; gray, non-reversed. (F) KEGG enrichment analysis of the 1,734 reversed-up phosphosites. Bubble size represents phosphosite count, and color indicates adjusted  $P$  value. (G) KSEA kinase activity analysis comparing the CCI state and the taVNS treatment effect. Z-scores were combined across mouse and human substrate annotations using Stouffer's weighted method.  $P < 0.05$ . (H) STRING protein-protein interaction network of representative reversed-up phosphoproteins (*Mus musculus*; interaction score  $\geq 0.4$ ), grouped by functional module: synaptic plasticity (blue), cAMP signaling (orange), Ca<sup>2+</sup>/excitability (green), inhibitory synapse (purple), and cytoskeleton (gray). Node

size indicates degree; edge thickness indicates combined interaction score. Anchor nodes are highlighted in red (Adcy9, Pde4b; Camk2a, Dlg4/PSD-95, Syn1; Cacna1c, Atp2b2; Gphn, Slc12a5/KCC2).

## Discussion

Chronic pain and depression are highly comorbid, with approximately 40% of chronic pain patients experiencing concurrent depressive symptoms[1,2]. Patients with this comorbidity report significantly fewer functional benefits from standard antidepressant pharmacotherapy compared to those without chronic pain, underscoring the need for novel therapeutic strategies. taVNS, as a non-invasive neuromodulation approach, has shown therapeutic potential for both pain and depression in clinical trials[10–12], yet the central mechanisms driving its dual efficacy have remained incompletely defined.

Pain–depression comorbidity is increasingly understood as a distributed network disorder rather than the consequence of dysfunction in a single brain region. Human neuroimaging and animal circuit studies have implicated the amygdala, prefrontal cortex, anterior cingulate cortex, nucleus accumbens, hippocampus, thalamic nuclei, and brainstem modulatory systems in the shared sensory, affective, motivational, and autonomic dimensions of chronic pain and depression[28,29]. Chronic neuropathic pain and depression frequently co-occur and are increasingly understood to involve overlapping neural circuit mechanisms[30]. Recent circuit-level studies have identified both protective and maladaptive pathways in this comorbidity. Protective or alleviating mechanisms include CeA enkephalinergic interneurons[31], the CeA–vIPAG pathway, and DRN serotonergic projections to SOM-positive CeA neurons, whereas maladaptive or exacerbating circuits include the BLA→ACC pathway, LC→rACC noradrenergic projection, and GABAergic CeA input to PF glutamatergic neurons[25,27,32–34]. As a peripheral intervention, taVNS raises a specific question: where within this distributed network do its antinociceptive and antidepressant actions converge? The CeA is well positioned to serve such an integrative role, receiving convergent nociceptive, affective, and neuromodulatory inputs. Our findings implicate the DRN–CeA serotonergic projection as one such convergence point, through which auricular stimulation can access pain- and affect-related limbic processing.

Our findings also build on prior work defining the central mechanisms of auricular vagal stimulation. Previous studies have shown that auricular or vagal stimulation can produce analgesic effects and engage brainstem-limbic circuits. Recent circuit-level work identified an auricular afferent route involving JNG and NTS neurons that recruits downstream vIPAG-projecting analgesic pathways[17], while other studies have implicated DRN serotonergic mechanisms in taVNS-induced analgesia[35]. However, these findings have largely been confined to single disease contexts or single levels of analysis, and how these elements are integrated within pain – depression comorbidity remains to be clarified.

The present study provides convergent multi-modal evidence for a peripheral-to-central serotonergic signaling pathway through which taVNS alleviates pain – depression comorbidity—auricular concha → JNG → NTS → DRN → CeA—and implicates CeA 5-HT<sub>1A</sub> receptor signaling and its downstream Gi → cAMP → PKA axis as a central candidate mechanism. Together, these findings connect auricular stimulation to DRN–CeA serotonergic recruitment, CeA neuronal response remodeling, and downstream molecular reprogramming within the same pain–depression comorbidity model.

Using dual retrograde tracing, we found that the auricular concha → NTS pathway (~50% JNG co-labeling) substantially predominates over the auricular concha → Sp5 pathway (~10–20%), consistent with recent comprehensive mapping of the auricular vagal afferent circuit[17]. Anterograde tracing with the transsynaptic tracer H129 further revealed NTS → DRN projections, providing the structural substrate for taVNS signals to access the central serotonergic system. Notably, the NTS occupies a unique position in vagal afferent processing: as the primary central recipient of vagal sensory input, it distributes signals to functionally diverse downstream targets. The recent demonstration that NTS<sup>POMC</sup> neurons project to the vIPAG to mediate descending analgesia[17], together with our evidence for NTS → DRN connectivity, raises the possibility that the NTS functions as a distribution hub from which taVNS engages parallel effector circuits. Whether distinct NTS neuronal subpopulations preferentially route signals to the vIPAG versus the DRN—or whether overlapping populations project to both—remains an important question for future cell-type-specific intersectional studies.

At the functional level, fiber photometry, a genetically encoded 5-HT sensor, CeA microdialysis, and Western blot collectively demonstrated that taVNS activates DRN 5-HT neurons, enhances CeA 5-HT release, restores basal CeA 5-HT tone, and upregulates DRN TPH2 and SERT expression. Two features of these findings merit discussion. First, the significantly stronger DRN and CeA responses under CCI comorbidity than under normal conditions indicate state-dependent recruitment—taVNS does not uniformly drive the serotonergic system, but preferentially engages it when the system is in a pathologically suppressed state. This is reminiscent of the "state-dependent efficacy" well-documented for other neuromodulation approaches. Deep brain stimulation is most effective when targeting dysfunctional circuits underlying specific pathological states[36], and repetitive transcranial magnetic stimulation—where state-dependency is a well-characterized conceptual framework—produces effects whose direction and magnitude depend on the baseline activation state of the targeted cortex[37]. Such state-dependence may also explain why taVNS produces robust clinical effects in patients with depression or pain despite inconsistent neurophysiological and behavioral responses in healthy volunteers[38]. Second, the concurrent upregulation of TPH2 and SERT in the DRN after 28-day taVNS suggests long-term restructuring of the serotonergic system beyond acute activation. The direction of SERT change is opposite to the SSRI strategy, which acutely blocks SERT to increase synaptic 5-HT. However, this

apparent paradox dissolves when taVNS and SSRIs are understood as acting through fundamentally different mechanisms: SSRIs increase 5-HT availability by preventing reuptake at the terminal, whereas taVNS appears to enhance the overall tone and functional capacity of the 5-HT system, as evidenced by concurrent upregulation of synthesis and transporter machinery at the source (DRN TPH2, SERT) and restoration of terminal serotonergic tone at the target (CeA extracellular 5-HT). This pattern is reminiscent of sustained invasive VNS, which progressively increases DRN 5-HT neuronal firing and enhances serotonergic transmission[39,40], and of regular exercise, which similarly recruits DRN 5-HT neurons and promotes serotonergic adaptations[41]. This "capacity-building" mode of action may carry therapeutic advantages over the "blockade" strategy, particularly for comorbid conditions requiring sustained multi-target modulation. The causal necessity of DRN serotonergic signaling was confirmed by two independent manipulations: Intra-DRN microinjection of PCPA to locally deplete 5-HT synthesis, and projection-specific chemogenetic silencing of DRN 5-HT neurons projecting to the CeA (hM4Di), each of which abolished all four behavioral indicators of taVNS efficacy (PWT, TST, OFT, SPT). Consistent with our findings, Shin et al.[35] recently demonstrated that taVNS induces analgesia in a partial sciatic nerve ligation model through DRN serotonergic mechanisms, providing independent support for the centrality of the DRN serotonergic system in mediating taVNS effects.

In vivo electrophysiology revealed that taVNS shifted CeA neuronal responses from predominantly excitatory (94% in Normal) to predominantly inhibitory (71.4% in CCI), and that this inhibitory effect was abolished by chemogenetic silencing of DRN 5-HT neurons. This state-dependent flip is particularly striking and warrants mechanistic interpretation. In Normal mice, where the DRN–CeA serotonergic tone is intact, taVNS-evoked 5-HT release likely acts on multiple receptor subtypes including excitatory 5-HT<sub>2A</sub> receptors on Crh<sup>+</sup> neurons, producing a net excitatory response. Under CCI, however, chronic alterations in serotonergic tone may reshape the functional balance of 5-HT receptor subtypes, a form of plasticity well-documented for the 5-HT<sub>1A</sub> receptor in response to sustained changes in 5-HT signaling and stress[42,43]. When taVNS restores 5-HT release in this remodeled state, the inhibitory 5-HT<sub>1A</sub>-mediated component may predominate, consistent with the pharmacological data showing that CeA WAY-100635 abolished taVNS-induced behavioral effects specifically in the CCI condition. Given that the CeA is predominantly GABAergic[24], the recorded population activity likely mainly reflects modulation of GABAergic neurons, although cell-type-specific confirmation will require future studies using Cre-dependent optrode strategies. Consistent with this, phosphoproteomics revealed that GABAergic synapse was enriched among reversed phosphosites at the phosphorylation but not protein expression level, suggesting that taVNS regulation of CeA GABAergic function operates primarily through post-translational modifications rather than changes in protein abundance—a distinction that may have implications for the temporal dynamics and reversibility of taVNS effects.

Local CeA injection of WAY-100635 abolished all behavioral effects of taVNS, pinpointing 5-HT1A as the effector receptor and providing the third independent loss-of-function validation. Together with PCPA-mediated 5-HT depletion and projection-specific hM4Di silencing, three mechanistically distinct manipulations converged on the same behavioral outcome, providing strong evidence for the causal necessity of the DRN 5-HT → CeA 5-HT1A signaling axis. Notably, disruption at any single point along this axis was sufficient to abolish the full spectrum of taVNS effects across both pain and depression domains. This suggests that the DRN → CeA serotonergic circuit functions as a bottleneck node in the taVNS effector network for pain – depression comorbidity—disruption at this single node collapses the entire downstream cascade, even though taVNS likely engages additional circuits in parallel. This bottleneck property is consistent with the broader network neuroscience concept that complex behavioral outputs can depend on a small number of highly connected hubs, whose disruption produces disproportionate effects relative to their anatomical scope[44].

The receptor-level specificity of 5-HT1A should be understood within the existing literature. Zhou et al.[25] showed that DRN 5-HT inhibits CeA SOM<sup>+</sup> neurons via 5-HT1A, that chronic pain weakens this inhibition to drive depressive-like behavior, and that 5-HT1A activation—but not SSRI paroxetine—reverses the depressive phenotype. Wang et al.[26] reported a complementary mechanism: acute stress-induced analgesia operates through 5-HT2A on CeA Crh<sup>+</sup> neurons, with 5-HT1A blockade ineffective. Thus, the same DRN → CeA projection engages distinct receptor subtypes on different cell populations to regulate depression (5-HT1A/SOM<sup>+</sup>) and acute analgesia (5-HT2A/Crh<sup>+</sup>) separately. Our findings extend this framework by suggesting that, in the context of taVNS-treated pain – depression comorbidity, CeA 5-HT1A signaling contributes not only to antidepressant-like effects but also to analgesic outcomes. This dual role of 5-HT1A may reflect the unique pharmacological profile of this receptor: it is the only known 5-HT receptor subtype that simultaneously mediates analgesic, antidepressant, and anxiolytic effects[45]. The recently developed functionally selective 5-HT1A Gi agonist ST171 demonstrates potent opioid-independent analgesia in acute and chronic pain models[46], further supporting 5-HT1A–Gi signaling as a convergence point for pain and mood regulation. The downstream signaling divergence—5-HT1A coupling to Gi/cAMP–PKA inhibition versus 5-HT2A coupling to Gq/PLC–IP3 activation[47]—is consistent with the phosphoproteomic data in this study, suggesting that the receptor subtype engaged by taVNS may dictate not only the direction of neuronal modulation but also the intracellular signaling architecture of the therapeutic response.

Proteomic and phosphoproteomic analyses extended the circuit findings to the molecular level and revealed an unexpected degree of molecular reorganization in the CeA. At the protein level, CCI established a neuroinflammatory program—centered on immune response, complement activation, and defense response—that was significantly reversed by taVNS. The prominence of neuroinflammation in the CeA

proteome is consistent with a recent human post-mortem study showing that chronic pain-associated gene expression changes prominently involve amygdalar microglial immune pathways[48], suggesting that the neuroinflammatory signature we observed in mice may have translational relevance. How enhanced serotonergic signaling leads to neuroinflammatory suppression is not resolved by our data, which do not identify a single causal cellular source. One plausible candidate mechanism involves microglial modulation: microglia express functional 5-HT receptors, and their polarization from pro-inflammatory to homeostatic states is modulated by serotonergic signaling[49]. Enhanced CeA 5-HT following taVNS may thus shift microglial phenotypes, reducing pro-inflammatory cytokine production, although direct demonstration in this model will require microglia-specific loss-of-function experiments. Additionally, taVNS-specific differentially expressed proteins enriched in ECM remodeling and TGF- $\beta$  pathways suggest that taVNS does not merely reverse pathology but actively initiates structural remodeling, potentially reflecting microglia-mediated perisynaptic ECM remodeling that facilitates synaptic plasticity[50]. At the phosphorylation level, a mirror complementary pattern emerged: CCI predominantly upregulated protein expression (75.1%) but downregulated phosphorylation (64.0%), with taVNS reversing both trends. This dissociation between protein abundance and phosphorylation state suggests that CCI and taVNS engage distinct layers of molecular regulation—transcriptional/translational control of protein levels on one hand, and kinase/phosphatase-mediated post-translational signaling on the other. KEGG enrichment placed the cAMP signaling pathway at the top of reversed-up phosphosites, and KSEA indicated that PKA may be a key kinase whose elevated activity under CCI was reversed by taVNS. PKA hyperactivation in the CeA drives pain-related central sensitization through NMDA receptor NR1 phosphorylation[51], and local PKA inhibition alleviates pain behaviors[52]. The convergence of 5-HT1A receptor pharmacology (Gi-coupled  $\rightarrow$  cAMP $\downarrow$   $\rightarrow$  PKA $\downarrow$ ) with the phosphoproteomic finding of PKA-axis reversal provides pathway-level support for the proposed signaling mechanism, although our data do not directly demonstrate that CeA 5-HT1A activation is upstream of all phosphoproteomic changes observed after taVNS, and direct testing of whether CeA 5-HT1A blockade prevents taVNS-induced PKA normalization remains an important next step.

The STRING-based functional module network further organized these phosphoproteomic findings into interconnected modules related to cAMP signaling, synaptic plasticity, Ca<sup>2+</sup> handling, inhibitory synapse organization, and cytoskeletal remodeling, thereby providing a biologically interpretable bridge between the phosphoproteomic data and the cellular effects observed in the CeA. Within the synaptic plasticity module, PSD-95 (Dlg4), CaMKII (Camk2a), and synapsin-1 (Syn1) are key regulators of excitatory synaptic architecture and activity-dependent signaling. In particular, synapsin-1 is a classical PKA-regulated synaptic phosphoprotein involved in synaptic vesicle mobilization and neurotransmitter release[53]. More broadly, PKA- and CaMKII-dependent phosphorylation of glutamatergic signaling components contributes to central sensitization, and PKA-dependent NMDA receptor

phosphorylation in the CeA has been implicated in pain-related synaptic plasticity [51,52], and PSD-95 itself is a recognized contributor to chronic pain through its interactions with NMDA receptors and downstream signaling partners[54]. Thus, restoration of phosphoregulation within this synaptic plasticity module, together with  $\text{Ca}^{2+}$ -handling nodes such as *Cacna1c* and *Atp2b2*, is consistent with taVNS counteracting CCI-associated excitability and synaptic remodeling in the CeA.

The inhibitory synapse module is particularly relevant to the state-dependent CeA neuronal response switch observed in this study. *KCC2* (*Slc12a5*) maintains the chloride gradient required for hyperpolarizing GABA<sub>A</sub> receptor-mediated inhibition, gephyrin (*Gphn*) organizes inhibitory postsynaptic scaffolds, and phosphorylation of GABA<sub>A</sub> receptors regulates receptor trafficking, expression, and scaffold interactions. Impairment of *KCC2*-mediated chloride extrusion and GABAergic inhibition has been implicated in neuropathic pain and neural disinhibition[55,56]. Gephyrin, as a core inhibitory postsynaptic scaffold, anchors and stabilizes GABAergic receptors at inhibitory synapses[57]. Thus, the *Gphn*–*Gabra1*–*Slc12a5*/*KCC2* module provides a molecular context for impaired inhibitory synapse organization and neural disinhibition in neuropathic pain. Given that the CeA is predominantly GABAergic, restoration of phosphoregulation within the *Gphn*–*Gabra1*–*Slc12a5*/*KCC2* module provides a molecular counterpart to our electrophysiological finding that taVNS shifts CeA neuronal responses from excitation toward inhibition under the CCI state. Collectively, this phosphorylation network suggests that taVNS-induced serotonergic recruitment is accompanied by coordinated restoration of synaptic phosphoregulation programs in the CeA, converging on excitability control and inhibitory synapse organization.

Resting-state fMRI provided systems-level corroboration. DRN–CeA functional connectivity showed a negative correlation in Normal mice, reversed to positive after CCI, and was restored toward baseline by taVNS. Because resting-state functional connectivity does not directly resolve synaptic directionality or receptor-level mechanisms, these data should be interpreted as network-level correlates rather than direct evidence of inhibitory or excitatory coupling. Nevertheless, the observed FC change pattern aligns with the circuit-level data: the negative FC in Normal mice is consistent with tonic 5-HT<sub>1A</sub>-mediated inhibitory coupling, its loss under CCI parallels diminished serotonergic tone, and its restoration by taVNS mirrors the recovery of DRN 5-HT activity and CeA 5-HT release documented by our functional experiments. Zhou et al.[25] reported that DRN–centromedial amygdala FC was specifically reduced in human patients with pain – depression comorbidity—but not in chronic pain alone or depression alone—with the degree of reduction correlating with depression severity. Despite inherent differences between rodent and human fMRI, both studies converge on the conclusion that altered DRN–CeA functional coupling is a characteristic network signature of pain – depression comorbidity, and our data extend this by demonstrating that taVNS can reverse this signature in a serotonergic-dependent manner. Across CCI and taVNS-treated animals,

normalization of DRN – CeA connectivity and restoration of CeA serotonergic tone were associated with behavioral outcomes, providing supportive cross-level evidence that circuit- and neurochemical-level restoration tracks behavioral status.

In summary, this study provides multi-scale evidence supporting a mechanistic framework for taVNS in pain – depression comorbidity. At the pathway level, taVNS signals access the central serotonergic system via the auricular concha → JNG → NTS → DRN route, a serotonergic arm that diverges at the NTS from the recently characterized glutamatergic NTS<sup>POMC</sup> → vIPAG analgesic pathway<sup>[17]</sup>. While the glutamatergic arm engages descending nociceptive inhibition, the serotonergic arm modulates limbic processing underlying both pain affect and depression—together suggesting that taVNS recruits multiple NTS-originating pathways tuned to distinct functional domains, which may help account for the multi-dimensional therapeutic profile of taVNS observed clinically across pain, mood, and autonomic symptoms. At the circuit level, taVNS preferentially recruits DRN → CeA serotonergic signaling in a state-dependent manner under comorbid conditions, with 5-HT1A as the necessary effector receptor and the DRN → CeA node functioning as a bottleneck for the dual behavioral effects. At the molecular level, taVNS reversed the CCI-associated neuroinflammatory program and was accompanied by restoration of cAMP/PKA-associated phosphorylation signaling; network-based analysis further organized these changes into synaptic phosphoregulation modules converging on excitability control and inhibitory synapse organization, providing candidate mechanisms that link receptor activation to downstream cellular effects—including the state-dependent switch of CeA neuronal responses—while serotonin-mediated microglial modulation may further contribute to neuroinflammatory resolution and ECM remodeling. These results offer mechanistic support for the clinical application of taVNS in comorbid pain – depression populations and identify the 5-HT1A → cAMP → PKA axis as a candidate molecular target for future translational investigation.

## **Limitations**

Several limitations of the present study should be acknowledged. First, the NTS → DRN segment of the afferent pathway was established anatomically but not functionally validated through selective manipulation. Second, CeA neuronal subtypes were classified by waveform analysis rather than genetic markers, limiting cell-type resolution. Third, the proteomic and phosphoproteomic findings are descriptive; targeted functional validation of specific inflammatory or synaptic candidate molecules in the CeA would strengthen causal claims at the molecular level.

## **Methods**

### **Animals and ethical approval**

Male C57BL/6 mice (20–25 g) were obtained from SPF (Beijing) Biotechnology Co., Ltd. [License No.: SCXK (Jing) 2016-0002]. Pet1-Cre mice were kindly provided by Professor Zhi Zhang's laboratory at Anhui Medical University. Mice were housed under specific-pathogen-free conditions at 20–26 °C and 40–60% humidity under a 12 h light/dark cycle, with food and water available ad libitum. Animals were allowed to acclimatize for 7 days before any experimental procedure. Mice were then randomly assigned to the indicated experimental groups. Mice in the CCI and taVNS groups were individually housed during the experimental period to minimize potential social influences on pain- and depression-related behaviors.

All animal procedures were approved by the Ethics Committee of the Institute of Acupuncture and Moxibustion, China Academy of Chinese Medical Sciences (Approval No.: D2021-11-12-2) and were conducted in accordance with the National Standard for Laboratory Animal Welfare and Ethics (GB/T 35892-2018).

### **Chronic constriction injury (CCI) model**

The CCI model was established as previously described[58]. Mice were anesthetized with sodium pentobarbital (30 mg kg<sup>-1</sup>, i.p.) and placed on a sterile surgical platform. A skin incision of approximately 5 mm was made along the course of the left sciatic nerve, and the muscle layer was bluntly separated to expose the nerve trunk. Four loose ligatures were placed around the sciatic nerve at approximately 1 mm intervals using 4-0 non-absorbable sutures, with care taken to slightly constrict the nerve without completely blocking nerve conduction. The wound was irrigated with sterile saline and closed in layers.

After surgery, mice were returned to their home cages and monitored until recovery from anesthesia. Behavioral assessments were performed 4 weeks after surgery to verify the development of pain- and depression-related phenotypes before the initiation of taVNS intervention.

### **Transcutaneous auricular vagus nerve stimulation (taVNS)**

Beginning 4 weeks after CCI surgery, mice in the taVNS group received taVNS once daily for 28 consecutive days. For each session, mice were anesthetized with isoflurane (2.0–3.0% for induction and 1.0–1.5% for maintenance), and respiration was monitored throughout the procedure. Self-adhesive conductive electrodes were placed bilaterally on the auricular concha. Electrical stimulation was delivered using a HANS-100A stimulator in the dense-and-disperse mode, alternating between 2 Hz and 15 Hz every 3 s, as symmetric biphasic rectangular pulses in constant-current mode (1 mA). Pulse width varied automatically with frequency (0.2–0.6 ms), and each session lasted 30 min. The 2/15 Hz combination has been widely adopted for analgesia, including in rodent models of CCI-induced neuropathic pain, where it produces robust and stable antinociceptive effects, and represents a well-established paradigm in electroacupuncture and transcutaneous electrical stimulation research

[59,60]. Stimulation was administered daily between 9:00 and 11:00 AM.

For acute stimulation during fiber photometry and in vivo electrophysiological recordings, taVNS was delivered using the same stimulation parameters.

### **Behavioral tests**

Behavioral assessments were performed at baseline (0W), 4 weeks after CCI surgery and before taVNS treatment (4W), and after 28 days of taVNS treatment (8W). All tests were conducted in a quiet room under controlled temperature ( $22 \pm 2$  °C). Mice were allowed to habituate to the testing room for at least 30 min before each test.

**Paw withdrawal threshold (PWT):** Mechanical allodynia was measured using a Dynamic Plantar Aesthesiometer (Ugo Basile, 37550). Mice were placed individually in transparent chambers on a metal mesh floor. A mechanical probe was applied to the plantar surface of the left hind paw with a force ramp of  $1 \text{ g s}^{-1}$  up to a cutoff of 25 g. The force that evoked paw withdrawal was automatically recorded. Each mouse was tested 3–5 times at intervals of at least 5 min, and the mean value was used for analysis.

**Tail suspension test (TST):** Mice were suspended by the tail using adhesive tape placed approximately 1 cm from the tail tip, with the head positioned 30–50 cm above the floor. The total test duration was 6 min. Immobility time during the final 4 min was quantified, with the first 2 min regarded as habituation. Increased immobility time was interpreted as behavioral despair-like behavior.

**Open field test (OFT):** Mice were gently placed in the center of a square arena ( $50 \times 50 \times 40$  cm) and allowed to explore freely for 5 min. Time spent in the center zone was recorded using an automated video-tracking system (Shanghai Jiliang Software Technology Co., Ltd.). Reduced center time was interpreted as decreased exploratory behavior and increased anxiety-like behavior. The arena was cleaned with 75% ethanol between trials.

**Sucrose preference test (SPT):** Mice were habituated to the drinking paradigm by exposure to two bottles of 2% sucrose solution for 24 h, followed by two bottles of water for 24 h. They were then presented with one bottle of 1% sucrose solution and one bottle of water for 48 h, with bottle positions switched every 24 h to avoid side bias. After habituation, mice were water-deprived for 20 h and then given free access to one bottle of 1% sucrose solution and one bottle of water for 2 h. Sucrose preference was calculated as  $\text{sucrose intake} / (\text{sucrose intake} + \text{water intake}) \times 100\%$ . Reduced sucrose preference was interpreted as anhedonia-like behavior.

### **Resting-state functional magnetic resonance imaging (rs-fMRI)**

Following the 28-day intervention period, mice from the Normal, CCI, and taVNS

groups underwent brain MRI scanning on a PharmaScan 70/16 US 7.0 T small-animal MRI system (Bruker, Germany) equipped with a mouse body coil and a surface coil for radiofrequency transmission and reception. Mice were pre-anesthetized with 2.5% isoflurane in oxygen, positioned prone on the animal bed, and maintained at 1.5–2.0% isoflurane during image acquisition. Respiratory rate was monitored in real time using PC-SAM32 software (Small Animal Instruments Inc.) and maintained at 60–80 breaths min<sup>-1</sup>. Body temperature was maintained using a SC100-S14P circulating water bath system (Thermo Scientific).

T2-weighted structural images were acquired using a T2\_TurboRARE sequence with the following parameters: repetition time (TR) = 4500 ms, echo time (TE) = 35 ms, slice thickness = 0.35 mm, field of view (FOV) = 20 × 20 mm, matrix = 256 × 256, and averages = 3. Resting-state BOLD functional images were acquired using a T2star\_FID\_EPI\_sat sequence with the following parameters: TR = 2000 ms, TE = 15 ms, slice thickness = 0.35 mm, FOV = 20 × 20 mm, matrix = 64 × 64, 300 repetitions, and a total scan time of 10 min.

Image preprocessing was performed using the RABIES (Rodent Automated Bold Improvement of EPI Sequences) pipeline[61]. Preprocessing steps included motion correction, spatial normalization to a standard mouse brain atlas, spatial smoothing, nuisance regression, and bandpass filtering (0.01–0.1 Hz). ROI-to-ROI functional connectivity analysis was then performed across 13 pain- and emotion-related brain regions, with the DRN and CeA defined as primary regions of interest. Pearson correlation coefficients were Fisher z-transformed for group-level statistical analysis.

## **Neural tract tracing**

### **Dual retrograde tracing of the vagal pathway**

To examine the afferent pathway linking the auricular concha to the brainstem, retro-rAAV-EGFP (BrainCase, PT-1990) was injected into the NTS and retro-rAAV-mCherry (BrainCase, PT-0100) was injected into multiple sites of the auricular concha. After 3 weeks of viral expression, mice were perfused and the jugular-nodose ganglion (JNG) was collected for fluorescence analysis. Co-labeling of EGFP and mCherry was quantified to estimate the proportion of JNG neurons contributing to the auricular concha-to-NTS pathway.

### **Dual retrograde tracing of the trigeminal pathway**

In a parallel experiment, retro-rAAV-EGFP (BrainCase, PT-1990) was injected into the spinal trigeminal nucleus (Sp5), and retro-rAAV-mCherry (BrainCase, PT-0100) was injected into multiple sites of the auricular concha. Co-labeling was assessed in both the JNG and trigeminal ganglion (TG) to compare labeling patterns associated with trigeminal and vagal afferent pathways.

### **H129ΔTK-tdTomato anterograde transsynaptic tracing**

To examine whether NTS neurons give rise to downstream labeling in the DRN, AAV-TK-GFP (helper virus; BrainVTA, PT-9342) was first injected into the NTS. After 21 days, H129ΔTK-tdTomato (BrainVTA, H03001) was injected into the same region. The TK helper virus supplied thymidine kinase in trans to permit H129ΔTK propagation. After the designated transport period, brain sections were examined for tdTomato-positive signals in the DRN.

### **WGA-FLP-based transsynaptic tracing of the DRN 5-HT→CeA projection**

To examine serotonergic projections from the DRN to the CeA, Pet1-Cre mice received rAAV-CAG-DIO-WGA-FLP-WPRE-hGH pA (BrainVTA, PT-0557) in the DRN and rAAV-EF1α-fDIO-EGFP-WPRE-hGH pA (BrainVTA, PT-1047) in the CeA. In this design, Cre-dependent WGA expression was restricted to DRN serotonergic neurons, and FLP delivered across synaptically connected neurons enabled EGFP expression in downstream CeA cells. EGFP-positive signals in the CeA were interpreted as evidence consistent with synaptically connected DRN 5-HT→CeA circuitry.

### **Stereotaxic surgery and viral injection**

Mice were anesthetized with sodium pentobarbital (30 mg kg<sup>-1</sup>, i.p.) and mounted on a digital stereotaxic apparatus (RWD Life Science). After exposure of the skull, bregma and lambda were identified as stereotaxic reference points. Glass micropipettes were used for intracranial injection. The stereotaxic arm was angled at 15° from the Z-axis for DRN injections and 90° for CeA injections. Coordinates relative to bregma were as follows: NTS, AP = -7.64 mm, ML = ±0.35 mm, DV = -5.10 mm; Sp5, AP = -6.48 mm, ML = ±2.0 mm, DV = -5.50 mm; DRN, AP = -5.10 mm, ML = 0 mm, DV = -2.40 mm; CeA, AP = -1.34 mm, ML = 2.55 mm, DV = -3.85 mm. All DV coordinates were measured from the dura.

Viral solution (300 nL) was delivered at a rate of 30 nL min<sup>-1</sup> using a Hamilton microsyringe (80100). After injection, the pipette was left in place for 10 min to minimize reflux and was then slowly withdrawn. Mice were allowed to recover for at least 3–4 weeks before subsequent experiments to ensure adequate viral expression.

### **Fiber photometry**

To record calcium activity of DRN serotonergic neurons, rAAV-EF1α-DIO-GCaMP6s-WPRE-hGH polyA (BrainVTA, PT-0071) was injected into the DRN of Pet1-Cre mice (NOR-GCaMP6s and CCI-GCaMP6s groups), and rAAV-EF1α-DIO-EGFP-WPRE-hGH polyA (BrainVTA, PT-0795) was used as a control (CCI-EGFP group). To monitor serotonin dynamics in the CeA,

pAAV-hSyn-5HT3.0-WPRE-hGH polyA (BrainVTA, PT-4724) was injected into the CeA of C57BL/6 mice (NOR-5HT3.0 and CCI-5HT3.0 groups), and the nonresponsive mutant sensor pAAV-hSyn-5HT3.0 mut-WPRE-PA (BrainVTA, PT-4722) was used as a control (CCI-MUT group).

Ceramic ferrules (Inper) were implanted immediately after viral injection at the corresponding coordinates and secured with dental cement. After 4 weeks of viral expression, fiber photometry recordings were performed using a mono-wavelength multi-channel fiber photometry system (Thinker Tech Nanjing Bioscience Inc.). Excitation power was maintained at 30–40  $\mu$ W to minimize photobleaching. Baseline compensation signals were collected for 100 s before connection of the patch cable to the implanted ferrule.

During recording, calcium signals in the DRN or serotonin sensor signals in the CeA were acquired during taVNS. Fluorescence changes were expressed as  $\Delta F/F$ , and responses within the stimulation epoch were quantified by the area under the curve (AUC).

### **In vivo multichannel electrophysiology**

Mice were deeply anesthetized with 10% urethane (Sigma, U2500) and fixed in a stereotaxic frame. Two skull screws were implanted for grounding. A cranial window of approximately  $2 \times 2$  mm was opened above the CeA target region, and the dura was carefully removed. A multichannel microelectrode array (Plexon) was then slowly advanced into the CeA. Neural signals were acquired using an OmniPlex neural signal acquisition system (Plexon, OPX).

CeA neuronal firing was recorded during taVNS stimulation. Firing rates were compared between the 10 s period preceding stimulation and the 10 s period following stimulation. Spike sorting was performed offline using Offline Sorter, and single-unit waveform properties were analyzed using NeuroExplorer. Based on previous studies[62,63], well-isolated units were classified according to trough-to-peak duration and firing frequency as narrow-waveform or broad-waveform units. Response direction for each unit was defined as increased firing, decreased firing, or no detectable change after taVNS.

### **Microdialysis**

Following the 28-day intervention period, a guide cannula (CMA, cat. no. 8010773) was implanted into the CeA of mice in the Normal, CCI, and taVNS groups under deep anesthesia. On the following day, a microdialysis probe (CMA, cat. no. 8010771) was inserted through the guide cannula and connected to a syringe infusion pump via polyethylene tubing. The probe was continuously perfused with artificial cerebrospinal fluid (ACSF) at a constant flow rate. After an equilibration period,

sequential dialysate fractions were collected and averaged to obtain a steady-state value for each analyte in each animal. Concentrations of 5-HT and 5-HIAA in the dialysates were quantified by high-performance liquid chromatography with electrochemical detection (HPLC-ECD) and expressed as ng mL<sup>-1</sup>. Probe placements were verified histologically after sample collection.

## **Western blot**

Following the 28-day intervention period, DRN tissue was collected from mice in the Normal, CCI, and taVNS groups. Total protein was extracted according to the manufacturer's instructions, and protein concentration was determined prior to denaturation in SDS-PAGE loading buffer at 100 °C for 10 min. Equal amounts of protein (50 µg per lane) were separated on 12% SDS-PAGE gels with a 5% stacking gel at 80 V during stacking and 120 V during separation. Proteins were then transferred onto 0.22 µm PVDF membranes (Millipore) by wet transfer at a constant current of 300 mA for 1.5 h.

Membranes were blocked with rapid blocking solution for 10 min and incubated overnight at 4 °C with primary antibodies diluted in 1% BSA/5% milk: anti-SERT (Bioss, bs-1893R; 1:500; 70 kDa) and anti-TPH2 (Bioss, bs-8729R; 1:500; 54 kDa). β-Actin (Abcam, ab8226; 1:3000; 42 kDa) was used as the loading control. After washing with TBST (3 × 5 min), membranes were incubated with HRP-conjugated goat anti-rabbit IgG secondary antibody (Abcam, ab6721; 1:5000) for 50 min at room temperature, followed by four additional washes in TBST (4 × 5 min). Protein bands were visualized using an ECL Plus Western Blot Kit and imaged with a ChemiScope 5100 chemiluminescence imaging system (Shanghai Qinxiang). Band intensities were quantified using Quantity One v4.6.2 and expressed as the ratio of target protein to β-actin.

## **Chemogenetic inhibition**

To test the requirement for DRN serotonergic signaling in the behavioral effects of taVNS, rAAV-EF1α-DIO-hM4D(Gi)-mCherry-WPREs (BrainVTA, PT-0043) was injected into the DRN of Pet1-Cre mice to express the inhibitory DREADD receptor selectively in serotonergic neurons. Control mice received rAAV-EF1α-DIO-mCherry-WPRE-hGH polyA (BrainVTA, PT-0013). Guide cannulae were implanted above the CeA. After 4 weeks of viral expression, mice underwent CCI surgery, and taVNS intervention was initiated at 4 weeks after surgery and continued for 28 days.

Clozapine N-oxide (CNO, 125 µg µL<sup>-1</sup>, 0.2 µL) was microinjected into the CeA through the implanted cannula at 100 nL min<sup>-1</sup>, 30 min before each daily taVNS session. The injection needle was left in place for 5 min after infusion to minimize reflux. Three groups were included: taVNS (CCI + taVNS), taVNS-hM4Di (CCI + taVNS + hM4Di + CNO), and taVNS-mCherry (CCI + taVNS + mCherry + CNO).

Behavioral tests were performed at 0W, 4W, and 8W.

For electrophysiological experiments, CNO was administered intraperitoneally (5 mg kg<sup>-1</sup>), and CeA recordings were performed 40 min later.

### **Pharmacological interventions**

Two pharmacological loss-of-function experiments were performed in CCI mice receiving taVNS. In the first experiment, the 5-HT synthesis inhibitor L-PCPA (4-chloro-DL-phenylalanine; MCE, 14173-39-8; 1 g L<sup>-1</sup>) or ACSF was microinjected into the DRN (0.2 µL at 100 nL min<sup>-1</sup>) through preimplanted guide cannulae 30 min before each taVNS session for 28 days (DRN-PCPA and DRN-ACSF groups). In the second experiment, the selective 5-HT<sub>1A</sub> receptor antagonist WAY-100635 (MCE, 162760-96-5; 0.5 g L<sup>-1</sup>) or ACSF was microinjected into the CeA under the same conditions (CeA-WAY and CeA-ACSF groups). Behavioral assessments (PWT, TST, OFT, and SPT) were performed at 0W, 4W, and 8W.

### **Quantitative proteomics**

Following the 28-day intervention period, bilateral CeA tissue was collected from mice in the Normal, CCI, and taVNS groups. Total protein was extracted using a BPP buffer–phenol extraction method. Protein precipitates were dissolved in lysis buffer containing 8 M urea and 1% SDS supplemented with protease inhibitor cocktail, and protein concentration was determined using the BCA assay (Thermo Scientific). For each sample, 100 µg of protein was reduced with TCEP, alkylated with iodoacetamide, and digested overnight at 37 °C with trypsin at an enzyme-to-protein ratio of 1:50. Peptides were desalted using HLB cartridges and dried by vacuum centrifugation.

Peptides were analyzed by data-independent acquisition (DIA) mass spectrometry using a Vanquish Neo UHPLC system coupled to an Orbitrap Astral mass spectrometer (Thermo, USA). Peptide separation was performed on a uPAC High Throughput column (75 µm × 5.5 cm, Thermo). DIA data were acquired over a mass range of 70–1050 m/z for MS1 and 150–2000 m/z for MS2. Raw data were processed using Spectronaut software (version 20) against the UniProt Mus musculus database, and the resulting quantitative protein matrix was used for downstream analysis. Differentially expressed proteins (DEPs) were identified using Student's t-test with fold-change thresholds of  $\geq 1.2$  for upregulated proteins and  $\leq 0.83$  for downregulated proteins, together with  $P < 0.05$ . DEPs were subjected to principal component analysis (PCA), volcano plot visualization, four-quadrant directional analysis, Gene Ontology (GO) enrichment, KEGG pathway enrichment, and protein–protein interaction (PPI) network analysis. Data analysis was performed using the Majorbio Cloud platform[64].

### **Phosphoproteomics and kinase-substrate enrichment analysis (KSEA)**

Phosphopeptides were enriched from the same CeA tissue samples used for proteomic analysis and were analyzed on the same DIA mass spectrometry platform using the same search settings described above<sup>[31]</sup>. Differentially expressed phosphosites were identified using Student's t-test with fold-change thresholds of  $\geq 1.5$  for upregulated sites and  $\leq 0.67$  for downregulated sites, together with  $P < 0.05$ . KEGG enrichment analysis was performed separately for reversed phosphosites and taVNS-unique differentially expressed phosphosites. Kinase-substrate enrichment analysis (KSEA) was performed to infer upstream kinase activity changes using combined mouse and human substrate annotations and Stouffer's weighted Z-score method.

## **Histological verification and immunofluorescence**

After completion of all experiments, mice were deeply anesthetized with sodium pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde (PFA). Brains were post-fixed in 4% PFA for 2 h at 4 °C and then cryoprotected in 30% sucrose until sinking. Coronal sections (40  $\mu$ m) were cut using a cryostat microtome (Leica, CM1860) and stored in PBS at 4 °C.

For immunofluorescence staining, sections were blocked in 0.3% Triton X-100 and 5% normal donkey serum (Jackson ImmunoResearch, AB\_2337258) for 1 h and then incubated overnight at 4 °C with anti-TPH2 antibody (Abcam, ab184505; 1:300). After washing in PBS (3  $\times$  10 min), sections were incubated with Alexa Fluor 546-conjugated donkey anti-rabbit IgG (Thermo, A10040; 1:300) for 2 h at room temperature in the dark, washed again in PBS, and counterstained with DAPI (ZLI-9557). Sections were mounted with anti-fade mounting medium and imaged using a confocal microscope (Nikon AX). Injection sites, fiber placements, electrode positions, and cannula placements were verified histologically. Co-localization of GCaMP6s and TPH2 was quantified using ImageJ.

## **Statistical analysis**

All statistical analyses were performed using GraphPad Prism 9.0. Data were first tested for normality using the Shapiro–Wilk test and for homogeneity of variance using the Brown–Forsythe test. For behavioral data collected at multiple time points (0W, 4W, and 8W), two-way repeated-measures ANOVA was used, followed by Tukey's post hoc test for multiple comparisons. For single time-point comparisons among three groups, including microdialysis measurements, Western blot data, rs-fMRI Fisher z values, and fiber photometry AUC values, one-way ANOVA followed by Tukey's post hoc test was used.

For electrophysiological analyses, pre- and post-stimulation firing rates were compared using paired t-tests, response-direction distributions across groups were compared using chi-square tests, and within-group directional bias was assessed using binomial tests. All quantitative data are presented as mean  $\pm$  SD.

For proteomic analysis, DEPs were defined using Student's t-test with fold change  $\geq 1.2$  or  $\leq 0.83$  and  $P < 0.05$ . For phosphoproteomic analysis, differentially expressed phosphosites were defined using Student's t-test with fold change  $\geq 1.5$  or  $\leq 0.67$  and  $P < 0.05$ . GO and KEGG enrichment analyses were corrected for multiple testing using the Benjamini–Hochberg method. For KSEA, significance was determined based on Z-score with  $P < 0.05$  considered statistically significant.

For cross-level correlation analyses, CeA 5-HT and DRN–CeA functional connectivity were each correlated with behavioral measures (PWT, TST, and SPT) using Spearman's rank correlation across CCI and taVNS-treated animals with complete paired measurements, with these correlations predefined on the basis of the proposed mechanism.

## Abbreviations

5-HIAA: 5-hydroxyindoleacetic acid; 5-HT: 5-hydroxytryptamine; 5-HT1A: 5-hydroxytryptamine receptor 1A; 5-HT2A: 5-hydroxytryptamine receptor 2A; AAV: adeno-associated virus; ABVN: auricular branch of the vagus nerve; ACSF: artificial cerebrospinal fluid; ANOVA: analysis of variance; AP: anteroposterior; AUC: area under the curve; BBB: blood–brain barrier; BCA: bicinchoninic acid; BOLD: blood-oxygen-level-dependent; BSA: bovine serum albumin; cAMP: cyclic adenosine monophosphate; CaMKII: calcium/calmodulin-dependent protein kinase II; CCI: chronic constriction injury; CeA: central amygdala; CNO: clozapine N-oxide; DAPI: 4',6-diamidino-2-phenylindole; DEP: differentially expressed protein; DIA: data-independent acquisition; DIO: double-floxed inverted orientation; DRN: dorsal raphe nucleus; DV: dorsoventral; ECL: enhanced chemiluminescence; ECM: extracellular matrix; EGFP: enhanced green fluorescent protein; FC: functional connectivity; FLP: flippase recombinase; fMRI: functional magnetic resonance imaging; FOV: field of view; GABA: gamma-aminobutyric acid; GFP: green fluorescent protein; GO: Gene Ontology; H129 $\Delta$ TK: thymidine kinase-deleted herpes simplex virus type 1 strain H129; hM4Di: inhibitory designer receptor exclusively activated by designer drugs; HPLC: high-performance liquid chromatography; HRP: horseradish peroxidase; JNG: jugular–nodose ganglion; KCC2: potassium-chloride cotransporter 2; KEGG: Kyoto Encyclopedia of Genes and Genomes; KSEA: kinase-substrate enrichment analysis; ML: mediolateral; MRI: magnetic resonance imaging; MUT: mutant; NMDA: N-methyl-D-aspartate; NOR: normal; NTS: nucleus tractus solitarius; OFT: open field test; PBS: phosphate-buffered saline; PCA: principal component analysis; PCPA: para-chlorophenylalanine; PFA: paraformaldehyde; PKA: protein kinase A; POMC: proopiomelanocortin; PPI: protein–protein interaction; PSD-95: postsynaptic density protein 95; PVDF: polyvinylidene difluoride; PWT: paw withdrawal threshold; rAAV: recombinant adeno-associated virus; ROI: region of interest; rs-fMRI: resting-state functional magnetic resonance imaging; SD: standard deviation; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SERT: serotonin transporter; SNRI: serotonin–norepinephrine reuptake inhibitor; SOM: somatostatin; Sp5: spinal

trigeminal nucleus; SPT: sucrose preference test; SSRI: selective serotonin reuptake inhibitor; STRING: Search Tool for the Retrieval of Interacting Genes/Proteins; taVNS: transcutaneous auricular vagus nerve stimulation; TBST: Tris-buffered saline with Tween 20; TCEP: tris(2-carboxyethyl)phosphine; TE: echo time; TG: trigeminal ganglion; TGF- $\beta$ : transforming growth factor beta; TPH2: tryptophan hydroxylase 2; TR: repetition time; TST: tail suspension test; UHPLC: ultra-high-performance liquid chromatography; vIPAG: ventrolateral periaqueductal gray; VNS: vagus nerve stimulation; WGA: wheat germ agglutinin; WPRE: woodchuck hepatitis virus post-transcriptional regulatory element.

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## Data Availability Statement

All data supporting the findings of this study — including behavioral recordings, fiber photometry traces, in vivo electrophysiology recordings, microdialysis measurements, histological images, and resting-state fMRI data — are available from the corresponding author upon reasonable request. Custom analysis code is available from the corresponding author upon reasonable request.

## Competing Interests

The authors have declared that no competing interests exist.

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