Title: Elimination of galanin synthesis in noradrenergic neurons reduces galanin in select brain areas and promotes active coping behaviors

Running Title: Noradrenergic-derived galanin modulates active coping

Authors: Rachel P. Tillage[1]*, Natale R. Sciolino[2]*, Nick W. Plummer[2], Daniel Lustberg[1], L. Cameron Liles[1], Kathleen G. Smith[2], Patricia Jensen[2#], David Weinshenker[1#]

[1]*Emory University, Department of Human Genetics, Atlanta, GA 30322*

[2]*National Institute of Environmental Health Sciences, Developmental Neurobiology Group, Research Triangle Park, NC*, 27709

*These authors contributed equally to the work

[#]Co-corresponding authors: David Weinshenker, Ph.D., Department of Human Genetics, Emory University School of Medicine, Whitehead 301, 615 Michael St., Atlanta, GA 30322, e-mail: dweinsh@emory.edu, and Patricia Jensen, Ph.D., Neurobiology Laboratory, National Institute of Environmental Health Sciences, National Institute of Health, Department of Health and Human Services, Research Triangle Park, NC 27709, e-mail: patricia.jensen@nih.gov

ABSTRACT: Accumulating evidence indicates that disruption of galanin signaling is associated with neuropsychiatric disease, but the precise functions of this neuropeptide remain largely unresolved due to lack of tools for experimentally disrupting its transmission in a cell type-specific manner. To examine the function of galanin in the noradrenergic system, widely thought to be an important source of galanin for modulation of emotional responses, we generated and crossed two novel knock-in mouse lines to create animals lacking galanin specifically in noradrenergic neurons ($Gal^{cKO\text{-}Dbh}$). We observed reduced levels of galanin peptide in pons, hippocampus, and prefrontal cortex of $Gal^{cKO\text{-}Dbh}$ mice, indicating that noradrenergic neurons are a major source of galanin to those brain regions, while midbrain and hypothalamic galanin levels were comparable to littermate controls. In these same brain regions, we observed no change in levels of norepinephrine or its major metabolite, suggesting that loss of galanin does not affect baseline noradrenergic synthesis or turnover. $Gal^{cKO\text{-}Dbh}$ mice had normal performance in tests of depression, learning, and motor-related behavior, but had an altered response in anxiety-related tasks. Specifically, $Gal^{cKO\text{-}Dbh}$ mice showed increased marble and shock probe burying and had a reduced latency to eat in a novel environment, indicative of a more proactive coping strategy. Together, these findings indicate that noradrenergic neurons provide a significant source of galanin to discrete brain areas, and noradrenergic-specific galanin opposes adaptive coping responses.

INTRODUCTION

The neuropeptide galanin was first discovered in the early 1980s in porcine gut. Subsequent studies revealed that galanin is expressed throughout the brain and body of both humans and rodents [1-4] and modulates a variety of physiological processes including feeding, nociception, seizures, stress responses, cognition, and mood [2,5]. Given this broad range of functions, it is not surprising that genome-wide association studies have implicated variants in genes encoding galanin and its receptors in conferring increased risk of depression and anxiety in humans [6-8]. The exact role of galanin in these complex disorders, however, has yet to be determined.

Preclinical studies examining the effects of galanin have relied heavily on global knockouts or intracerebroventricular infusion of galanin agonists or antagonists. These experimental strategies lack regional specificity and often generate conflicting results. For example, galanin agonists administered into the rodent brain have been reported to elicit an anxiolytic effect in some tests (e.g. conflict test and elevated zero maze), and no effect in other anxiety assays (elevated plus maze, light-dark exploration, and open field) [9-12]. Likewise, galanin agonists also produce mixed effects on behavior in the forced swim and tail suspension tests [13-17]. Furthermore, global galanin knockout mice exhibit a wide variety of phenotypes, including endocrine, neurological, and behavioral deficits [18-23]. These findings suggest that galanin's effects are specific to the cell type, brain region, circuit, and/or receptor(s) being engaged. Thus, strategies to manipulate galanin in a cell type-specific manner will help unravel its distinct functional roles in diverse physiological processes and disease states.

Galanin is expressed abundantly in a subset of noradrenergic neurons located in the locus coeruleus (LC) and subcoeruleus (SubC) in both rodents and humans [24-29]. Intriguingly, transgenic mice overexpressing galanin in all noradrenergic neurons exhibit seizure resistance, mild cognitive deficits, and resilience against yohimbine-induced anxiety-like behavior [30-32]. Because the transgene is expressed in all neurons that produce norepinephrine (NE) and

epinephrine, and ectopically in some non-NE neurons (e.g. piriform cortex, entorhinal cortex) [30], it is difficult to ascribe phenotypes to the subset of NE neurons that endogenously express galanin. The role of noradrenergic-derived galanin in normal and impaired brain function therefore remains unclear (for reviews see [2,33-35].

To begin to address this important question, we combined two new mouse alleles - a conditional knockout allele of *Gal* and a knock-in cre driver allele under control of the noradrenergic-specific *Dbh* promoter - to generate a mouse model in which galanin is selectively disrupted in noradrenergic neurons. Using this model, we measured the proportion of noradrenergic neuron-derived galanin in discrete brain regions and determined the consequences of its loss on behaviors relevant to anxiety, depression, cognition, and gross motor function. Our biochemical findings reveal that noradrenergic neurons provide a significant source of galanin to the cortex and hippocampus, and that loss of galanin has no effect on NE levels or turnover. Furthermore, we demonstrate that noradrenergic neuron-specific galanin signaling plays a role in the regulation of defensive coping behaviors in the context of anxiogenic environmental manipulations.

MATERIALS AND METHODS

Animals

All procedures related to the use of animals were approved by the Animal Care and Use Committee of the NIEHS and Emory University and were in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Mice were maintained on a 12/12 h light-dark cycle with access to food and water *ad libitum* unless stated otherwise.

Generation of mouse lines

To generate the Gal^{cKO} allele ($Gal^{tm1a(KOMP)Pjen}$), we obtained a targeting vector (Project ID: CSD82928) containing loxP-flanked *Gal* exon 3 and FRT-flanked *LacZ* and *Neo* cassettes from the KOMP Repository (https://www.komp.org). Linearized vector was electroporated into

G4 embryonic stem cell [36], and homologous recombinants were identified by Southern blotting with a *Neo* probe and long range PCR (see Supplementary Information for details). Recombinant cells were injected into B6(Cg)-Tyr^{c-2J}/J blastocysts to produce chimeric mice. Heterozygous offspring of the chimeras were crossed to B6.Cg-*Tg(ACTFlpe)9205Dym*/J mice [37; Jackson Lab stock no. 005703] to excise the FRT-flanked *LacZ* and *Neo* cassettes and generate the conditional allele. The Gal^{cKO} mice were thereafter maintained by backcrossing to C57BL/6J mice. *Cre*-mediated recombination of this allele deletes exon 3 and introduces a frameshift if exon 2 is spliced to exon 4, thus eliminating expression of both galanin and galanin message associated protein (GMAP), which are encoded by the same mRNA.

To generate the Dbh^{cre} allele, we employed homologous recombination in G4 embryonic stem cells to insert a rox-flanked transcriptional stop cassette, *cre* cDNA, rabbit β-globin polyadenylation cassette, and attB/attP-flanked neomycin resistance cassette into the *Dbh* locus, immediately following the start codon. A recombinant clone was transiently transfected with pPGKPhiC31obpa [38] to excise the neomycin resistance cassette before cells were injected into B6(Cg)-Tyr^{c-2J} blastocysts to produce chimeric mice. Following germline transmission, we crossed heterozygotes with B6;129-*Tg(CAG-dre)1Afst* mice to permanently excise the rox-flanked stop cassette and generate the Dbh^{cre} mouse line. The Dbh^{cre} mice were thereafter maintained by backcrossing to C57BL/6J.

Dbh^{cre}*; RC::LTG* mice were generated by crossing heterozygous Dbh^{cre} to homozygous *RC::LTG* mice [39]. $Gal^{cKO\text{-}Dbh}$ mutants were created by crossing $Dbh^{cre/+}$;$Gal^{cKO/+}$ double heterozygotes or $Dbh^{cre/+}$;Gal^{cKO} homozygotes to Gal^{cKO} homozygotes (**Fig. 1a**). $Gal^{cKO\text{-}Nestin}$ mutants were created by crossing $Nestin^{cre/+}$;Gal^{cKO} homozygotes to Gal^{cKO} homozygotes ($Nestin^{cre/+}$ breeders were obtained from The Jackson Laboratory, Bar Harbor, ME, strain #003771).

Immunohistochemistry

Colchicine, a disrupter of microtubule polymerization, was used to amplify galanin peptide signal for experiments requiring immunofluorescent cell body detection, as previously described [25,26]. Colchicine stereotaxic delivery and tissue collection details can be found in Supplementary Information. Immunohistochemistry was performed as previously described [40,41]. Antibodies are summarized in **Supplementary Table 1** (Supplementary Information).

***In situ* hybridization**

In situ hybridization was performed using BaseScope™ Probe BA-Mm-Gal-E3-1ZZ-st (Catalog #712941, Lot #18067A; Advanced Cell Diagnostics, Newark CA), which targets nucleotides 228-266 of mouse *Gal* (exon 3). Tissue was labeled according to the manufacturer's instructions and then processed for immunohistochemistry as described above.

Galanin Enzyme-linked Immunosorbent Assay (ELISA):

Samples were processed as detailed in Supplementary Information. Samples were reconstituted in 250 µL EIA buffer (Peninsula Laboratories, San Carlos, CA), and processed according to the manufacturer's instructions (Galanin Rat and Mouse ELISA kit, S1208, Peninsula Laboratories, San Carlos, CA). Wells were read at 450 nm, averaged across duplicates, and a curve of best fit was used to calibrate to standards.

High Performance Liquid Chromatography (HPLC):

Samples were processed as detailed in Supplementary Information. NE and its primary metabolite MHPG were measured by with electrochemical detection using an ESA 5600A CoulArray detection system, as previously described [43]. Analytes were identified by matching criteria of retention time and sensor ratio measures to known standards (Sigma-Aldrich, St. Louis, MO). Compounds were quantified by comparing peak areas to those of standards on the dominant sensor.

Behavior

Adult male and female mice were tested between 3 and 8 months of age. Mice were group-housed for the duration of behavioral testing, unless stated otherwise. Tests were

separated by at least 4 days. Details of behavioral tasks can be found in Supplemental Information.

Statistical Analysis

Data were analyzed via unpaired t-test, one-way or two-way ANOVA with *post hoc* Tukey's test for multiple comparisons when appropriate. Significance was set at $p<0.05$ and two-tailed variants of tests were used throughout. Data are presented as mean ± SEM. Calculations were performed, and figures created using Prism Version 7 (GraphPad Software, San Diego, CA).

RESULTS

***Gal*$^{cKO\text{-}Dbh}$ mice lack galanin mRNA and protein in noradrenergic neurons**

To characterize the role of galanin in noradrenergic neurons, we first generated a knock-in allele that expresses cre recombinase under control of the noradrenergic-specific dopamine ***β***-hydroxylase promoter (*Dbh*^{cre}). To evaluate the expression pattern and activity of *Dbh*^{cre}, we crossed it to the cre-responsive reporter *RC::LTG* [39] which drives EGFP expression upon *cre* recombination. We observed EGFP expression in tyrosine hydroxylase+ (TH) noradrenergic neurons in the peripheral and central nervous system of *Dbh*^{cre}*; RC::LTG* double heterozygotes (**Supplementary Fig. 1** and data not shown). Similar to some *Dbh-cre* transgenic alleles [46,47], we also observed scattered EGFP+ cells in the adult cortex that likely reflect early transient expression of *Dbh* (**Supplementary Fig. 2**). Importantly for the present study, the EGFP+ cortical neurons were negative for *Gal* mRNA expression (**Supplementary Fig. 2**).

We next generated a conditional knockout allele of *Gal* (*Gal*^{cKO}) and crossed it with *Dbh*^{cre} to create the experimental mice referred to as *Gal*$^{cKO\text{-}Dbh}$ mutants (**Fig. 1**). To confirm that mutants lack galanin expression selectively in noradrenergic neurons, we used *in situ* hybridization to detect *Gal* mRNA in adult brains of *Gal*$^{cKO\text{-}Dbh}$ mutant and littermate control mice. *Gal* mRNA was abundant in LC and SubC noradrenergic neurons of control mice, but completely absent in these nuclei in *Gal*$^{cKO\text{-}Dbh}$ mutants (**Fig. 1**). Importantly, *Gal* mRNA expression remained intact in TH-negative neurons in C2/A2 and other non-noradrenergic brain

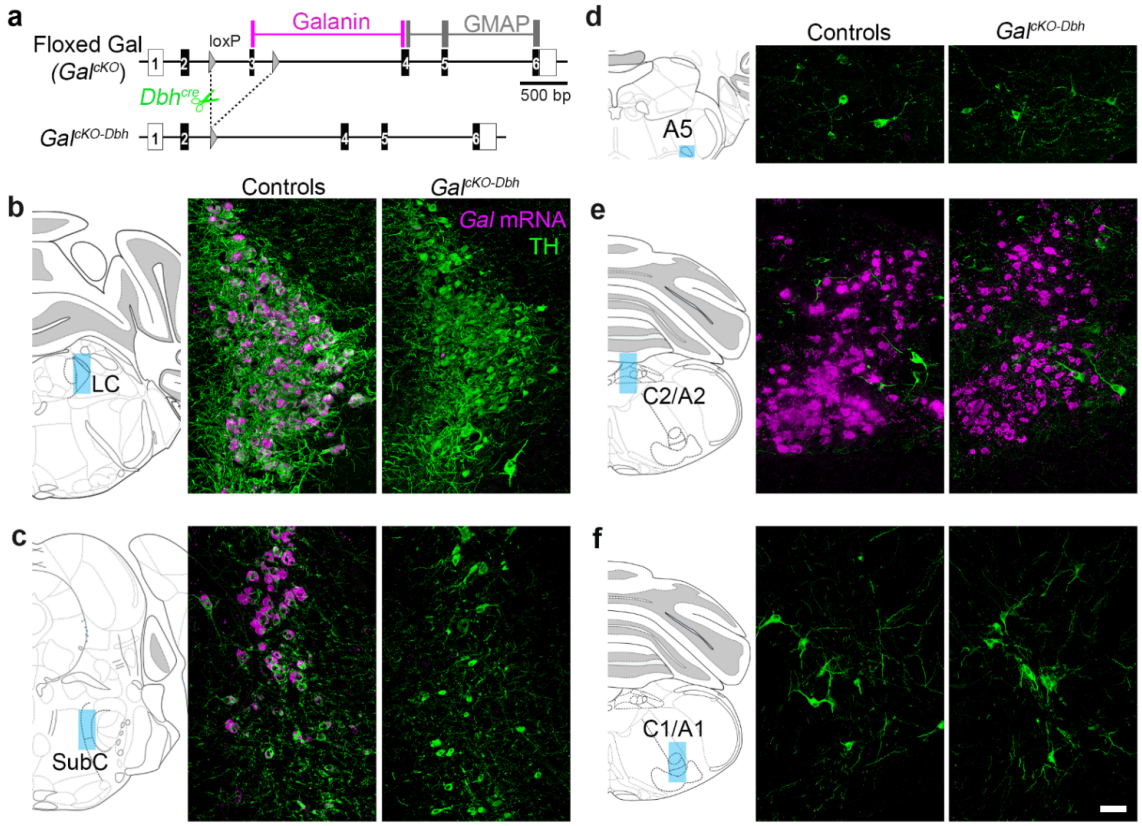

Figure 1. *Gal* conditional knockout allele permits selective disruption of galanin synthesis in noradrenergic neurons. (a) Schematic diagram of *Gal*^{cKO} allele. Recombination by *Dbh*^{cre} leads to disruption of galanin synthesis in NE neurons. (b-f) *Left*. Schematic illustration of coronal mouse brain showing location of images. *Right*. Representative coronal brain sections stained for *Gal* mRNA (riboprobe; magenta) and tyrosine hydroxylase (TH antibody; green). *Gal* mRNA is absent from the SubC and LC of mutant mice (*Gal*$^{cKO\text{-}Dbh}$). Scale bar, 50 μm. LC, locus coeruleus. SubC, sub-coeruleus.

regions, including the bed nucleus of the stria terminalis (BNST), lateral preoptic area, lateral hypothalamus and inferior olive (**Fig. 1 and Supplementary Fig. 3**).

Next, we examined galanin protein expression by immunohistochemistry. To induce accumulation of the peptide in neuronal somata and thus improve its visualization, we injected the axonal transport blocker colchicine (10 µg) into the lateral ventricle of adult *Gal*$^{cKO\text{-}Dbh}$ mutant and littermate control mice. Like the pattern of mRNA expression, galanin was evident in TH+ neurons of the LC and SubC of littermate controls but abolished in almost all noradrenergic neurons in *Gal*$^{cKO\text{-}Dbh}$ mutants (**Supplementary Fig. 4** and data not shown). In both *Gal*$^{cKO\text{-}Dbh}$ mutant and littermate control mice, we observed galanin immunoreactivity in non-noradrenergic populations, including the inferior olive (**Supplementary Fig. 4** and data not shown). Interestingly, galaninergic fibers persisted in the LC region of *Gal*$^{cKO\text{-}Dbh}$ animals (**Supplementary Fig. 4**), revealing that LC cells are innervated by galanin-containing neurons from non-noradrenergic sources. Combined, these findings indicate that *Gal*$^{cKO\text{-}Dbh}$ mice have a selective disruption of galanin synthesis in noradrenergic neurons.

Noradrenergic neurons provide a significant proportion of galanin to select brain regions

To assess the proportion of galanin in the brain that comes from noradrenergic neurons, we measured galanin protein levels in dissected tissue from the prefrontal cortex (PFC), dorsal and ventral hippocampus, hypothalamus, midbrain, and pons by ELISA. As a negative control and for comparison, we also generated and tested mice lacking galanin throughout the brain (*Gal*$^{cKO\text{-}nestin}$). A one-way ANOVA comparing *Gal*$^{cKO\text{-}Dbh}$ to littermate control and *Gal*$^{cKO\text{-}nestin}$ mice revealed significant differences between genotypes for each brain region (PFC, $F(2,11)=7.496$,

p=0.009; dorsal hippocampus, F(2,12)=33.09, p<0.001; ventral hippocampus, F(2,12)=4.164, p=0.042; hypothalamus, F(2,12)=7.068, p=0.008; midbrain, F(2,11)=35.49, p<0.001; pons, F(2,13)=15.17, p<0.001). As expected, *Gal*$^{cKO\text{-}nestin}$ mice had a significant reduction in galanin levels compared to littermate controls in all brain regions examined (Tukey's post hoc: PFC, p=0.035; dorsal hippocampus, p<0.001; ventral hippocampus p=0.078; hypothalamus, p=0.008; midbrain, p<0.001; pons, p<0.001) (**Fig. 2**), validating the effectiveness and specificity of our biochemical assay. Interestingly, *Gal*$^{cKO\text{-}Dbh}$ mice had significantly decreased galanin in the PFC (p=0.012), pons (p=0.013), dorsal hippocampus (p<0.001) and a trending decrease in ventral hippocampus (p=0.058) compared to littermate controls, but no difference in the midbrain (p=0.791) or hypothalamus (p=0.847) (**Fig. 2**). The reduced levels of galanin in the PFC and hippocampus of *Gal*$^{cKO\text{-}Dbh}$ mutants was not significantly different from that of *Gal*$^{cKO\text{-}nestin}$ mice (PFC, p=0.85; dorsal hippocampus, p=0.394; ventral hippocampus p=0.984) (**Fig. 2a, c, d**). Taken together, these

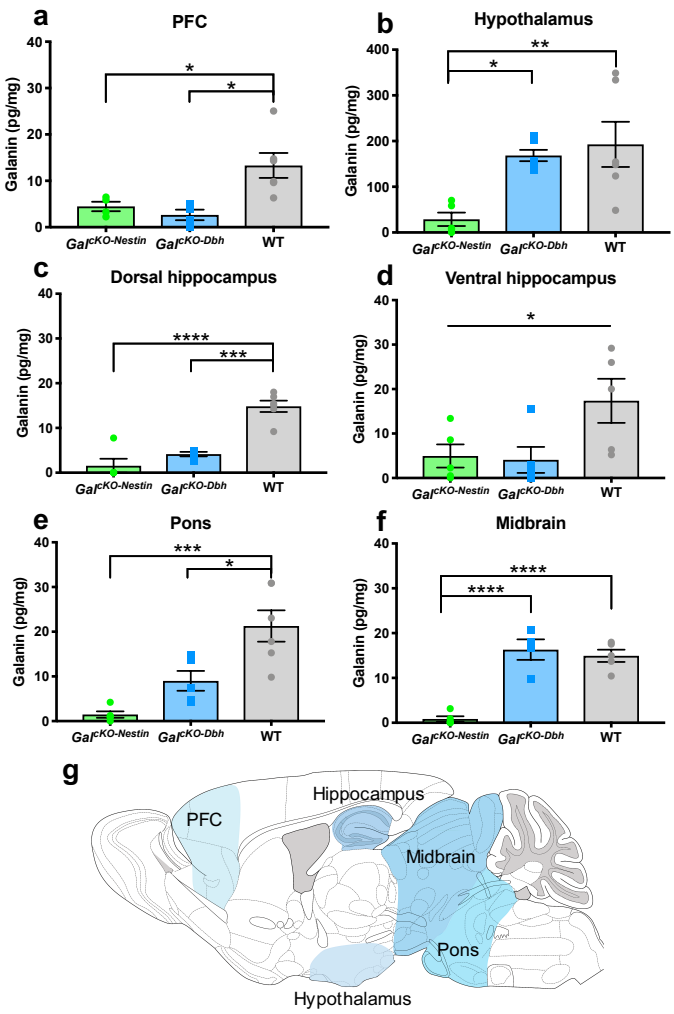

Figure 2. *Gal*$^{cKO\text{-}Dbh}$ mice have decreased galanin in the hippocampus, prefrontal cortex, and pons. Tissue galanin levels were measured by galanin ELISA. *Gal*$^{cKO\text{-}Dbh}$ mice showed significantly decreased galanin in the PFC (a), dorsal and ventral hippocampus (c, d), and pons (e), with no differences in the hypothalamus (b) or midbrain (f) compared to littermate controls. Tissue was collected via rapid dissection of whole regions (see methods for more detail) (g). Whole brain galanin knockout mice (*Gal*$^{cKO\text{-}nestin}$) were used as a negative control. In the PFC and hippocampus, *Gal*$^{cKO\text{-}Dbh}$ galanin levels were reduced to an equivalent level as seen in the negative control samples (a, c, d). N = 4-6 mice per group. Data were analyzed by one-way ANOVA with Tukey's test for multiple comparisons. Error bars show SEM. *p< 0.05 **p<0.01 ***p<0.001 ****p<0.0001

findings reveal that noradrenergic neurons provide a significant source of galanin to the cortex and hippocampus.

Normal NE abundance and turnover in *Gal*$^{cKO\text{-}Dbh}$ mutants

To determine whether loss of noradrenergic-derived galanin affects NE metabolic activity, we measured concentrations of NE, its major metabolite MHPG (3-Methoxy-4-hydroxyphenylglycol), and turnover ratio of MHPG:NE in dissected tissue from the PFC and hippocampus by HPLC. In both regions, we observed no difference between *Gal*$^{cKO\text{-}Dbh}$ mice and littermate controls in the concentrations of either NE (PFC, t=0.626, p=0.541; hippocampus, t=0.345, p=0.735), MHPG (PFC, t=1.069, p=0.303; hippocampus, t=1.003, p=0.333), or MHPG:NE ratio (PFC, t=1.099, p=0.290; hippocampus, t=1.474, p=0.163) (**Fig. 3a-f**).

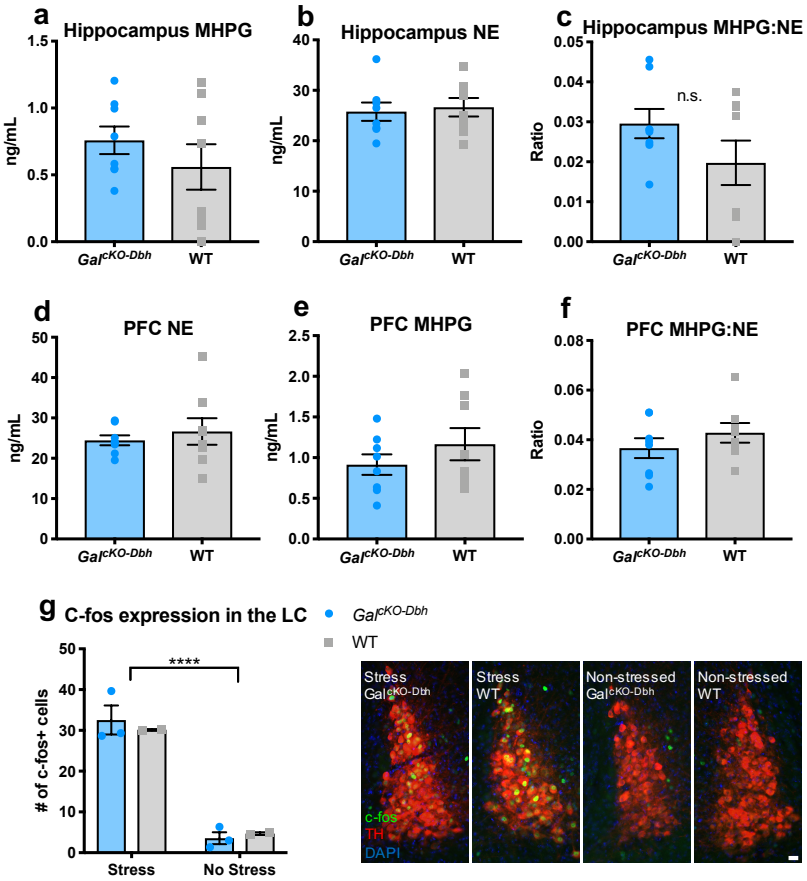

Figure 3. NE levels and stress-induced LC activation are normal in *Gal*$^{cKO\text{-}Dbh}$ mice. NE and the major metabolite of NE, MHPG, were measured via HPLC at baseline in two major projection targets of the LC, the hippocampus (a-c) and the PFC (d-f). No differences were seen between the groups in the raw measurements or the MHPG:NE ratio. N = 8 mice per group. Fos immunohistochemistry and quantification did not show any differences between *Gal*$^{cKO\text{-}Dbh}$ mice and WT littermate control mice at baseline or after acute restraint stress (g) *Right*. Representative LC sections stained for Fos (antibody; green) and tyrosine hydroxylase (TH antibody; red). N = 3 slices per animal, 2-3 animals per group. Error bars show SEM. Scale bar, 20 µm.

Given that somatodendritic release of galanin is thought to inhibit LC activity [48-50], we next wanted to determine if LC activity is influenced by the absence of noradrenergic-derived

galanin. To do this, we measured Fos, an immediate early gene used as a proxy for neuronal activation, at baseline and after acute restraint stress. A two-way ANOVA comparing *Gal*$^{cKO\text{-}Dbh}$ mutants and controls at baseline and after stress showed that, as expected, stress increased Fos expression in the LC ($F(1,6)=119.7$, $p<0.0001$). However, there was no genotype effect ($F(1,6)=0.065$, $p=0.806$), indicating that Fos expression was comparable between *Gal*$^{cKO\text{-}Dbh}$ mutants and littermate controls (**Fig. 3e**). Together, these findings suggest that NE transmission remains intact when galanin is absent from noradrenergic neurons.

Gal$^{cKO\text{-}Dbh}$ mice display a more proactive coping strategy in anxiogenic tasks measuring active defensive behaviors

Next, we assessed the behavior of adult *Gal*$^{cKO\text{-}Dbh}$ mutants and littermate control mice in a battery of anxiety-, depression-, learning-, and motor-related tests. We observed no differences between *Gal*$^{cKO\text{-}Dbh}$ and littermate control mice in canonical tests of anxiety-like behavior (elevated plus maze, $t=0.937$, $p=0.359$; zero maze, $t=0.095$, $p=0.925$; open field, $t=0.177$, $p=0.861$) (**Fig. 4a-c**) or depressive-like behavior (tail suspension test, $t=1.366$, $p=0.185$; forced swim test, $t=1.55$, $p=0.135$; sucrose preference, $F(1,16)=0.004$, $p=0.948$) (**Fig. 4d-f**). In addition, *Gal*$^{cKO\text{-}Dbh}$ exhibited normal cognitive responses in contextual and cued fear conditioning (**Fig. 4g**), as well as circadian locomotor activity (**Fig. 4h**). Mutants also showed no difference in repetitive/compulsive behavior during the nestlet shredding task ($t=0.484$, $p=0.634$) (**Supplementary Fig. 5a**).

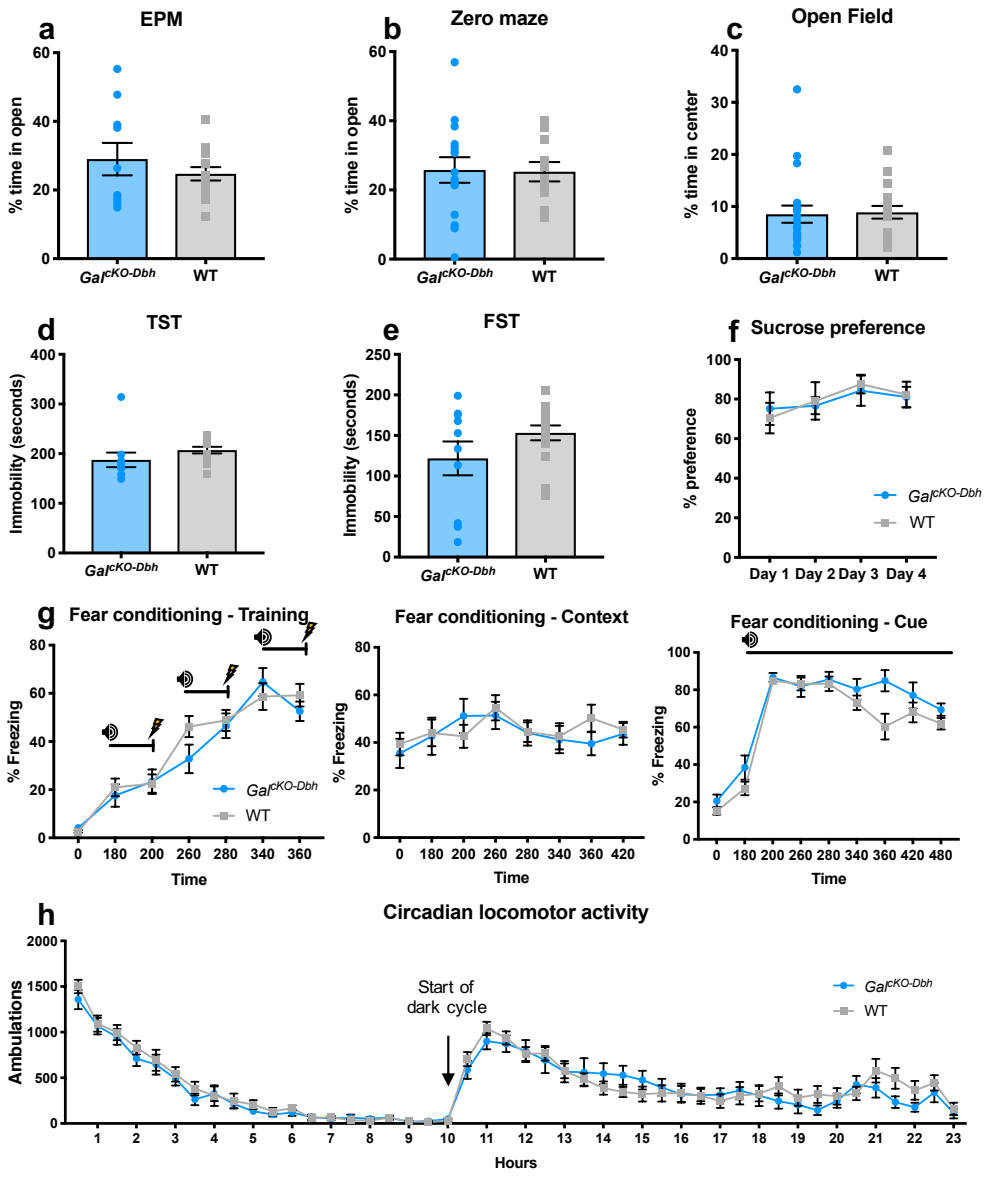

Figure 4. *Gal*$^{cKO\text{-}Dbh}$ display normal behavior at baseline in canonical tests for anxiety- and depression-like behavior, locomotion, and associative learning. There were no differences between *Gal*$^{cKO\text{-}Dbh}$ and WT littermate control mice in elevated plus maze (EPM) (a), zero maze (b), open field (c), tail suspension test (TST) (d), forced swim test (FST) (e), sucrose preference (f), fear conditioning (g), or locomotor activity (h). N = 10-20 per group; Mixed males and females. Error bars show SEM.

Having established that *Gal*$^{cKO\text{-}Dbh}$ mutants preform normally in standard tests of anxiety and depression that are reliant on *passive* behavioral responses (e.g. immobility, avoidance of open spaces, freezing), we next examined non-canonical anxiety tasks that assess *active* coping behaviors (e.g. digging). Using the marble burying assay, we observed that *Gal*$^{cKO\text{-}Dbh}$

mice buried significantly more marbles than littermate control mice (t=3.85, p<0.001) (**Fig. 5a**), indicating an enhancement of proactive defensive behavior. Similarly, in the shock probe defensive burying assay, *Gal*$^{cKO\text{-}Dbh}$ mice showed increased time spent in active digging (t=2.191, p=0.045) (**Fig. 5b**), and a trend towards a decrease in passive freezing (t=1.601, p=0.13) (**Fig. 5c**). Additional analysis of concurrent exploratory behaviors revealed *Gal*$^{cKO\text{-}Dbh}$ mice and littermate controls exhibited similar time spent grooming (t=0.627, p=0.54) and number of rearing events (t=0.143, p=0.888) (**Supplementary Fig. 5b, c**). There was no difference in the number of times the mice were shocked by the probe (t=0.104, p=0.918) (**Supplementary Fig. 5d**), indicating that alterations in behavior were not caused by differences in higher numbers of shocks to one group. We next examined performance in the novelty-suppressed feeding task, a conflict test that pits a mouse's innate fear of an unfamiliar environment against a desire to feed. *Gal*$^{cKO\text{-}Dbh}$ mice were quicker to start eating (t=2.348, p=0.025) (**Fig. 5d**), but ate a similar overall amount (t=1.331, p=0.198) (**Fig. 5e**), suggesting the *Gal*$^{cKO\text{-}Dbh}$ phenotype was not driven by increased appetite. Taken together, these data indicate

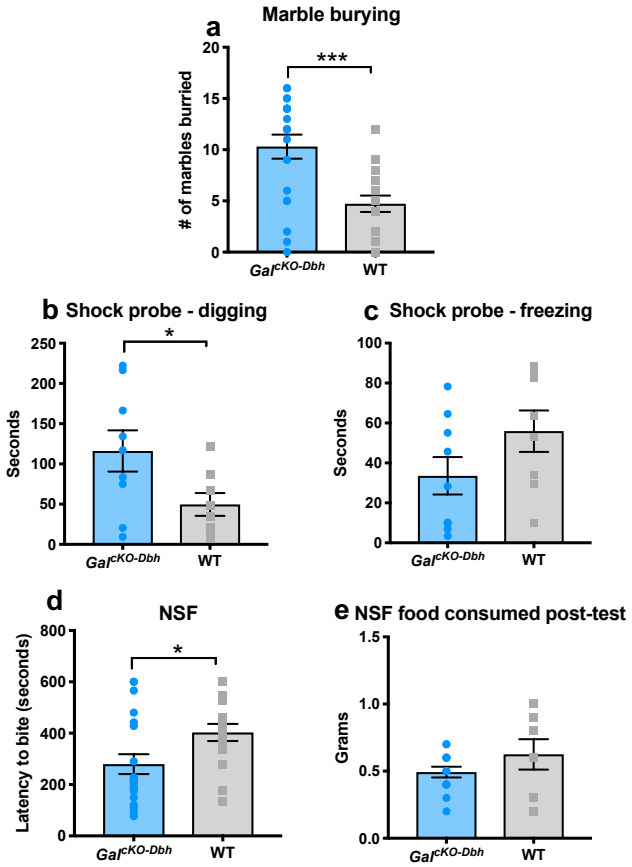

Figure 5. *Gal*$^{cKO\text{-}Dbh}$ display increased digging behavior in the marble burying and shock probe defensive burying tasks and decreased latency to feed in the novelty-suppressed feeding task. In the marble burying assay (a), *Gal*$^{cKO\text{-}Dbh}$ mice buried significantly more marbles than WT littermate control mice. *Gal*$^{cKO\text{-}Dbh}$ mice showed increased digging in the shock probe defensive burying task and a trend towards decreased freezing (b, c). In the novelty-suppressed feeding task (NSF), *Gal*$^{cKO\text{-}Dbh}$ mice showed a decrease in latency to feed but consumed the same amount of food as WT controls in the hour after the test (d, e). N = 10-20 per group; Mixed males and females. Error bars show SEM. *p < 0.05, ***p<0.001

that selective galanin gene inactivation from noradrenergic neurons shifts defensive behavior to an active coping strategy under anxiogenic conditions.

DISCUSSION

We have generated the first cell type-specific galanin knockout mouse by selectively deleting the *Gal* gene in noradrenergic neurons, leading to a loss of Gal mRNA and protein in these cells (**Fig. 1 and Supplementary Fig. 4**). Our results indicate that most of the galanin in the hippocampus and PFC, as well as about half the galanin in the brainstem, is derived from noradrenergic neurons, whereas alternate sources are responsible for hypothalamic and midbrain galanin. Measurement of tissue NE and MHPG levels in LC projection regions and Fos expression in the LC suggest that the noradrenergic system is functioning normally in $Gal^{cKO\text{-}Dbh}$ mice, at least under baseline conditions and following a mild acute stressor. Finally, we found that $Gal^{cKO\text{-}Dbh}$ mice display a more active coping strategy in three independent tasks measuring active defensive behaviors in an anxiogenic context (marble burying, shock probe defensive burying, novelty suppressed feeding), but their performance in other tests relevant to anxiety, depression, gross motor function, and cognition are indistinguishable from littermate controls.

Prior studies using slice electrophysiology show that galanin has an inhibitory effect on LC neuron activity [34,51]. Additionally, it has been suggested that galanin can be released somatodentritically from large dense core vesicles in LC-NE neurons and thereby produce an autoinhibitory effect on LC neuron activity, although this hypothesis has yet to be tested directly [48,52]. While the lack of NE-LC system deficits in the $Gal^{cKO\text{-}Dbh}$ mice may seem surprising, it is important to keep in mind that the measures for noradrenergic system function used in the present study were indirect and may have lacked the sensitivity to detect subtle changes. Although steady-state tissue levels of NE and MHPG have been used to estimate NE turnover, *in vivo* microdialysis is a more direct measure of NE overflow and transmission. Likewise, while Fos is a validated proxy for neuronal activity, it is a binary response that lacks the temporal

resolution and sensitivity of electrophysiology. Despite these methodological considerations, our data indicate that the noradrenergic system is not grossly altered in the *Gal*$^{cKO\text{-}Dbh}$ mice, and that their behavioral phenotypes are caused by galanin depletion rather than alterations in NE signaling. Because *Gal*$^{cKO\text{-}Dbh}$ mice lack galanin expression in the LC throughout development, it is possible there may be compensatory mechanisms that allow LC activity regulation in the absence of autoinhibitory galanin release. Additionally, our immunohistochemical data uncovered a previously unrecognized galaninergic input to LC neurons that arises from a non-noradrenergic source(s) (**Supplementary Fig. 4**) that persists in *Gal*$^{cKO\text{-}Dbh}$ mice and could play a role in regulating LC activity.

Previous studies using the neurotoxin 6-hydroxydopamine (6-OHDA) to lesion the LC suggested that the majority of galanin in the hippocampus and PFC comes from noradrenergic sources by examining the overlap between DBH-positive and galanin-positive fibers or measuring galanin peptide levels in brain regions innervated by the LC [29,53-55]. However, these experiments had only been performed in rats, and neurotoxin lesioning is not cell type-specific, is rarely complete, can lead to axonal sprouting, and causes neuroinflammation. Overcoming these limitations, here we showed that *Gal*$^{cKO\text{-}Dbh}$ mice have decreased tissue levels of galanin peptide in LC projection fields, including the dorsal and ventral hippocampus, PFC, and pons (**Fig. 2**). Additionally, galanin levels in the hippocampus and PFC of the *Gal*$^{cKO\text{-}Dbh}$ mutants was equivalent to that observed in mice lacking galanin in all neurons and glia, supporting the idea that galanin in these regions comes primarily from noradrenergic sources. The trace amounts of galanin we detected in the *Gal*$^{cKO\text{-}nestin}$ samples likely comes from circulating galanin in blood vessels, but given that the amounts were stable across tissues and mice, it likely had no significant impact on the interpretation of our results. One previous study showed that intracerebroventricular 6-OHDA treatment in rats eliminated DBH-positive fibers in both the dorsal and ventral hippocampus, but abolished galanin-positive fibers in only the dorsal hippocampus, suggesting that galanin in the ventral hippocampus may come from non-

noradrenergic neurons [54]. We observed galanin depletion in both the dorsal and ventral hippocampus of *Gal*$^{cKO\text{-}Dbh}$ mice, possibly indicative of a species differences in the source of galanin in the ventral hippocampus. Further, our data suggest that, in mice, galanin in the midbrain and hypothalamus is derived from non-noradrenergic sources, consistent with the abundant expression of galanin in many hypothalamic neurons [25,29]. By contrast, a previous study in rats reported decreased galanin in the ventral tegmental area following LC lesion using 6-OHDA [53]. Because our midbrain dissection encompassed a much larger area, it is possible that a decrease in VTA galanin was obscured by non-noradrenergic galanin in surrounding tissue.

The performance of *Gal*$^{cKO\text{-}Dbh}$ mice was normal in all behavioral assays examined except for ethological-based anxiety tests that measure active, innate patterns of species-typical defensive behaviors. Specifically, *Gal*$^{cKO\text{-}Dbh}$ mutants buried more marbles, likely a result of increased digging [56], and showed increased digging in the shock probe defensive burying task. Increased digging is sometimes interpreted as increased anxiety-like behavior, and could indicate that endogenous noradrenergic-derived galanin may have an anxiolytic effect. However, our other findings do not support this interpretation, as we did not see any differences in canonical approach-avoidance tests for anxiety-like behavior such as the open field, elevated plus maze, and zero maze. These canonical tasks rely on innate, passive avoidance behavior to measure anxiety, whereas marble burying and shock probe burying are defensive tasks that rely on an active response (e.g., digging) as the measure of anxiety behavior, and may elicit increased levels of stress compared to approach-avoidance tasks [57]. Increased digging in mice has also been interpreted as a repetitive, perseverative behavior, but we did not observe any other evidence of repetitive behaviors in these mice, such as increased nestlet shredding [56,58,59]. Additionally, in the novelty-suppressed feeding task, we found that *Gal*$^{cKO\text{-}Dbh}$ mutants start eating sooner than controls, traditionally interpreted as decreased anxiety- or depressive-like behavior. This task is a conflict task that pits the desire for food against the fear of being in a

novel environment, and therefore involves a component of motivation that the other tasks do not possess. Combined, these behavioral data indicate a role for noradrenergic-derived galanin in regulating active coping strategies to environments that elicit immediate or potential threat.

Neuropeptides, such as galanin, are preferentially released when neurons fire at high frequencies, and previous research has repeatedly suggested a role for galanin under challenging conditions that strongly activate noradrenergic neurons [2,35,60]. Therefore, it is possible that stronger behavioral phenotypes would emerge if the noradrenergic systems of *Gal*$^{cKO\text{-}Dbh}$ mutants were challenged by increasing the intensity and/or duration of stress during or prior to behavioral testing. It may also be informative to test the *Gal*$^{cKO\text{-}Dbh}$ mutants following chronic voluntary exercise, which is widely reported to increase galanin in the LC and has anxiolytic/antidepressant properties [61-63]. Future studies using environmental manipulations, as well as optogenetic strategies for manipulating noradrenergic neuron activity, may help uncover a role for NE-derived galanin in modulating defensive behaviors often associated with anxiety and depression.

Collectively, our studies using the *Gal*$^{cKO\text{-}Dbh}$ mutant model reveal that galanin plays an important role within the NE system by organizing a characteristic repertoire of defensive behaviors. This model is an invaluable tool to probe the many other functions hypothesized to be under the control of NE-derived galanin. Similar strategies targeting other cell type-specific galaninergic populations will help elucidate the broader neuroanatomy of the galanin system and its contribution to many physiological processes and disease states.

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We thank Philip V. Holmes, Jessica Hooversmith, Hannah Yoder, and Diane D'Agostin for their technical assistance. Valuable support was provided by the NIEHS Fluorescence Microscopy and Imaging and Transgenic Cores. RPT, NRS, PJ, and DW conceived, designed, and supervised the project. PJ and NWP created the *Dbh*^{cre} and *Gal*^{cKO} mouse lines. Immunohistochemistry, *in situ* hybridization, and image acquisition was performed by RPT, NRS, and KGS. Behavioral and neurochemical experiments were performed by RPT under the guidance of DW. DL assisted with neurochemical experiments. Mouse husbandry and genotyping at Emory University were performed by CJ. RPT, NRS, PJ, and DW wrote the manuscript with input from co-authors.

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FIGURE LEGENDS

Figure 1. *Gal* conditional knockout allele permits selective disruption of galanin synthesis in noradrenergic neurons. (a) Schematic diagram of *Gal*^{cKO} allele. Recombination by *Dbh*^{cre} leads to disruption of galanin synthesis in NE neurons. (b-f) *Left*. Schematic illustration of coronal mouse brain showing location of images. *Right*. Representative coronal brain sections stained for *Gal* mRNA (riboprobe; magenta) and tyrosine hydroxylase (TH antibody; green). *Gal* mRNA is absent from the SubC and LC of mutant mice (*Gal*$^{cKO\text{-}Dbh}$). Scale bar, 50 µm. LC, locus coeruleus. SubC, sub-coeruleus.

Figure 2. *Gal*$^{cKO\text{-}Dbh}$ mice have decreased galanin in the hippocampus, prefrontal cortex, and pons. Tissue galanin levels were measured by galanin ELISA. *Gal*$^{cKO\text{-}Dbh}$ mice showed significantly decreased galanin in the PFC (a), dorsal and ventral hippocampus (c, d), and pons (e), with no differences in the hypothalamus (b) or midbrain (f) compared to littermate controls. Tissue was collected via rapid dissection of whole regions (see methods for more detail) (g). Whole brain galanin knockout mice (*Gal*$^{\text{cKO-nestin}}$) were used as a negative control. In the PFC and hippocampus, *Gal*$^{cKO\text{-}Dbh}$ galanin levels were reduced to an equivalent level as seen in the negative control samples (a, c, d). N = 4-6 mice per group. Data were analyzed by one-way ANOVA with Tukey's test for multiple comparisons. Error bars show SEM. *$p< 0.05$ **$p<0.01$ ***$p<0.001$ ****$p<0.0001$

Figure 3. NE levels and stress-induced LC activation are normal in *Gal*$^{cKO\text{-}Dbh}$ mice. NE and the major metabolite of NE, MHPG, were measured via HPLC at baseline in two major projection targets of the LC, the hippocampus (a-c) and the PFC (d-f). No differences were seen between the groups in the raw measurements or the MHPG:NE ratio. N = 8 mice per group. Fos immunohistochemistry and quantification did not show any differences between *Gal*$^{\text{cKO-Dbh}}$ mice and WT littermate control mice at baseline or after acute restraint stress (g) *Right*.

Representative LC sections stained for Fos (antibody; green) and tyrosine hydroxylase (TH antibody; red). N = 3 slices per animal, 2-3 animals per group. Error bars show SEM. Scale bar, 20 µm.

Figure 4. *Gal*$^{cKO\text{-}Dbh}$ display normal behavior at baseline in canonical tests for anxiety- and depression-like behavior, locomotion, and associative learning. There were no differences between *Gal*$^{cKO\text{-}Dbh}$ and WT littermate control mice in elevated plus maze (EPM) (a), zero maze (b), open field (c), tail suspension test (TST) (d), forced swim test (FST) (e), sucrose preference (f), fear conditioning (g), or locomotor activity (h). N = 10-20 per group; Mixed males and females. Error bars show SEM.

Figure 5. *Gal*$^{cKO\text{-}Dbh}$ display increased digging behavior in the marble burying and shock probe defensive burying tasks and decreased latency to feed in the novelty-suppressed feeding task. In the marble burying assay (a), *Gal*$^{cKO\text{-}Dbh}$ mice buried significantly more marbles than WT littermate control mice. *Gal*$^{cKO\text{-}Dbh}$ mice showed increased digging in the shock probe defensive burying task and a trend towards decreased freezing (b, c). In the novelty-suppressed feeding task (NSF), *Gal*$^{cKO\text{-}Dbh}$ mice showed a decrease in latency to feed but consumed the same amount of food as WT controls in the hour after the test (d, e). N = 10-20 per group; Mixed males and females. Error bars show SEM. *$p < 0.05$, ***$p<0.001$