

Brief Communications

Parabrachial Calcitonin Gene-Related Peptide Neurons Mediate Conditioned Taste Aversion

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Conditioned taste aversion (CTA) is a phenomenon in which an individual forms an association between a novel tastant and toxin-induced gastrointestinal malaise. Previous studies showed that the parabrachial nucleus (PBN) contains neurons that are necessary for the acquisition of CTA, but the specific neuronal populations involved are unknown. Previously, we identified calcitonin gene-related peptide (CGRP)-expressing neurons in the external lateral subdivision of the PBN (PBel) as being sufficient to suppress appetite and necessary for the anorexigenic effects of appetite-suppressing substances including lithium chloride (LiCl), a compound often used to induce CTA. Here, we test the hypothesis that PBel CGRP neurons are sufficient and necessary for CTA acquisition in mice. We show that optogenetic activation of these neurons is sufficient to induce CTA in the absence of anorexigenic substances, whereas genetically induced silencing of these neurons attenuates acquisition of CTA upon exposure to LiCl. Together, these results demonstrate that PBel CGRP neurons mediate a gastrointestinal distress signal required to establish CTA.

Key words: CGRP; ChR2; conditioned taste aversion; CTA; parabrachial; PBN

Introduction

Conditioned taste aversion (CTA) develops when consumption of a novel taste is followed by transient gastrointestinal malaise (Welzl et al., 2001). Although the neural basis of CTA is unknown, previous research demonstrated that lesions of the parabrachial nucleus (PBN) in rodents can cause deficits in CTA (Dilorenzo, 1988; Flynn et al., 1991; Reilly et al., 1993; Grigson et al., 1998). However, because the PBN contains subpopulations of neurons that regulate multiple functions including both taste (Rosen et al., 2011; Tokita and Boughter, 2012; Tokita et al., 2012) and appetite suppression (Becskei et al., 2007; DiPatrizio and Simansky, 2008; Wu et al., 2009; Carter et al., 2013), the molecular identity of neurons that underlie these effects is unknown.

Anatomically, the parabrachial neurons that mediate CTA may be located specifically in the lateral division of the PBN. Stereotaxically guided electrolytic lesions of only the lateral PBN attenuate CTA (Agüero et al., 1993a,b). Furthermore, injection of lithium chloride (LiCl), a compound routinely used to cause gastrointestinal malaise and induce CTA (Rowland et al., 2004),

activates Fos, a surrogate marker of neuronal activity, specifically in the external lateral subdivision of the PBN (PBel) (Yamamoto et al., 1992; Sakai and Yamamoto, 1997; St Andre et al., 2007).

Previously, we demonstrated that these Fos-positive neurons expressed calcitonin gene-related peptide (CGRP) and that their stimulation was sufficient to induce appetite suppression (Carter et al., 2013). Importantly, inhibition of CGRP neurons reduced appetite suppression caused by injection of LiCl. Therefore, we hypothesized that neural activity in PBel CGRP neurons is necessary and sufficient to form a CTA. We tested this hypothesis using *Calca*^{Cre/+} mice (*Calca* is the gene that encodes CGRP) and genetically encoded neural actuators, demonstrating a critical role for PBel CGRP neurons in mediating CTA.

Materials and Methods

Mice. All experiments were approved by the University of Washington Institutional Animal Care and Use Committee and were performed in accordance with the guidelines described in the U.S. National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. We used exclusively heterozygous male *Calca*^{Cre/+} mice, aged 7–9 weeks at the start of experimental procedures and no more than 12 weeks at the end of experimental procedures. These mice had been backcrossed onto a C57BL/6 background for more than five generations. Before stereotaxic surgery, mice were group housed and maintained with a rodent diet (Picolab, catalog #5053) and water available *ad libitum* with a 12 h light/dark cycle at 22°C.

Virus production. Cre-dependent adeno-associated virus pAAV mCherry and ChR2-mCherry (driven by the E1 α promoter) DNA plasmids were kindly provided by Dr. Karl Deisseroth, and Cre-dependent pAAV hM4Di-mCherry (driven by the human synapsin promoter) DNA plasmids were kindly provided by Dr. Bryan Roth. The plasmid encoding AAV1-CBA-DIO-GFP-TetTox-WPRE-pA virus was prepared by inserting a GFP-TetTox cassette obtained from Dr. Susan Dymecki (Kim et al., 2009) into a generic AAV-CBA-DIO-WPRE-pA vector. AAVs were prepared in human embryonic kidney 293T cells with AAV1 coat serotype,

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purified by sucrose and CsCl gradient centrifugation steps, and resuspended in HEPES buffered saline solution at a titer of $\sim 2 \times 10^9$ viral genomes per microliter. Viral aliquots were stored at -80°C before stereotaxic injection.

Stereotaxic surgery. At the start of surgical procedures, mice were anesthetized with isoflurane and placed on a small stereotaxic frame (David Kopf Instruments). Stereotaxic coordinates for the anterior–posterior plane were normalized using a correction factor [$F = (\text{Bregma} - \text{Lambda distance})/4.21$] and based on the coordinates of Paxinos and Franklin (2013). Virus was injected unilaterally (on the left side) or bilaterally in the PBN [anteroposterior (AP), -4.9 mm; mediolateral (ML), 1.4 mm; dorsoventral (DV), 3.8 mm] at a rate of $0.2 \mu\text{l}/\text{min}$ for 2.5 min ($0.5 \mu\text{l}$ total volume). Note that the viral injection coordinates target the most anterior aspect of the PBN; however, the virus diffuses posteriorly to hit all lateral PBN subnuclei. This injection site improves the accuracy of injecting into the lateral PBN between the superior cerebellar peduncle (scp) and lateral wall of the pons. Also note that the presence of the scp fortuitously limits the spread of virus from the PBN region, thus preventing unintended transduction of other nearby *Calca*-expressing brain regions. In histological analysis of brain tissue following behavioral experiments, we found no expression of reporter proteins outside the PBel region.

After viral injection, mice used for optogenetic experiments also received unilateral surgical implantation of a mono fiber-optic cannula (Doric Lenses) above the PBN (AP, -5.2 mm; ML, 1.6 mm; DV, 3.0 mm). Cannulae were affixed to the skull with C&B Metabond (Parkell) and dental acrylic.

Behavioral experiments. Animals were allowed to acclimate to lickometer cages for at least 1 week before experimental procedures, with dispensers for water and food pellets on the floor.

At the onset of the dark cycle of Day 0, a liquid food dispenser was added containing vanilla-flavored Ensure. One hour after the onset of the dark cycle, mice received either injection of LiCl (84 mg/kg; 0.20 M at 10 ml/kg; Fisher Scientific, catalog #L121) or unilateral photostimulation of PBel CGRP neurons at 30 Hz (for 5 min). The amount of Ensure consumed was calculated 15 h later (4 h after the onset of the light cycle), and the Ensure food dispenser was removed. These procedures were repeated on subsequent days except that mice did not receive further treatments of LiCl or photostimulation. For pharmacogenetic inhibition experiments, HM4Di-mCherry-transduced mice received an injection of clozapine *N*-oxide (CNO; Sigma; 1 mg/kg) at the onset of the dark cycle on the first day.

Photostimulation. Mice were allowed at least 5 d to acclimate to fiber-optic cables (1.5 m long, $200 \mu\text{m}$ diameter; Doric Lenses) coated with opaque heat-shrink tubing before experimental sessions. During photostimulation experiments, light pulse trains (10 ms pulses) were programmed using a waveform generator (Agilent Technologies, catalog #33220A) that provided input to a blue light laser (473 nm; LaserGlow). We adjusted the light power of the laser such that the light power exiting the fiber-optic cable was 20 mW. Using an online light transmission calculator for brain tissue (www.stanford.edu/group/dlab/cgi-bin/graph/chart.php), we estimate the light power at the PBel to be 36.2 mW/mm². Note that this is probably a high estimation because some light is probably lost at the interface between the fiber-optic cable and the implanted fiber-optic cannula.

Histology. Mice were anesthetized with buprenorphine and perfused transcardially with PBS, pH 7.4 , followed by 4% paraformaldehyde in PBS. The brains were extracted, allowed to postfix overnight in the same fixative at 4°C , and cryoprotected in 30% sucrose dissolved in PBS for an additional 24 h at 4°C . Each brain was sectioned at $30 \mu\text{m}$ on a cryostat (Leica Microsystems) and collected in cold $1 \times$ PBS.

For immunohistochemistry experiments, sections were washed three times in PBS with 0.2% Triton X-100 (PBST) for 10 min at room temperature. Sections were then incubated in a blocking solution composed of PBST with 3% normal donkey serum (Jackson ImmunoResearch, catalog #017-000-121) for 1 h. For primary antibody exposure, sections were incubated in rabbit anti-c-Fos ($1:2000$; Calbiochem, catalog #PC38) in blocking solution at 4°C for ~ 20 h. After three 10 min washes in PBST, sections were incubated in Alexa Fluor 488 donkey anti-rabbit IgG ($1:200$; Jackson ImmunoResearch, catalog #711-545-152) in block solution

for 1 h at room temperature. Finally, sections were washed three times in PBS.

Sections were mounted in PBS onto SuperFrost Plus glass slides (VWR, catalog #48311-703) and coverslipped with Dapi Fluoromount-G (Southern Biotech, catalog #0100-20). Slides were stored in the dark at 4°C before microscopy and image acquisition.

Quantification of colocalization of Fos and mCherry in the PBN (see Fig. 2A–C) was performed on adjacent sections from approximately -4.90 to -5.50 mm from Bregma (exactly 21 sections per mouse). All quantification analysis was performed by an investigator (M.E.C.) blinded to the identity of the conditions used to induce Fos.

Analysis was performed using a Nikon upright epifluorescent microscope with a QImaging camera. Images were minimally processed using Photoshop CS5 (Adobe Systems) to enhance the brightness and contrast for optimal representation of the data. All digital images were processed in the same way between experimental conditions to avoid artificial manipulation between different datasets.

Statistics. All data were analyzed using Prism 6.0 (GraphPad Software). Data were exported into Illustrator CS5 (Adobe Systems) for preparation of the figures.

Results

Optogenetic stimulation of PBel CGRP neurons is sufficient to induce CTA

To test the role of PBel CGRP neurons in CTA, we first developed a paradigm to induce CTA by acclimating mice to lickometer chambers for 7 d and subsequently allowing them access to a novel tastant, vanilla-flavored Ensure, at the onset of the dark (active) period, when mice typically consume the most food (Fig. 1A). One hour later, we administered LiCl (84 mg/kg, i.p.) or saline and allowed mice *ad libitum* access to Ensure and mouse chow over the next 5 d. Before injection of LiCl or saline, there was no significant difference in food consumption (measured by number of licks) between the two experimental groups during the first hour of Ensure exposure ($n = 8$ mice per condition, $p = 0.56$). However, LiCl-injected animals developed a CTA and consumed significantly less Ensure than saline-injected animals during the first 3 d following LiCl injection ($n = 8$ mice per condition; interaction of condition \times day, $F_{(4,56)} = 21.72$, $p < 0.0001$; Fig. 1B). To test whether the pairing of LiCl and a familiar tastant could induce CTA, we repeated the previous experiment but exposed mice to Ensure *ad libitum* for 5 d before LiCl injection. This paradigm did not produce a significant CTA to Ensure, demonstrating the necessity that the tastant be novel ($n = 8$ mice per condition; no interaction of condition \times day, $F_{(4,56)} = 1.23$, $p = 0.2913$; Fig. 1C). We also found that daily pretreatment with LiCl for 5 d before exposure to the novel tastant blocked CTA ($n = 8$ mice per condition; no interaction of condition \times day, $F_{(4,56)} = 1.015$, $p = 0.4076$; Fig. 1D). Together, these results show that pairing Ensure with LiCl injection causes a CTA for Ensure, but the association occurs only if both the tastant and the visceral malaise induced by LiCl are novel.

To determine whether PBel CGRP neurons are sufficient to induce CTA, we unilaterally injected AAV carrying a Cre-dependent channelrhodopsin-2 transgene (AAV1-DIO-ChR2-mCherry) (Yizhar et al., 2011) or control transgene (AAV1-DIO-mCherry) into the PBNs of *Calca*^{Cre/+} mice (Fig. 1E). Instead of LiCl, we paired access to Ensure with *in vivo* photostimulation of CGRP neurons for 5 min at 30 Hz. Stimulation of these neurons produced a significant CTA ($n = 9$ mice per condition; interaction of condition \times day, $F_{(4,64)} = 29.61$, $p < 0.0001$; Fig. 1F) in a manner that mimicked LiCl-mediated CTA (Fig. 1B). These effects were lost when mice received daily activation (5 min per day) of CGRP neurons in the PBel before exposure to Ensure ($n = 9$ mice per condition; no interaction of condition \times day, $F_{(4,64)} = 0.5866$,

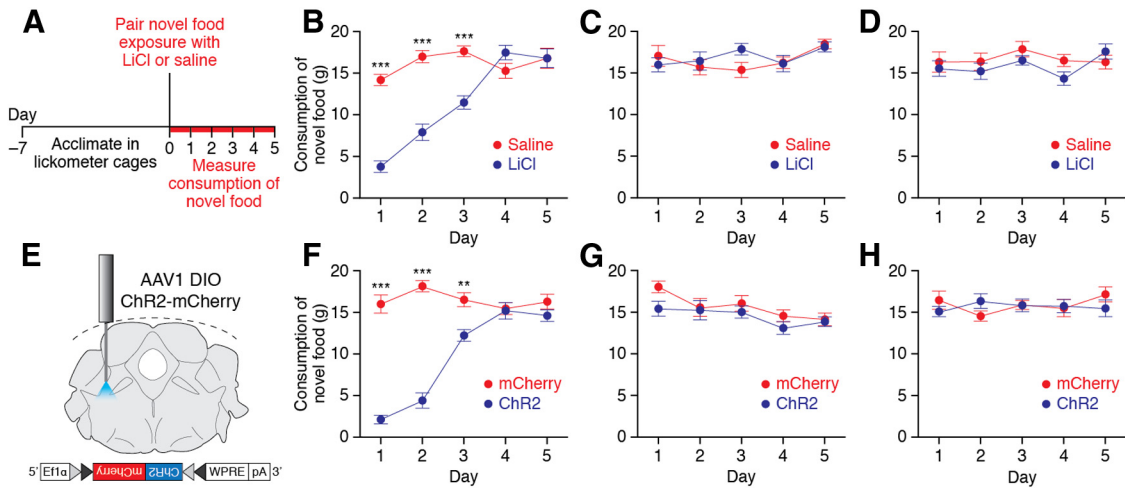


Figure 1. Optogenetic stimulation of PBel CGRP neurons is sufficient to mimic LiCl-induced CTA. **A**, Paradigm used to cause CTA. **B**, Pairing LiCl with novel tastant causes CTA ($n = 8$ mice per condition). **C**, Daily exposure to novel tastant 5 d before exposure to LiCl prevents CTA ($n = 8$ mice per condition). **D**, Daily exposure to LiCl 5 d before exposure to novel tastant prevents CTA ($n = 8$ mice per condition). **E**, Placement of fiber-optic implant in the PBel in a *Calca^{Cre/+}* animal injected with AAV-DIO-ChR2-mCherry. **F**, Pairing photostimulation of PBel CGRP neurons with novel tastant causes CTA ($n = 9$ mice per condition). **G**, Daily exposure to PBel CGRP photostimulation 5 d before exposure to novel tastant prevents CTA ($n = 9$ mice per condition). **H**, Daily exposure to LiCl 5 d before exposure to novel tastant prevents PBel CGRP neuron-mediated CTA ($n = 9$ mice per condition). All values represent the mean \pm SEM. $**p < 0.001$, $***p < 0.0001$ (Bonferroni *post hoc* test between conditions).

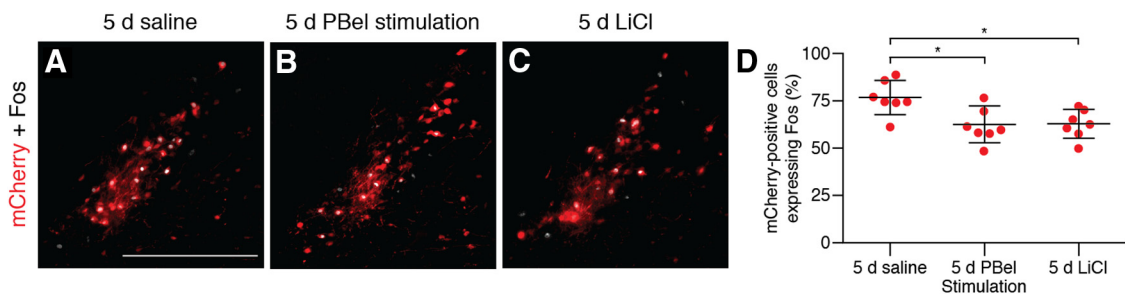


Figure 2. Prior LiCl or PBel CGRP stimulation reduces subsequent PBel CGRP activation during CTA. **A–C**, Representative histological examples of coincidence of mCherry (red) and Fos (white) expression in animals following LiCl injection after 5 d daily exposure to saline (**A**), PBel CGRP photostimulation (**B**), or LiCl administration (**C**). Scale bar, 500 μ m. **D**, Quantification of Fos expression 2 h after injection with LiCl ($n = 7$ mice per condition). Values represent the mean \pm SD. $*p < 0.05$ (Tukey's multiple comparisons test between conditions).

$p = 0.6735$; Fig. 1G). These effects were also lost when mice received daily pretreatment of LiCl for 5 d ($n = 9$ mice per condition; no interaction of condition \times day, $F_{(4,64)} = 0.1357$, $p = 0.2586$; Fig. 1H). Together, these results indicate that photoactivation of CGRP neurons is sufficient to induce CTA if it is novel, or prevent CTA if it is administered before exposure to a novel taste.

Importantly, there was no effect of ChR2-mCherry expression on body weight in the days leading up to acquisition of the CTA (no interaction of experimental group \times day, $F_{(55,576)} = 0.006$, $p > 0.99$). Additionally, there were no significant differences in body weight between mice with induced CTA and control mice with no induced CTA (no interaction of experimental group \times day, $F_{(44,480)} = 0.0044$, $p > 0.99$), demonstrating that CTA caused an aversion specific to Ensure and not an aversion to eating in general. This result is consistent with our previous finding that the decrease in food intake during stimulation of PBel CGRP neurons is rapidly reversible and does not cause a general, long-term reduction of feeding (Carter et al., 2013).

Repeated daily PBel CGRP neuron stimulation or LiCl administration slightly reduces subsequent activation of PBel CGRP neurons

To examine whether daily stimulation of PBel CGRP neurons for 5 d blocked subsequent CTA by reducing neural activity in PBel

neurons, we exposed *Calca^{Cre/+}* mice transduced with AAV-DIO-mCherry to 5 d of intraperitoneal saline administration, 5 d of PBel photostimulation, or 5 d of LiCl administration. Then the injected animals were injected with LiCl and killed 2 h later to examine the expression of Fos in mCherry-positive neurons (Fig. 2A–C). We found a modest but significant reduction in the percentage of mCherry-positive neurons expressing Fos in animals exposed to either photostimulation of CGRP neurons in the PBel or LiCl administration compared with animals exposed to saline administration ($n = 7$ mice per condition; difference between treatments, $F_{(2,18)} = 62.90$, $p < 0.0085$; Fig. 2D), suggesting that the PBel may be a potential site of plasticity for previous exposure to stimuli that cause CTA. However, because these neurons continue to show expression of Fos following these treatments, these results also demonstrate that LiCl can continue to activate PBel CGRP neurons after multiple days of exposure.

PBel CGRP neural activity is necessary for a normal CTA response to LiCl

To determine whether PBel CGRP neurons are necessary for LiCl-mediated CTA, we bilaterally transduced these neurons with AAV carrying a Cre-dependent hM₄Di-mCherry transgene (Armbruster et al., 2007) or mCherry control transgene and administered CNO (1 mg/kg; i.p.) during novel exposure to Ensure

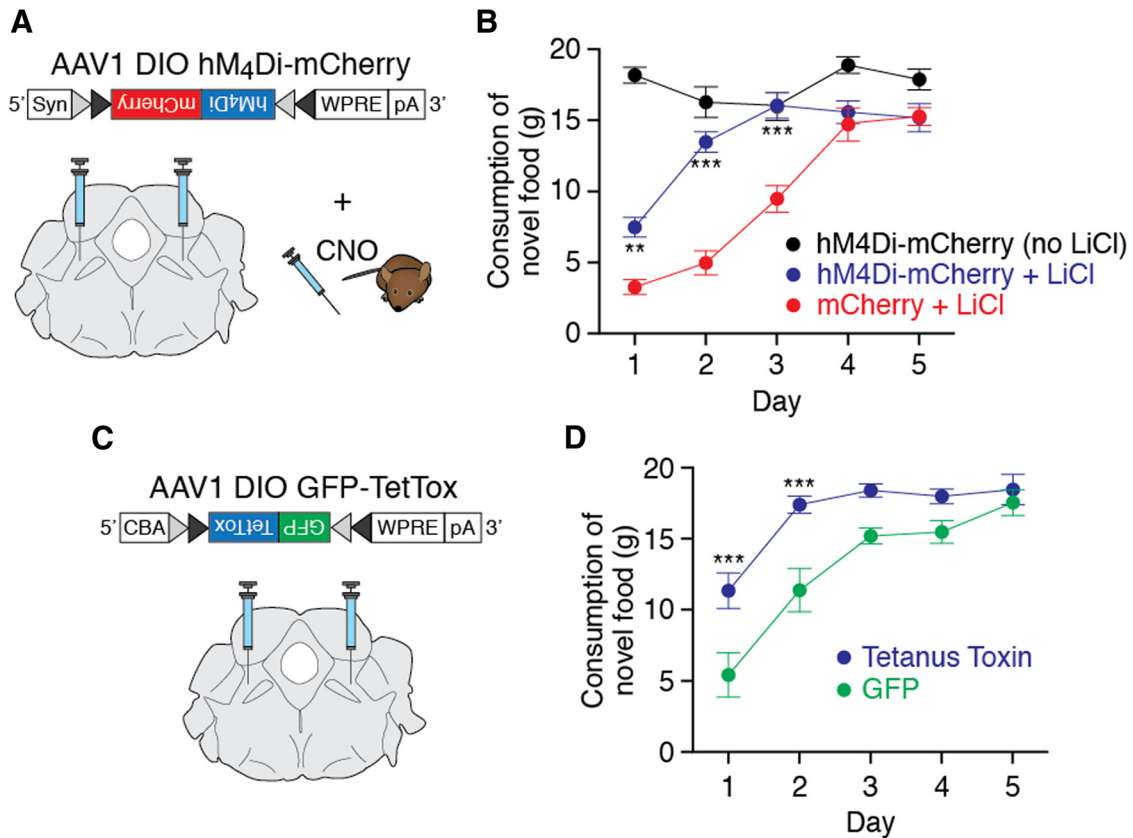


Figure 3. Genetic inhibition or silencing of PBel CGRP neurons attenuates LiCl-mediated CTA. **A**, AAV-DIO-hM₄Di-mCherry transgene bilaterally injected into the PBN. **B**, Pharmacogenetic inhibition of CGRP neurons during pairing of LiCl and novel tastant attenuates CTA ($n = 9$ mice per condition). **C**, AAV-DIO-GFP-TetTox transgene bilaterally injected into the PBN. **D**, TetTox-induced silencing of PBel CGRP neurons attenuates LiCl-mediated CTA ($n = 9$ mice per condition). All values represent the mean \pm SEM. $**p < 0.001$; $***p < 0.0001$ (Bonferroni *post hoc* test between conditions).

paired with injection of LiCl (Fig. 3A). Inhibition of CGRP neurons with CNO in animals expressing hM₄Di-mCherry in CGRP neurons significantly attenuated the CTA response compared with control mice expressing only mCherry ($n = 9$ mice per condition; interaction of viral genotype \times day, $F_{(8,96)} = 12.34$, $p < 0.0001$; Fig. 3B), although it did not completely block the CTA response on the first day.

To independently confirm these results, we also bilaterally transduced PBel CGRP neurons with AAV carrying a Cre-dependent tetanus toxin (AAV-DIO-GFP-TetTox) transgene (Kim et al., 2009) or a GFP control virus (Fig. 3C). When expressed in neurons, TetTox blocks synaptic transmission without resulting in cell death (Yamamoto et al., 2003). Consistent with hM4Di-mediated inactivation, we found that LiCl-mediated CTA was significantly attenuated in mice expressing TetTox in CGRP neurons compared with control mice with GFP only ($n = 9$ mice per condition, interaction of viral genotype \times day, $F_{(4,80)} = 2.491$, $p < 0.0497$; Fig. 3D). These results demonstrate that inhibition or silencing of CGRP neurons causes a significant reduction in CTA for Ensure paired with LiCl-induced malaise.

Discussion

Together, these findings demonstrate that PBel CGRP neurons are sufficient to cause CTA and are necessary for a normal CTA response to LiCl. Interestingly, our finding that CTA caused by optogenetic stimulation of PBel CGRP neurons could be blocked both by prior photostimulation and also by previous LiCl administration indicates that mice may interpret PBel CGRP stimulation and LiCl adminis-

tration as being equivalent. Thus, our results build upon previous findings that implicate the lateral PBN in regulating CTA by demonstrating that PBel CGRP neurons mediate a gastrointestinal distress signal required to establish CTA.

Inhibiting PBel CGRP neural activity (Fig. 3A,B) or silencing PBel CGRP synaptic activity (Fig. 3C,D) reduced, but did not completely abolish, the acquisition of CTA. These results suggest that additional circuits exist, independent of PBel CGRP neurons, which participate in LiCl-mediated CTA. Administration of LiCl, as well as other abdominal irritants and emetic agents, causes expression of Fos in the nucleus of the solitary tract (NTS) and area postrema (Yamamoto et al., 1992; Thiele et al., 1996; Sakai and Yamamoto, 1997). Lesioning or suppressing neural activity in the area postrema attenuates LiCl-induced CTA and suppresses Fos expression in downstream brain areas (Wang et al., 1997; Spencer et al., 2012). Therefore, projections from the NTS or area postrema that bypass the PBel could provide alternate neural pathways by which visceral signals could integrate to form a CTA.

However, it is worth noting that it is technically more difficult to inhibit a neural population than to active one. Activating a fraction of relevant neurons is often sufficient to achieve a maximal effect, whereas nearly all of the neurons have to be inhibited for complete suppression. Because the PBel is an elongated structure, it is possible that we did not transduce all CGRP-expressing neurons, and any unsilenced neurons were sufficient to induce a residual CTA.

Our study examined the effect of inhibiting/silencing PBEL CGRP neurons on acquisition, but not subsequent expression, of CTA. The acquisition and expression of a CTA can be defined as two separate behavioral processes (Parker, 2003), and it is possible that the PBEL is also necessary for the expression of a previously established CTA. Therefore, future studies should focus on the role of the PBEL in the reduction of food intake in the days following expression of a CTA in addition to the induction of the CTA itself.

Although prior activation of PBEL CGRP neurons for 5 d modestly reduces Fos expression caused by LiCl (Fig. 2), Fos expression continues to occur even in the absence of CTA, suggesting that the association between a novel tastant and environmental stimulus occurs primarily in a downstream location. A likely region is the amygdala, where visceral malaise and sensory taste signals are thought to converge (Koh et al., 2003; Reilly and Bornova, 2005). We showed previously that PBEL CGRP neurons send dense projections to the central amygdala (CeA; Carter et al., 2013), a region that is also necessary and sufficient for the acquisition of CTA (Bahar et al., 2003; Ma et al., 2011; Kwon and Houpt, 2012). Therefore, we propose that PBEL CGRP neurons constitute a necessary and sufficient hub for acquiring a CTA, whereas the association occurs downstream, presumably in the CeA.

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