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# Suprachiasmatic vasopressin and the circadian regulation of voluntary locomotor behavior

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## Abstract

A role for arginine vasopressin in the circadian regulation of voluntary locomotor behavior (wheel running activity) was investigated in the golden hamster, *Mesocricetus auratus*. Spontaneous nocturnal running was suppressed in a dose-dependent manner by systemic injections of vasopressin, and also in a concentration-dependent manner by microinjections directly into the hypothalamic suprachiasmatic nucleus. Pre-injections of a vasopressin V1 receptor antagonist into the nucleus reduced the suppression of behavior by vasopressin. Ethogram analyses revealed that peripheral drug injections predominantly increased grooming, flank marking, and sleep-related behaviors. Central injections did not induce sleep, but increased grooming and periods of 'quiet vigilance' (awake but not moving). Nocturnal behavioral profiles following either peripheral or central injections were similar to those shown by untreated animals in the hour prior to the onset of nocturnal wheel running. Site control vasopressin injections into the medial preoptic area or periaqueductal gray increased flank marking and grooming, but had no significant effect on locomotion, suggesting behavioral specificity of a vasopressin target near the suprachiasmatic nucleus. Both peripheral and central administration increased FOS-like immunoreactivity in the retinorecipient core of the suprachiasmatic nucleus. The distribution of FOS-positive cells overlapped the calbindin subregion, but was more extensive, and most calbindin-positive cells did not co-express FOS. We propose a model of temporal behavioral regulation wherein voluntary behavior, such as nocturnal locomotor activity, is inhibited by the activity of neurons in the suprachiasmatic ventrolateral core that project to the posterior hypothalamus and are driven by rhythmic vasopressin input from the dorsomedial shell.

## Introduction

Arginine vasopressin (AVP) has numerous important roles in the central regulation of behavior. Specific brain sites of action have been implicated in the control of some defined behaviors, including motility and passive avoidance (Appenrodt & Schwarzberg, 1999; Schwarzberg & Appenrodt, 1999), aggression (Ferris & Potegal, 1988; Ferris *et al.*, 1997, 1999; Ferris, 2000; Gutzler *et al.*, 2010), the stress response (Koob *et al.*, 1985; Engelmann *et al.*, 2000), learning and memory (Koob *et al.*, 1985; Van Wimersma Greidanus & Veldhuis, 1985; Bluthe & Dantzer, 1992; Paban *et al.*, 1999), flank marking (Ferris *et al.*, 1984, 1985, 1996; Albers & Ferris, 1985; Albers *et al.*, 1986; Irvin *et al.*, 1990; Hennessey & Albers, 1992; Bamshad & Albers, 1996), and social recognition (Albers *et al.*, 1992; Bluthe & Dantzer, 1992; Landgraf *et al.*, 1995; Albers & Bamshad, 1998).

The anterior hypothalamus is important for many of these responses. However, the suprachiasmatic nucleus (SCN) presents a unique site as the location of a central biological clock controlling locomotor behavior and physiology (Moore & Eichler, 1972; Stephan & Zucker, 1972; Ralph *et al.*, 1990). It also is the source of rhythmic AVP in the hypothalamus and cerebrospinal fluid (Schwartz & Reppert, 1985; Kalsbeek *et al.*, 1995; Kalsbeek & Buijs, 1996; Van Esseveldt *et al.*, 1999) and *in vitro* (Earnest & Sladek, 1986, 1987; Gillette & Reppert, 1987; Murakami *et al.*, 1991; Watanabe *et al.*, 1993; Shinohara *et al.*, 1994; Gerkema *et al.*, 1999; Jansen *et al.*, 2000; Van der Zee *et al.*, 2002).

A role for suprachiasmatic AVP neurons in the temporal programming of locomotor behaviors is suggested by studies in various mammals [see Van Esseveldt *et al.* (2000) for a review]. Differences in AVP content within the SCN are associated with the age-related decline in locomotor rhythmicity in rats (Roozendaal *et al.*, 1987) and humans (Hofman & Swaab, 1994). AVP content, number of cells and release from the SCN are correlated with differences in locomotor patterns between strains of mice (Bult *et al.*, 1993), and among individual voles (Gerkema *et al.*, 1994; Jansen *et al.*, 1999, 2000, 2003; Van der Zee *et al.*, 1999). The amplitude of circadian rhythms is reduced in AVP-deficient rats as compared with their wild-type controls *in vivo* (Brown & Nunez, 1989) and *in vitro* (Ingram *et al.*, 1996), although rhythm production is not eliminated in these animals (Groblewski *et al.*, 1981; Danguir, 1983; Brown & Nunez, 1989; Murphy *et al.*, 1996; Wideman *et al.*, 2000).

In hamsters, AVP-producing cells are located in the dorsomedial 'shell' of the SCN [see Moore *et al.* (2002) for a review]. These give rise to efferent fibers that target other hypothalamic and thalamic nuclei, but a major target is the SCN itself (Castel *et al.*,

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1990; Reuss, 1996; Dai *et al.*, 1997; Abrahamson & Moore, 2001; Leak & Moore, 2001; Moore *et al.*, 2002). In the hamster, SCN neurons are activated by AVP via a V1-like receptor, with higher sensitivity during the night than during the day (Liou & Albers, 1989). Furthermore, V1 receptor expression is rhythmic in the SCN, with expression being higher at night, in anti-phase with the rhythm of AVP content and release (Young *et al.*, 1993). However, the function of rhythmic intra-SCN AVP activity is not known. We now report that AVP regulates the temporal expression of voluntary locomotor behavior [wheel running activity (WRA)] via a subpopulation of SCN cells near the core of the nucleus.

## Materials and methods

### Animals and animal care

Male golden hamsters (*Mesocricetus auratus*) were obtained from Charles River Canada, Quebec, and were 60–70 days of age at the beginning of each experiment. All animals were housed individually in translucent polypropylene cages with food, water and a running wheel (17 cm in diameter) freely available. Room temperature was maintained at 21 ± 2 °C. Experiments involving animals were approved by the University of Toronto Animal Care Committee, and were in accordance with established guidelines of the Canadian Council on Animal Care and the Province of Ontario. Animals were monitored by observation twice daily and by daily analysis of the activity records throughout each experiment.

### Behavior recording and analysis

#### Wheel running behavior

WRA was recorded continuously with Dataquest III or VitalView (Phillips-Respironics, Bend, OR, USA). WRA was accumulated in 6-min bins for presentation and analysis. During photic entrainment, animals were maintained on a light cycle consisting of 14 h of light and 10 h of dark [light/dark (LD) 14 : 10]. Pharmacological and behavioral tests were performed under constant conditions [constant dark (DD)]. Manipulations were performed 0.5 h after nocturnal locomotor onset, circadian time (CT) 12.5, with activity onset of each cycle defined as CT12. The onset on the day of the injection was predicted by use of an eye-fitted line through the recorded onsets on the previous 3 days. Each record was checked immediately before an injection to verify that the animal had begun to run at the expected time on that day. Inhibition of WRA was measured by comparing WRA on the night of a given treatment with the averaged WRA for the previous three cycles. For this part of the analysis, WRA data for each cycle were collapsed into 18-min bins, beginning at CT12. Data for 24 bins (total of 432 min) following each CT12 were analysed.

#### Effects on circadian timing (phase shifts)

Phase shifts were defined as the difference between the timing of CT12 on the day after an injection (day 1) projected from the daily onsets prior to and following day 1. To determine phase shifts, eye-fitted lines were drawn through the recorded onsets on the 7 days prior to injection and days 4–10 post-injection, and then extrapolated to day 1 to project the unperturbed and perturbed timing of CT12 on that day. Lines were produced independently by two individuals, and then averaged to ensure the reliability of the measurements.

#### Ethogram analysis

Direct observations of behavior were made in an effort to characterise and compare the effects of both central and peripheral AVP administration. For collection of these data, animals were maintained in LD 14 : 10 until they were entrained (2–3 weeks), and then released into DD. After free-running for 7 days in DD, observations were made at two time points under dim red light (47 lux). The first observation was made at the end of the subjective day, starting 1 h before predicted activity onset. Behavior was observed and recorded every 30 s for 1.0 h. Five circadian cycles were recorded, and the data were averaged for each animal. The second was in the early subjective night, starting 40 min after injections of saline ($n$ = 5) or AVP ($n$ = 5). The lag time between injection at CT12.5 and the observation was based on previous observations showing that WRA inhibition reached a maximum at ~30 min after injection. The same procedure was used to observe the behavior of animals in response to injections of AVP (1 μg/μL in 0.5 μL; $n$ = 5) or saline ($n$ = 5) into a site immediately dorsal to the SCN.

#### Behaviors

Behavioral categories were determined during preliminary observations of animals undisturbed in their running wheel cages. These were: (i) wheel running on the inside of the wheel; (ii) drinking from the water bottle; (iii) eating labotatory chow; (iv) exploring the wheel, i.e. sniffing, biting or showing interest in the wheel; (v) grooming the fur, face, and paws, and biting at the flanks; (vi) climbing or digging along the walls within the cage, rearing, or hanging onto (swinging from) the metal bars of the cage top; (vii) nest maintenance, e.g. digging in the nest, removing feces, or manipulating nesting materials; and (viii) sleeping – curled up in a ball. For the central injections, the category of sitting or quiet vigilance (animal awake in the nest but not moving) was also included.

### Statistics

The primary effects of AVP agonists and antagonists on WRA (see Results – experiments 1–3) were analysed with a two-way ANOVA (drug dose/concentration × time). $F$-statistics and significance are presented in the text. Two-tailed $t$-tests were used to analyse follow-up experiments, which were limited to single drug doses and latencies (see Results – experiments 4–6).

### Protocols and procedures

Following entrainment to LD 14 : 10, animals were placed in DD and their circadian rhythms were allowed to free-run for 7 days. Initial recordings served to verify the stability of the rhythms. Circadian onsets of activity were predictable to within 5 min each cycle. Both peripheral and central injections were administered at CT12.5, 0.5 h after activity onset, unless specified otherwise. The time required to perform peripheral injections was ~30 s. Central injections were performed over a 2–3-min period. Immediately after each injection, the animal was returned to its home cage. Each animal was given two injections, and the order of control and drug administration was counterbalanced. The time between injections was 7 days. All injections were performed under dim red light (~2 lux at the animal's location). 8-arginine-vasopressin (AVP), an agonist at V1 (V1a), V2 and V3 (V1b) receptors, and [d($CH_8$)$_5^1$,Tyr(Me)$^2$, Arg$^8$]-vasopressin [Manning compound (MC)], a selective V1 receptor antagonist, were obtained from Bachem Bioscience (King of

Prussia, PA, USA). Drugs were dissolved in physiological saline (0.9%) for all peripheral and central injections. Peripheral injections of 0.2 mL were administered subcutaneously between the scapulae by use of 1-mL syringe with a 27-gauge needle. For central microinjections, a 30-gauge microinjector (Plastics One, Roanoke, VA, USA) was attached to a 5-μL Hamilton syringe with a 10-cm length of Teflon injector tubing. The injector was inserted snugly into an indwelling cannula guide that had been implanted stereotaxically so that, when it was fully inserted, the tip of the injector was located immediately dorsal to the target site. A volume of 0.5 μL of drug or control solution was injected over a period of 1 min, and left in place for 1 min to allow fluid to diffuse away from the injector tip.

#### *Dose–response curve for peripheral administration*

Animals were injected subcutaneously with AVP in saline or saline alone (counterbalanced). Six doses of AVP were administered – 100 μg/kg ($n$ = 10), 50 μg/kg ($n$ = 8), 10 μg/kg ($n$ = 7), 5 μg/kg ($n$ = 7), 2 μg/kg ($n$ = 8), and 1 μg/kg ($n$ = 6). Injections (0.2 mL) were made at CT12.5 with the aid of a dim safety light (< 5 lux). Actograms were examined for phase shifts.

#### *Dose–response curve for central administration*

A central dose–response curve was determined in order to compare the effects of AVP injected centrally into the SCN with peripheral administration. Animals received central injections of AVP or saline control (counterbalanced). Five different concentrations of AVP were examined – 1 μg/μL, 0.1 μg/μL, 0.01 μg/μL, 10 ng/μL, and 4 ng/μL ($n$ = 6 per group). The concentrations were calculated to fall in the range that would be produced in the SCN following systemic administration if AVP were determined by body weight, and were able to distribute freely throughout the animal. Animals received injections at CT12.5 under dim red light (< 5 lux). Actograms were examined for phase shifts.

#### *Central injections of a V1 antagonist*

To establish that AVP suppression of locomotor behavior was receptor-mediated, we pre-injected animals with one of two concentrations of a V1 antagonist (MC) before an AVP injection. Animals implanted with a cannula aimed at the SCN were injected at CT12.5 with 0.5 μL of saline or MC in saline at either 300 or 900 nM. This was followed 2 min later by AVP. Each animal was given both drug and vehicle injections, counterbalanced, with 10 days between injections ($n$ = 9 per concentration). The order of administration of the antagonist and vehicle was varied to avoid order effects.

#### *Antagonist microinjections at CT4 and CT9*

To examine the relationship between endogenous AVP and locomotor WRA, MC (0.9 mM) was injected into the SCN at the reported peak of AVP release, CT4 ($n$ = 8), or at CT9 ($n$ = 4). The purpose was to test the idea that natural AVP rhythmicity contributes to the inactive portion of the locomotor rhythm in the hamster. For each time point, animals were given two injections spaced 10–14 days apart, of either antagonist or vehicle, in a counterbalanced design.

#### *Site specificity*

To determine whether AVP-induced inhibition of WRA is specific to the SCN, animals were implanted with cannula guides aimed at either the medial preoptic area (MPOA; $n$ = 5) or the periaqueductal gray (PAG; $n$ = 4). Each animal received an injection of AVP at CT12.5.

### *Cannulations*

Animals were anesthetised with a ketamine/xylazine pre-anesthetic (80 and 5 mg/kg, respectively), and anesthesia was maintained with isoflurane inhalant anesthetic (0.15%). Twenty-two-gauge stainless steel guide cannulae (Plastics One) were aimed at the SCN or control site (MPOA or PAG). The cannulae were implanted at a 10° angle relative to vertical. The stereotaxic coordinates (mm) relative to bregma were as follows – SCN, AP = +0.6, ML = +1.7, and DV = −7.3 (from dura); MPOA, AP = +1.1, ML = +1.7, and DV = −7.3 (from dura); PAG, AP = −5.0, ML = +0.7, and DV = −2.8 (from dura). These coordinates were chosen so that the internal injection cannula was positioned immediately dorsal to the injection site.

Cannulae were secured to the skull with jeweler's screws and dental cement. Bupivicaine (0.015 mL) was applied topically at the site. Animals were kept in a post-operative room for 2 days for observation. Animals received ketoprofen analgesic (5 mg/kg; once daily for 2 days) were then placed in a recording cabinet with a 14 : 10 LD cycle for 2–3 weeks. After entrainment to the LD cycle had been established, the lighting was switched to DD for 1 week prior to the first injection. Animals were maintained in DD for the remainder of the experiment.

### *Immunohistochemistry and histology*

#### *Injection site verification*

Animals were anesthetised with sodium pentabarbitol (100 mg/kg, intraperitoneal), and perfused transcardially with 150 mL of cold saline followed by 100 mL of cold 4% paraformaldehyde (pH 7.4). Brains were cryopreserved in 30% sucrose/phosphate-buffered saline, and then frozen and sectioned at 40 μm. Nissl-stained sections were examined for the dorsoventral extent of tissue damage, including lesions resulting from the presence of the indwelling cannula and injector assembly. The midpoint of this damage in the mediolateral and rostrocaudal directions, together with the dorsoventral position, determined the injection site reported. These sites are shown in Fig. 3.

*FOS analysis.* Animals were anesthetised and perfused as for the injection site verification. Perfusions were performed in the dark at CT13.5 (1 h after AVP or control injections). Free-floating sections were washed in cold phosphate buffer (PB) (pH 7.4), and incubated with polyclonal rabbit anti-cFOS diluted 1 : 10 000 with 0.3% Triton X-100 (Sigma) in PB and 1% normal horse serum (Vector), biotinylated goat anti-rabbit antibody (Vector) diluted 1 : 200 in PB and 1% normal goat serum, and avidin–horseradish peroxidase complex (Vectastain Elite ABC kit; Vector). FOS was visualised with 3,3′-diaminobenzidine (Sigma) in PB (pH 7.8), 0.01% $H_2O_2$, and 8% $NiCl_2$.

#### *Co-localisation of FOS and calbindin-D28k*

Animals were killed at CT13.5 to CT14 (1 h after AVP or control injections), and brain tissue sections were prepared as above. Free-floating sections were double-labeled, first with monoclonal anti-calbindin-D28k (1 : 10 000; Sigma-Aldrich) and donkey anti-mouse fluorescein isothiocyanate (FITC) (1 : 200; Jackson ImmunoResearch Laboratories, PA, USA), and then with cFOS antibody (1 : 5000; Santa Cruz, CA, USA) and donkey anti-rabbit Texas Red

(1 : 200; Jackson ImmunoResearch Laboratories). Histological images were obtained with a WaveFX-X1 Spinning Disc Confocal System (Quorum Technologies, Guelph, Ontario, Canada) on a Zeiss platform (Carl Zeiss, Toronto, Ontario, Canada) with VOLOCITY 3D image analysis software (Perkin Elmer, Waltham, MA, USA).

## Results

### *Locomotor behavior suppression following peripheral injections of AVP*

Subcutaneous administration of AVP significantly decreased WRA as compared with saline ($F_{5,40} = 7.441$, $P < 0.001$). Animals stopped running on the wheels between 30 and 60 min after AVP injections. After treatment with saline, WRA was not significantly perturbed. Example actograms are shown in Fig. 1A. Over the course of the subjective night (CT12–CT24), the amount of WRA decreased in all treatment conditions for saline and various doses of AVP. The amount of inhibition observed after an AVP injection was dose-dependent ($F_{1,40} = 10.192$, $P < 0.001$; Fig. 1B). *Post hoc* analysis (Scheffé test) confirmed that doses of 50 and 100 μg/kg (as compared with saline at the same time points) significantly and consistently inhibited WRA for 198 and 216 min respectively. The overall main effect of time ($F_{23,920} = 42.522$, $P < 0.001$) is a statistical description of the fact that WRA decreases over time, and applies to all experiments in this study. This decrease is characteristic of normal wheel running behavior in untreated hamsters. However, in this experiment, the decrease in WRA was also different between saline and AVP, as seen from the main effect of the drug and from the drug × time interaction ($F_{23,920} = 9.269$, $P < 0.001$).

Following the inhibitory response to AVP, WRA recovered to higher than control levels later in the subjective night. This rebound can be seen in the behavioral records (Fig. 1A) and in the cumulative wheel running data (Fig. 1B) at ~180 min following the systemic injections of 50 μg/kg. A similar rebound from inhibition with the 100 μg/kg dose occurred at ~288 min after the time of injection, but was less pronounced. This may have been attributable to the longer duration of influence of AVP at the higher dose, which could have delayed recovery until much later in the subjective night. Neither AVP nor saline produced phase shifts.

### *Locomotor behavior suppression following SCN injections of AVP*

Central microinjections of AVP into the SCN had main effects that were similar to those of peripheral administration, specifically a significant inhibition of WRA as compared with saline ($F_{4,25} = 7.714$, $P < 0.01$). Example actograms are shown in Fig. 2A. After a brief increase in WRA resulting from post-injection excitation, WRA decreased in all treatment conditions. The effect of AVP was concentration-dependent ($F_{4,25} = 10.201$, $P < 0.001$; Fig. 2B). In both the control condition and with low drug concentrations (4 and 10 ng/μL), WRA had recovered to pre-injection levels by ~18 min. However, the higher concentrations (0.01, 0.1, and 1 μg/μL) significantly inhibited WRA. For central AVP injections, there was a drug × time interaction ($F_{23,575} = 2.771$, $P < 0.001$), indicating a differential decrease in activity after AVP treatments as compared with saline.

The latency to recover to saline control levels increased with higher concentrations of AVP. Average recovery latencies were approximately 54, 108 and 234 min for the three effective concentrations (0.01, 1, and 1.0 μg/μL, respectively) following the first point of maximal inhibition (36 min post-injection). The general trend observed was that the higher the dosage of AVP, the greater and longer the inhibition. A small rebound in activity was observed following suppression by the 1 and 0.01 μg/μL AVP concentrations. As in the peripheral condition, no consistent pattern of phase shifting was observed after treatment with either AVP or saline.

### *Reduction of AVP-induced inhibition by an antagonist at the V1 receptor*

To confirm that AVP suppression of locomotor behavior was receptor-mediated, we injected a V1 antagonist (MC) into the SCN prior

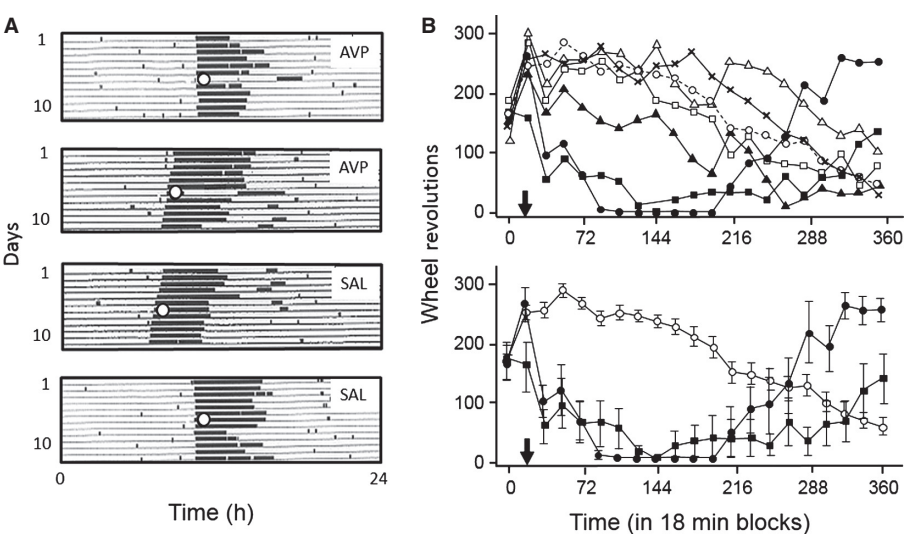


FIG. 1. Inhibition of WRA by subcutaneous injection of AVP at CT12.5. (A) Running wheel data from example animals given 100 μg/kg AVP or saline vehicle. Times and days of the injections are indicated by circles on each record. (B) Dose–response relationship for subcutaneous. injections. Graphs show the average number of wheel turns for each 18-min bin. Upper panel – error bars have been omitted for clarity. Significant inhibition is produced by 50 μg/kg and 100 μg ~30 min post-injection. Lower panel – the most effective doses (50 and 100 μg/kg), plus the saline group with standard error of the mean bars shown. The arrows at 30 min indicate the injection time. (B) Upper – saline (○); AVP 1 μg/kg (×); AVP 2 μg/kg (Δ); AVP 5 μg/kg (□); AVP 10 μg/kg (▲); AVP 50 μg/kg (•); AVP 100 μg/kg (■). (B) Lower – saline (○); AVP 50 μg/kg (•); AVP 100 μg/kg (■).

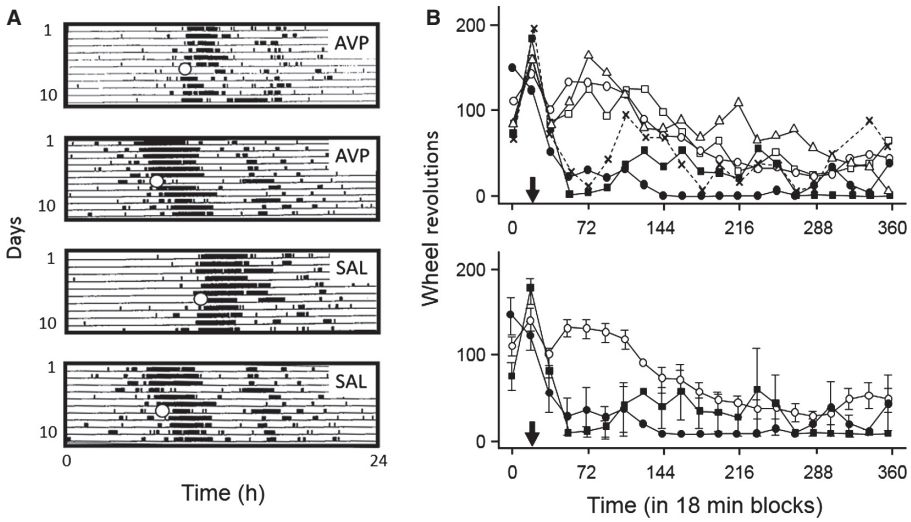


FIG. 2. Inhibition of WRA by central injections of AVP at CT12.5. (A) Running wheel data from example animals given 1 μg/μL AVP in 0.5 μL of saline vehicle or 0.5 μL of saline vehicle alone. Times and days of the injections are indicated by circles on each record. (B). Concentration–response relationship for central injections of AVP at CT12.5. Graphs show the average number of wheel turns for each 18-min bin. Upper panel – five concentrations of AVP are shown, plus the saline control. Errors bars have been omitted for clarity. Lower panel – the most effective doses (0.1 and 1.0 μg/μL AVP) are shown plus the saline group with standard error of the mean. Arrows at 30 min indicate the injection time. (B) Upper – saline (○); AVP 4 ng/μL (Δ); AVP 10 ng/μL (□); AVP 0.01 μg/μL (×); AVP 0.1 μg/μL (■); AVP 1.0 μg/μL (●). (B) Lower – saline (○); AVP 0.1 μg/μL (■); AVP 1.0 μg/μL (●).

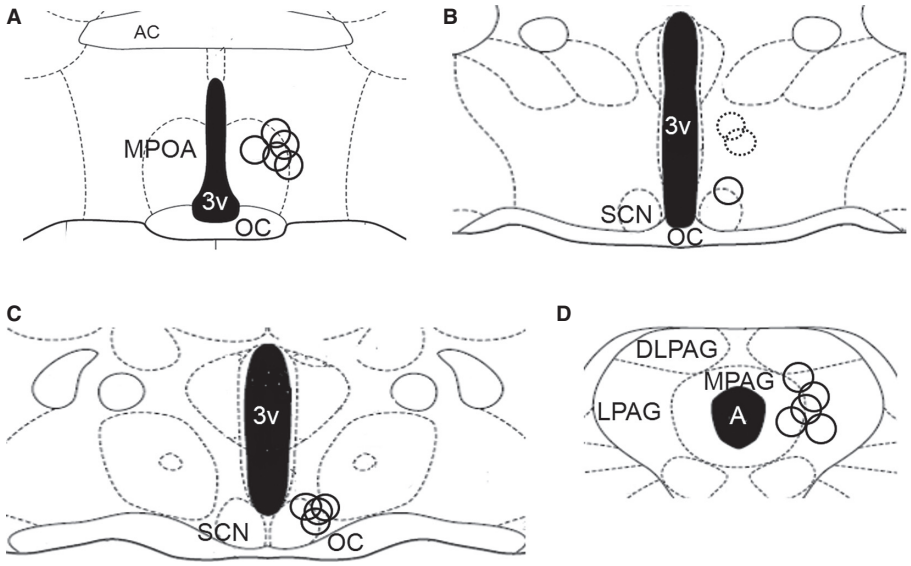


FIG. 3. Injection sites. (A) MPOA. (B and C) SCN. (D) PAG. Open ovals indicate injector tip placements into targets. Dashed circles indicate missed target sites. 3v, third ventricle; A, aqueduct; AC, anterior commissure; DLPAG, dorsal PAG; LPAG, lateral PAG; MPAG, medial PAG; OC, optic chiasm.

to a central AVP injection. Two concentrations of the antagonist were tested. Both concentrations significantly reduced the response to AVP, although neither blocked the effect completely (Fig. 4A and B). WRA began to decrease within the first 18-min block following the injection of either AVP or AVP+MC. After ~36 min, however, animals given MC prior to AVP began WRA once again. This reversal occurred sooner in animals that were given a higher concentration of antagonist. Although the total amount of WRA was reduced in all groups on the day following the injection as compared with the previous 3 days, MC prevented WRA from remaining at levels as low as those observed in the saline+AVP condition. In the saline+AVP condition, WRA remained suppressed for several hours, and then showed a slight rebound. Significant main effects of treatment ($P < 0.05$) and a treatment × time interaction ($P < 0.05$) were found. SCN injections of the antagonist (900 nM) did not reverse the behavioral response to peripheral AVP (50 μg/kg) injections ($P > 0.5$, Student's $t$-test; $n = 4$ per group).

### *General behavioral consequences of AVP injections*

To assess the behavioral specificity of exogenous AVP, we performed ethological analyses comparing the effects of peripheral and central AVP injections with the vehicle controls (Fig. 5). As the experiments were timed according to the onset of WRA, an initial ethogram was obtained for all animals during the hour prior to their predicted WRA onset (Fig. 5A). During this time, animals were generally awake and moving in their cage, and performed a variety of maintenance behaviors.

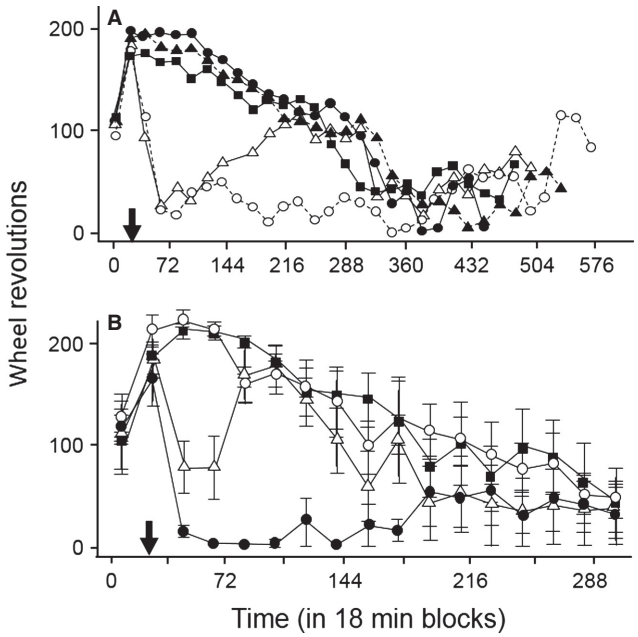


FIG. 4. Reversal of AVP-induced locomotor suppression by a V1 antagonist at CT12.5. Effects on the SCN with pre-injection of (A) 300 nM or (B) 900 nM vs. saline are shown, along with the baseline wheel running patterns for the 2 days before the injections. Error bars have been omitted from the lower concentration data (A) for greater clarity of the antagonist effect. (A) Saline (▲); AVP 0.1 μg/μL (○); anti-AVP 300 nM (■); AVP 0.1 μg/μL+anti-AVP 300 nM (Δ); untreated (●). (B) Saline (■); AVP 0.1 μg/μL (●); anti-AVP 900 nM (○); AVP 0.1 μg/μL+anti-AVP 900 nM (Δ).

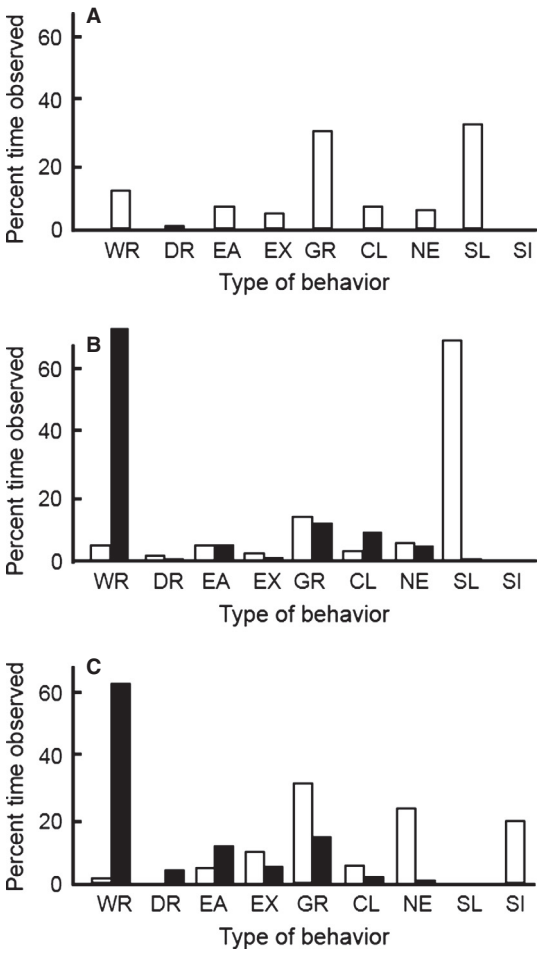


FIG. 5. Ethogram analyses. (A) Unperturbed behavioral profile 1 h prior to nocturnal activity (WRA) onset (average of 5 days). (B) Behavioral profile 1 h after WRA onset (dark histograms) and following peripheral 100 μg/kg AVP injection (open histograms). (C) Behavioral profile 1 h after WRA onset (dark histograms), and following 1.0 μg/μL AVP being delivered to the SCN (open histograms). Observations commenced 40 min after the injections, and were made every 30 s for the next hour. All data were collected in constant dim red light. The performance of each behavior is plotted as a portion of the total number of observations made. $n = 5$ animals per condition. WR, wheel running; DR, drinking; EA, eating; EX, exploring; GR, grooming; CL, climbing or rearing; NE, nest building or maintenance; SL, sleeping, SI, sitting or quiet vigilance.

*Peripheral administration*

Animals were observed within their home cages for 1 h, beginning at 40 min after the time of injection. Ethograms derived for the saline control group reflected the dominant WRA activity at this time (Fig. 5B). Peripheral injections of AVP appeared to induce more sleep at a time when hamsters are normally active (CT13–CT14). Although sleep was not confirmed by electroencephalography, these animals adopted a sleep-like posture, usually, but not always, sitting on the nest with eyes closed. These animals also spent some time engaging in nest building and maintenance behavior (i.e. digging in the corner where they slept and removing fecal pellets). In both treatment conditions, animals also groomed, and climbed within their home cage. Qualitatively, the behavioral profile following AVP treatment was similar to that recorded in the hour prior to activity onset, except that more grooming was observed before WRA onset.

*Central injection*

As central AVP also reduced WRA, we obtained a behavioral profile for animals following injection of 1 μg/1 μL AVP directly into the SCN. Control animals that received injections of saline spent >60% of the observation time engaged in WRA. AVP essentially eliminated WRA while increasing maintenance behaviors such as grooming and nest cleaning as compared with the saline controls (Fig. 5C). Although animals in the central AVP condition did not spend their time sleeping, as did AVP-treated animals in the peripheral AVP condition, they were often sitting quietly on their nests, and were generally less active than saline-treated controls. Immobile animals oriented towards slight movements or noises outside the cage. Animals did not appear to be in distress after AVP injections. In general, the WRA was replaced with activities associated with home cage maintenance.

### *AVP induction of FOS expression in the SCN*

Because Fos induction in the SCN in response to AVP injections had been described in the rat (Lança *et al.*, 1999), the hamster SCN was examined for a link between cell activation (FOS response) and the behavioral effects of AVP. As demonstrated by Lança *et al.* (1999), peripheral administration of AVP induced a FOS immunoreaction in the ventrolateral part of the SCN (VLSCN). Representative sections are shown in Fig. 6. The distribution of AVP-induced FOS was distinctly different from that of light-induced FOS in the SCN (compare Fig. 6B with 6C). The AVP-responsive region overlapped with the calbindin-expressing region (compare Fig. 6C with 6D). However, FOS expression and calbindin expression were not co-localised to the same cell population (Fig. 6E–G). Less than 20% of

the calbindin cells showed a FOS signal after AVP treatment. Moreover, FOS-positive cells were more widely distributed than calbindin-positive cells. Although we did not address this issue specifically, the distribution of AVP-induced FOS more closely approximates a region where circadian rhythms of ERK protein phosphorylation are regulated by direct input from the eye (pERK region) (Lee *et al.*, 2003; Webb *et al.*, 2013). The model suggests a common mechanism by which voluntary locomotor behaviors such as WRA might be suppressed in the subjective day by the SCN circadian clock as well as by spontaneous or light-induced input from the retina.

### *Effect of the V1 antagonist during the subjective day*

Because MC significantly reduced the AVP inhibition of WRA in the subjective night (CT12.5), we tested the hypothesis that endogenous AVP contributes directly to the spontaneous suppression of WRA in the subjective day, when AVP release from the SCN is high. The antagonist or saline was given at CT4, and each animal received both injections spaced 10–14 days apart (counterbalanced). Visual examination of the actograms (not shown) indicated that the antagonist did not induce WRA at either CT.

### *Behavioral effects of AVP injected into the MPOA orthe PAG*

Flank marking and grooming were observed following injections of AVP into the MPOA and the PAG at CT12.5. Injections at both sites failed to significantly reduce overall WRA in the CT13–CT15 interval (30–150 min following an injection). Each individual's WRA during this interval was subtracted from the animal's average activity between CT13 and CT15 for the 3 days prior to injection, to give a difference score. The average scores were then compared among AVP-treated and saline-treated groups for the two sites. No significant drug effect was found in either the MPOA (AVP, 753 ± 46 min; saline, 732 ± 29 min; $n = 5$, $P = 0.72$) or the PAG (AVP, 663 ± 60 min; saline, 772 ± 39 min; $n = 4$, $P = 0.44$). In addition, injections into the hypothalamus that missed all three sites produced neither flank marking nor grooming, and had no effect on WRA.

## Discussion

It has been suggested that endogenous AVP is responsible for regulating, at least in part, the rhythmic expression of voluntary motor activity in rodents (Van Esseveldt *et al.*, 2000; Jansen *et al.*, 2003). Overall, our findings support this model. The most robust and reproducible results from the present experiments showed that AVP, when administered either systemically or centrally, directly into the SCN, produced locomotor inhibition in a dose-dependent or concentration-dependent manner. This effect was reduced by pre-injection of a V1 receptor antagonist into the SCN. Injections of AVP into other brain sites did not produce the same suppression. Therefore, an AVP target in the SCN is effective in mediating the suppression of locomotor behavior. This target may be delineated by the induction of FOS immunoreactivity in the SCN following AVP treatment. A circumscribed and reproducible reaction was found in the SCN core with a distribution that was coextensive with the retina-driven, or pERK, region, and that overlapped with the calbindin-expressing cells in the SCN core.

The only qualitative difference in outcome between the two routes of administration in our experiments was behavioral, in that systemic injections produced sleep-like behavior (animals that were unmoving, with eyes closed), whereas central injections produced a

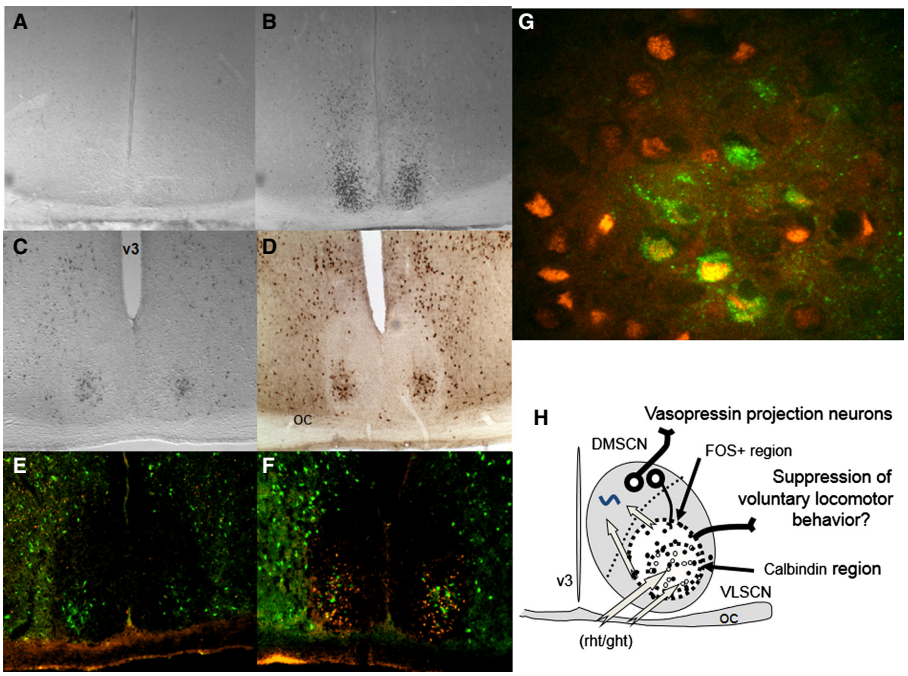


FIG. 6. Functional organisation in the SCN. (A) FOS background (subcutaneous saline control at CT12.5). (B) Light-induced FOS (CT12.5). (C) AVP-induced FOS (subcutaneous 100 mg/kg, CT12.5). (D) Calbindin-D28k immunoreactivity in an adjacent SCN section. (E) Section double-labeled with calbindin–FITC and FOS–Texas Red following saline injection. (F) Section double-labeled with calbindin–FITC and FOS–Texas Red following peripheral AVP injection. (G) Close-up (×200) of double-labeled section adjacent to F. (H) Model – suggested role for intra-SCN vasopressin in relation to the known functional anatomy of the nucleus. AVP-producing cells in the dorsomedial SCN (DMSCN) suppress behavior by activating cells located in the VLSCN core that are distinct from the calbindin-expressing cells. Photic signals via the retinohypothalamic tract (rht) reach the VLSCN, affecting numerous cell types, and may co-activate the AVP-responsive group (see text). ght, geniculohypothalamic tract; OC, optic chiasm; v3, third ventricle.

state of quiet vigilance (animals that were inactive, sitting in a nest, but demonstrably attentive to external stimuli). For peripheral injections, it was not possible to determine a specific target for AVP. However, differences in the behavioral responses to intra-SCN and subcutaneous injections are probably attributable to actions of AVP at systemic sites and/or brain sites other than the SCN. Systemically administered AVP certainly reaches many of the sites along with uncharacterised locations that have been shown previously to mediate other behavioral responses to AVP.

The behavioral response to AVP injections directly into the SCN differed from those reported previously for central administration to other brain sites. In our experiments, the behavioral profiles following injection during the subjective night were similar to those that are typically seen during the subjective day, when animals are often sitting in their nests but are not asleep. After specifically SCN-targeted injections, the immobile animals remained responsive to noise and movement outside the cage. Published reports have described stereotypic behaviors (grooming and flank marking), aggressive behaviors or sleep when AVP is injected into specific brain regions (see Introduction). Our injections into the SCN produced few or none of these behaviors, so that locomotor suppression was not caused by the induction of, or masking by, another activity. Conversely, control injections into the MPOA and the PAG both resulted in grooming and flank marking, consistent with earlier reports, but did not significantly reduce WRA. Taken together, these results indicate that: (i) there is a functional specificity linked to a target cell population within the SCN that is responsible for mediating the AVP suppression of WRA; and (ii) other stereotypic behaviors are triggered at sites outside the SCN.

This conclusion is supported by the appearance of FOS in a subregion of the nucleus following AVP injections. AVP-induced FOS was found within a small population of cells near the VLSCN core of the nucleus. This area overlapped with the calbindin-D28k-rich mid-posterior subregion, which also shows a FOS response to nocturnal light pulses (Silver *et al.*, 1996; LeSauter *et al.*, 2002). However, the cells expressing AVP-induced FOS were distinct from calbindin-expressing cells, and appeared to occupy a part of the SCN described previously as the 'pERK' region (Lee *et al.*, 2003). Expression of pERK in these cells is regulated rhythmically by the retina, and the cells are distinct from those expressing either calbindin or AVP. AVP-expressing fibers also do not have significant appositions with calbindin-expressing cells in the SCN (LeSauter *et al.*, 2002).

Because exogenous AVP does not produce circadian phase shifts (Albers *et al.*, 1984), it is unlikely that the cells that responded to AVP with FOS expression in our experiments are part of a photic entrainment pathway; it is more likely that they form part of a circadian output that regulates WRA, and possibly voluntary activities in general. This raises the possibility that the AVP-responsive SCN cells in these experiments might also be instrumental in direct negative masking effects of light on locomotor behavior, a possibility that is currently being investigated.

An interesting phenomenon for which we have only a speculative explanation was a rebound in WRA that occurred later in the subjective night in many AVP-treated animals. In those animals, WRA was suppressed immediately after injection, but returned to a rate in the late subjective night that was higher than predicted by activity over the previous days (Figs 1A and 2A). This was not quantified, owing to the variability in timing and magnitude of this WRA; nonetheless, its influence can be seen in the cumulative data in Figs 1B and 2B. A plausible explanation for the rebound may be that daily or circadian WRA is conserved across different entrainment conditions and genetic effects on period (Osiel *et al.*, 1998). Additional data from our laboratory (D. A. Golombek, unpublished) have demonstrated that restriction of WRA during the early subjective night results in rebound WRA in the late subjective night within the same cycle. It is possible that AVP suppresses WRA but not the motivation to run, and restricting the behavior prevents the dissipation of the motivation when the animals run. This possibility is also discussed below with regard to the rapid onset of WRA at the end of the subjective day.

A current view of the functional organisation of the SCN is that the generation of rhythmicity occurs in the dorsomedial shell, whereas the photic input required for entrainment of the clock is received by the ventrolateral core (Hamada *et al.*, 2001), where it is integrated with other regulatory inputs, and transferred to the pacemaker cells in the shell. Rhythmic expression of the c-*fos* gene and the clock genes (e.g. *mper1*, *mper2*, and *bmal1*) occurs spontaneously in the SCN shell. In the core region, these genes are activated by light during the subjective night (Hamada *et al.*, 2001; Dardente *et al.*, 2002).

An intriguing possibility, therefore, is that the activity of a group of cells in the SCN core is regulated by both rhythmic AVP from pacemaker cells in the shell and by rhythmic and/or light-driven input from the retina. This is consistent with the fact that both light and AVP produce suppression of voluntary locomotor behavior. The arrangement that emerges from these studies is represented in Fig. 6H. It seems likely that the core of the nucleus contains functionally heterogeneous groups of cells that act as gateways between both output from and input to the pacemaker cells. Central to this model is the idea that both light and AVP activate subsets of these cells that contribute to locomotor suppression (see LeSauter & Silver, 1999). These AVP-sensitive cells would be expected to be rhythmically active, being driven by the rhythmic release of endogenous AVP and/or light. The anti-phase sensitivity of V1 receptors would make them more responsive to exogenous AVP at night, although it is not clear how nocturnal AVP release might fit into a model of circadian regulation.

The organisational model is consistent with results from various circadian resetting experiments, in which both locomotor effects and the pattern of FOS in the SCN have been investigated. Stimuli that evoke circadian phase shifts at night while, at the same time, suppressing locomotion (e.g. light, nerve growth factor, and glutamate) produce a widespread FOS response in the SCN, especially in the core region. On the other hand, agents that block light-induced phase shifts while suppressing locomotion [e.g. glutamate antagonists (e.g. Abe *et al.*, 1991), and serotonin 5HT1B agonists (e.g. Pickard & Rea, 1997)] produce a FOS response that is restricted to the core region. We have now shown that AVP induces a FOS response only in a subregion of the core while suppressing locomotion, but has a negligible effect on circadian phase. Thus, the two behavioral responses, suppression of WRA and phase shifting, may involve the activation of different subsets of SCN cells.

To understand further what this locomotor inhibition might mean, and the specific role of AVP in the SCN, we examined the overall behavioral response to AVP. In our experiments, AVP applied to the SCN caused animals to become motionless but to appear vigilant at a time when ambulatory behavior is usually at its peak. Qualitatively, this was similar to the 'sitting awake on the nest' behavior that we observed during the hour prior to WRA onset at the end of the subjective day. Animals remained responsive to slight movement and to sound, whereas behaviors that require voluntary locomotion (exploration, WRA, and scent marking) were performed minimally. Because endogenous AVP is released during the day, a role for AVP in the SCN might be to inhibit the animal's drive to move about and explore during the daytime. We therefore suggest that the

abrupt onset of locomotion and the intense WRA at the beginning of the night in hamsters could be, in part, a rebound after release from AVP inhibition.

A neural connection has been described between the SCN and the ascending hippocampal synchronising pathway that controls voluntary motor movements such as wheel running (Abrahamson *et al.*, 2001). Activation of the ascending pathway by electrical stimulation of the posterior hypothalamus induces voluntary locomotor behaviors such as WRA (Oddie *et al.*, 1996). This raises the possibility that the AVP-induced suppression of locomotor behavior is mediated by the activation of an inhibitory connection to this ascending motor control pathway. If this is so, AVP antagonists applied to the SCN might decrease inhibition during the daytime, thereby permitting increased activity in the ascending cholinergic pathway and producing the small phase shifts observed. Although it is not clear why the antagonist did not induce WRA in the daytime, it is possible that, while cholinergic activity might be increased, other systems (e.g. dopamine) involved in driving locomotor behavior may also need to be activated for WRA to occur.

## Conclusions

The locomotor rhythm of the golden hamster is characterised by exquisitely precise and accurate timing of the nocturnal onset. Furthermore, most of the animals' spontaneous locomotor behavior is consolidated within the first few hours after activity onset. This suggests that there has been a strong selective pressure to remain inconspicuous or unexposed in the home nest until an optimal time each day, locomotion-based behaviors being rapidly performed thereafter. The daytime release of AVP in the SCN may contribute to a general strategy for avoiding predation and other risks by withholding locomotor behavior and confining it to a few optimal hours per day. At the same time, light produces behavioral inhibition as well as circadian entrainment. We showed many years ago that daily WRA is highly conserved in golden hamsters across different light cycles and circadian phenotypes (Osiel *et al.*, 1998). The current findings therefore suggest that a population of cells in the SCN core might play multiple roles in the regulation of voluntary locomotor behavior by providing a common pathway for regulation by the clock and by input from the retina.

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## Abbreviations

AVP, 8-arginine vasopressin; CT, circadian time; DD, constant dark; ERK, extracellular signal-related kinase; FITC, fluorescein isothiocyanate; LD, light/dark; MC, Manning compound; MPOA, medial preoptic area; PAG, periaqueductal gray; PB, phosphate buffer; SCN, suprachiasmatic nucleus; VLSCN, ventrolateral part of the suprachiasmatic nucleus; WRA, wheel running activity.

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