Psychoneuroendocrinology 119 (2020) 104720

Contents lists available at ScienceDirect

Psychoneuroendocrinology

journal homepage: www.elsevier.com/locate/psyneuen

The supramammillary nucleus controls anxiety-like behavior; key role of GLP-1R

L. López-Ferreras[a,c], K. Eerola[a,d], O.T. Shevchouk[a], J.E. Richard[a], F.H. Nilsson[a], L.E. Jansson[a], M.R. Hayes[b], K.P. Skibicka[a,c,e,*]

[a] Institute for Neuroscience and Physiology, University of Gothenburg, Sweden
[b] Department of Psychiatry, University of Pennsylvania, Philadelphia, PA, USA
[c] Wallenberg Centre for Molecular and Translational Medicine, University of Gothenburg, Sweden
[d] Research Centre of Integrative Physiology and Pharmacology, Institute of Biomedicine, University of Turku, Finland
[e] Department of Nutritional Sciences, Pennsylvania State University, University Park, PA, USA

ARTICLE INFO

Keywords:
GLP-1
Supramammillary
Anxiety
Exendin-4
Exendin-9

ABSTRACT

Anxiety disorders are among the most prevalent categories of mental illnesses. The gut-brain axis, along with gastrointestinally-derived neuropeptides, like glucagon-like peptide-1 (GLP-1), are emerging as potential key regulators of emotionality, including anxiety behavior. However, the neuroanatomical substrates from which GLP-1 exerts its anxiogenic effect remain poorly characterized. Here we focus on a relatively new candidate nucleus, the supramammillary nucleus (SuM), located just caudal to the lateral hypothalamus and ventral to the ventral tegmental area. Our focus on the SuM is supported by previous data showing expression of GLP-1R mRNA throughout the SuM and activation of the SuM during anxiety-inducing behaviors in rodents. Data show that chemogenetic activation of neurons in the SuM results in an anxiolytic response in male and female rats. In contrast, selective activation of SuM GLP-1R, by microinjection of a GLP-1R agonist exendin-4 into the SuM resulted in potent anxiety-like behavior, measured in both open field and elevated plus maze tests in male and female rats. This anxiogenic effect of GLP-1R activation persisted after high-fat diet exposure. Importantly, reduction of GLP-1R expression in the SuM, by AAV-shRNA GLP-1R knockdown, resulted in a clear anxiolytic response; an effect only observed in female rats. Our data identify a new neural substrate for GLP-1 control of anxiety-like behavior and indicate that the SuM GLP-1R are sufficient for anxiogenesis in both sexes, but necessary only in females.

1. Introduction

Anxiety disorders have a staggering prevalence in the western world with nearly a quarter of the adult population being affected by an anxiety disorder at some point during their lifetime. Indeed, anxiety disorders are the most common mental health disorder category in the western world (Kessler et al., 2005; Kessler and Wang, 2008). Anxiety disorders are also on the rise in children and adolescence, with 31 % of youth in the US diagnosed with an anxiety disorder, according to the National Institute of Mental Health. Some studies suggest that obesity or diabetes may be a risk factor for anxiety, and many, but not all, studies indicate a positive corellation between metabolic syndrome and anxiety disorders (Gariepy et al., 2010; Li et al., 2008; Smith et al., 2013; Strine et al., 2008). This link is perhaps not surprising, as there are many overlapping neural circuits and neurochemicals identified as critical for the control of both feeding and anxiety behaviors (Singh, 2014).

The supramammillary nucleus (SuM) recently emerged as a potential new component of the behavioral avoidance system (Pedersen et al., 2017). Indirect evidence also suggests that it may be involved specifically in the control of anxiety behaviors. Increased neuronal activation throughout the SuM was identified in rats undergoing the open field (Ito et al., 2009) or elevated plus-maze (Silveira et al., 1993) tests, two well-validated rodent tests of anxiety. Reversal of fear-induced c-Fos neuronal activation in the SuM by an anxiolytic drug, diazepam (Beck and Fibiger, 1995), and an anxiolytic effect of SuM lesions, in rats with elevated baseline anxiety (Aranda et al., 2006) further suggest that the SuM is a key nucleus of the anxiety-regulating neural circuit.

* Corresponding author at: Department of Physiology/Metabolic Physiology, Institute of Neuroscience and Physiology, The Sahlgrenska Academy at the University of Gothenburg, Medicinaregatan 11, PO Box 434, SE-405 30 Gothenburg, Sweden.
E-mail address: Karolina.Skibicka@neuro.gu.se (K.P. Skibicka).

https://doi.org/10.1016/j.psyneuen.2020.104720
Received 4 February 2020; Received in revised form 30 April 2020; Accepted 18 May 2020

We recently found expression of the glucagon-like peptide-1 receptor (GLP-1R) throughout the SuM (Lopez-Ferreras et al., 2019). GLP-1 is a gut/neuro-peptide made primarily in the intestine and the caudal brainstem (Holst, 2007). It is critical for maintenance of glucose and body weight homeostasis, and GLP-1R agonists are FDA-approved for use in type 2 diabetes and obesity (Muller et al., 2018). However, there is also strong evidence that GLP-1 and its CNS receptors have a less investigated, but essential, role in emotional reactivity (Ghosal et al., 2017; Kinzig et al., 2003; Zheng et al., 2019a).

GLP-1R manipulations in the SuM result in differential feeding and food reinforcement effects in males compared to females (Lopez-Ferreras et al., 2019). This differential behavioral outcome was linked to a neuroanatomical connection between the SuM and the lateral hypothalamus, a major feeding control hub. Whether SuM neurons or SuM GLP-1R affect anxiety, and whether they do so in a sex divergent manner, remains unknown. In fact, to the best our knowledge, the contribution of the SuM, or the GLP-1 system, to emotional reactivity has not been investigated in female rodents, despite key evidence that sex differences are present in these behaviors. In fact, women are much more likely to suffer from mood and anxiety disorders than men, as indicated by the twofold higher prevalence of anxiety disorders in women in the US (Kessler et al., 2012). Moreover, metanalysis of the literature suggests that the positive relationship between obesity and anxiety disorders may be stronger in women compared to men (Barry et al., 2008; Gariepy et al., 2010; McLean et al., 2011; McLean and Hope, 2010). While many different factors have been suggested to contribute to this sex difference, the exact neurobiological mechanisms mediating it are poorly understood.

Identifying the neural bases of anxiety-like behaviors is essential for understanding mechanisms that contribute to normal and pathological anxiety responses. To gain basic understanding to the contribution of SuM neurons to anxiety-like behavior control, chemogenetic activation of SuM neurons was employed. Next, we determined whether SuM downstream targets support a potential role in anxiety-like behavior by applying retrograde neural tract tracing to detect connections from the SuM to a brain area well-known for its role in anxiety, the lateral habenula (LHb), utilizing a designer retrograde viral vector (Tervo et al., 2016). Considering the recently established role of SuM GLP-1R in feeding control, along with a wealth of previous data indicating an important role for the GLP-1 system in emotionality control, we first examined whether GLP-1R binding is detected on the SuM neurons, and next determined whether SuM GLP-1R are necessary and sufficient for anxiety-like behavior control in male and female rats, by utilizing pharmacological and genetic receptor manipulations.

2. Materials and methods

2.1. Animals

Male and female Sprague-Dawley rats were purchased from Charles River, Germany (5 weeks old at arrival) and housed in individual cages with *ad libitum* access to chow and water (unless otherwise stated) in a 12 -h light/dark cycle. All studies were carried out with ethical permissions from the Animal Welfare Committee of the University of Gothenburg, in accordance with legal requirements of the European Community (Decree 86/609/EEC). All efforts were made to minimize suffering.

2.2. Brain cannulation

A combination of ketamine (Ketaminol® Vet, Intervet International BV, AN Boxmeer, Holland) (18.75 mg/kg) and xylazine (Rompun® Vet, Bayer Animal Health GmbH, Leverkusen Germany) (2.5 mg/kg) was administered intraperitoneally to achieve surgical anesthesia. Guide cannulae (26-gauge cannula; Plastic One Roanoke, VA) targeting the SuM were implanted with the following coordinates adapted from (Vogel et al., 2016): 4.7 mm posterior to bregma, on midline, and 7.1 mm ventral from the surface of the skull, with injector aimed 9.1 mm ventral to the skull. Dental acrylic and jeweler's screws were used to attach the cannulae to the skull. Cannula placement confirmation was verified post mortem. Seven rats had a misplaced cannula. In five of these rats injectors targeted the interpeduncular nucleus (IPN) instead of the SuM. Since the IPN is positioned close to the SuM and it is a GLP-1R-expressing site (Tuesta et al., 2017), all behavioral data from the IPN-injected rats were analyzed and presented in Fig. S5.

2.3. Drugs

Exendin-4 (Ex4, a GLP-1R agonist) and exendin-9 (Ex9, a GLP-1R antagonist) were purchased from Tocris (Bristol, UK) and dissolved in artificial cerebrospinal fluid (aCSF; Tocris), which was also used as vehicle. Aliquots were stored at −20 °C. 0.03 μg of Ex4 was infused into the SuM or IPN in a volume of 0.3 μl, and testing was conducted 20 min after (Lopez-Ferreras et al., 2019). For Ex9, rats fasted overnight were given a 20 min chow meal immediately prior to the injection. The rats received 10 μg of Ex9 in a volume of 0.3 μl and their behavior was measured 10 min after the injection (Hayes et al., 2009). Clozapine N-Oxide (CNO; Larodan AB, Solna, Sweden) was freshly dissolved in saline (B. Braun, Melsungen, Germany, also used as vehicle) and given by intraperitoneal injection in a concentration of 3 mg/kg (Terburg et al., 2018). Testing was conducted 20 min after injection. All treatments were counterbalanced in a Latin square design and carried out early in the light phase. FAM-labeled Ex4 (FAM-Ex4) was purchased from BioNordika (Stockholm, Sweden), dissolved and used as described for Ex4 above (in aCSF, 0.03 μg in 0.3 μl). FAM-Ex4 binds to GLP-1R on the cell surface and the FAM label allows for green florescent signal detection corresponding to bound molecules of FAM-Ex4. Three hours after intra-SuM injection, under deep anesthesia brains were extracted and frozen immediately. This timing was based on recently published studies (Reiner et al., 2016; Terrill et al., 2016) using this technique.

2.4. Neural tract tracing

To uncover neural pathways *via* which SuM activation may affect anxiety-like behaviors, a retrograde AAV vector, expressing EGFP under the enhanced synapsin promoter (AAV2(Retro)-eSyn-EGFP, 1.2×10^{13} GC/mL; Vector Biolabs, Malvern, PA, USA; (Tervo et al., 2016)) was injected unilaterally into the LHab, a brain nucleus hypothesized to be downstream of the SuM, and previously linked to emotionality behavior control (Geisler and Trimble, 2008; Gill et al., 2013; Jhou et al., 2009). The viral infusions were carried out under anesthesia (ketamine:xylazine 3:1) using a Hamilton Neuros 10 μL syringe with a 33 gauge needle (Hamilton Co. Reno, NV, USA). The final target volume was 0.3 μl, infused over 3 min. The needle was then left for an additional 10 min to allow diffusion of the virus into parenchyma surrounding the injection site. The following coordinates were used for the LHab viral tracer infusion: AP -3.60, ML + 0.70, and DV -5.10 mm from the surface of the skull. Three weeks after the viral infusions, brains were extracted after myocardial perfusion and 20 μm coronal slices were collected and mounted as previously described (Lopez-Ferreras et al., 2019). Two to three consecutive slices were imaged as 10x tile images at different coronal levels, in multiple layers of 1 − 3 μm (5–6 levels), were taken using a Zeiss LSM700 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Images were then converted with the Maximum Projection-software (Zen software, Carl Zeiss Microscopy GmbH, Jena, Germany) for increased depth range.

Cell counting: Coronal brain atlas figures (Paxinos & Watson 5th edition) were superimposed on the fluorescent images of the SuM sections (2–3 sections were analyzed for each SuM level in each subject, a total of five rats, two males and three females were included in this analysis) according to the corresponding antero-posterior level using GIMP-software (www.gimp.org). After addition of a transparent image

layer, red circles were plotted on top of neurons, defined by spherical EGFP expression greater than 70 μM^2 (Meitzen et al., 2011). Images containing only the plotted circles in the specific parts of the SuM were exported as jpeg images for analysis. Fiji software (Schindelin et al., 2012) was used to automatically count the plotted circles after first conversion of the images into 8-bit gray scale and then by running the particle analysis program.

2.5. Anxiety-like behavior testing

The elevated plus maze (EPM) and the open field (OF) behavioral tests were used to measure anxiety-like behavior. These tests are based on a rodent's inborn aversion expressed as passive avoidance behavior to open spaces perceived as less safe. The EPM test consists of a plus-shaped maze where two arms are enclosed and the other two are open. The total time the animal spends in the open arms is inversely proportional to its anxiety behavior (Belzung and Griebel, 2001). For this study, the Med-Associates (Georgia, USA) apparatus was used. The animal's position, as well as its locomotor activity were measured by beam breaks of infrared lasers located at the entry and in the middle of each arm and recorded with the MED-SYST-8A PCI software package, provided by Med-Associates. The OF test measures the animal's exploratory activity in an open area surrounded by walls. In this case, the more anxious animals will spend less time in the center, preferring to stay close to the walls. The OF arena was equipped with a grid of photocells which detect the animal's position and movement. Med-Associates Activity Monitor software was used for the OF tests, thus all scoring was conducted automatically by the software using the data sent by the grid photocells. For all the experiments carried out in this study, the EPM test was conducted for 5 min, and immediately after, the animals were moved to the OF for 30 min (Alvarez-Crespo et al., 2012). The minimum viral incubation, when relevant, and post-surgery period before the anxiety testing commenced, was three weeks.

2.6. Chemogenetic activation of SuM neurons

To test the effect of neuronal activation in the SuM, a Designer Receptors Exclusively Activated by Designer Drugs (DREADD)-based chemogenetic approach was used (Urban and Roth, 2015). The DREADD system allows for long-lasting neuronal activation following administration of the DREADD ligand, clozapine N-oxide (CNO) (Fortress et al., 2015). In summary, DNA coding for the DREADD and a fluorescent tag, in this case hM3D and mCherry, were inserted into a viral vector under a neuron specific promoter (AAV2-hSyn-hM3D (Gq)-mCherry, Addgene, USA) which was injected into the SuM. An incubation period of a minimum of three weeks was allowed for DREADD expression in the brain tissue. Rats were anesthetized with a mix of ketamine:xylazine 3:1 (3:1) and the virus was infused directly into the SuM, or IPN, through the guide cannula (0.3 µl over 3 min). The microinjector was then left in place for 10 min. In order to activate the infected neurons, three weeks after infusion, CNO was injected intraperitoneally as described above, and food choice or anxiety-like behavior tests were carried out. In order to evaluate effects of CNO itself, a second group of animals (CNO control group), with no viral injection, was also tested.

2.7. Food choice testing after chemogenetic activation of SuM neurons

Rats received CNO or vehicle injection and were immediately given a free choice of chow and peanut butter in their home cage. Food intake was measured 6 and 24 h following injections.

2.8. High-fat/high-sugar (HFHS) diet

The role of GLP-1R activation in the SuM was further investigated in rats challenged with a HFHS diet. Following three weeks of maintenance on a free choice of lard, 30 % sucrose solution and chow diet, the effect of GLP-1R activation with Ex4 on anxiety-like behavior was evaluated as described above.

2.9. Detection of GLP-1R on chemogenetically-targeted SuM neurons

We previously identified GLP-1R mRNA in the SuM of male and female rats (Lopez-Ferreras et al., 2019). Here we determined whether GLP-1R protein is expressed on SuM neurons by evaluating binding of FAM-labeled GLP-1R agonist in the SuM, and specifically on the neurons expressing mCherry-DREADD in female rat after a 2 h food restriction. Brains were isolated and frozen 3 h after SuM FAM-Ex4 injection. Ten µm sections were cut on a cryostat and mounted using Vectashield H-1200 mounting medium (Vector Labs, Burlingame, CA, USA).

2.10. GLP-1R knockdown

GLP-1R expression in the SuM was knocked down using an adeno-associated virus expressing a short hairpin RNA targeting GLP-1R transcripts (AAV-GLP-1R-shRNA, serotype 1, titer = 5.22^{e12}; vector cloned and packed in collaboration with the Viral Vector Core at the University of Pennsylvania). For details see (Schmidt et al., 2016). We have previously shown a reduction in GLP-1R expression *in vivo* in the lateral hypothalamus using this construct (Lopez-Ferreras et al., 2018; Lopez-Ferreras et al., 2019). As a control group, rats matched for body weight to the knockdown group, were infused with a GFP-AAV expressing a scrambled shRNA (titer = 5.0^{e12}). Viral infusions were carried out as above (under anesthesia, 0.3 µl over 3 min infusion followed by 10 min diffusion). Three weeks later, anxiety-like behavior was measured. Daily body weight and food intake, which were not altered by SuM GLP-1 knockdown, as well as knockdown efficiency are reported in (Lopez-Ferreras et al., 2019). Male and female rats had significantly reduced SuM GLP-1R expression, and also impaired anorexic response to intra-SuM Ex4 injection, with no sex differences observed, confirming knockdown strategy was effective in both sexes (see Fig 10 in (Lopez-Ferreras et al., 2019)).

2.11. Statistical analysis

All the data are presented as mean ± SEM. Statistical significance was analyzed using Student's *t*-test for comparisons of two groups, or two-way ANOVA with post-hoc Holm-Sidak tests when appropriate (GraphPad Prism 7 and 8 Software, Inc, USA). P-values lower than 0.05 were considered statistically significant.

3. Results

3.1. Chemogenetic activation of SuM neurons decreases anxiety-like behavior in OF, but not EPM

Activation of SuM neurons by DREADDs increased the time spent in the center of the arena in the OF test in both male and female rats (Fig. 1A). Two-way ANOVA indicated a significant effect of CNO: $F_{(1,36)} = 6.22$, $P < 0.05$; but no sex effect: $F_{(1,36)} = 0.043$, $P > 0.05$; and no interaction between these two factors: $F_{(1,36)} = 0.13$, $P > 0.05$. Increased locomotor activity was also observed in both sexes (Fig. 1B; significant effect of CNO: $F_{(1,36)} = 14.5$, $P < 0.0005$ and sex: $F_{(1,36)} = 9.04$, $P < 0.005$; and no interaction between these two factors: $F_{(1,36)} = 0.53$, $P > 0.05$). Chemogenetic activation of SuM neurons did not alter 6 h chow intake (Fig. 1C; no significant effect of CNO: $F_{(1,35)} = 1.08$, $P > 0.05$, a significant effect of sex: $F_{(1,35)} = 4.74$, $P < 0.05$; but no interaction between these two factors: $F_{(1,35)} = 0.11$, $P > 0.05$), but increased the amount of chow consumed at 24 h by females, and not males (Fig. 1D; two-way ANOVA indicated no significant effect, but a potential trend, of CNO: $F_{(1,35)} = 2.78$, $P = 0.10$, a significant effect of

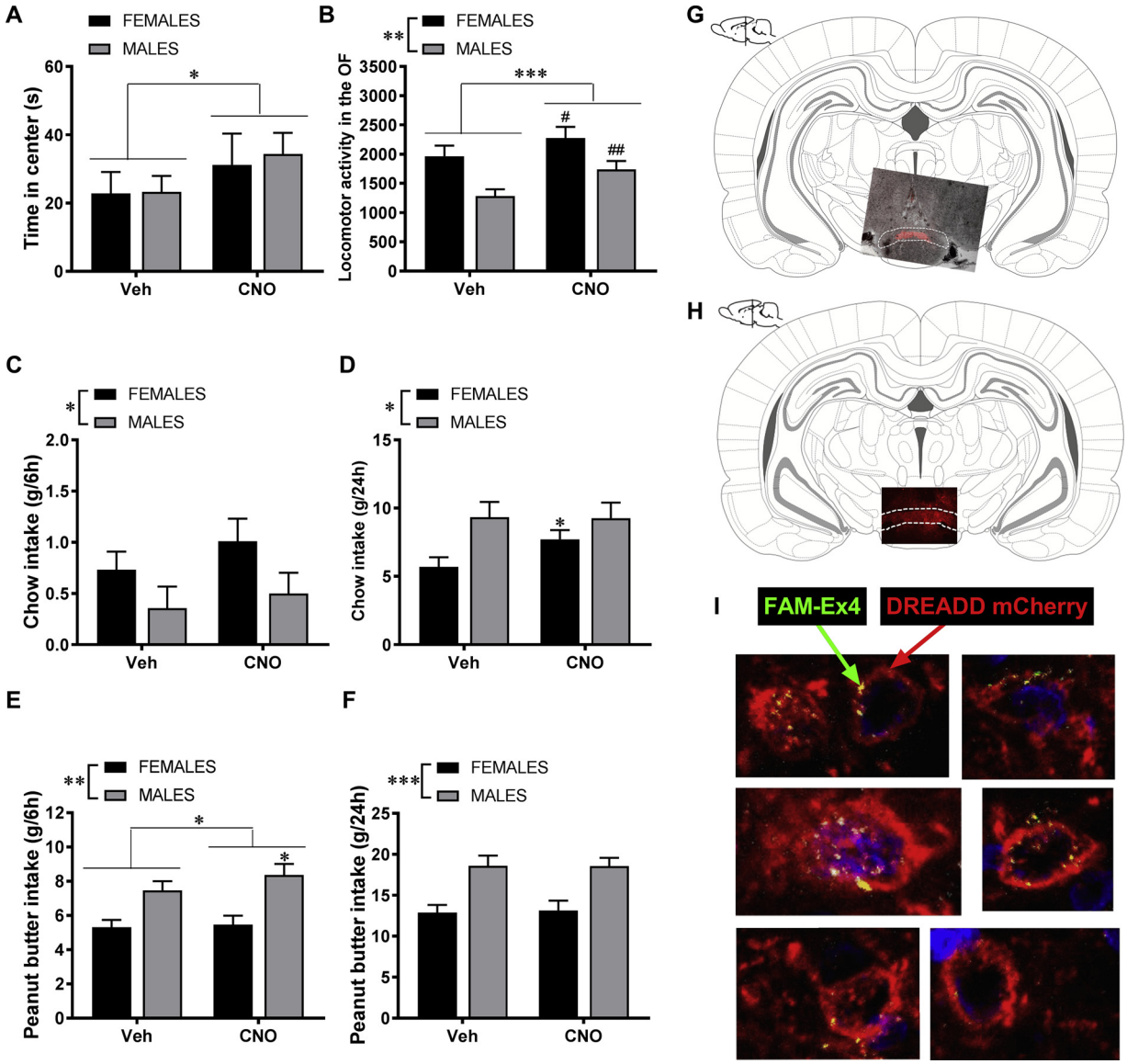

Fig. 1. Chemogenetic activation of SuM neurons. Activation of SuM neurons by intraperitoneal CNO injections in female and male rats expressing DREADDs selectively on SuM neurons is anxiolytic, as shown by an increase in the time spent in the center of the open field (OF) (A). In addition, locomotor activity was increased (B). Activation of SuM neurons did not alter chow intake during the 6 h period after CNO injection in either sex (C). However, female rats increased chow intake after 24 h with no effect in males (D). Yet, the consumption of the more palatable food, peanut butter, was increased after 6 h only in male rats (E). This orexigenic effect did not persist to the 24 h measurement (F). Representative confocal and rat atlas images for DREADD injection site in male (G) and female (H) rats. Bregma -4.56 to -4.8. Neurons expressing DREADDs are indicated in red (mCherry). Many of the SuM neurons targeted by the CNO-DREADD strategy express GLP-1R as indicated by binding of the FAM-labeled exendin-4 (Ex4; green) (I). Data are expressed as mean ± SEM. n = 19 for male rats and n = 17-19 for female rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ main factor effect (sex or CNO). # $p < 0.05$, ## $p < 0.01$ (for post-hoc comparisons). Coronal brain atlas figures are from Paxinos & Watson 5th edition. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

sex: $F_{(1,35)} = 4.74$, $P < 0.05$; and a trend for an interaction between these two factors: $F_{(1,35)} = 3.15$, $P = 0.08$). In contrast, intake of palatable food was increased in males, but not females, 6 h after chemogenetic activation of SuM neurons (Fig. 1E; a significant effect of CNO: $F_{(1,35)} = 5.26$, $P < 0.05$, a significant effect of sex: $F_{(1,35)} = 11.81$, $P < 0.005$; and a trend to interaction between these two factors: $F_{(1,35)} = 2.61$, $P = 0.11$). This orexigenic effect did not persist through the 24 h measurement point (Fig. 1F; no significant effect of CNO: $F_{(1,34)} = 0.06$, $P > 0.05$, a significant effect of sex: $F_{(1,34)} = 14.58$, $P < 0.0005$; and no interaction: $F_{(1,34)} = 0.06$, $P > 0.05$). Since male rats weight significantly more than female rats, we additionally analysed the food intake results per 100 g of body weight to attempt to account for body weight differences between the sexes. It is important to note that in the literature evaluating sex differences in feeding or metabolism this type of normalization is a hotly debated topic, considering that muscle mass (or more metabolically active tissue mass) and fat mass proportion is vastly different in males and females. However, we, and the field in general, agree that while there is no ideal way to compare male and female intake, it can be useful for the reader to have both ways of data presentation available, hence normalized data are presented in Fig S1. CNO injections in male and female rats without viral DREADD expression did not change anxiety-like behavior or feeding (Figs. S2 and S3).

3.2. GLP-1R are present on chemogenetically-targeted SuM

FAM-based green fluorescence was co-localized to DREADD-expressing SuM neurons (Fig. 1I), indicating that SuM neurons, activation

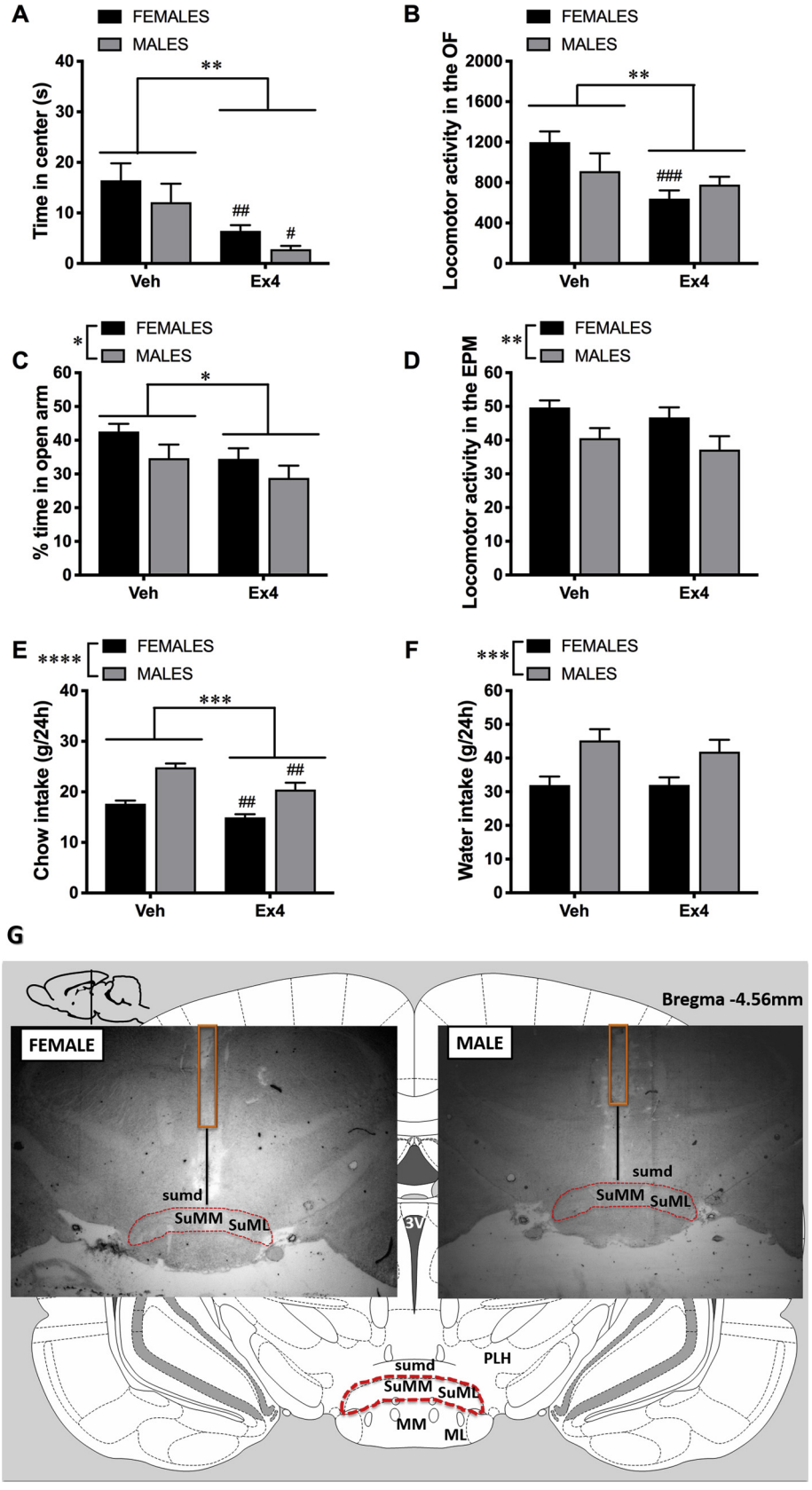

Fig. 2. SuM GLP-1R activation is anxiogenic. Activation of the GLP-1R in the SuM, by Ex4 microinjection directly into this area, increased anxiety-like behavior in both female and male rats in the OF test (A). This effect was robust, and also detected in the EPM test, where Ex4-treated rats spent significantly less time in the open arm of the maze (C). While an anxiogenic effect was detected in both sexes, intra-SuM Ex4 application significantly reduced locomotor activity in the OF test in females, with no change in locomotion detected in males (B). However, when locomotion was assessed during the EPM test, no effect was detected in either sex (D). As expected, Ex4 reduced chow intake 24 h post injection (E). Water intake was not altered (F). Representative image for injection site in both female and male rats (G). Data are expressed as mean ± SEM. n = 9-11 for male rats and n = 17-18 for female rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ main factor effect (sex or drug). # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ (for post-hoc comparisons). Medial SuM: SuMM; lateral SuM: SuML; dorsal SuM: sumd; posterior lateral hypothalamus: PLH; mammillary nuclei, comprising pars medialis (MM) and pars lateralis (ML); 3V: third ventricle. Coronal brain atlas figures are from Paxinos & Watson 5th edition.

of which was anxiolytic, are potentially responsive to GLP-1 and its analogues. These results clearly indicate that GLP-1R capable of binding Ex4 are present on SuM neurons, future studies are necessary to evaluate whether other SuM cell types, namely astrocytes and microglia, are also capable of directly responding to GLP-1R agonists.

3.3. SuM GLP-1R activation increases anxiety-like behavior

GLP-1R activation in the SuM, with Ex4, was anxiogenic, measured by a significant decrease in the time spent in the center of the OF arena in both males and females (Fig. 2A; significant effect of Ex4: $F_{(1, 52)} = 12.10$, $P < 0.005$; no effect of sex: $F_{(1, 52)} = 2.07$, $P > 0.05$; no interaction; $F_{(1, 52)} = 0.016$, $P > 0.05$). During the OF test female, but not male rats, displayed reduced locomotor activity (Fig. 2B; $F_{(1, 50)} = 8.7$, $P < 0.005$; no effect of sex: $F_{(1, 50)} = 0.39$, $P > 0.05$; and a trend to significant interaction; $F_{(1, 50)} = 3.28$, $P = 0.07$). The anxiogenic effect of GLP-1R activation in the SuM, detected in the OF test, was confirmed by the EPM test (Fig. 2C; significant effect of Ex4: $F_{(1, 52)} = 4.41$, $P < 0.05$; significant effect of sex: $F_{(1, 52)} = 4.19$, $P < 0.05$; and no interaction; $F_{(1, 52)} = 0.11$, $P > 0.05$). However, in contrast to the hypolocomotion detected in females during the OF test, Ex4 treatment was not associated with changes in locomotor activity during the EPM test, although females moved more than males in both drug and control conditions (Fig. 2D; no significant effect of Ex4: $F_{(1, 52)} = 1.02$, $P > 0.05$; but a significant effect of sex: $F_{(1, 52)} = 8.88$, $P < 0.005$; no interaction; $F_{(1, 52)} = 0.005$, $P > 0.05$). Furthermore, Ex4 treatment in the SuM was also anorexic, with both males and females significantly reducing their 24 h chow intake (Fig. 2E; significant effect of Ex4: $F_{(1, 48)} = 17.33$, $P < 0.0005$; significant effect of sex: $F_{(1, 48)} = 55.57$, $P < 0.0005$; and no interaction; $F_{(1, 48)} = 0.99$, $P > 0.05$), confirming recently published data (Lopez-Ferreras et al., 2019). In contrast, we did not find any significant effect of Ex4 on 24 h water intake (Fig. 2F; no effect of Ex4 $F_{(1, 50)} = 0.3$, $P > 0.05$; a significant effect of sex: $F_{(1,50)} = 15.00$, $P < 0.0005$; and no interaction; $F_{(1, 50)} = 0.33$, $P > 0.05$). As expected male rats ate more chow and drank more water compared to females during the 24 h period of measurement, irrespective of drug treatment (Fig. 2E–F). These data are also presented as intake per 100 g of body weight (Fig S4). This data transformation does not change the main conclusion – Ex4 injected into the SuM is anorexic in both sexes, and the effect size is similar in both males and females.

3.4. Anxiogenic effect of SuM GLP-1R activation persists in HFHS diet-fed rats

Intra-SuM administration of Ex4 elicited anxiety-like behavior in the OF test ($F_{(1, 14)} = 18.98$, $P < 0.001$) in both males and females (Fig. 3A; no effect of sex ($F_{(1, 14)} = 0.20$, $P > 0.05$), and no interaction of Ex4 and sex: ($F_{(1, 14)} = 0.39$, $P > 0.05$)). Consistently with the results obtained in chow-maintained rats, Ex4-treated HFHS-fed female, but not male rats, displayed reduced locomotor activity (Fig. 3B; overall effect of Ex4: $F_{(1, 14)} = 11.48$, $P < 0.005$). Under HFHS diet, in contrast to chow experiment, there was a significant effect of sex: $F_{(1, 14)} = 5.26$, $P < 0.05$; and also a trend for interaction; $F_{(1, 14)} = 3.0$, $P = 0.1$). SuM GLP-1R activation was also anxiogenic when HFHS-fed rats were tested in the EPM (Fig. 3C). Ex4 significantly reduced the % time spent in open arms of the maze ($F_{(1, 14)} = 13.78$, $P < 0.005$), with no effect of sex ($F_{(1, 14)} = 0.82$, $P > 0.05$) and no interaction ($F_{(1, 14)} = 0.29$, $P > 0.05$). There was a trend to Ex4 associated reduction in locomotor activity $F_{(1, 14)} = 3.73$, $P = 0.07$), with an effect of sex ($F_{(1, 14)} = 10.26$, $P < 0.01$) and a potential trend for an interaction ($F_{(1, 14)} = 2.9$, $P = 0.1$), but no effect was detected in males or females separately (Fig. 3D).

3.5. Sex divergence in the effect of meal-associated endogenous SuM GLP-1 on anxiety

Food intake is associated with short-latency intestinal GLP-1 production and activation of hindbrain GLP-1 neurons with subsequent GLP-1 release throughout the brain (Kanoski et al., 2016; Vrang et al., 2003). In order to determine whether this meal-associated endogenously produced GLP-1 exerts its anxiogenic effect by acting on SuM GLP-1R, rats were offered an *ad libitum* chow meal with subsequent GLP-1R antagonist administration into the SuM. Male and female rats, food restricted overnight, chose to eat comparable amounts of chow on average during the 20 min pre-injection feeding period (males 3.5 ± 0.18 g, females 2.8 ± 0.14 g). Surprisingly, this treatment resulted in an anxiogenic effect (overall effect of Ex9: $F_{(1, 52)} = 4.78$, $P < 0.05$; no significant effect of sex: $F_{(1, 52)} = 0.5$, $P > 0.05$) but interestingly only in male rats (Fig. 4A). Significant interaction of Ex9 and sex ($F_{(1, 52)} = 8.77$, $P < 0.005$) strongly suggests a sex difference in the role of SuM GLP-1R in anxiety-like behavior. The anxiogenic effect of Ex9 in male rats was associated with reduced locomotor activity during the OF test, while no effect on activity was detected in female rats (Fig. 4B; trend for an overall effect of Ex9: $F_{(1, 52)} = 3.49$, $P = 0.06$; significant effect of sex: $F_{(1, 52)} = 4.12$, $P < 0.05$). Here again a significant interaction of Ex9 and sex ($F_{(1, 52)} = 7.29$, $P < 0.01$) strongly suggests a sex difference in the effect of SuM GLP-1R in locomotor activity control. In contrast no overall effect of Ex9 or interaction were detected during the EPM test ($F_{(1, 52)} = 0.97$, $P > 0.05$; $F_{(1, 52)} = 0.47$, $P > 0.05$ respectively) and neither males nor females altered their open arm behavior during this test after intra-SuM Ex9 injections (Fig. 4C). Although during the EPM test females spent more time in the open arm compared to males, irrespective of drug treatment, thus there was a significant overall effect of sex ($F_{(1, 52)} = 18.44$, $P < 0.0001$). The EPM total locomotor activity results closely followed those obtained during OF, with only males moving less in the EPM apparatus (Fig. 4D; no significant effect of Ex9: $F_{(1, 52)} = 0.29$, $P > 0.05$; a significant effect of sex: $F_{(1, 52)} = 6.29$, $P < 0.05$; and interaction; $F_{(1, 52)} = 6.0$, $P < 0.05$).

3.6. SuM GLP-1R are necessary for normal expression of anxiety-like behavior

SuM GLP-1R knockdown, resulted in a clear anxiolytic effect in female rats in the OF test (Fig. 4E), consistent with an anxiogenic effect of Ex4 shown by the current study. However, surprisingly, male rats again responded with a significant increase in anxiety-like behavior (Fig. 4E), in line with the acute blockade (Ex9) results. Thus, whether chronically reduced GLP-1R levels in the SuM were associated with increased or reduced anxiety was determined by the sex of the subject. These observations are fully supported by the two-way ANOVA analysis which indicated a significant interaction between sex and GLP-1R knockdown ($F_{(1, 32)} = 11.43$, $P < 0.005$), and no overall effect of knockdown ($F_{(1, 32)} = 0.05$, $P > 0.05$) or sex ($F_{(1, 32)} = 0.39$, $P > 0.05$). GLP-1R knockdown did not affect locomotor activity during the OF test (Fig. 4F) in either sex or drug condition (knockdown: $F_{(1, 33)} = 0.9$, $P > 0.05$; sex ($F_{(1, 33)} = 1.8$, $P > 0.05$; interaction: $F_{(1, 33)} = 0.8$, $P > 0.05$). The anxiolytic effect detected in females is robust since GLP-1R knockdown also increased the amount of time female rats chose to spend in the open arms of the EPM (Fig. 4G; significant interaction between sex and GLP-1R knockdown ($F_{(1, 32)} = 7.4$, $P < 0.05$), and no overall effect of knockdown ($F_{(1, 32)} = 0.01$, $P > 0.05$) or sex ($F_{(1, 32)} = 2.45$, $P > 0.05$)). In contrast to the OF results, EPM test did not indicate any changes in anxiety-like behavior in males. There was no effect on total locomotor activity in the EPM test (Fig. 4H) in either sex or drug condition (knockdown: $F_{(1, 32)} = 0.1$, $P > 0.05$; sex: $F_{(1, 32)} = 0.1$, $P > 0.05$; interaction: $F_{(1, 32)} = 0.68$, $P > 0.05$).

3.7. SuM innervation of the lateral habenula is sex divergent

In addition to sex differences in the anxiogenic effect of SuM GLP-1R activation or blockade, we also evaluated neurocircuit sex differences between SuM efferent connections to an anxiety-controlling brain nucleus, LHab (Geisler and Trimble, 2008; Gill et al., 2013; Jhou et al., 2009). Thus, using retrograde tracing (Fig. 5A), neurons projecting from SuM to LHb were quantified in male and female rats. Delivery of the retrograde virus AAV2 (Retro)-eSyn-EGFP to the LHab was confirmed as shown in Fig. 5B and C. Expression of EGFP was detected throughout the SuM (Fig. 5N) confirming SuM projections to the LHab.

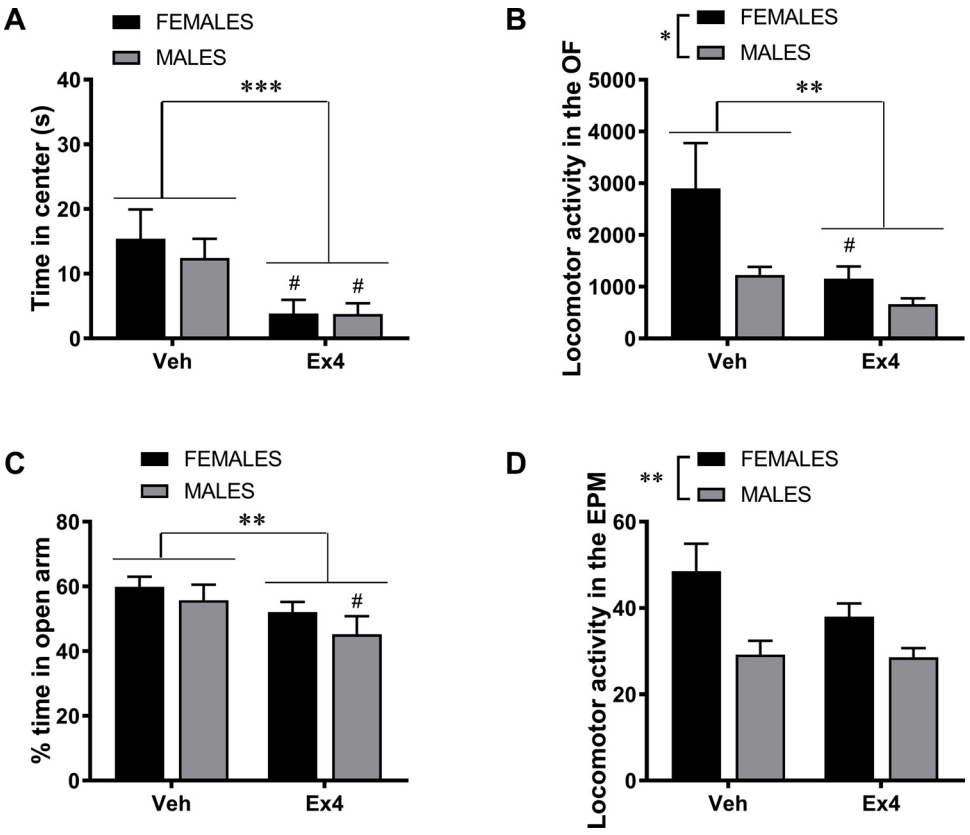

Fig. 3. Anxiogenic effect of SuM GLP-1R activation persists in rats fed an obesogenic diet. Ex4 administration directly into the SuM of female and male rats maintained on a HFHS diet increased anxiety-like behavior in the OF and EPM tests (A, C respectively). Locomotor activity was reduced only in females when tested during the OF test (B) but not in the EPM (D). No change in locomotion was detected in male rats in either test (B, D). There was a significant effect of sex on locomotor activity driven by higher activity in females compared to males irrespective of drug treatment (B, D). Data are expressed as mean ± SEM. n = 9 for male rats and n = 7 for female rats. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ main factor effect (sex or drug). # $p < 0.05$ (for post-hoc comparisons).

Females show a higher number of SuM neurons projecting to the LHab compared to males (D). This difference is driven by the caudal SuM (Fig. 5M).

3.8. GLP-1R activation, or blockade, in the IPN does not affect anxiety-like behaviors or feeding

IPN recently emerged as an important CNS site for GLP-1's effect on nicotine reward (Tuesta et al., 2017). It is located directly on the caudal tail of the SuM, expresses GLP-1R, and has neuroanatomical connections partly overlapping with those of the SuM (Pan and McNaughton, 2004). However, in contrast to the results obtained here for the SuM, GLP-1R activation (Fig S5A–F) or blockade (Fig S5G–J) in the IPN did not alter anxiety-like behavior, locomotor activity, or chow intake. Chow or peanut butter intake were also not altered by chemogenetic activation of the IPN (Fig S5K–L).

4. Discussion

Based on results obtained in this study, the SuM emerges as an essential component of the brain's anxiety behavior control network. Increasing activity of the SuM neurons had an anxiolytic and orexigenic effect. In contrast, activating SuM GLP-1R resulted in a potent anxiogenesis, and anorexia. The anxiogenic effect of SuM GLP-1R activation was robust, and persisted in rats fed an obesogenic diet; these effects were present in both males and females. Importantly, GLP-1R signaling in the SuM was necessary for normal expression of anxiety-like behavior. In females, chronic reduction in SuM GLP-1R, by virally mediated knockdown, was anxiolytic. In males however, pharmacological acute reduction in GLP-1R activation resulted in anxiogenesis. Sex divergence was also identified in the link between the SuM and the LHb. While SuM neurons innervating the LHb were identified in both males and females, significantly more LHb-projecting neurons were found in the females. Effects on anxiety-like behavior and food intake were site-specific, as identical manipulation just caudal to the SuM, in the IPN, had no effect on the behaviors measured.

While the amygdala, bed nucleus stria terminalis, or the prefrontal cortex are well established and investigated for their contribution to the generation of anxiety responses, by human and rodent studies (Charney, 2003; Davis et al., 1997a, b; Schmidt et al., 2018), our study is the first to show that manipulation of SuM neurons is sufficient to alter the behavioral expression of anxiety. Previous reports only indirectly evaluated the position of the SuM in the brain's anxiety network, and presented evidence for SuM activation by various conditions associated with stress, fear, and anxiety. However, only neuronal activation was examined in these studies, for example c-fos expression associated with locomotor activity or spatial exploration, leaving the potential for an indirect or unspecific association. The specific components of the anxiety response controlled by SuM were not examined, and a potential role in counter-regulatory/coping response was not refuted. Moreover, all the previous (c-fos) studies were done only in male rats.

Activation of SuM neurons was orexigenic, with a sex divergence in effect onset and palatability, where male rats increased intake of palatable food and female rats increased intake of less palatable chow. We previously found that SuM-directed delivery of GLP-1 or Ex4 reduces food intake and motivated behavior for sucrose in male rats (Vogel et al., 2016). Moreover, we have recently found that chronic reduction in GLP-1R expression in the lateral hypothalamus of males or females results in a profound weight gain and hyperphagia, but the same manipulation produces a rather small effect when targeting the SuM, with no changes in body weight or food intake (Lopez-Ferreras et al., 2018; Lopez-Ferreras et al., 2019). Thus, the anxiolytic effect of SuM GLP-1R knockdown, detected here in female rats, is not linked with changes in weight or amount of food consumed.

The contrasting effect on emotionality and ingestive behavior of broad chemogenetic activation of neurons in the SuM, and intra-SuM GLP-1R agonist administration, suggest a potential inhibitory effect of

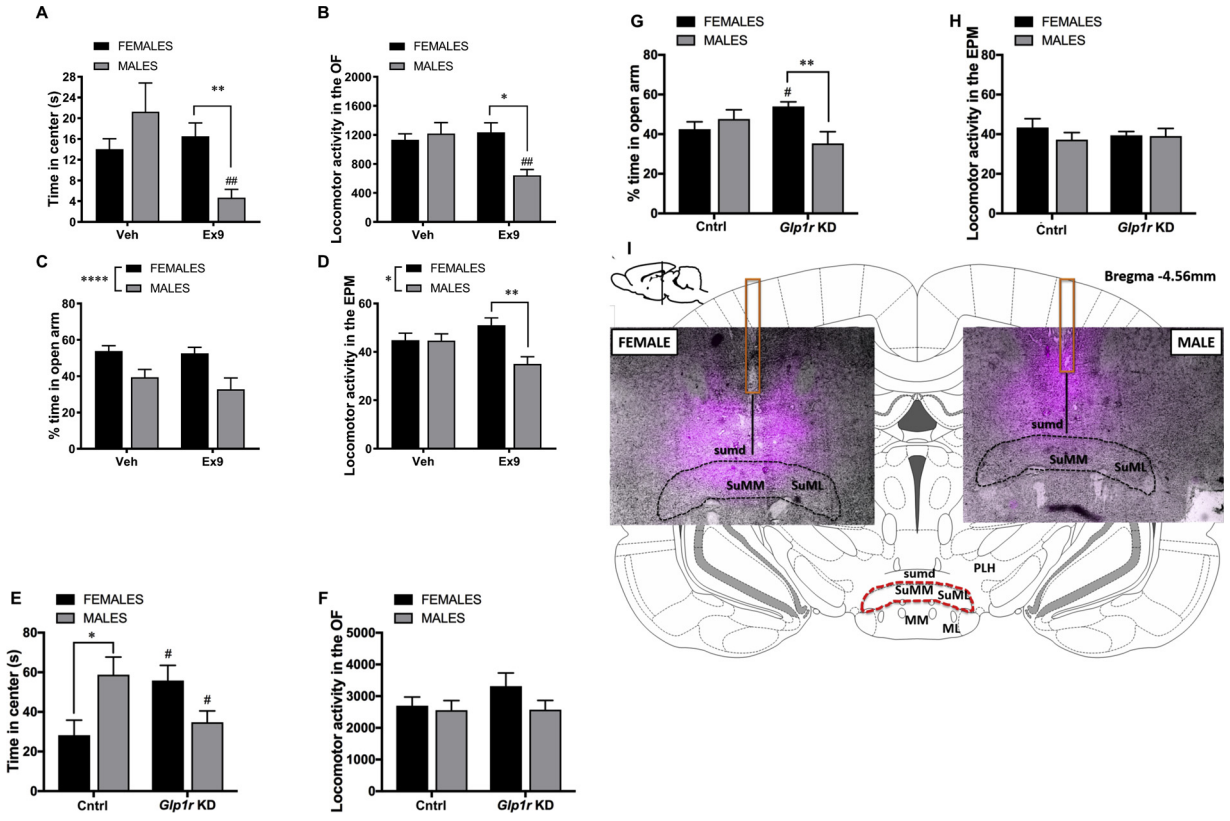

Fig. 4. Acute pharmacological SuM GLP-1R blockade or chronic SuM GLP-1R knockdown, has a sex divergent effect on anxiety. Post-meal Ex9 administration into the SuM increased anxiety-like behavior in male rats in the OF test (A). Locomotor activity was also reduced in males (B). No changes in anxiety-like behavior during the OF test were detected in females after GLP1-R blockade in the SuM (A, B). No differences in anxiety-like behavior after Ex9 microinjection were detected in the EPM test in either sex (C). Ex9 did not alter locomotor activity during the EPM test, yet females moved significantly more than males under the Ex9 condition (D). Data are expressed as mean ± SEM. n = 9 for male rats and n = 18 for female rats. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. ## $p < 0.01$ (for post-hoc comparisons of Ex9 effect). Chronic blockade of endogenous GLP-1 signal by viral knockdown of GLP-1R (*Glp1r* KD) in the SuM was anxiolytic in females who spent two-fold more time in the central region of the OF (E) and also more time in the open arm of the EPM (G). However, an increase in anxiety-like behavior was detected in male rats in the OF (E) but not the EPM test (G). For either sex, knockdown of GLP-1R in the SuM had no effect on locomotor activity nor measured during the OF (F) or the EPM (H). Representative image of injection site for females and males (I). Data are expressed as mean ± SEM. * $p < 0.05$, ** $p < 0.01$. # $p = 0.05$. n = 9 for male rats and n = 10 for female rats. Medial SuM: SuMM; lateral SuM: SuML; dorsal SuM: sumd; posterior lateral hypothalamus: PLH; mammillary nuclei, comprising pars medialis (MM) and pars lateralis (ML). Coronal brain atlas figures are from Paxinos & Watson 5th edition.

GLP-1R activation. While an excitatory effect of GLP-1R activation is commonly reported (Anderberg et al., 2017; Richard et al., 2014), emerging data indicate the possibility of inhibitory or a heterogeneous, inhibitory and excitatory, cellular responses to GLP-1R activation depending on the brain area investigated (Acuna-Goycolea and van den Pol, 2004). These electrophysiological results are supported by signaling studies indicating that GLP-1R are not only coupled to $G_{\alpha(s)}$ but also to $G_{\alpha(i)}/G_{\alpha(o)}$ type G proteins (Bavec et al., 2003; Hallbrink et al., 2001). Different G-protein subfamilies can be induced by targeting distinct domains within GLP-1R, allowing the native GLP-1 peptide to act on the same receptor to activate different second messenger systems (Bavec et al., 2003; Hallbrink et al., 2001).

Many preclinical studies found that CNS-directed injections of GLP-1, or it's analogues, in rodents may increase anxiety (Anderberg et al., 2016; Gulec et al., 2010; Kinzig et al., 2003), while others show no changes in anxiety-like behavior (Krass et al., 2012, 2015). Yet, other studies, have even found an anxiolytic effect (Komsuoglu Celikyurt et al., 2014; Sharma et al., 2015). Of note, nearly all (but one) studies to date evaluated the effect of GLP-1R agonists exclusively on male rats, in contrast to the current study. While at first glance this may present a very inconsistent effect of GLP-1R agonists on anxiety-like behavior, in general the studies that found an anxiogenic effect of GLP-1R activation tended to study healthy rats in acute experiments, this is also the context of the current study. An anxiolytic effect, or no effect on anxiety-like behavior, was more common in studies that either applied GLP-1R agonists in a chronic manner, and/or applied the agonists to obese or diabetic rodents; and this effect was not dependent on the specific anxiety test used. The latter result is of course not surprising, since application of GLP-1 analogues in this context is expected to improve the glucoregulation, body weight and fat, and overall health of the obese or diabetic animal, thus improvements in mood can be driven by improvements in metabolic health. Moreover, the idea that acute *vs.* chronic effect of GLP-1R agonists on mood is divergent was directly shown by Anderberg et al. (2016), where acute GLP-1R stimulation led to increased anxiety-like behavior, while chronic stimulation of these receptors led to reduced depression-like behavior, but no changes in anxiety-like behavior. The divergence of acute and chronic treatment was also supported by gene expression and neurotransmitter changes (Anderberg et al., 2016). Whole body knockout of GLP-1R in mice resulted in a mixed emotionality phenotype, where each of the three anxiety-like behavior tests used in the study suggested a different effect on the anxiety parameter (MacLusky et al., 2000). Recently, however, chronic reduction in GLP-1R in bed nucleus stria terminalis in rats, *via* the receptor knockdown approach utilized in the current study, resulted in a consistently reduced anxiety-like behavior in rats (Zheng et al., 2019b), similarly to what was observed in female SuM GLP-1R knockdown rats in the current study.

In addition to changes in anxiety-like behavior, SuM Ex4 delivery reduced locomotor activity, but did so specifically in the OF test, and only in female rats. Since there is a significant difference in the duration

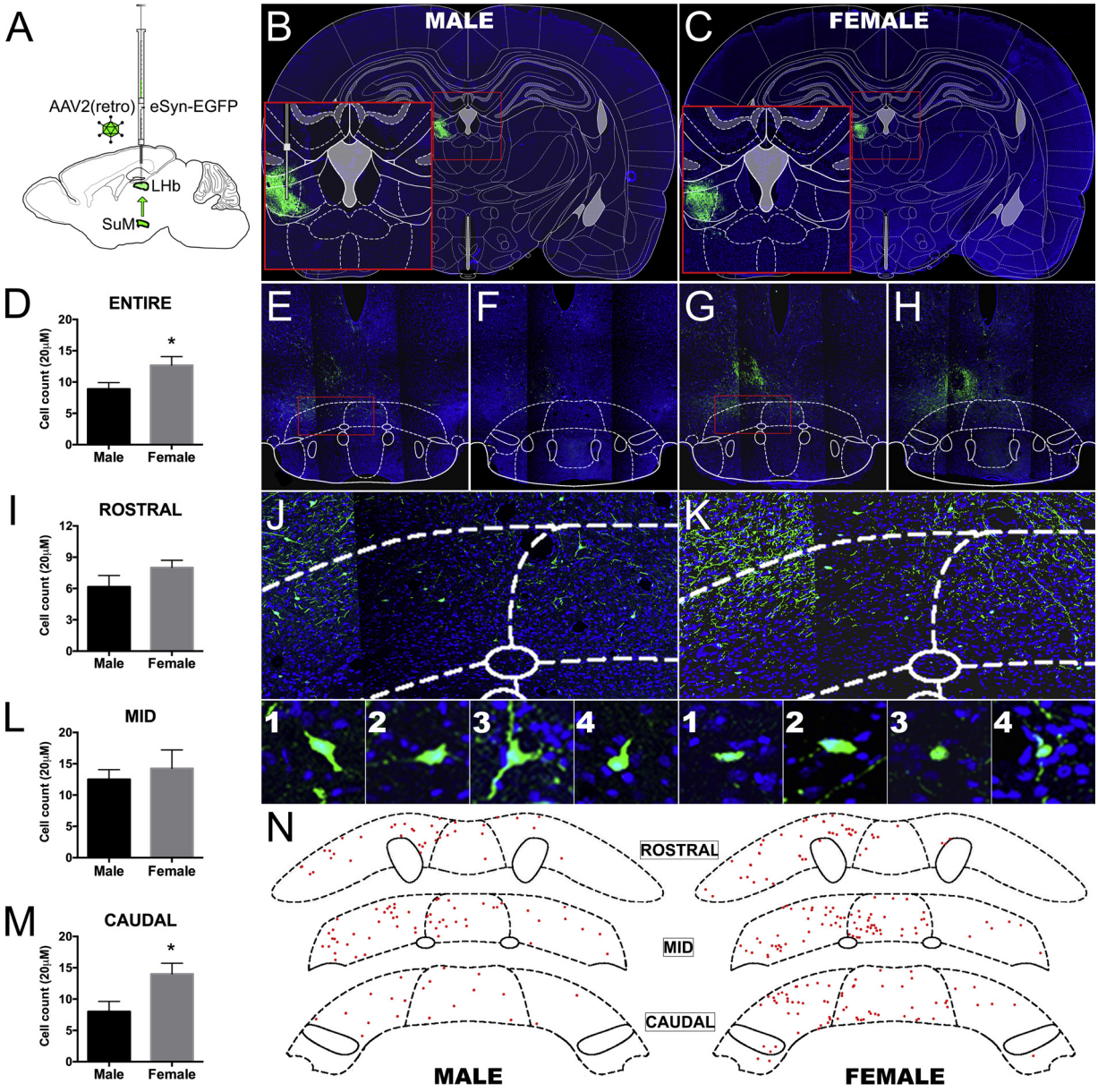

Fig. 5. SuM innervates the lateral habenula (LHb). Retrograde viral tracer (A) was injected into the LHb of male (B) and female (C) rats. Representative confocal images for males (E, F, J) and females (G, H, K) SuM as well as cell body maps (N) corresponding to the confocal images (J and K) highlight sex differences in the SuM to LHb connectivity. Higher magnification images (1-4) show clear cell bodies with green fluorescent retrogradedly carried EGFP. For both sexes, panels 1-3 are taken from the lateral SuM and panel 4 is from the medial SuM. Comparison of the number of cell bodies of neurons projecting to the LHb from the SuM of males and females (D) indicates that more LHb-projecting neurons are found in females compared to males. More LHb-projecting neurons are found in the caudal SuM of females (M). In contrast, rostral and medial SuM contained similar numbers of EGFP-labeled neurons in males and females (I, L). Cell nuclei are labeled in blue with DAPI. Two to three sections were analyzed for each SuM level; total of five rats, two males and three females were included in this analysis. For N, rostral starts at bregma -4.3, mid at -4.5 and caudal at -4.7. * $p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

of the two anxiety-like behavior tests (5 min for EPM *vs* 30 min for OF), it is likely that the difference is largely a result of small changes in activity accumulated over time. We cannot exclude the possibility that the effect on locomotion is indirect, *via* increasing active periods of wakefulness, since one previous study reported that in mice, chemoactivation of SuM glutamatergic neurons increases wakefulness (Pedersen et al., 2017). However, that the effect of GLP-1R activation on locomotion is context dependent is likely, since previous results from our lab demonstrate that the same doses of Ex4 as those used here, delivered to the SuM, did not alter activity in a familiar environment (Lopez-Ferreras et al., 2019). If GLP-1R activation increased wakefulness consequent changes in locomotion, regardless of the context, would be expected. Unlike females, the anxiogenic effect of SuM Ex4 administration in males was not coupled with hypolocomotion in the current study.

Sex differences may result from differential levels of sex steroid signaling, since estrogen receptors, ERα and ERβ are present in the SuM of females (Leranth et al., 1999; Mitra et al., 2003). Exogenous estrogen application reduces anxiety-like behavior in rodents (Walf and Frye, 2009). Moreover, mice show lower anxiety-like behavior in the EPM when tested in the high (behavioral estrus) *versus* the low (diestrus), estrogen phase of the estrous cycle (Galeeva and Tuohimaa, 2001; Walf and Frye, 2009), although not all studies detect estrous cycle driven changes in anxiety-like behavior. In addition to estrogen, progesterone

fluctuations have also been associated with altered anxiety in humans or anxiety-like behavior in rodents; though a more complex and potentially cyclic relationship between progesterone, stress, and anxiety (see (Wirth, 2011) for a review) exists compared to that established for estrogens (Reynolds et al., 2018). In humans a much higher prevalence of anxiety disorders is reported in women compared to men. However, not all preclinical studies reflect these differences. Similarly, in the current study, females displayed more anxiety-like behavior compared to males in only one experiment, where baseline testing was not associated with microinjection-based treatment. Thus, it is possible that the mild additional stress associated with microinjections, despite the extensive habituation, is still sufficient to interfere with detection of sex differences. It is also possible that the large differences found in the clinic are associated with gender and social expectations of gender rather than biological sex.

We did not detect any effect of HFHS-diet feeding on anxiety-like behavior of male or female rats, based on similar EPM and OF behavior of vehicle-treated rats fed either chow or HFHS. Although, chow and HFHS testing were performed as two separate experiments, with the main aim of assessing differences in Ex4 responses, thus direct diet-effect comparisons are not fully supported by this design. Preclinical literature on the potential influence of obesogenic diet on anxiety is mixed, with no effect, increased or reduced anxiety found in different studies. One recent study, in an effort to explain these conflicting results, revealed that the amount of time mice were allowed to consume the obesogenic diet was a crucial and often underreported factor (Sweeney et al., 2017). They found that initially HFHS-diet consumption was anxiolytic, followed by a period with no effect on anxiety behaviors (at 2 months on diet), culminating with an anxiogenic effect at nearly 4 months on the diet, but only in subjects that displayed symptoms of metabolic syndrome on the diet. Rats in the current study were fed the HFHS diet for 5 weeks before anxiety-like behavior testing commenced.

The LHab contributes to behavioral expression of anxiety behaviors and is a key component of the habenular avoidance system (Geisler and Trimble, 2008; Gill et al., 2013; Jhou et al., 2009). Its direct afferents to the amygdala or bed nucleus stria terminalis further support its potential modulatory role in anxiety (Geisler and Trimble, 2008). Current data indicate that a potential for direct neural communication between the SuM and LHab exists, but that the extent of that connection is modulated by sex, with a stronger SuM-LHab link detected in female rats.

It should also be considered that factors other than direct influence on emotionally charged inputs are affected by SuM manipulations to finally affect anxiety-like behavior. Recently SuM glutamatergic neurons emerged as a key component of the sleep-wake and arousal systems (Pedersen et al., 2017). It would be reasonable to hypothesize that signals associated with satiety are also utilized to engage neurocircuitry associated with post-meal suppression of arousal (an expected part of the satiety sequence). We should note that the meal offered to the rats designated for the SuM GLP-1R blockade experiment was preceded by a period of overnight food restriction, so the data cannot be directly compared to results derived from non-food deprived rats (note that fasting signals like ghrelin and food deprivation have been shown to reduce anxiety (Alvarez-Crespo et al., 2012)). Moreover, a recent study reveals the SuM as a key node to control theta-frequency spike time coordination, an essential process that enables animals to navigate to desired locations in space (Ito et al., 2018). Thus, it is possible that SuM integrates a wide range of input including arousal, satiety, and novelty, and all these factors modulate the anxiety responses elicited by this nucleus.

In summary, current data indicate that SuM GLP-1R are necessary and sufficient for normal expression of anxiety-like behavior in both sexes. However, the consequences of reduced SuM GLP-1R signaling are sex divergent. In contrast to GLP-1R-specific SuM activation, the behavioral outcome of neuronal SuM activation is reduced anxiety-like behavior. Collectively our results highlight SuM as a novel node in the central nervous system network underlying emotionality regulation, a node that is neuroanatomically predisposed to modulate sex divergent responses.

Financial disclosures

MRH is a partial owner of Cantius Therapeutics, LLC, and receives research funding from Zealand Pharma,Eli Lilly and Company, Boehringer Igelheim and Novo Nordiskthat was not used to support these studies. All the other authors declare no conflict of interest.

Author contributions

LLF contributed to the design, acquisition, analysis, and interpretation of all *in vivo* data. KE contributed to the design, acquisition, analysis, and interpretation of all retrograde tracer data. OTS contributed to the design, acquisition, analysis, and interpretation of DREADD images. JER, FHN, and LEJ contributed to the design, acquisition, analysis, and interpretation of select *in vivo* data. KPS and MRH had substantial contributions to the conception and design of the work. All authors contributed to drafting the work or revising it critically for important intellectual content; and approved the final version to be published. All autors agree to be accountable for all aspects.

Acknowledgements

This research was funded by the Wallenberg Foundation (WCMTM), Swedish Research Council (2014-2945 and 2018-00660 to KPS; and 2013–7107 to PR), Novo Nordisk Foundation Excellence project grant (to KPS), Ragnar Söderberg Foundation (KPS), Harald Jeanssons Stiftelse and Greta Jeanssons Stiftelse (KPS), and Magnus Bergvalls Stiftelse (KPS), and National Institute of HealthNIH-DK115762 (MRH). We would also like to thank Graziella DiGiacomo for her technical assistance with cryo-sectioning.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.psyneuen.2020.104720.

References

Acuna-Goycolea, C., van den Pol, A., 2004. Glucagon-like peptide 1 excites hypocretin/orexin neurons by direct and indirect mechanisms: implications for viscera-mediated arousal. J. Neurosci. 24, 8141–8152.

Alvarez-Crespo, M., Skibicka, K.P., Farkas, I., Molnar, C.S., Egecioglu, E., Hrabovszky, E., Liposits, Z., Dickson, S.L., 2012. The amygdala as a neurobiological target for ghrelin in rats: neuroanatomical, electrophysiological and behavioral evidence. PLoS One 7, e46321.

Anderberg, R.H., Richard, J.E., Hansson, C., Nissbrandt, H., Bergquist, F., Skibicka, K.P., 2016. GLP-1 is both anxiogenic and antidepressant; divergent effects of acute and chronic GLP-1 on emotionality. Psychoneuroendocrinology 65, 54–66.

Anderberg, R.H., Richard, J.E., Eerola, K., Lopez-Ferreras, L., Banke, E., Hansson, C., Nissbrandt, H., Berqquist, F., Gribble, F.M., Reimann, F., Wernstedt Asterholm, I., Lamy, C.M., Skibicka, K.P., 2017. Glucagon-like peptide 1 and its analogs act in the dorsal raphe and modulate central serotonin to reduce appetite and body weight. Diabetes 66, 1062–1073.

Aranda, L., Santin, L.J., Begega, A., Aguirre, J.A., Arias, J.L., 2006. Supramammillary and adjacent nuclei lesions impair spatial working memory and induce anxiolitic-like behavior. Behav. Brain Res. 167, 156–164.

Barry, D., Pietrzak, R.H., Petry, N.M., 2008. Gender differences in associations between body mass index and DSM-IV mood and anxiety disorders: results from the National Epidemiologic Survey on Alcohol and Related Conditions. Ann. Epidemiol. 18, 458–466.

Bavec, A., Hallbrink, M., Langel, U., Zorko, M., 2003. Different role of intracellular loops of glucagon-like peptide-1 receptor in G-protein coupling. Regul. Pept. 111, 137–144.

Beck, C.H., Fibiger, H.C., 1995. Chronic desipramine alters stress-induced behaviors and regional expression of the immediate early gene, c-fos. Pharmacol. Biochem. Behav. 51, 331–338.

Belzung, C., Griebel, G., 2001. Measuring normal and pathological anxiety-like behaviour

in mice: a review. Behav. Brain Res. 125, 141–149.
Charney, D.S., 2003. Neuroanatomical circuits modulating fear and anxiety behaviors. Acta Psychiatr. Scand. Suppl. 38–50.
Davis, M., Walker, D.L., Lee, Y., 1997a. Amygdala and bed nucleus of the stria terminalis: differential roles in fear and anxiety measured with the acoustic startle reflex. Philos. Trans. R. Soc. Lond., B, Biol. Sci. 352, 1675–1687.
Davis, M., Walker, D.L., Lee, Y., 1997b. Roles of the amygdala and bed nucleus of the stria terminalis in fear and anxiety measured with the acoustic startle reflex. Possible relevance to PTSD. Ann. N. Y. Acad. Sci. 821, 305–331.
Fortress, A.M., Hamlett, E.D., Vazey, E.M., Aston-Jones, G., Cass, W.A., Boger, H.A., Granholm, A.C., 2015. Designer receptors enhance memory in a mouse model of Down syndrome. J. Neurosci. 35, 1343–1353.
Galeeva, A., Tuohimaa, P., 2001. Analysis of mouse plus-maze behavior modulated by ovarian steroids. Behav. Brain Res. 119, 41–47.
Gariepy, G., Nitka, D., Schmitz, N., 2010. The association between obesity and anxiety disorders in the population: a systematic review and meta-analysis. Int. J. Obes. (Lond.) 34, 407–419.
Geisler, S., Trimble, M., 2008. The lateral habenula: no longer neglected. CNS Spectr. 13, 484–489.
Ghosal, S., Packard, A.E.B., Mahbod, P., McKlveen, J.M., Seeley, R.J., Myers, B., Ulrich-Lai, Y., Smith, E.P., D'Alessio, D.A., Herman, J.P., 2017. Disruption of glucagon-like peptide 1 signaling in sim1 neurons reduces physiological and behavioral reactivity to acute and chronic stress. J. Neurosci. 37, 184–193.
Gill, M.J., Ghee, S.M., Harper, S.M., See, R.E., 2013. Inactivation of the lateral habenula reduces anxiogenic behavior and cocaine seeking under conditions of heightened stress. Pharmacol. Biochem. Behav. 111, 24–29.
Gulec, G., Isbil-Buyukcoskun, N., Kahveci, N., 2010. Effects of centrally-injected glucagon-like peptide-1 on pilocarpine-induced seizures, anxiety and locomotor and exploratory activity in rat. Neuropeptides 44, 285–291.
Hallbrink, M., Holmqvist, T., Olsson, M., Ostenson, C.G., Efendic, S., Langel, U., 2001. Different domains in the third intracellular loop of the GLP-1 receptor are responsible for Galpha(s) and Galpha(i)/Galpha(o) activation. Biochim. Biophys. Acta 1546, 79–86.
Hayes, M.R., Bradley, L., Grill, H.J., 2009. Endogenous hindbrain glucagon-like peptide-1 receptor activation contributes to the control of food intake by mediating gastric satiation signaling. Endocrinology 150, 2654–2659.
Holst, J.J., 2007. The physiology of glucagon-like peptide 1. Physiol. Rev. 87, 1409–1439.
Ito, M., Shirao, T., Doya, K., Sekino, Y., 2009. Three-dimensional distribution of Fos-positive neurons in the supramammillary nucleus of the rat exposed to novel environment. Neurosci. Res. 64, 397–402.
Ito, H.T., Moser, E.I., Moser, M.B., 2018. Supramammillary nucleus modulates spike-time coordination in the prefrontal-thalamo-Hippocampal circuit during navigation. Neuron 99, 576–587 e575.
Jhou, T.C., Fields, H.L., Baxter, M.G., Saper, C.B., Holland, P.C., 2009. The rostromedial tegmental nucleus (RMTg), a GABAergic afferent to midbrain dopamine neurons, encodes aversive stimuli and inhibits motor responses. Neuron 61, 786–800.
Kanoski, S.E., Hayes, M.R., Skibicka, K.P., 2016. GLP-1 and weight loss: unraveling the diverse neural circuitry. Am. J. Physiol. Regul. Integr. Comp. Physiol. 310, R885–895.
Kessler, R.C., Wang, P.S., 2008. The descriptive epidemiology of commonly occurring mental disorders in the United States. Annu. Rev. Public Health 29, 115–129.
Kessler, R.C., Chiu, W.T., Demler, O., Merikangas, K.R., Walters, E.E., 2005. Prevalence, severity, and comorbidity of 12-month DSM-IV disorders in the National Comorbidity Survey Replication. Arch. Gen. Psychiatry 62, 617–627.
Kessler, R.C., Petukhova, M., Sampson, N.A., Zaslavsky, A.M., Wittchen, H.U., 2012. Twelve-month and lifetime prevalence and lifetime morbid risk of anxiety and mood disorders in the United States. Int. J. Methods Psychiatr. Res. 21, 169–184.
Kinzig, K.P., D'Alessio, D.A., Herman, J.P., Sakai, R.R., Vahl, T.P., Figueiredo, H.F., Murphy, E.K., Seeley, R.J., 2003. CNS glucagon-like peptide-1 receptors mediate endocrine and anxiety responses to interoceptive and psychogenic stressors. J. Neurosci. 23, 6163–6170.
Komsuoglu Celikyurt, I., Mutlu, O., Ulak, G., Uyar, E., Bektas, E., Yildiz Akar, F., Erden, F., Tarkun, I., 2014. Exenatide treatment exerts anxiolytic- and antidepressant-like effects and reverses neuropathy in a mouse model of type-2 diabetes. Med. Sci. Monit. Basic Res. 20, 112–117.
Krass, M., Runkorg, K., Vasar, E., Volke, V., 2012. Acute administration of GLP-1 receptor agonists induces hypolocomotion but not anxiety in mice. Acta Neuropsychiatr. 24, 296–300.
Krass, M., Volke, A., Runkorg, K., Wegener, G., Lund, S., Abildgaard, A., Vasar, E., Volke, V., 2015. GLP-1 receptor agonists have a sustained stimulatory effect on corticosterone release after chronic treatment. Acta Neuropsychiatr. 27, 25–32.
Leranth, C., Shanabrough, M., Horvath, T.L., 1999. Estrogen receptor-alpha in the raphe serotonergic and supramammillary area calretinin-containing neurons of the female rat. Exp. Brain Res. 128, 417–420.
Li, C., Barker, L., Ford, E.S., Zhang, X., Strine, T.W., Mokdad, A.H., 2008. Diabetes and anxiety in US adults: findings from the 2006 Behavioral Risk Factor Surveillance System. Diabet. Med. 25, 878–881.
Lopez-Ferreras, L., Richard, J.E., Noble, E.E., Eerola, K., Anderberg, R.H., Olandersson, K., Taing, L., Kanoski, S.E., Hayes, M.R., Skibicka, K.P., 2018. Lateral hypothalamic GLP-1 receptors are critical for the control of food reinforcement, ingestive behavior and body weight. Mol. Psychiatry 23, 1157–1168.
Lopez-Ferreras, L., Eerola, K., Mishra, D., Shevchouk, O.T., Richard, J.E., Nilsson, F.H., Hayes, M.R., Skibicka, K.P., 2019. GLP-1 modulates the supramammillary nucleus-lateral hypothalamic neurocircuit to control ingestive and motivated behavior in a sex divergent manner. Mol. Metab. 20, 178–193.
MacLusky, N.J., Cook, S., Scrocchi, L., Shin, J., Kim, J., Vaccarino, F., Asa, S.L., Drucker, D.J., 2000. Neuroendocrine function and response to stress in mice with complete disruption of glucagon-like peptide-1 receptor signaling. Endocrinology 141, 752–762.
McLean, C.P., Hope, D.A., 2010. Subjective anxiety and behavioral avoidance: gender, gender role, and perceived confirmability of self-report. J. Anxiety Disord. 24, 494–502.
McLean, C.P., Asnaani, A., Litz, B.T., Hofmann, S.G., 2011. Gender differences in anxiety disorders: prevalence, course of illness, comorbidity and burden of illness. J. Psychiatr. Res. 45, 1027–1035.
Meitzen, J., Pflepsen, K.R., Stern, C.M., Meisel, R.L., Mermelstein, P.G., 2011. Measurements of neuron soma size and density in rat dorsal striatum, nucleus accumbens core and nucleus accumbens shell: differences between striatal region and brain hemisphere, but not sex. Neurosci. Lett. 487, 177–181.
Mitra, S.W., Hoskin, E., Yudkovitz, J., Pear, L., Wilkinson, H.A., Hayashi, S., Pfaff, D.W., Ogawa, S., Rohrer, S.P., Schaeffer, J.M., McEwen, B.S., Alves, S.E., 2003. Immunolocalization of estrogen receptor beta in the mouse brain: comparison with estrogen receptor alpha. Endocrinology 144, 2055–2067.
Muller, T.D., Clemmensen, C., Finan, B., DiMarchi, R.D., Tschop, M.H., 2018. Anti-obesity therapy: from rainbow pills to polyagonists. Pharmacol. Rev. 70, 712–746.
Pan, W.X., McNaughton, N., 2004. The supramammillary area: its organization, functions and relationship to the hippocampus. Prog. Neurobiol. 74, 127–166.
Pedersen, N.P., Ferrari, L., Venner, A., Wang, J.L., Abbott, S.B.G., Vujovic, N., Arrigoni, E., Saper, C.B., Fuller, P.M., 2017. Supramammillary glutamate neurons are a key node of the arousal system. Nat. Commun. 8, 1405.
Reiner, D.J., Mietlicki-Baase, E.G., McGrath, L.E., Zimmer, D.J., Bence, K.K., Sousa, G.L., Konanur, V.R., Krawczyk, J., Burk, D.H., Kanoski, S.E., Hermann, G.E., Rogers, R.C., Hayes, M.R., 2016. Astrocytes regulate GLP-1 receptor-mediated effects on energy balance. J. Neurosci. 36, 3531–3540.
Reynolds, T.A., Makhanova, A., Marcinkowska, U.M., Jasienska, G., McNulty, J.K., Eckel, L.A., Nikonova, L., Maner, J.K., 2018. Progesterone and women's anxiety across the menstrual cycle. Horm. Behav. 102, 34–40.
Richard, J.E., Farkas, I., Anesten, F., Anderberg, R.H., Dickson, S.L., Gribble, F.M., Reimann, F., Jansson, J.O., Liposits, Z., Skibicka, K.P., 2014. GLP-1 receptor stimulation of the lateral parabrachial nucleus reduces food intake: neuroanatomical, electrophysiological, and behavioral evidence. Endocrinology 155, 4356–4367.
Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682.
Schmidt, H.D., Mietlicki-Baase, E.G., Ige, K.Y., Maurer, J.J., Reiner, D.J., Zimmer, D.J., Van Nest, D.S., Guercio, L.A., Wimmer, M.E., Olivos, D.R., De Jonghe, B.C., Hayes, M.R., 2016. Glucagon-like Peptide-1 receptor activation in the ventral tegmental area decreases the reinforcing efficacy of cocaine. Neuropsychopharmacology 41, 1917–1928.
Schmidt, C.K., Khalid, S., Loukas, M., Tubbs, R.S., 2018. Neuroanatomy of anxiety: a brief review. Cureus 10, e2055.
Sharma, A.N., Pise, A., Sharma, J.N., Shukla, P., 2015. Glucagon-like peptide-1 (GLP-1) receptor agonist prevents development of tolerance to anti-anxiety effect of ethanol and withdrawal-induced anxiety in rats. Metab. Brain Dis. 30, 719–730.
Silveira, M.C., Sandner, G., Graeff, F.G., 1993. Induction of Fos immunoreactivity in the brain by exposure to the elevated plus-maze. Behav. Brain Res. 56, 115–118.
Singh, M., 2014. Mood, food, and obesity. Front. Psychol. 5, 925.
Smith, K.J., Beland, M., Clyde, M., Gariepy, G., Page, V., Badawi, G., Rabasa-Lhoret, R., Schmitz, N., 2013. Association of diabetes with anxiety: a systematic review and meta-analysis. J. Psychosom. Res. 74, 89–99.
Strine, T.W., Mokdad, A.H., Balluz, L.S., Gonzalez, O., Crider, R., Berry, J.T., Kroenke, K., 2008. Depression and anxiety in the United States: findings from the 2006 behavioral risk factor surveillance system. Psychiatr. Serv. 59, 1383–1390.
Sweeney, P., O'Hara, K., Xu, Z., Yang, Y., 2017. HFD-induced energy states-dependent bidirectional control of anxiety levels in mice. Int. J. Obes. (Lond) 41, 1237–1245.
Terburg, D., Scheggia, D., Triana Del Rio, R., Klumpers, F., Ciobanu, A.C., Morgan, B., Montoya, E.R., Bos, P.A., Giobellina, G., van den Burg, E.H., de Gelder, B., Stein, D.J., Stoop, R., van Honk, J., 2018. The basolateral amygdala is essential for rapid escape: a human and rodent study. Cell 175, 723–735 e716.
Terrill, S.J., Jackson, C.M., Greene, H.E., Lilly, N., Maske, C.B., Vallejo, S., Williams, D.L., 2016. Role of lateral septum glucagon-like peptide 1 receptors in food intake. Am. J. Physiol. Regul. Integr. Comp. Physiol. 311, R124–132.
Tervo, D.G., Hwang, B.Y., Viswanathan, S., Gaj, T., Lavzin, M., Ritola, K.D., Lindo, S., Michael, S., Kuleshova, E., Ojala, D., Huang, C.C., Gerfen, C.R., Schiller, J., Dudman, J.T., Hantman, A.W., Looger, L.L., Schaffer, D.V., Karpova, A.Y., 2016. A designer AAV variant permits efficient retrograde access to projection neurons. Neuron 92, 372–382.
Tuesta, L.M., Chen, Z., Duncan, A., Fowler, C.D., Ishikawa, M., Lee, B.R., Liu, X.A., Lu, Q., Cameron, M., Hayes, M.R., Kamenecka, T.M., Pletcher, M., Kenny, P.J., 2017. GLP-1 acts on habenular avoidance circuits to control nicotine intake. Nat. Neurosci. 20, 708–716.
Urban, D.J., Roth, B.L., 2015. DREADDs (designer receptors exclusively activated by designer drugs): chemogenetic tools with therapeutic utility. Annu. Rev. Pharmacol. Toxicol. 55, 399–417.
Vogel, H., Wolf, S., Rabasa, C., Rodriguez-Pacheco, F., Babaei, C.S., Stober, F., Goldschmidt, J., DiMarchi, R.D., Finan, B., Tschop, M.H., Dickson, S.L., Schurmann, A., Skibicka, K.P., 2016. GLP-1 and estrogen conjugate acts in the supramammillary nucleus to reduce food-reward and body weight. Neuropharmacology 110, 396–406.
Vrang, N., Phifer, C.B., Corkern, M.M., Berthoud, H.R., 2003. Gastric distension induces c-Fos in medullary GLP-1/2-containing neurons. Am. J. Physiol. Regul. Integr. Comp. Physiol. 285, R470–478.

Walf, A.A., Frye, C.A., 2009. Effects of two estradiol regimens on anxiety and depressive behaviors and trophic effects in peripheral tissues in a rodent model. Gend. Med. 6, 300–311.
Wirth, M.M., 2011. Beyond the HPA Axis: progesterone-Derived neuroactive steroids in human stress and emotion. Front. Endocrinol. (Lausanne) 2, 19.
Zheng, H., Reiner, D.J., Hayes, M.R., Rinaman, L., 2019a. Chronic suppression of glucagon-like peptide-1 receptor (GLP1R) mRNA translation in the rat bed nucleus of the stria terminalis reduces anxiety-like behavior and stress-induced hypophagia, but prolongs stress-induced elevation of plasma corticosterone. J. Neurosci.
Zheng, H., Reiner, D.J., Hayes, M.R., Rinaman, L., 2019b. Chronic suppression of glucagon-like peptide-1 receptor (GLP1R) mRNA translation in the rat bed nucleus of the stria terminalis reduces anxiety-like behavior and stress-induced hypophagia, but prolongs stress-induced elevation of plasma corticosterone. J. Neurosci. 39, 2649–2663.