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Defining the Role of Locus Coeruleus Noradrenergic Neurons in the Modulation of Homeostatic Feeding

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Abstract

The locus coeruleus (LC) is a hindbrain structure that is the major source of norepinephrine (NE) input to the cortex and other forebrain areas. It is involved in processes of arousal, attention, and stress. It has been shown that the locus coeruleus norepinephrine system is also involved in the modulation of feeding. In this experiment, we used triple transgenic mice expressing the hM3Dq receptor in LC-NE neurons. These mice and littermate controls were overnight fasted then injected with vehicle or deschloroclozapine (DCZ), which is an inert ligand that solely activates the designer hM3Dq receptor. The mice were then immediately placed in a feeding assay, where their food intake was measured at set time points over three hours. We found that activation of LC-NE neurons via administration of DCZ to the triple transgenic mice suppressed feeding compared to those injected with vehicle, especially at 3-hours post-injection. Additionally, we found that LC activation suppressed feeding in female mice more than in male mice. The results suggest that this dose of DCZ is effective at inducing a change in behavior in mice. The data also supports the idea that the LC-NE system is involved in the modulation of homeostatic feeding.

Keywords: locus coeruleus, norepinephrine, chemogenetics, feeding

Introduction

Locus Coeruleus-Norepinephrine System

The locus coeruleus (LC) is a group of neurons in the hindbrain, or brainstem, that produce the neurotransmitter norepinephrine (NE). These neurons project to various forebrain and hindbrain areas and receive inputs from many different brain regions (1,2). The LC is a bilateral structure, meaning it is present in both hemispheres of the brain. Rhombomeres are the segmental units of the developing hindbrain, each with its own genetic marker (1). The locus coeruleus is derived from rhombomere 1 cells, and this rhombomere also develops into portions of the dorsal subcoeruleus and A7 nuclei of the hippocampus (1).

Locus coeruleus neurons have two modes of firing: tonic and phasic. Tonic firing is characterized by a continual and regular pattern (2). This tonic discharge is implicated in the modulation of behavioral states of arousal and alertness and increases in response to stress (2,3). Phasic firing is a short-lived spike in response to relevant sensory stimuli that enables focusing on a specific task (2). Tonic activation of locus coeruleus neurons through chemogenetic and optogenetic manipulation has been shown to increase anxiety-like behavior, while inhibition of the LC-NE neurons decreases this anxiety-like behavior (4). Additionally, the LC suppresses feeding when optogenetically stimulated and chemogenetically activated by the ligand clozapine-N-oxide (CNO) (5,6). Studying the LC-NE system can have implications in treatment for anxiety, obesity, and disorders related to deficits in sensory processing, such as PTSD, ADHD, autism, and schizophrenia (7).

Designer Receptors Exclusively Activated by Designer Drugs (DREADDS)

Designer Receptors Exclusively Activated by Designer Drugs (DREADDs) are used to selectively activate or inhibit specific types of cells *in vivo*. G protein-coupled receptors are mutated to be activated by inert ligands but not their native ligands (8). One widely used DREADD is the hM3Dq receptor, a G_q-coupled human M3 muscarinic receptor (8). When the hM3Dq receptor is stimulated, the G_q subunit activates the enzyme phospholipase C (9). Phospholipase C activates second messengers within the cell and causes calcium to flow into the cytoplasm (8,9). This G_q-mediated signal transduction pathway leads to excitation of the neuron (9). The native human muscarinic 3 receptor is activated by acetylcholine (ACh), however this receptor was modified to be activated by the inert ligand clozapine-N-oxide (CNO) (8). CNO is extensively used as a designer drug to activate DREADDs, but it metabolizes into the psychoactive ligand clozapine, which has off-target effects at endogenous receptors (10). DREADDs and their designer drugs are used to target specific neural circuits and link their activation to behaviors (9). Advantages of chemogenetic activation include that it is noninvasive and can activate deep brain structures, while other methods such as optogenetics can be potentially harmful to the brain tissues and may be spatially restricted (9). Additionally, pharmacological activation of certain receptors cannot precisely target specific brain regions in the same manner as chemogenetic manipulation.

To address the problems associated with CNO metabolism into clozapine, a new DREADD agonist, deschloroclozapine (DCZ), has been synthesized (10). DCZ binds to the hM3Dq receptor with a 100-fold higher affinity than CNO, so a lower dose of DCZ is needed to have the same effects as a higher dose of CNO (10). DCZ also has a very low affinity for endogenous receptors and its metabolites do not have off-target effects either (10). When systemically administered, DCZ is able to rapidly enter the brain and activate neurons in both

mice and monkeys (10). DCZ has been used to manipulate behaviors by activating specific neuron populations in monkeys (10). However, the appropriate doses to initiate a change in behavior in mice have not been characterized, so in this experiment I will investigate if a 10 μg/kg dose of DCZ is able to sufficiently activate the LC through the hM3Dq receptor.

LC-hM3Dq Mouse Line

In order to express the hM3Dq receptor only in locus coeruleus noradrenergic neurons, we used a mouse line that uses two recombinase-dependent knock-in alleles (11). En1 is a gene that defines rhombomere 1, a segment of the developing hindbrain that develops into the majority of the locus coeruleus and portions of the dorsal subcoeruleus and A7 of the hippocampus (1). Dopamine-beta hydroxylase (Dbh) is the enzyme responsible for synthesizing NE from dopamine, so the Dbh gene is expressed in all NE-producing neurons. In the specific mouse line used for this experiment, the En1 gene drives Cre recombinase expression and the Dbh gene drives Flp recombinase expression. These genes ensure that Cre and Flp are only expressed in the noradrenergic neurons of the locus coeruleus. Cre and Flp recombine the alleles at their specific cut sites, LoxP sites for Cre recombination and FRT sites for Flp recombination, to result in the expression of hM3Dq fused with the mCherry fluorophore for visualization (11). When DCZ is injected systemically into these mice, it will only activate the LC-NE neurons, as these are the only neurons expressing the hM3Dq receptor (Fig. 1).

The RC::FL-hM3Dq allele contains an epitope that localizes the receptor to the soma and dendrites of neurons (11). This was verified in LC neurons, as mCherry fluorescence was confined to the soma and dendrites in the LC, indicating that the hM3Dq receptor was localized to these regions as well (11). The ability of this allele to modulate behavior *in vivo* when

activated by CNO was tested (11). As shown previously, optogenetic activation of LC neurons causes increased anxiety behavior (4). When CNO was administered to the LC^{hM3Dq} mice, anxiety was induced in three assays of anxiety – the elevated plus maze, light dark box, and open-field test (11). This indicates that the LC^{hM3Dq} mouse line can effectively manipulate behavior when activated through administration of a ligand of the hM3Dq receptor.

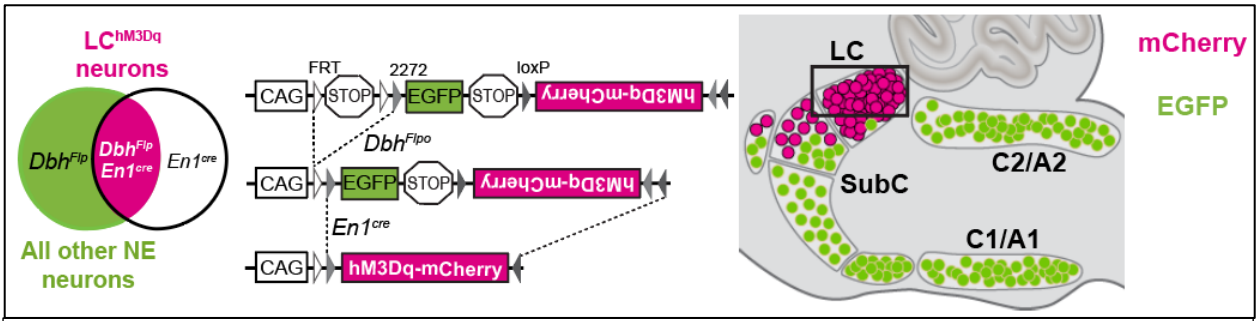

Figure 1. Dbh^{Flpo} and $En1^{cre}$ drive the expression of the hM3Dq receptor in this triple transgenic mouse line. *Left.* LC^{hM3Dq} neurons express both Dbh^{Flpo} and $En1^{cre}$. *Middle.* Schematic of the RC::FL-hM3Dq allele, depicting how Flp and Cre recombinase recombine the DNA for proper expression of the hM3Dq receptor. *Right.* Schematic showing the portions of the midbrain that express mCherry fused to the hM3Dq receptor, which includes the LC and portions of the subcoeruleus and A7 of the hippocampus. All other NE-producing neurons express green fluorescent protein (EGFP). Schematic illustration from Sciolino et al. 2019 (5).

Neural Circuits of Feeding Behavior

The hypothalamus and brainstem are two brain areas that have been implicated in the regulation of appetite and feeding (12). Destruction of the ventromedial hypothalamus, hypothalamic paraventricular nucleus, or arcuate nucleus of the hypothalamus results in overeating and obesity (12). Additionally, activation of the lateral hypothalamus has increased feeding and hoarding when both hungry and sated (12). On the contrary, feeding is decreased when neurons between the brainstem and hypothalamus are cut (12).

The neurotransmitter norepinephrine is thought to be involved in the circuits of feeding. Activation of the α1, β2, and β3-adrenoreceptors decreases eating while activation of the α2-

adrenoreceptors increases eating (12). Norepinephrine and other monoamine neurotransmitters (dopamine and serotonin) are targets of some anti-obesity drugs (12). Locus coeruleus norepinephrine-producing neurons project to the hypothalamus and specifically those areas implicated in feeding, including the paraventricular nucleus, arcuate nucleus, and lateral hypothalamic area (LHA) (1). Therefore, the LC is likely involved in feeding as the source of NE input into the hypothalamus.

It has been found that optogenetic and chemogenetic activation of LC-NE neurons reduce food intake (5). When overnight fasted LC^{hM3Dq} mice and controls were injected with CNO, the LC^{hM3Dq} mice ate less over a 20-minute period than the control mice (5). Another study also showed that chemogenetic activation of the hM3Dq receptor in LC neurons by CNO lead to reduced food intake over a longer 3-hour period (6). In this study, the Cre-responsive hM3Dq receptor was inserted in an adeno-associated virus (AAV) and injected into mice that express Cre recombinase in the tyrosine hydroxylase gene (6), which is the rate-limiting enzyme in the process that synthesizes norepinephrine. Brief and continuous optogenetic stimulation also confirmed that activation of LC-NE neurons suppresses feeding (5). Flp-dependent AAVs expressing either ChrimsonR-tdT, a cation channel activated by red-light, or the control fluorophore tdTomato were injected into the LC of Dbh^{Flpo} mice, which express Flp in NE neurons (5). Photostimulation lead to decreased food intake in the $LC^{ChrimsonR}$ mice and not the $LC^{tdTomato}$ controls, again demonstrating that LC-NE neurons are involved in the suppression of feeding (5).

Optogenetic activation of a more specific circuit, LC-NE neurons projecting to the LHA, also suppresses feeding (5). In one experiment, AAVs expressing Cre-dependent channelrhodopsin-2 (ChR2) or constitutive yellow fluorescent protein (EYFP) were injected into

the LC of mice expressing the Cre driver in the Dbh gene (5). Channelrhodopsin-2 is a cation channel that opens in response to blue light and depolarizes the cell (13). ChR2 or EYFP were expressed in LC-NE neurons, as these neurons expressed Cre and could therefore recombine the viral DNA to express these proteins. Optogenetic activation of the LC-NE terminals in the LHA using blue light (465 nm) over a 30-minute period resulted in decreased feeding in fasted mice expressing ChR2 but not fasted mice expressing EYFP (5). Therefore, the LC-NE to LHA circuit is involved in feeding behavior.

In this current experiment, fasted LC^{hM3Dq} mice and littermate controls were injected with vehicle or DCZ then their food intake was recorded at set times over a 3-hour period. I predicted that injection with DCZ would suppress food intake in the triple heterozygous mice of the strain $En1^{Cre}$; Dbh^{Flpo}; RC::FL-hM3Dq, as was seen in these mice when injected with CNO. This feeding assay was used to determine an effective dose of DCZ that suppresses feeding to guide future experiments.

Materials and Methods

Animals

Procedures involving use of mice were approved by the University of Connecticut Institutional Animal Care and Use Committee. Adult male and female mice between the ages of 3 to 5 months were used for this experiment. Nine triple transgenic $En1^{Cre}$; Dbh^{Flpo}; RC::FL-hM3Dq mice and 15 littermate controls were used. One LC^{hM3Dq} mouse was excluded from analyses, as it was a statistical outlier. The triple transgenic mice were generated by crossing $En1^{Cre}$ mice to double transgenic Dbh^{Flpo}; RC::FL-hM3Dq mice[7]. Mice were group housed and kept on a reverse l2:12-hour light:dark cycle, with lights off at 9:00 AM. Experiments were

performed during the active dark period. Mice had access to *ad libitum* food, except when overnight fasted the day prior to testing.

Drugs

Deschloroclozapine (DCZ) from Hello Bio (HB8555) was used for this experiment. DCZ was dissolved in DMSO in a 1 mM stock DCZ. On the day of experiments, the drug was brought to volume in 0.9% physiological saline to make a dose of 10 μg/kg DCZ. Vehicle was made by adding saline to <1% DMSO. The body weight of the mice was measured the morning of the trial, and the compounds were injected at a volume of 0.1 mL/10 g body weight. Drug or vehicle was injected via intraperitoneal (i.p.) route immediately before behavioral trials.

Food-Intake Test

The food intake test took place in a place preference arena, with one corner containing an empty food dish and the opposite corner containing a dish filled with standard food. The side in which the food was placed was counterbalanced for each treatment group. The arena was placed inside a Med Associates sound attenuating cubicle during testing. Mice were habituated to the arena and an i.p. saline injection prior to testing in a non-fasted state. Mice were fasted for 24 hours in their own cages a day before testing to motivate feeding. The mice were injected with DCZ (10 μg/kg, i.p.) or vehicle immediately before testing then given 3 hours to explore the arena. Food was weighed before the experiment and before each time point. Food was removed to be weighed at 10 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours into the experiment, then replaced with new food that was also weighed. Mice were returned to *ad libitum* food in their

group housed cages after testing. All mice were tested with both vehicle and DCZ, with trials separated by one week.

All trials were recorded using Noldus GigE cameras that were mounted vertically on the ceiling of the Med Associates cubicles. Noldus Ethovision XT software was used to track the movement of the mice in the arena. The live video tracking was monitored periodically throughout the trials to ensure no problems arose with any of the mice.

Statistical Analysis

All statistical analyses were performed using the GraphPad Prism software. All data in the figures are expressed as mean ± standard error (SEM). Food intake data is presented in grams (g) or percent change in food intake from vehicle to DCZ. ANOVAs and t-tests were used to assess differences between groups. All analyses were two-tailed. Tukey's method was used to identify any extreme outliers, and it identified one LC^{hM3Dq} male mouse that was removed from all analyses.

Results

LC^{hM3Dq} mice and littermate controls were fasted for 24 hours then injected with DCZ (10 μg/kg, i.p.) or vehicle. They were immediately placed in an arena and food intake was measured after 10 minutes, 30 minutes, 1 hour, 2 hours, and 3 hours (Fig. 2A). We found that there was a trend towards a reduction in feeding in LC^{hM3Dq} mice receiving DCZ compared to vehicle, however this result was not statistically significant (Fig. 2B). There was no significant difference between control mice receiving DCZ or vehicle, indicating that DCZ did not have off-target effects that impact feeding (Fig. 2B). When the data was expressed as DCZ-induced

percent change in food intake, we found a trend towards a decrease in feeding in LC^{hM3Dq} mice compared to controls (Fig 2B). There was an average of an 11% decrease in food intake in LC^{hM3Dq} mice compared to approximately a 4% increase in food intake in control mice (Fig. 2B).

At each time point throughout the experiment, there was a trend towards lower food intake in LC^{hM3Dq} mice receiving DCZ compared to those receiving vehicle (Fig. 2C). This reduction in food intake was most apparent at the 3-hour timepoint (Fig. 2C). There was no difference in food intake between control mice receiving DCZ or vehicle at any time point (Fig. 2C). Next, we analyzed if there were any sex differences in the suppression of feeding by the LC. We found a significant difference in percent change in food intake between LC^{hM3Dq} male and female mice (Fig. 2D). Female LC^{hM3Dq} mice had nearly a 20% decrease in feeding when the LC was activated, while male LC^{hM3Dq} mice only had about a 2% decrease in feeding (Fig. 2D). There was no significant difference in food intake between male and female control mice (Fig. 2D). All of our data suggest that chemogenetic activation of LC-NE neurons suppresses feeding.

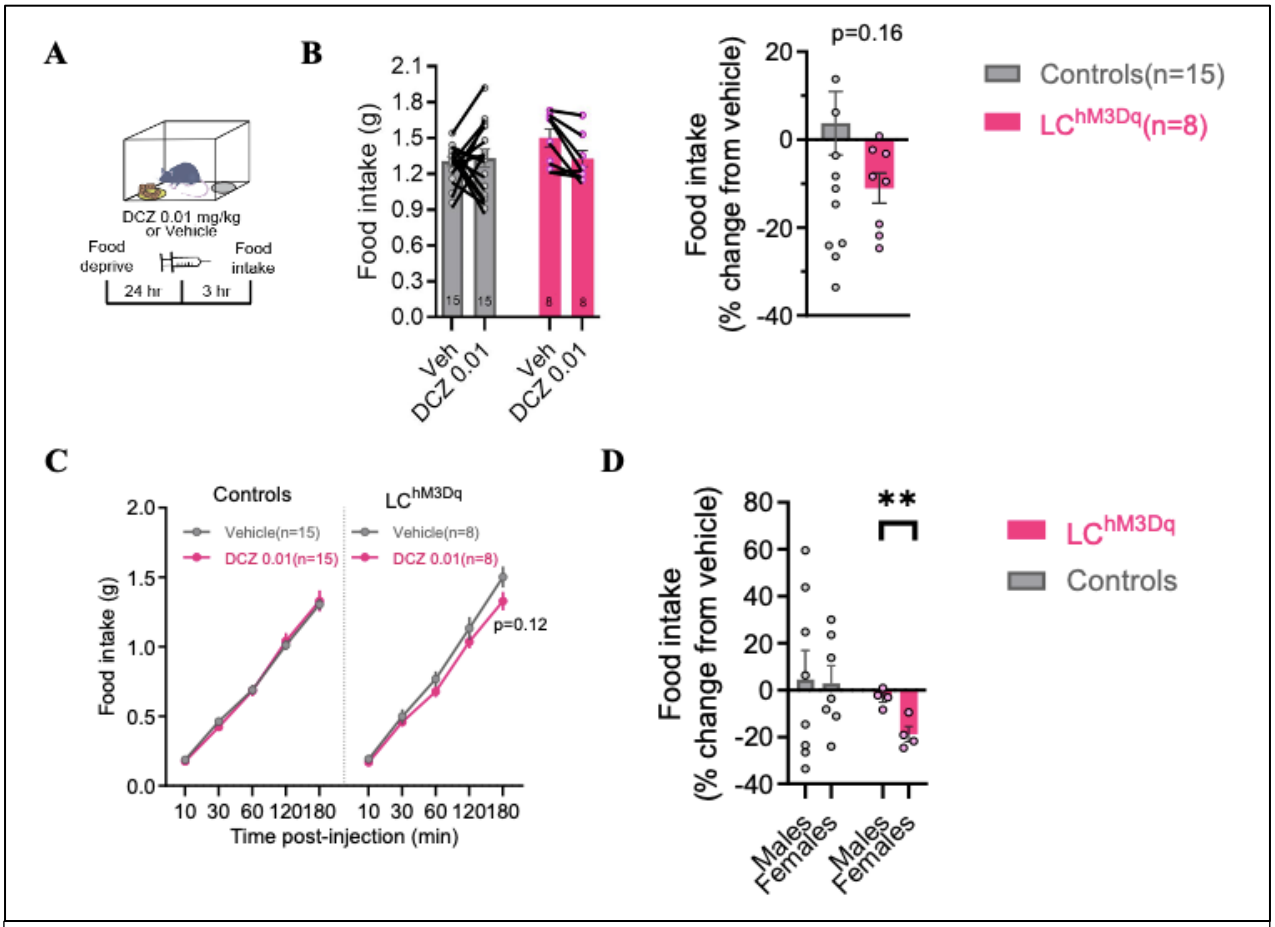

Fig 2. Chemogenetic activation of the LC shows a trend towards suppressing food intake, especially at 3 hours and in female mice. (A) Timeline of the food intake experiment. Mice were food deprived for 24 hours, injected with DCZ or vehicle, then their food intake was measured over the next 3 hours. (B) *Left*. Average food intake in control mice and LC^{hM3Dq} mice receiving vehicle and DCZ. *Right.* Food intake presented as percent change from vehicle. Two-tailed, unpaired t-test between LC^{hM3Dq} mice and control mice: p=0.16. n=13 control mice and n=8 LC^{hM3Dq} mice. (C) Food intake over the span of 3 hours in control and LC^{hM3Dq} mice receiving DCZ or vehicle. Two-way repeated measures ANOVA: 180 mins, LC^{hM3Dq} mice (p=0.12). (D) Sex differences in DCZ-mediated percent change in food intake. Unpaired, two-tailed t-test between male and female LC^{hM3Dq} mice: p=0.0065**. n=8 control males, n=7 control females, n=4 LC^{hM3Dq} males, n=4 LC^{hM3Dq} females.

Discussion

It is known that the locus coeruleus is involved in arousal and alerting animals to salient stimuli (3,7). Previous studies have also shown that activation of the LC suppresses feeding (5,6). This current experiment further characterized this using a different designer drug,

deschloroclozapine. Our approach used a LC^{hM3Dq} mouse line to ensure that only the noradrenergic-producing neurons of the LC would be activated through stimulation of the hM3Dq receptor. This chemogenetic manipulation was noninvasive and spatially precise.

Our data showed a trend towards decreased feeding when the LC was activated, especially at the 3-hour mark of the trials. This indicates that this dose of DCZ has long-lasting effects in the brain, and the amount of time that these effects last should be characterized in another experiment. Additionally, we found a significant difference in food intake between male and female LC^{hM3Dq} mice. This could indicate a potential sex difference in locus coeruleus signaling and suppression of feeding. Since this was a small sample size, a future experiment with a larger sample size is needed to confirm these results. The results suggest that chemogenetic activation of LC-NE neurons suppresses homeostatic feeding.

Future experiments in the lab will build upon this preliminary experiment, as it was the first to find a dose of DCZ that is sufficient in activating the LC and initiating a change in behavior in mice. This dose will be used to chronically activate the LC over the course of four days. LC^{hM3Dq} and control mice will be injected with DCZ (10 μg/kg, i.p.) twice daily and their food intake and weight will be measured daily and compared to baseline. After characterizing this chronic dosing, mice at different stages in obesity will be chronically injected with DCZ (10 μg/kg, i.p.) to determine if activating the LC can reduce food intake and cause weight loss in obese mice. This current experiment and the future experiments could thus have implications in the treatment of obesity.

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