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A circulating ghrelin mimetic attenuates light-induced phase delay of mice and light-induced Fos expression in the suprachiasmatic nucleus of rats

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**Abstract**

Anatomical evidence suggests that the ventromedial arcuate nucleus (vmARC) is a route for circulating hormonal communications to the suprachiasmatic nucleus (SCN). Whether this vmARC-SCN connection is involved in the modulation of circadian activity of the SCN is not yet known. We recently demonstrated, in rats, that intravenous injection of a ghrelin mimetic, GHRP-6, during the daytime activated neurons in the vmARC and reduced the normal endogenous daytime Fos expression in the SCN. In the present study we show that i.v. administration of GHRP-6 decreases light-induced Fos expression at ZT13 in the rat SCN by 50%, indicating that light-induced changes in the SCN Fos expression can also be reduced by GHRP-6. Because it is difficult to study light-induced phase changes in rats, we examined the functional effects of GHRP-6 on light-induced phase shifts in mice and demonstrated that peripherally injected GHRP-6 attenuates light-induced phase delays at ZT13 by 45%. However, light-induced Fos expression in the mice SCN was not blocked by GHRP-6. These results illustrate that acute stimulation of the ghrelinergic system may modulate SCN activity, but that its effect on light-induced phase shifts and Fos expression in the SCN might be species related.

**Introduction**

Daily rhythms of behavior and physiology of mammals are generated by the central biological clock located in the suprachiasmatic nucleus (SCN). The activity of the SCN can be entrained by external photic stimuli as well as adjusted by non-photic factors. Under a normal 12/12 (light/dark (LD)) cycle, the circadian rhythm of locomotor activity is synchronized with the LD cycle. Light exposure during the (subjective) dark period will shift the endogenous activity rhythm backward or forward, depending on the timing of the light. Most non-photic stimuli producing behavioral arousal, such as novel wheel access and social interaction, can only shift the locomotor activity during the subjective daytime and are less effective during the subjective night. However, non-photic stimuli can interfere with the photic response during the subjective night, and behavioral arousal has been shown to attenuate light-induced phase shifts. The most important areas relaying non-photic information to the SCN,
by their serotonergic or NPYergic and GABAergic afferents, respectively, are thought to be the midbrain raphe and the intergeniculate leaflet of the thalamus (IGL) \(^{360, 382-386}\). NPY can inhibit photic phase shifts both in vivo and in vitro \(^{387-390}\), and this effect was confirmed in NPY-/- mice in which such influence is abolished \(^{391}\). Furthermore, application of Y5 receptor antagonists can reverse the inhibitory effect of NPY \(^{387, 392}\). Systemic administration of the ghrelin mimetic GHRP-6, whose physiological analogy to ghrelin has been well confirmed \(^{328, 393}\), can activate ARC NPY neurons of such different species as rat, mouse and hamster \(^{394-396}\). The recent finding that in the ventromedial arcuate nucleus (vmARC) Agouti-related protein (AGRP)/NPY neurons project to the SCN-opened up the possibility that part of the NPY terminals in the SCN might be derived from another source than the IGL and may come from the ARC \(^{243}\). The importance of ghrelin-induced feeding via the ARC was confirmed by intranuclear injection \(^{397}\), although several other hypothalamic nuclei also express abundant ghrelin receptors \(^{398}\). Circulating GHRP-6 reduces daytime endogenous Fos immunoreactivity in the rat SCN \(^{243}\), suggesting that ghrelin might be able to influence the activity of the SCN as a non-photic stimulus. Indeed, a recent study on mice showed ghrelin-induced phase advance when applied at circadian time 6, both in in vitro cultured SCN slices and in in vivo fasting condition \(^{399}\). This raises the question whether ghrelin, as a strong hunger signal, can disturb light entrainment effects on the biological clock.

In the present study, we confirmed the activation of the vmARC AGRP/NPY neurons after i.v. injection of GHRP-6 by Fos expression in these neurons. In order to investigate whether GHRP-6 could also modulate the photic activation of the SCN first we studied the light-induced Fos immunoreactivity pattern at ZT13 in the rat SCN, with or without a prior i.v. injection of GHRP-6. To confirm that the reduced light-induced Fos expression after prior GHRP-6 treatment was an indication of a reduced capacity of light to shift the phase of the SCN, we also examined, at ZT 13, the effect of GHRP-6 on the light-induced phase shift in locomotor activity. Since rats are known to be less reliable for determining light-induced phase shifts, these studies were performed in mice.

**Materials and Methods**

**Animals**

Experiments 1 and 2 were aimed at investigating the effect of GHRP-6 on light-induced changes in SCN activity. These experiments were conducted in the Netherlands, with the approval of the Animal Care Committee of the Royal Netherlands Academy of Arts and Sciences. Sixteen male Wistar rats weighing 250–350g (Harlan Nederland, Horst, The Netherlands) were housed at room temperature with a 12hr/12hr light/dark regimen (lights on at 7:00 AM.). Animals were housed in separate cages one week before their operation. Food and water were available ad libitum.
Experiments 3 and 4 were performed with mice in accordance with the principles of laboratory animal care set forth in NIH publication 86-23 (revised 1985) and the French national laws. Forty-two male C57BL/6J mice (Charles River laboratories, L’arbresle, France) were purchased when 8 weeks old, housed at room temperature and exposed to a 12hr/12hr light/dark cycle for 3 weeks before the start of the experiments. Food and water were available ad libitum.

**Rat experiments**

To administer GHRP-6 systematically, and to avoid stress due to handling, restraint or anesthesia, an intravenous silicone catheter was implanted through the right jugular vein according to the method of Steffens 400, when the body weight of the rats reached 300g. After surgery, the rats were given at least 10 days to recover. Handling, vehicle (saline) injection and sham blood sampling (i.e., blood was withdrawn and immediately returned) were carried out regularly, starting three days before the experiment, to familiarize the rats with the experimental procedures. On day 9, the rats were connected permanently to a drug administration catheter for 24 hrs, which was attached to a metal collar and kept out of reach of the rats by means of a counterbalanced beam. This allowed all manipulations to be carried out without handling the rat. All of the experiments were performed in the rat’s home cage. On day 10, the drug administration catheter was connected to a syringe containing the infusion solution.

Experiment 1 investigated the activation of AGRP/NPY neurons by GHRP-6. Eight rats were divided into two groups, one receiving a GHRP-6 (0.5ml; 50μg /300g body weight, n=4) and the other receiving vehicle (Saline, 0.5ml/300g body weight, n=4) by infusion into the jugular vein catheter both at ZT2. The dose of 50μg GHRP-6 was derived from similar studies showing activation of ARC neurons with intravenous injection 401, 402. The physiological relevance of this dose was validated by their common effects on Fos induction and growth hormone releasing hormone 328, 393. Moreover, in our previous experiments, a higher dose, e.g. 100μg/300g, did not induce more Fos immunoreactivity in the ARC. The infusion speed was kept at 0.1ml/min. Ninety min after the i.v. infusion, all rats were sacrificed by intra-atrial perfusion under deep anesthesia preceded by a lethal dose of i.v. sodium pentobarbital i.v. with saline, followed by a solution of 4% paraformaldehyde in 0.1M phosphate buffer (pH 7.4) at 4°C.

For Experiment 2, 8 rats were randomly divided in two groups. Ten min before ZT13, one group was infused with GHRP-6 (0.5ml; 50μg /300g body weight, n=4) and the other with vehicle (Saline, 0.5ml/300g body weight, n=4) into the jugular vein catheter. At ZT13, 200 lux light exposure was applied to all the rats for 10 min. Ninety min after the light exposure all rats were sacrificed under the same conditions as the animals in Experiment 1.
After perfusion, all the brains were removed and kept in 4% paraformaldehyde at 4°C for overnight post-fixation. After equilibration for 48 hrs with 30% sucrose in 0.1M Tris-buffered saline (TBS), brains were cut with a cryostat into three series of 30μm sections, and one series of sections dedicated to immunocytochemical staining were collected and rinsed in 0.1 M TBS.

To visualize the action of GHRP-6 on AGRP/NPY neurons in vmARC, we used double-labeling immunofluorescence. Sections were incubated overnight at 4°C with goat anti-Fos (1:1500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and rabbit anti-AGRP (1:1000; Phoenix Pharmaceuticals, Belmont, CA) primary antibody. Sections containing the SCN were incubated only with rabbit anti-AGRP (1:1500).

For Fos and AGRP double staining, sections were then rinsed in 0.1M TBS, incubated for 1 hr in biotinylated horse anti-goat IgG, and then in streptavidin–Cy5 (for Fos) and donkey anti-rabbit-Cy3 (for AGRP) also for 1 hr, rinsed, mounted on gelatin-coated glass slides, dried, covered with glycerol in 0.1M PBS (pH 9.0), and put under a confocal laser scanning microscope. For AGRP immunostaining in the SCN, sections were rinsed and incubated in biotinylated secondary antibody and then incubated in avidin–biotin complex (ABC, Vector) for 1 hr. The reaction product was visualized by incubation in 1% diaminobenzidine (DAB) (0.05% nickel ammonium sulfate was added to the DAB solution to darken the reaction product, DAB/Ni) with 0.01% hydrogen peroxide for 5-7 min. Sections were mounted on gelatin-coated glass slides, dried, run through ethanol and xylene and covered for observation by light microscope.

To assess Fos immunoreactivity in the central nervous system (CNS), brain sections were incubated with goat anti-Fos primary antibody overnight at 4°C, tissues were rinsed and incubated in biotinylated secondary antibody (horse anti-goat IgG) for 1 hr; rinsed and incubated in avidin–biotin complex (ABC, Vector) for 1 hr, and visualized by DAB reaction.

For counting the Fos-immunoreactive neurons in the SCN, tiled images were captured by a computerized image analysis system consisting of an Axioskop 9811-Sony XC77 black and white video camera (Sony Corp., Tokyo, Japan). In the images, both sides of the middle portion of the SCN were manually outlined. The Fos-positive nuclear profiles were automatically segmented by a dedicated macro written within the ImagePro programming environment. For each rat, three sections were measured 90μm apart (from bregma -1.20 to -1.40 mm); the mean number of Fos positive nuclear profiles from these three sections was calculated. All values are expressed as the mean ± SEM/per section, and data were analyzed using one-way ANOVA. Statistical significance was set at $P < 0.05$.

**Mice experiments**

After adaptation to their cages, equipped with a running wheel, forty-two mice were
transferred to constant darkness by not allowing the lights to come on at the normal time, which was designated as ZT₀, the onset of the subjective day.

In Experiment 3, twenty-four mice were divided at random into 4 groups to investigate the effect of GHRP-6 on photic phase shifts. On the first day of darkness, group 1 received an i.p. saline injection (0.2 ml, n=6), and group 2 a GHRP-6 (0.2 ml, 1000μg/kg; n=6) i.p. injection 15 min before being exposed to 100 lux of light for 10 min at ZT₁₃. In order to investigate the GHRP-6 or saline effect per se, the control groups 3 and 4 received the same volume and dose of GHRP-6 or saline respectively by i.p. injections 15 min before ZT₁₃ without light exposure.

Wheel running activity data were recorded by a PC equipped with an acquisition card (Dispsi Industrie, Chatillon, France). Phase shifts were calculated by projecting the phase of onset of nocturnal locomotor activity during the first 10 days in constant darkness to the mean onsets during the last 10 days under a light-dark cycle (Clocklab software, Actimetrics, Evanston, IL). All values are expressed as mean ± SEM.

In Experiment 4, eighteen mice were divided into 4 groups to study the GHRP-6 effect on light-induced Fos expression in SCN. On the first day of darkness half of the animals were exposed to 100 lux of light for 10 min at ZT₁₃. Within both groups (i.e. light exposed (n=5) and animals maintained in darkness (n=4)), half of the animals received the i.p. GHRP-6 pretreatment, whereas the remaining animals received vehicle. This part of the protocol was similar to that of Experiment 3. Mice were then all perfused within 60 min after the onset of light or without light. Perfusion, post-fixation, immunocytochemical staining all followed the same methods as in experiment 2. For counting Fos immunoreactivity positive cells, three adjacent sections of 30μm were measured in the mid area of the SCN.

Both phase-shifting and Fos counting data in mice were analyzed by a two-way ANOVA (Group (2 levels), i.e., light versus dark; and Treatment (2 levels), i.e., GHRP-6 versus saline). If significant main or interaction effects were detected, it was followed by a post-hoc LSD test. The differences were considered significant when P values were lower than 0.05.

Results

Experiment 1 GHRP-6 activates AGRP/NPY neurons in the arcuate nucleus.
Following the i.v. saline control injections into the circulation via the jugular vein, almost no Fos-immunoreactive neurons in the ARC could be detected. Furthermore, AGRP-immunoreactive cell bodies in the vmARC were also difficult to visualize. Only AGRP-immunoreactive fibers were visible in the vmARC and other areas receiving a vmARC projection. In contrast, after GHRP-6 injections, not only many vmARC neurons showed strong Fos expression but also many of them were clearly AGRP-immunopositive, confirming the activating effect of ghrelin on AGRP/NPY neurons.
The co-localization of Fos and AGRP could be more readily shown by confocal immunofluorescent microscopy (Fig. 1). Consistent with previous studies in the hypothalamus, Fos activation by GHRP-6 stimulation was limited to the vmARC and was not observed in other areas such as PVN, dorsomedial and ventromedial nuclei. In the brain stem, Fos positive cells in the area postrema were observed after GHRP-6 stimulation but not in the saline control animals. This latter observation is consistent with other studies using a similar dose of GHRP-6. However, since the area postrema has no direct projection to the SCN, this area was not further processed. Under these conditions, with GHRP-6 induced Fos expression in the hypothalamus limited to vmARC neurons, several AGRP terminals were also found in the ventral SCN (data not shown), as further evidence for the vmARC-SCN projection. Due to the dense IGL-NPY projection, it was not possible to identify whether these AGRP fibers also contain NPY or whether the amount of NPY staining in the SCN had changed.

Figure 1 Systemic administration of GHRP-6 activates AGRP neurons in vmARC which project to ventral SCN. Double-labeling immunofluorescence showing AGRP cell bodies (Cy3-red) in the ventromedial part of the ARC after GHRP-6 stimulation, the same area also presents most of the GHRP-6 inducible Fos immunoreactivity (Cy5-blue), the majority of AGRP cell bodies being colocalized with Fos. The arrow shows a typical double labeled neuron. Scale bar: 100μm.
Therefore we only applied the AGRP staining in our study, with the knowledge that AGRP nearly completely co-exists with NPY in vmARC.

**Experiment 2 GHRP-6 reduces light-induced Fos expression in rat SCN in the early subjective night**

Light exposure at ZT13 resulted in a large amount of Fos expression in the SCN. Fos positive neurons were mainly clustered in the ventral part of the SCN. Along the rostral-caudal axis, Fos positive neurons were mainly distributed in the middle part of the nuclei (therefore we selected sections from bregma -1.20 to -1.40 for cell counting) (Fig. 2). The light-induced number of Fos immunoreactive cells was 60.0 ± 9.9 per section after saline control injections, while after 50μg GHRP-6 injection, the light-induced number of Fos immunoreactive cells was significantly reduced to 28.8 ± 3.0 per section, (P= 0.02; Fig. 3).

**Figure 2** GHRP-6 interferes with light-inducible Fos immunoreactivity in the rat SCN. 90 min following a 10-min light stimulation of 200 lux at ZT13, saline control pre-treated animals show many Fos immunoreactive cells in SCN (A). The light-induced Fos reaction is attenuated by around 50% by the GHRP-6 pretreatment (B). Scale bar: 100μm.

**Figure 3** GHRP-6 inhibited light-induced Fos expression in rat but not mouse SCN (mean ± SEM.). * The GHRP-6 + light group is significantly different from the saline + light group, P=0.02.
Experiments-3 and -4 GHRP-6 decreases photic phase-shifts but not SCN Fos expression in the early subjective night in mice

Photic stimulation at ZT13 following saline administration resulted in a phase delay of 63.6 ± 6.5 min; administration of GHRP-6 prior to light exposure resulted in a light-induced phase delay of 34.8 ± 2.2 min. Injection of saline or GHRP-6 alone at ZT13 (i.e. without light exposure) did not result in a phase shift (5.0 ± 9.5 min vs 3.6 ± 9.0 min) (Fig. 4). The two-way ANOVA indicated a significant effect of Group (i.e. light vs dark exposure) (F(1,17)=48.3; p<0.001), but the effect of Treatment did not reach statistical significance (F(1,17)=3.8; p=0.07), nor did the interaction of Treatment x Group (F(2,17)=3.2; p=0.09). The light-induced phase-shift was significant in both the saline-treated (p<0.001) and the GHRP-6 treated (p=0.002) animals. On the other hand, the light-induced phase shift was significantly smaller in the GHRP-6 group.
than in the saline group (p=0.02). These results demonstrate that GHRP-6 reduced the light-induced phase delay (Fig. 5).

Light-induced Fos immunoreactive cells in mice SCN with saline injection was 114.1 ± 11.5 per section, with GHRP-6 injection, 121.4 ± 9.3 per section. Without light treatment, there is sparse endogenous Fos expression in SCN with GHRP-6 or saline treatment (16.33 ± 1.42 vs 15.58 ± 1.89 per section). Two-way ANOVA indicated a significant effect of Group (i.e. light exposure vs dark) (F(1,14)=124.20; p<0.001), but no effect was showed with Treatment (F(1,14)=2.65; p=0.13), also no effect of Treatment x Group (F(2,14)=2.9; p=0.11), which means GHRP-6 pretreatment can not interfere light induced Fos expression in mice SCN.

Discussion

VmARC relays circulating ghrelin signal to SCN

In our previous study, we showed that the efferent projections of the arcuate nucleus also reach the SCN. In addition, we showed that animals kept in LD, that received a GHRP-6 injection in the early daytime (i.e. ZT2) showed a reduction of endogenous Fos expression in the ventral SCN. Considering that the vmARC can sense circulating GHRP-6, and has projections to the SCN, we assumed that this Fos reduction in the SCN was due to a direct GHRP-6 stimulation on vmARC neurons and subsequent vmARC signaling to the SCN. In the present study we therefore aimed at investigating the possibility that circulating GHRP-6 can indeed inhibit SCN activity and thus may counteract the effect of nocturnal light exposure. Indeed GHRP-6 pretreatment at ZT13 significantly reduced (50%) the light induced Fos activation in rat. In addition, light-induced phase-shifts experiment with mice showed likewise that the light induced phase shift had decreased by 45% after GHRP-6 injection. However, in spite of the GHRP-6 induced reduction of the light-induced phase shifts, mice did not show a reduction in SCN Fos expression. Interestingly this observation agrees with a study...
of Edelstein et al.\textsuperscript{378} showing a reduction of a light-induced phase shift by non-photic stimuli in hamsters, but no reduction of the light-induced Fos or PER1 expression. The present observation that in contrast to the mouse, in the rat, GHRP-6 interferes with the light-induced Fos activity may indicates that the mechanisms of non-photic interference with SCN activity may be species-related.

Ghrelin receptors are present in many areas in the central nervous system, including in the SCN. Recently, evidence has been presented that ghrelin injected into the general circulation may also pass the blood brain barrier\textsuperscript{404}. In addition, there are reports that ghrelin is produced within the hypothalamus\textsuperscript{405-407}. However, considering the sensitivity to ghrelin, clearly the neurons in the vmARC are the most prominent group in the brain that are activated by both peripheral and central ghrelin stimulation\textsuperscript{359, 397, 408}, which supports the proposal that the vmARC may be viewed as a hormonal sensor in the hypothalamus\textsuperscript{316, 317}. In addition, ghrelin-driven feeding behavior is independent of the feeding schedule, which means animals may respond to ghrelin with or without food deprivation. Herein the function of GHRP-6 is not different from insulin, leptin and glucose - all substances that have been shown to be able to influence vmARC neuronal activity\textsuperscript{319, 320, 409, 410}. By its efferent projections the vmARC can subsequently affect the SCN and other structures within and outside of the hypothalamus\textsuperscript{243}. It is important to consider that ghrelin receptors have been described in other areas of the CNS, including the SCN\textsuperscript{321, 398}, indicating that if GHRP-6 is able to penetrate the blood brain barrier it may also affect these other brain areas directly.

Several arguments favor an explanation of the present data whereby GHRP-6 acts at the level of the vmARC. Firstly, since the vmARC is outside the blood brain barrier\textsuperscript{313, 411, 412}, the Fos expression of its neurons more than likely indicates a direct activation by circulating GHRP-6, a result which is supported by \textit{in vitro} electrophysiological recordings of vmARC neurons\textsuperscript{413}. Indirect evidence supporting this explanation comes from a recent study on accessibility of circulating leptin to the hypothalamus, which showed that ARC neurons expressing the long form of the leptin receptor have more specific and direct contact with circulating leptin compared to other hypothalamic neurons that have no direct access to the circulation\textsuperscript{414}; Secondly, injection of GHRP-6 into the cerebral ventricles not only results in Fos expression in the vmARC but also Fos expression in the paraventricular nucleus (PVN), dorsomedial nucleus (DMH), lateral hypothalamus, and the nucleus of the solitary tract\textsuperscript{415}. Because this response cannot be observed after i.v. injections, these data indicate that ghrelin receptors in other CNS areas other than circumventricular organs\textsuperscript{344} such as the vmARC\textsuperscript{321, 398} cannot be easily reached by peripheral ghrelin or GHRP-6. Thirdly, binding of GHRP-6 to its receptor, either in the CNS from intracerebral administration, or in the ARC from the peripheral circulation, only activates neurons as indicated by Fos induction or by electrophysiological recording of activity\textsuperscript{416}. Since the activation of
the vmARC is known to induce an inhibitory effect in its projection areas, such as the lateral ARC, it is likely that also the projection of the vmARC to the SCN has an inhibitory effect on its target neurons. Consequently, the inhibition of Fos expression in the SCN after i.v. administration of GHRP-6 can be considered as an indirect effect from circulating GHRP-6 stimulation, via the vmARC. Lastly, the modulating effect of the blood brain barrier is also illustrated by a recent publication by Yannielli et al., who observed a direct phase shifting effect of ghrelin on the SCN in vitro, but failed to observe a similar phase shift in vivo.

Circulating ghrelin indirectly affects the SCN as a non-photic stimulus

Fos is a well-known neuronal marker that reflects the activation of SCN neurons after a light stimulus. However, the decreased light-induced Fos expression following GHRP-6 pretreatment does not necessarily mean that the effect of light on the circadian activity of SCN neurons is blocked. Therefore, we examined to what extent a GHRP-6 injection was able to prevent the light-induced phase shift at ZT13. Clearly, the bolus injection of GHRP-6 into the circulation in mice had a functional consequence, i.e. it caused a reduction of the light-induced phase delay at ZT13, without effect on Fos expression in SCN. Consequently just like other non-photic stimuli, such as locomotor activity, ghrelin may modulate the light-induced phase shift of the SCN without affecting Fos expression. The mechanisms for this influence on the locomotor outcome via the SCN still needs to be further understood. Clearly there must be slight differences among rodents’ SCN organization that need to be explored in order to understand this interesting effect in mice.

A non-photic stimulus either results in a phase shift during the subjective day, or in a blocking of the light effect during the subjective night. In laboratory animals, most of these well known non-photic inputs to the SCN are related to locomotion, either by direct behavioral manipulation such as wheel running or by non-photic inputs mimicked by neurotransmitters injected into the SCN. The attenuation of the light-induced phase delay by circulating GHRP-6 indicates that it can be categorized as a non-photic stimulus, which might reach the SCN via sensory circumventricular organs such as the ARC. Several arguments plead for an important “relay” role of the ARC AGRP/NPY neurons. Firstly, NPY and GABA can interfere with and suppress the light-induced phase shifting via direct actions on SCN neurons. Secondly, AGRP/NPY neurons of the vmARC project to the SCN and are activated by circulating GHRP-6. Thirdly, NPY/AGRP in the vmARC is also colocalized with GABA. Fourthly, since NPY innervation is still observed in the SCN after lesioning of the IGL, the vmARC is another possible source for NPY innervation of the SCN. The small caliber of the AGRP fibers derived from the vmARC may indicate why after IGL lesioning some of us observed the loss of all NPY innervation in the SCN of rats. The total amount
of the IGL-derived NPY terminals may by far outnumber NPY terminals originating from the vmARC, but together NPY and GABA may disrupt the stable pacemaker organized by the cellular synchrony across the SCN.

The observed reduction of light-induced phase delays by GHRP-6 agrees with the previously observed reduction in light-induced phase delays due to direct NPY injections into the SCN, i.e. 45% reduction with GHRP-6 versus 70% with NPY. The lesser inhibition can be explained by the fact that NPY injected directly in the SCN will reach all NPY receptors, including those from the IGL, while the endogenous neurotransmitter released from vmARC terminals in the SCN will only reach a selection of these receptors. The present results provide an anatomical basis by which metabolic stimuli can affect the functionality of the SCN. Light induced phase shifts have already been shown to be modulated by changes in glucose availability, such as observed during diabetes-related hyperglycemia, insulin-induced hypoglycemia, blockade of glucose utilization or caloric restriction. Yet it has been difficult to specify the mechanism by which these changes in glucose availability affected the SCN. Gold-thioglucose lesions of the glucose-receptive neurons in the VMH and ARC prevent the metabolic modulation of photic resetting during shortage of glucose availability. Thus these data also suggest an effect on the SCN via a relay in the VMH and/or ARC and consequently, give support to our hypothesis for an NPYergic vmARC-SCN projection that would be specifically activated by metabolic stimuli.

Although the environmental light/dark cycle provides the principal entrainment to circadian rhythms, many more factors can also influence the phase of the SCN or its outputs via direct effects on SCN. For a nocturnal animal, this means the light inhibition on its locomotion must be removed to allow the motivation of feeding be expressed through active behavior. Several studies indicate the importance of sensory circumventricular organs for communication with the SCN. The present study allows us to hypothesize that after sensing blood borne metabolic signals, the output from one of the circumventricular organs, for instance the vmARC may send this metabolic feedback information to the SCN, which could influence the synchrony of the SCN on physiology and behavior. For example the suppression of light information to the SCN by the “hunger signal” ghrelin may allow the animal to become more active in order to search for food. This hypothesis is supported by the results of a study on leptin, the natural antagonist of ghrelin, which demonstrates that its normal signaling in the hypothalamus, especially in the vmARC, is necessary to maintain a normal level of locomotion at night. The present study would indicate that the vmARC-SCN connection may also be involved in the effect of leptin on locomotion. In conclusion, the demonstration that intravenously injected GHRP-6 modifies the response of the SCN to light is evidence for the existence of neuronal pathways that transmit peripheral circulating information to the SCN.