ORIGINAL ARTICLE

GHS-R1a signaling in the DMH and VMH contributes to food anticipatory activity

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BACKGROUND: Rats that have restricted access to food at a fixed time point of the circadian phase display high levels of food anticipatory activity (FAA). The orexigenic hormone ghrelin has been implicated in the regulation of FAA. However, it is not known via which brain area ghrelin exerts this effect. Growth hormone secretagogue receptor 1a (GHS-R1a) is highly expressed in the hypothalamus, including the dorsomedial hypothalamus (DMH) and the ventromedial hypothalamus (VMH). These two hypothalamic areas have been reported to play a role in FAA.
AIM OF THE STUDY: To examine the role of GHS-R1a signaling in the DMH and VMH in FAA.
DESIGN: Adeno-associated virus expressing a shRNA directed against GHS-R1a was used to establish local knockdown of GHS-R1a in the DMH and VMH in rats. Rats were subsequently subjected to a restricted feeding schedule (RFS).
RESULTS: Under *ad libitum* conditions, knockdown of GHS-R1a in the VMH increased food intake and body weight gain. In addition, GHS-R1a knockdown in VMH and DMH reduced body temperature and running wheel activity (RWA). When rats were subjected to a RFS, the main effect of GHS-R1a knockdown in both DMH and VMH was a decrease in RWA and an attenuation of body weight loss. Rats with knockdown of GHS-R1a in DMH and VMH showed a delay in onset of FAA. In addition, GHS-R1a knockdown in DMH resulted in a reduction of FAA amplitude.
CONCLUSION: This is the first study to investigate the effect of local hypothalamic knockdown of GHS-R1a on FAA. Our results implicate hypothalamic GHS-R1a signaling in the regulation of FAA. Nevertheless, some FAA remained, suggesting that a distributed network of brain areas and signaling pathways is involved in the development of FAA.

INTRODUCTION

The orexigenic peptide ghrelin is the endogenous ligand of growth hormone secretagogue receptor 1a (GHS-R1a). It is produced in the stomach and released into the circulation.[1] Ghrelin not only stimulates growth hormone release[1] but also acts as a potent stimulator of appetite and causes an increase in fat mass.[2–4] Ghrelin plasma levels are increased in anorexia nervosa patients and also after fasting.[5] In contrast, weight gain and obesity are associated with reduced plasma ghrelin levels.[5] Ghrelin's orexigenic effect is at least partly mediated by Agouti-related peptide and Neuropeptide Y in the arcuate nucleus.[4,6] In addition to the arcuate nucleus, GHS-R1a is also expressed in other hypothalamic regions, such as the ventromedial hypothalamus (VMH) and dorsomedial hypothalamus (DMH).[7,8]

Rats subjected to a restricted feeding schedule (RFS), with access to food for a limited period at a fixed time point of their circadian phase, show hyperactivity preceding their expected meal, a behavior referred to as food anticipatory activity (FAA).[9] During a RFS, circadian rhythms in behavior and clock gene expression uncouple from the master clock, the suprachiasmatic nucleus, and cycle in relation to food availability.[10] Plasma ghrelin levels showed entrainment to habitual meal patterns in humans and rats,[11,12] and central administration of ghrelin increased FAA.[13] In contrast, FAA was attenuated by GHS-R1a antagonism[14]

and in GHS-R1a − / − mice.[13–16] Ghrelin thus appears to have a role in FAA, although the question remains via which brain nuclei ghrelin exerts its effect on FAA.

Lesioning studies and investigation of the expression of immediate early genes have implicated several brain areas in the network that regulates FAA.[17–20] These include several areas of the hypothalamus, a brain region well known for its role in the regulation of energy balance and autonomic behaviors, and efferent projections of the suprachiasmatic nucleus extend to many other hypothalamic sites.

The VMH was the first brain area to become activated during anticipation after a shift in meal time,[21] and it has been proposed to amplify food entrainable rhythms.[22] The DMH also showed increased Fos expression during FAA[17–19,23,24] and clock gene rhythms shifted or changed in the DMH and VMH in rodents on RFS.[24–27] However, the role of DMH in FAA is controversial, as one study has reported a decrease in FAA after lesioning DMH,[23] whereas other studies still observed FAA in DMH-lesioned rodents.[27–30] Although lesions of VMH abolished FAA in rats on RFS,[31,32] FAA eventually re-established after a longer recovery period.[33,34]

Taken together, previous studies have implicated GHS-R1a, as well as DMH and VMH, in FAA, but the present study is the first to examine the role of GHS-R1a in the VMH and DMH in FAA. An AAV vector containing a short hairpin RNA (shRNA) directed against rat

GHS-R1a was injected in the VMH and DMH to determine the effects of local knockdown of GHS-R1a on running wheel activity (RWA), food intake, body weight gain and FAA in rats.

MATERIALS AND METHODS

Cell lines and plasmids

Human embryonic kidney 293T cells were cultured at 37 °C and 5% CO_2 in Dulbecco's Modified Eagles Medium (Gibco, Paisley, Scotland) supplemented with 10% (v/v) fetal calf serum (Integro, Zaandam, The Netherlands), 2 mM glutamine (Gibco), 100 U ml^{-1} penicillin, 100 units ml^{-1} streptomycin and non-essential amino acids (Gibco).

Bioinformatics analysis (www.biopredsi.org and www.invitrogen.com) revealed three potential functional shRNA sequences targeting the rat GHS-R1a gene (Table 1). As a control (pAAV-shCON), we designed oligonucleotides directed against the Renilla gene (NW_001321314), which is not expressed in the rat. The annealed oligonucleotides (Sigma, Zwijndrecht, The Netherlands) were ligated into the pAAV-shbase plasmid (a kind gift from RJ Dileone[35]). Expression of shRNA was under the control of a mouse U6 promoter and the plasmid coexpressed enhanced green fluorescent protein (GFP) driven by a CMV promoter hybridized to a β-actin intron, and a terminator sequence.

Rat GHS-R1a complementary DNA (cDNA) was amplified from hypothalamic rat cDNA. Primers were designed based on the published sequence (NM_032075.3) and contained attB-sites to allow Gateway cloning, a Kozak sequence for expression in mammalian cells, and a Shine–Dalgano sequence for expression in Escherichia coli (Table 1). GHS-R1a cDNA was cloned into the Gateway entry vector pDONR201 (Invitrogen, Carlsbad, CA, USA) and subsequently into a pBabe-puro vector (Invitrogen) containing Renilla cDNA in order to get a rat GHS-R1a-Renilla fusion plasmid.

Experiment 1: Luciferase assay—in vitro knockdown

Human embryonic kidney 293T cells were cultured in a 24-well plate and were transfected using polyethylenimine) with 5 ng pcDNA4/TO-luc (a kind gift from M van der Wetering), 500 ng pBabe GHS-R1a-Renilla plasmid and 2400 ng pAAV-shRNA (molar ratio 1:4) per well. Transfections were performed in duplo for the various pAAV-shRNA and pAAV-shbase. Three days after transfection, cells were lysed in passive lysis buffer and analyzed with a dual luciferase reporter assay according to the manufacturer's protocol (Promega, Madison, WI, USA). Firefly luciferase activities were measured using a Viktor 96-well plate reader (Perkin Elmer, Waltham, MA, USA). All values were normalized to luciferase values and expressed as a percentage of pAAV-shbase, which does not contain a shRNA insert.

Virus production and purification

Virus production and purification for AAV-shGHS-R1a no. 1, AAV-shGHS-R1a no. 3 and AAV-shCON were performed as described previously.[36] Following purification, the viral titer, in genomic copies per ml (gc ml^{-1}), was determined by real-time quantitative PCR in a LightCycler (Roche, Indianapolis, IN, USA) using primers for GFP (Table 1).

Animals

Male Wistar rats (Charles River, Sulzfeld, Germany) weighing 200–225 g upon arrival were individually housed in transparent acrylic cages in an ambient temperature (21 ± 2 °C) and humidity-controlled room on a 12 h/12 h light–dark cycle with lights on (Zeitgeber Time, ZT0) at 1900 hours (experiment 2) or 0700 hours (experiment 3). Rats were allowed to acclimatize for at least 1 week after arrival and had ad libitum access to water and food unless mentioned otherwise. All described procedures were approved by the ethical committee on use and care of animals of the University of Utrecht, The Netherlands. For ethical reasons, the experiment had to be terminated when rats lost more than 20% of their initial body weight.

Surgical procedures

Following acclimatization, rats were injected bilaterally with AAV virus in the VMH (coordinates: AP −2.6 mm from bregma, ML ±1.2 mm from bregma, DV −9.7 mm below the skull, under an angle of 5°) or DMH (coordinates: AP −2.8 mm from bregma, ML ±1.2 mm from bregma, DV −9.0 mm below the skull, under an angle of 5°). Per site, 1 µl of virus (1 × 10^{12} gc ml^{-1}) was delivered at a rate of 0.2 µl min^{-1}, after which the needles remained in place for an additional 10 min. Surgery was performed under fentanyl/fluanisone (0.1 ml per 100 g body weight, intramuscular; Hypnorm, Janssen Pharmacuetica, Beerse, Belgium) and midazolam (0.05 ml per 100 g body weight, intraperitoneal; Dormicum, Hoffman-LaRoche, Mijdrecht, The Netherlands) anesthesia. In addition, rats received saline (6 ml, subcutaneous) postoperatively and carprofen (Rimadyl, Pfizer Animal Health, Capelle a/d Ijssel, The Netherlands, 0.01 ml per 100 g subcutaneous) as analgesic both preoperatively and once a day postoperatively for 2 days.

Experiment 2

To determine in vivo knockdown, rats in experiment 2 received intra-VMH injections with AAV-shGHS-R1a no. 1 (n = 3) or AAV-shGHS-R1a no. 3 (n = 3) on one side, and an injection with AAV-shCON on the other side of the brain. In this way, each rat served as its own control for the establishment of in vivo knockdown. Rats were killed 5 weeks after surgery.

Experiment 3

Baseline measurements of body weight, food intake and water intake were taken in the week before surgery in order to divide rats (n = 90) into three experimental groups (AAV-shGHS-R1a no. 1, AAV-shGHS-R1a no. 3, AAV-shCON). Two weeks after surgery, rats were transferred to cages with a running wheel. After a habituation period of 2 weeks, rats were subjected to a RFS, in which they had limited access to food for 2 h in the middle of the light period from ZT6 to ZT8, which induced FAA in the hours before access to food. To control for differences in general RWA, measures of FAA were normalized as a percentage of total daily RWA. After 10–14 days on

Table 1. Overview of oligonucleotides used in this study

shGHS-R1a no. 1	Top	5′-TTTGGACAAAGTCGAGCATCAACACTTCCTGTCATGTTGATGCTCGACTTTGTCCTTTTT-3′
	Bottom	5′-CTAGAAAAAGGACAAAGTCGAGCATCAACATGACAGGAAGTGTTGATGCTCGACTTTGTCC-3′
shGHS-R1a no. 2	Top	5′-TTTGGCAGTGTTCAAACTGCTAGGCTTCCTGTCACCTAGCAGTTTGAACACTGCCTTTTT-3′
	Bottom	5′-CTAGAAAAAGGCAGTGTTCAAACTGCTAGGTGACAGGAAGCCTAGCAGTTTGAACACTGCC-3′
shGHS-R1a no. 3	Top	5′-TTTGCGAGGAGCCGGAGCCTAACGCTTCCTGTCACGTTAGGCTCCGGCTCCTCGCTTTTT-3′
	Bottom	5′-CTAGAAAAAGCGAGGAGCCGGAGCCTAACGTGACAGGAAGCGTTAGGCTCCGGCTCCTCGC-3′
shCon	Top	5′-TTTGAATTATAATGCTTATCTACTTCCTGTCATAGATAAGCATTATAATTCTTTTT-3′
	Bottom	5′-CTAGAAAAAGAATTATAATGDTTATCTATGACAGGAAGTAGATAAGCATTATAATTC-3′
cDNA GHS-R1a	Forward	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATACCACCATGTGGAACGCGACCCCCAGC-3′
	Reverse	5′GGGGACCACTTTGTACAAGAAAGCTGGGTCTGTGTTGATGCTCGACTTTG-3′
qPCR GHS-R1a	Forward	5′-CTCTGAAGGATGAGAGTTCCCGGGC-3′
	Reverse	5′-AAGTCCCGCTTGGCTACGGCT-3′
qPCR CycA	Forward	5′-AGCCTGGGGGAGAAAGGATT-3′
	Reverse	5′-AGCCACTCGTCTTGGCAGT-3′
GFP	Forward	5′-CACAGACTTGTGGGAGAAGC-3′
	Reverse	5′-CCCCTGAACCTGAAACATAAA-3′

Overview of the oligonucleotides that were used to obtain cDNA of rat GHS-R1a, to obtain shRNA directed against GHS-R1a and to perform qPCR.

RFS, depending on the amount of weight loss, rats were killed at ZT6 without receiving their expected meal.

Collection of blood and tissues
At the end of each experiment animals were killed by decapitation. Brains were quickly removed, immediately frozen and stored at $-80\,°C$. Trunk blood was collected in heparinized tubes containing 83 μmol ethylene-diaminetetraacetic acid and 1 mg aprotinin, and placed on ice. Following centrifugation, plasma was stored at $-20\,°C$ until further analyzed.

qPCR—in vivo knockdown
To establish in vivo knockdown, series of 16-μm coronal sections of the hypothalamus were sliced on a cryostat (Leica, Rijswijk, The Netherlands), thaw-mounted onto RNAse-free 1.0 polyethylene naphthalene membrane slides (Zeiss, Oberkochen, Germany) and stored at $-80\,°C$ until processing. Laser Capture Microdissection (PALM Robomover, Zeiss) was applied to dissect eight VMH sections with the highest GFP density. RNA was extracted from these sections with an RNeasy Plus Micro kit (Qiagen, Hilden, Germany). Expression of GHS-R1a was then detected with real-time quantitative PCR in a LightCycler (Roche using primers for each short hairpin sequence (Table 1). GHS-R1a expression was first normalized to the expression of a household gene (CycA, Table 1). Next, the normalized GHS-R1a expression on the side with AAV-shGHS-R1a was expressed as percentage of expression of GHS-R1a on the side with AAV-shCON.

In situ hybridization
To verify the injection sites, coronal sections (20 μm) were cut on a cryostat in series of 10 and collected on SuperFrost Plus slides (Menzel Gläser, Braunschweig, Germany). One series was used for in situ hybridization with a 720-basepair-long digoxigenin-labeled GFP riboprobe (NCBI gene DQ768212). Other series were used for Nissl or haemaluin-eosine staining. In situ hybridization was essentially conducted as described previously.[37]

Data analysis
All data are expressed as mean ± s.e.m. Body weight, food intake and water intake were measured daily. Food intake data were also collected by the automated system scales (Department Biomedical Engineering, UMC Utrecht, The Netherlands), which recorded the weight of food hoppers automatically every 12 s. A meal was defined as an episode with a minimal consumption of 0.3 g chow and a minimal intermeal interval of 5 min.[38] RWA was continuously registered by a Cage Registration Program (Department Biomedical Engineering, UMC Utrecht, The Netherlands). SPSS 15.0 for Windows software was applied for statistical analyses. Data points that exceeded three interquartile ranges from the edge of a boxplot were considered outliers and removed from the analysis. Differences between groups were calculated using multivariate analysis of variance (ANOVA) with a simple first contrast comparing sh1 and sh3 with shCON. Cumulative anticipatory activity was analyzed by a repeated-measures ANOVA with time as a within-subjects factor and a predefined simple contrast comparing sh1 and sh3 with shCON. In case of a significant group or time*group interaction, a multivariate ANOVA was run per time-point with a predefined simple contrast comparing sh1 and sh3 with shCON. Statistical significance was set at $P < 0.05$.

RESULTS

Experiment 1: In vitro knockdown efficiency
Once AAV vectors containing the different shRNAs were constructed, the efficiency of these shRNAs to downregulate rat GHS-R1a was determined with a renilla luciferase assay. pAAV-shGHS-R1a no. 1 showed the highest percentage of knockdown, namely 98% compared with pAAV-shbase, followed by pAAV-shGHS-R1a no. 3, which exhibited 92% knockdown. pAAV-shGHS-R1a no. 2 only produced 33% knockdown of GHS-R1a (Figure 1a). Based on these results, AAV virus was produced by AAV-shGHS-R1a no. 1 and no. 3. pAAV-shCON, which contains shRNA directed
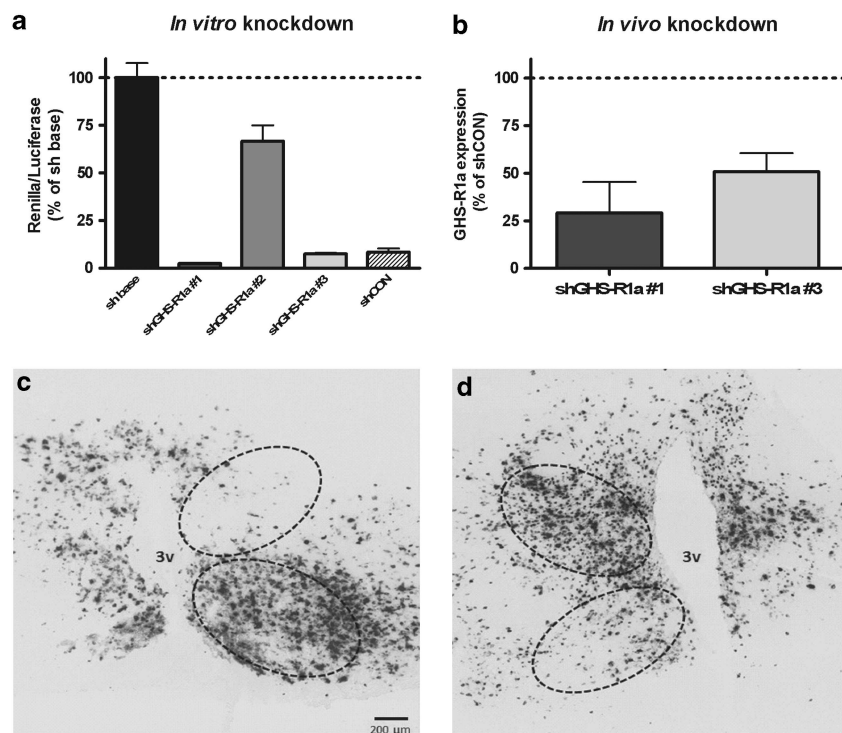

Figure 1. In vitro and in vivo knockdown efficiency of shGHS-R1a constructs. Graphical representation of the in vitro knockdown efficiency of a cDNA GHS-R1a-Renilla fusion construct by pAAV-shGHS-R1a (no. 1–3) and pAAV-shCON relative to a pAAV-shbase, which does not contain a shRNA sequence (a). In vivo knockdown efficiency of AAV-shGHS-R1a no. 1 and no. 3 in the VMH as measured by qPCR for GHS-R1a, which is expressed as percentage of GHS-R1a expression of the AAV-shCON-injected side and controlled for the expression of the household gene CycA (b). Values represent average ± s.e.m. Typical examples of GFP expression of rats that were bilaterally hit in the VMH (c) or DMH (d). The upper ellipses represent the DMH area and the lower ellipses represent the VMH area. 3v stands for third ventricle.

against Renilla, exhibited 92% knockdown of the GHS-R1a-Renilla fusion construct, and was, hence, a functional pAAV-shRNA.

Experiment 2: *In vivo* knockdown efficiency

To examine *in vivo* knockdown efficiency, rats were bilaterally injected in the VMH with AAV-shGHS-R1a no. 1 ($n = 3$) or no. 3 ($n = 3$) on one side and AAV-shCON on the other side. Figure 1b illustrates that AAV-shGHS-R1a no. 1 induced a 71% knockdown, whereas AAV-shGHS-R1a no. 3 downregulated GHS-R1a mRNA by 49%.

Experiment 3: Hypothalamic injections of pAAV-shGHS-R1a

Analysis of GFP expression in the rat brains revealed that 67 rats were bilaterally injected in the hypothalamus, of which 25 received pAAV-shCON (con), 21 received pAAV-shGHS-R1a no. 1 (sh1) and 21 received pAAV-shGHS-R1a no. 3 (sh3). Rats with DMH as the primary hypothalamic nucleus hit included 8 sh1 rats and 11 sh3 rats. The VMH was the main hypothalamic target for 13 sh1 rats and 10 sh3 rats. Of these rats, 11 (5 sh1, 6 sh3) showed some GFP expression in the DMH as well.

Figures 1c and d represent typical examples of injections targeting the VMH and the DMH. In addition, the DMH and VMH were hit unilaterally in five (two sh1, three sh3) and six rats (two sh1, four sh3), respectively. Furthermore, in 12 rats (9 sh1, 3 sh3), no GFP expression was detectable in the VMH or DMH; these were considered missed injections. Rats with unilateral or missed injections were not included in the analysis owing to small group sizes. Rats with missed injections lacked a clear phenotype and rats with unilateral injections of AAV-shGHS-R1a displayed intermediate phenotypes. Average values of all parameters measured of these groups and of the VMH rats that also showed GFP in the DMH are depicted in Supplementary Table S1.

Effect of hypothalamic GHS-R1a knockdown during *ad libitum* conditions

As AAV-mediated protein expression is usually observed 2 weeks after injection,[39] the effects of hypothalamic GHS-R1a knockdown under *ad libitum* conditions on body weight gain, food intake and RWA were examined in the third week following surgery.

Body weight gain. As depicted in Figure 2a, body weight gain was significantly increased in rats with knockdown of GHS-R1a in the VMH, whereas no significant effect of injection was detected in the DMH group.

Food intake and meal patterns. In line with an increase in body weight gain, daily average food intake was increased in rats with hypothalamic GHS-R1a knockdown in the VMH, but this effect was lacking in the DMH group (Figure 2b). The increase in food intake in the VMH group was due to an increase in meal size without an effect on total meal frequency. In the DMH group, there was no effect on total meal frequency or meal size. The circadian pattern of meal frequency was unaffected by GHS-R1a knockdown in either DMH or VMH (Supplementary Figure S1).

Running wheel activity. RWA was decreased in rats with knockdown of GHS-R1a in the VMH in the dark phase, but not in the light phase. In the DMH group, there was a significant
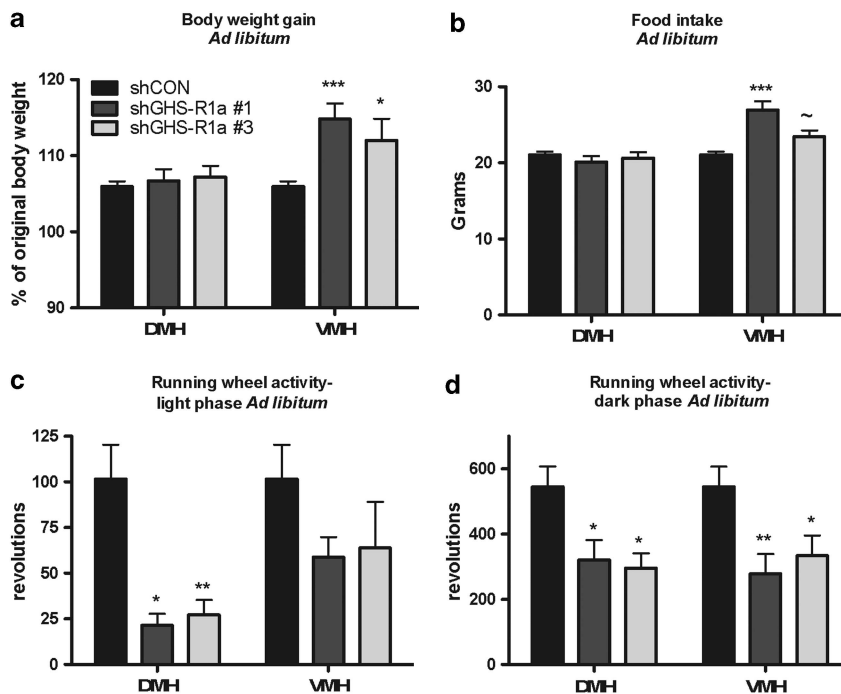

Figure 2. Effects of GHS-R1a knockdown in DMH and VMH during *ad libitum* feeding. Effects of knockdown of GHS-R1a in the DMH and VMH by pAAV-shGHS-R1a no. 1 and no. 3 compared with pAAV-shCON 3 weeks after surgery on body weight gain (VMH: $F = 10.848$, $P < 0.001$, shCON 105.90 ± 0.72, sh1 114.82 ± 2.02 ($P < 0.001$), sh3 $112.02 \pm 2.81\%$ ($P < 0.05$), DMH: $F = 0.401$, $P = 0.672$) (**a**), food intake (VMH: $F = 20.173$, $P < 0.001$, shCON 21.07 ± 0.39, sh1 26.94 ± 1.11 ($P < 0.001$), sh3 23.47 ± 0.76 ($P = 0.063$) grams, DMH: $F = 0.667$, $P = 0.519$, shCON 21.07 ± 0.39, sh1 20.08 ± 0.81, sh3 20.62 ± 0.77 g) (**b**), and running wheel activity (light phase (VMH: $F = 1.538$, $P = 0.226$, shCON 101.48 ± 18.92, sh1 58.75 ± 10.91, sh3 63.85 ± 25.04 revolutions, DMH: $F = 5.829$, $P < 0.01$, shCON 101.48 ± 18.92, sh1 21.39 ± 6.36 ($P < 0.05$), sh3 27.14 ± 8.09 ($P < 0.01$) revolutions) (**c**), dark phase (VMH: $F = 4.926$, $P < 0.05$, shCON 544.74 ± 62.46, sh1 278.59 ± 59.75 ($P < 0.01$), sh3 333.41 ± 62.10 ($P < 0.05$) revolutions, DMH: $F = 4.567$, $P < 0.05$, shCON 544.74 ± 62.46, sh1 321.11 ± 59.38 ($P < 0.05$), sh3 295.55 ± 45.74 ($P < 0.05$) revolutions) (**d**). Values represent absolute averages ± s.e.m. A multivariate ANOVA was conducted with a predefined simple contrast comparing each pAAV-shGHS-R1a with the control group. $^{\sim}P < 0.10$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
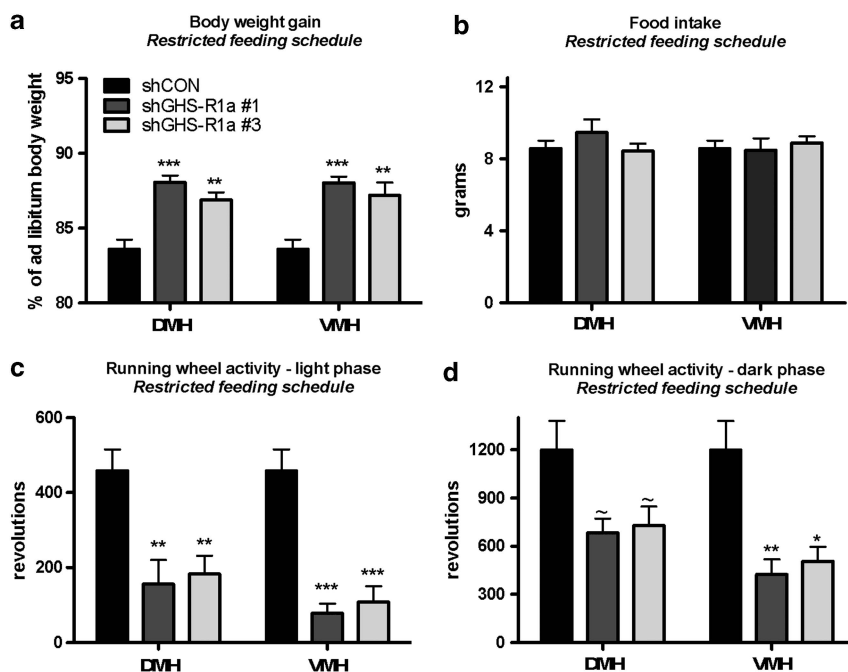
Figure 3. Effects of GHS-R1a knockdown in DMH and VMH during a RFS. Effects of knockdown of GHS-R1a in the DMH and VMH by pAAV-shGHS-R1a no. 1 and no. 3 compared with pAAV-shCON during the RFS on body weight gain (VMH: $F = 13.402$, $P < 0.001$, shCON 83.59 ± 0.64, sh1 88.04 ± 0.40 ($P < 0.001$), sh3 87.19 ± 0.87 ($P < 0.01$) % of pre-RFS body weight, DMH: $F = 11.417$, $P < 0.001$, shCON 83.59 ± 0.64, sh1 88.07 ± 0.46 ($P < 0.001$), sh3 86.89 ± 0.52 ($P < 0.01$) % of pre-RFS body weight) (**a**), food intake (VMH: $F = 0.099$, $P = 0.906$, shCON 8.58 ± 0.44, sh1 8.47 ± 0.67, sh3 8.87 ± 0.39 g, DMH: $F = 0.733$, $P = 0.487$, shCON 8.58 ± 0.44, sh1 9.46 ± 0.73, sh3 8.44 ± 0.40 g) (**b**), and running wheel activity (light phase)(VMH: $F = 16.085$, $P < 0.001$, shCON 458.35 ± 56.92, sh1 58.75 ± 10.91 ($P < 0.001$), sh3 108.44 ± 41.97 ($P < 0.001$) revolutions, DMH: $F = 7.480$, $P < 0.01$, shCON 458.35 ± 56.92, sh1 155.79 ± 65.13 ($P < 0.01$), sh3 182.65 ± 49.06 ($P < 0.01$) revolutions) (**c**); dark phase (VMH: $F = 6.556$, $P < 0.01$, shCON 1198.54 ± 181.35, sh1 278.59 ± 59.75 ($P < 0.01$), sh3 505.31 ± 91.96 ($P < 0.05$) revolutions, DMH: $F = 2.443$, $P = 0.099$, shCON 1198.54 ± 181.35, sh1 683.55 ± 88.74 ($P = 0.089$), sh3 730.22 ± 115.94 ($P = 0.082$) revolutions) (**d**)). Values represent absolute averages $\pm$ s.e.m. A multivariate ANOVA was conducted with a predefined simple contrast comparing each pAAV-shGHS-R1a with the control group. $\sim P < 0.10$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

reduction in RWA in both the light phase and the dark phase (Figures 2c and d).

Figures 4a and b depict the circadian rhythms of RWA per group. Both the VMH and the DMH group were hypoactive compared with the control rats. However, when normalizing RWA per hour for total RWA, the circadian rhythms of RWA were identical for control rats and rats with knockdown of GHS-R1a in the DMH or VMH (Figures 4c and d).

Effect of hypothalamic GHS-R1a knockdown during RFS

Body weight gain. As illustrated in Figure 3a, in response to a RFS, rats with knockdown of GHS-R1a in VMH or DMH lost less weight than control rats.

Food intake and meal patterns. Average daily food intake did not differ between control rats and rats with knockdown in VMH or DMH (Figure 3b). In addition, no changes were observed in meal frequency or meal size in the VMH or DMH group (Supplementary Figure S1). All groups spent on average an equal amount of time eating during the 120-min window of food availability (VMH: $F = 0.201$, $P = 0.818$, shCON 71.00 ± 4.18, sh1 74.18 ± 5.00, sh3 69.49 ± 3.48 min, DMH: $F = 0.374$, $P = 0.690$, shCON 71.00 ± 4.18, sh1 74.40 ± 7.41, sh3 76.64 ± 3.34 min).

Running wheel activity. In both the VMH and DMH groups, RWA during RFS was diminished in the light phase (Figure 3c). The dark phase RWA during RFS (Figure 3d) was reduced in the VMH group and showed a trend towards reduction in the DMH group.

Anticipatory running wheel activity. Comparison of the circadian rhythms of RWA during RFS showed that VMH and DMH groups were hypoactive compared with control rats and show reduced levels of anticipatory RWA not only preceding food access, but also preceding the dark phase (Figures 4e and f). To control for the reduced levels of RWA in general, RWA was also expressed as a percentage of total RWA per hour (Figures 4g and h).

To examine whether there was also a difference in relative anticipatory RWA, cumulative percentages of total RWA during the first and last 6 h of the light phase were calculated, to investigate, respectively, anticipation to food (Figures 5a and b) and anticipation to the dark phase (Figures 5c and d). In both DMH and VMH groups, anticipation to the dark phase was unaffected by GHS-R1a knockdown. On the other hand, GHS-R1a knockdown in DMH and VMH attenuated the time of onset of FAA (indicated by the reduced cumulative percentage of total RWA at ZT1–5). Knockdown of GHS-R1a in the DMH, but not the VMH, caused a decrease in amplitude of FAA as well (indicated by the decreased value at ZT6).

DISCUSSION

In this study, we demonstrate the importance of GHS-R1a expressed in VMH and DMH for driving FAA during food restriction. We determined the effect of hypothalamic knockdown of GHS-R1a using viral-mediated RNA interference. Two of the three constructed pAAV-shGHS-R1a were shown to efficiently knock down GHS-R1a both *in vitro* and *in vivo*, sh1 being more efficient than sh3. These two viruses and a control virus were
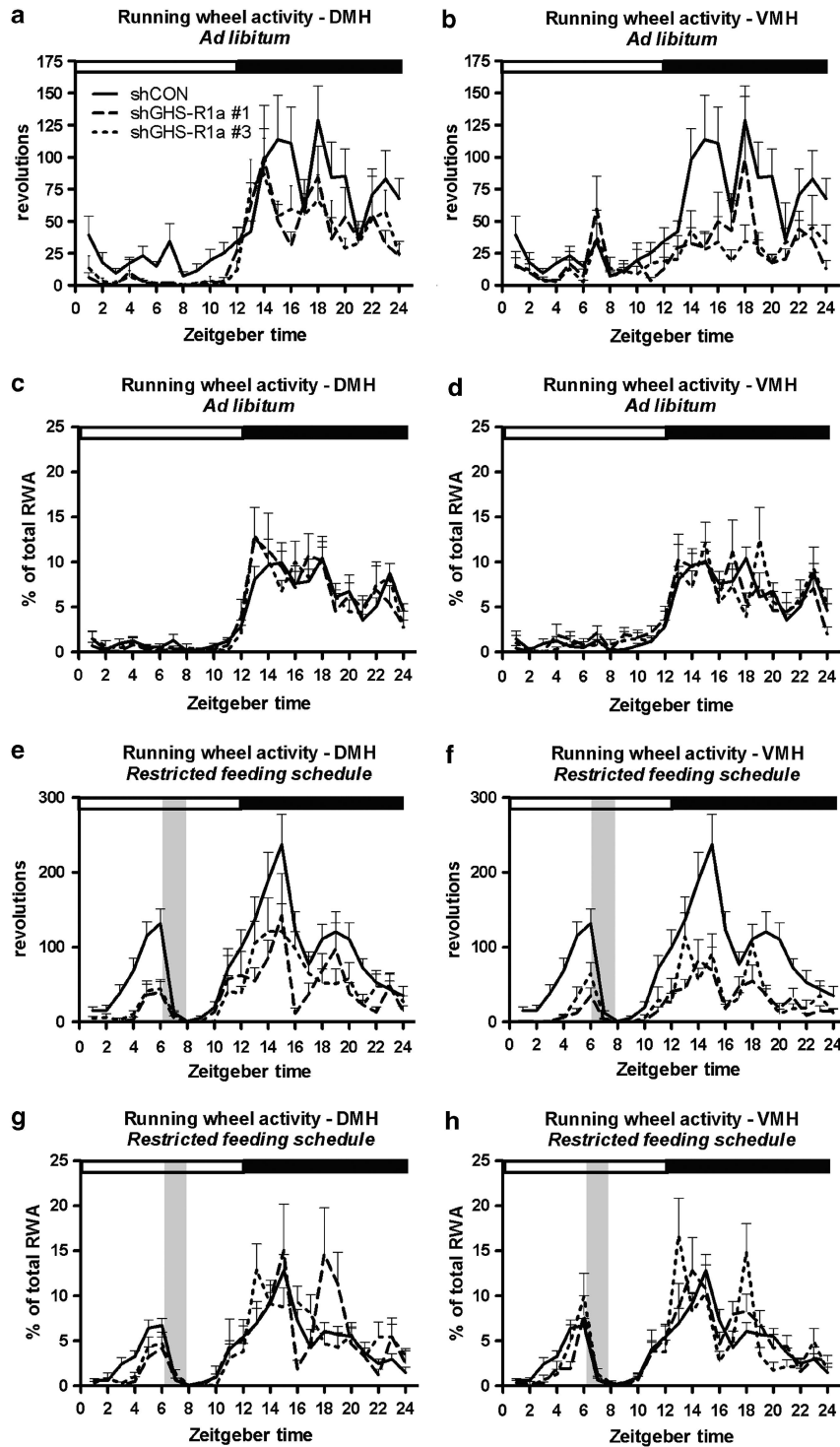
Figure 4. Effects of GHS-R1a knockdown in DMH and VMH on RWA. Absolute (**a**, **b**, **e**, **f**) and normalized (**c**, **d**, **g**, **h**) values of RWA were averaged per hour for the last 3 days of *ad libitum* feeding (**a–d**) and for the last 3 days of the RFS (**e–h**) in rats with GHS-R1a knockdown in DMH (**a**, **c**, **e**, **g**) and VMH (**b**, **d**, **f**, **h**). The gray vertical bar in (**e–h**) indicates the period during which rats had access to food when subjected to the RFS. Values represent absolute averages ± s.e.m. per group.

injected in the medial hypothalamus targeting the DMH or the VMH. In line with its increased *in vitro* and *in vivo* knockdown efficiency, physiological effects of sh1 were larger than those of sh3. Furthermore, rats with unilateral injections of shGHS-R1a showed intermediate phenotypes, while rats with missed injections lacked a phenotype (see Supplementary Table S1). Although some rats in the VMH group also showed GFP expression in the

DMH (which could be caused by the injection needle that passed the DMH in order to reach the VMH) no additive phenotype could be observed in these rats (Supplementary Table S1), indicating that the VMH was the primary target.

Under *ad libitum* conditions, body weight gain was increased in rats with knockdown of GHS-R1a in the VMH, presumably due to an increase in food intake. In addition, DMH and VMH knockdown
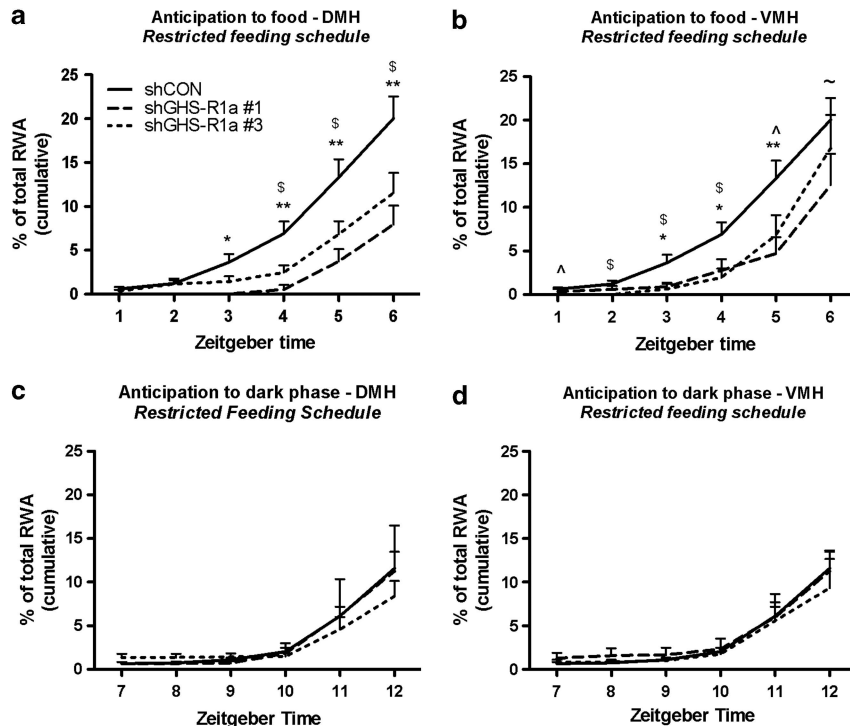
Figure 5. Effects of GHS-R1a knockdown in the DMH and VMH on FAA. Cumulative RWA, expressed as percentage of total RWA, was depicted per hour in anticipation to food (ZT1-6, **a**, **b**) and to the dark phase (ZT7-12, **c**, **d**) in rats with knockdown of GHS-R1a in the DMH (**a**, **c**) and VMH (**b**, **d**). Values represent absolute averages ± s.e.m. A repeated-measures ANOVA was conducted. In case of a significant time*group interaction (VMH: ZT1–6, $F = 2.247$, $P < 0.05$, ZT7–12, $F = 0.488$, $P = 0.656$, DMH: ZT1–6, $F = 4.526$, $P < 0.01$, ZT7–12, $F = 0.186$, $P = 0.883$), a multivariate ANOVA was conducted with a predefined simple contrast comparing each pAAV-shGHS-R1a with the control group. $\sim P < 0.10$, $*P < 0.05$, $**P < 0.01$ for shGHS-R1a no. 1 compared with shCON. $\wedge P < 0.10$, $\$P < 0.05$ for shGHS-R1a no. 3 compared with shCON.

of GHS-R1a led to reductions in RWA. When subjected to a RFS, rats with GHS-R1a knockdown in DHM or VMH lost less body weight than control rats, most likely due to decreased energy expenditure by reduced RWA, as food intake was similar among groups. As we did not measure other components of energy expenditure, such as resting metabolic rate, we cannot exclude that these contributed to the attenuated body weight loss. Even after normalization to total RWA, both onset and amplitude of anticipatory FAA were reduced in the DMH group, whereas the VMH group exhibited only a delay in time of onset of FAA. In contrast, anticipation to the dark phase remained unaffected in both groups. As meal frequency patterns, RWA patterns and anticipation to the dark period did not change in rats with knockdown of GHS-R1a in DMH or VMH, the effect on FAA seemed to be specific and not due to a general deficit in rhythmicity.

Although FAA was attenuated in rats with GHS-R1a knockdown in DMH as well as VMH, some anticipatory RWA remained. This could have several reasons. First, in contrast to knockout models, AAV-mediated knockdown of GHS-R1a did not result in complete ablation of a gene. Second, GHS-R1a signaling in the DMH and VMH is presumably part of a distributed network of brain areas that control FAA. Thus, interfering with one node of this network is likely to attenuate, but not completely prevent, the development of FAA.

In the present study, the strongest effect of hypothalamic GHS-R1a knockdown was the observed hypoactivity. This is in line with the finding that GHS-R1a antagonism reduced drug-induced locomotor activity,[40,41] although central administration of ghrelin has been reported both to decrease[42] as well as increase[43] locomotor activity. In addition to the reduction in total RWA, rats with knockdown of GHS-R1a in DMH also exhibited a decrease in normalized FAA. Similarly, GHS-R1a $-/-$ mice were shown to exhibit a reduction in FAA.[13–16] However, a decrease in general locomotor activity was not reported in GHS-R1a $-/-$ mice.[13,15,44]

This suggests opposite effects of ghrelin on locomotor activity in different nuclei that counteract the effect in the observed in the present study. Taken together, these findings suggest that GHS-R1a signaling in the DMH and VMH has a role in FAA.

Remarkably, in contrast to GHS-R1a $-/-$ mice[13–16] and to our results, ghrelin $-/-$ mice displayed normal levels of FAA.[36,45] A study comparing ghrelin $-/-$ with GHS-R1a $-/-$ mice reported that GHS-R1a $-/-$ mice failed to increase anticipatory locomotor activity in response to food restriction, whereas ghrelin $-/-$ mice did increase anticipatory locomotor activity.[46] The discrepancies in these studies suggest that another, to date unknown, ligand of GHS-R1a may be involved in the expression of FAA, or can replace ghrelin's role in FAA. Moreover, in contrast to knockout models, AAV-mediated shRNA injection provided local knockdown without affecting development. Hence, brain-area-specific physiological effects might not be visible in knockout models.

Ghrelin is known as an orexigenic hormone[1–4] and GHS-R1a signaling in the VMH has been shown to be involved in ghrelin's orexigenic effect.[47] GHS-R1a $-/-$ mice did not exhibit increase in food intake or body weight gain, they rather showed reduced body weight gain.[15,16,44,48,49] Therefore, the appetite-stimulating effect of GHS-R1a knockdown in VMH is remarkable. However, the direct effect of ghrelin on VMH neurons is predominantly stimulatory[50] and stimulation of the VMH resulted in a reduction in food intake.[51] Hence, suppression of the stimulatory effect of ghrelin on VMH neurons by AAV-shGHS-R1a could lead to decreased satiety and the observed increase in food intake. In the DMH, and under RFS conditions in both DMH and VMH, knocking down of GHS-R1a had no effect on food intake or meal patterns. This indicates that ghrelin modulates food intake and FAA via separate pathways.

Cellular toxicity has been reported following injection of AAV-shRNA into the liver,[52] red nucleus,[53] VTA/substantia nigra[54] and

the striatum.[55,56] Such damage might provide a phenotype itself that could be mistaken for effects of the shRNA. In this study, we were unable to detect any cellular damage in brain sections. In addition, the dose of AAV used in this study has been injected in the hypothalamus by others without reported side effects.[37,57] Moreover, any toxic effects of expression of shRNA in hypothalamic neurons would have been induced in control rats as well. Therefore, the fact that we observed differences between AAV-shCON and AAV-shGHS-R1a groups supports an effect of the shRNA that is independent of tissue damage. However, as the induction of cellular toxicity has been reported to be sequence-specific,[52] we cannot fully exclude the possibility that the observed phenotypes by injecting AAV-shGHS-R1a in VMH and DMH are in part due to cellular damage.

Lesioning of the DMH led to lower levels of locomotor activity, reduced nocturnality ratio and hypophagia under *ad libitum* conditions.[23,28,29,58,59] Although, in the present study, we observed hypoactivity in rats with knockdown of GHS-R1a in the DMH, food intake and circadian rhythms were unaltered. Ablation of VMH was previously shown to result in hypoactivity, attenuated nocturnality ratio, hyperphagia and obesity.[22,34,60] The reduced activity, diminished FAA and hyperphagia, observed in the present study, therefore correspond to the phenotype of rats with a VMH lesion. In contrast, nocturnality ratio remained identical to shCON rats. Taken together, the phenotypes induced by injection of AAV-shGHS-R1a in the DMH/VMH are not identical to phenotypes due to lesioning of these brain areas. This further supports the idea that the effects in this study were mediated by knockdown of GHS-R1a rather than cellular damage.

In conclusion, although studies in GHS-R1a−/− mice had already implicated GHS-R1a signaling in the regulation of FAA,[13–16] it was still unknown via which brain area this effect could be mediated. In the present study, the predominant effect of GHS-R1a knockdown in the medial hypothalamus during RFS was a decrease in RWA, which attenuated body weight loss. A previous study has identified ghrelin signaling within the medial hypothalamus as a potential node in the network that regulates FAA.[61] In agreement with this, GHS-R1a knockdown induced a reduction in FAA amplitude in the DMH, and a delay in onset of FAA in VMH as well as DMH. Together, this implicates mediobasal hypothalamic GHS-R1a signaling as an important part of the network that regulates FAA.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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